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Protection of Chickens against Very Virulent Infectious Bursal Disease Virus (IBDV) and Marek's Disease Virus (MDV) with a Recombinant MDV Expressing IBDV VP2

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To develop a herpes virus vaccine that can induce immunity for an extended period, a recombinant Marek's disease (MD) virus (MDV) CVI-988 strain expressing infectious bursal disease virus (IBDV) host-protective antigen VP2 at the *US2* site (rMDV) was developed under the control of an SV40 early promoter. Chickens vaccinated with the rMDV showed no clinical signs and no mortality and 55% of the chickens were considered protected histopathologically after challenge with very virulent IBDV (vvIBDV), whereas all of the chickens vaccinated with the conventional IBDV vaccine showed no clinical signs and were protected. Chickens vaccinated with the CVI-988 or chickens in the challenge control showed severe clinical signs and high mortality (70–75%) and none of them were protected. Also, the rMDV conferred full protection to chickens against vvMDV just as the CVI-988 strain did, whereas 90% of the challenge control chickens died of MD. Antibody levels against IBDV and MDV following the vaccination increased continuously for at least 10 weeks. No histopathological lesions in the rMDV-vaccinated chickens and no contact transmission of the rMDV to their penmates were confirmed. These results demonstrate that an effective and safe recombinant herpesvirus-based IBD vaccine could be constructed by expressing the VP2 antigen at the *US2* site of the CVI-988 vaccine strain.

INTRODUCTION

Infectious bursal disease (IBD) causes considerable economic losses in the poultry industry by inducing severe clinical signs, immunosuppression, and high mortality (≥50%) in chickens (Lukert and Saif, 1997). The bursa of Fabricius (BF) is a target organ for the IBD virus (IBDV) (Käufer and Weiss, 1980). B lymphocytes are destroyed by IBDV infection (Tanimura *et al.,* 1995; Tsukamoto *et al.,* 1995), followed by severe immunosuppression in chickens (Lukert and Saif, 1997). IBDV is a member of the *Birnaviridae* family, whose genome consists of two segments of double-stranded RNA (dsRNA) (Kibenge *et al.,* 1988): segment B encodes the putative dsRNA polymerase VP1 (Spies and Müller, 1990; Spies *et al.,* 1987), and segment A encodes two proteins, small and large. A small protein, VP5, is present in infected cells but is not essential for viral replication in cell culture (Mundt *et al.,* 1997). A large protein is processed to three mature viral proteins (VP2, VP3, and VP4). Both VP2 and VP3 are major structural proteins of IBDV particles (Bottcher *et al.,* 1997), and VP4 may be a protease (Kibenge *et al.,* 1988). VP2 is the conformational host-protective antigen of IBDV (Becht *et al.,* 1988; Fahey *et al.,* 1989, 1991b) and also an apoptotic inducer (Fernandez-Arias *et al.,* 1997). VP3 is the group-specific antigen but the protection roles need to be determined (Öppling *et al.,* 1991a; Reddy *et al.,* 1992).

Although IBD can be protected by several live IBDV vaccines, it is often difficult to protect field chickens with the use of live vaccines because the vaccines are susceptible to maternal antibodies (Lukert and Saif, 1997; Tsukamoto *et al.,* 1995b). In addition, some live vaccines cause moderate bursal atrophy (Mazariegos *et al.,* 1990), which may allow opportunistic bacterial infections (Lukert and Saif, 1997). Some characteristics of live IBDV vaccines such as antigenicity and virulence are not stable (Muskett *et al.,* 1985). Hence, the development of safer and more efficacious vaccines against IBDV is required in the field.

Marek's disease (MD) is a transmissible malignant T-cell lymphoma of chickens and is the most common lymphoproliferative disease in chickens. MD is caused by serotype 1 MD viruses (MDV) (Calnek and Witter, 1997). Although MDV has been classified as a gammaherpesvirus on the basis of its biological properties, the gene structural data suggest that it should be classified as an alpha-herpesvirus. MD has been controlled in the poultry industry for more than 25 years by serotype 1, 2, and 3 live MDV vaccines (Calnek and Witter, 1997). The field experience has clearly demonstrated the safety and effectiveness of the MDV vaccines as a viral vector.

The MDV vaccines have some ideal characteristic as

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AB024076.

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a recombinant vaccine vector. (i) The MDV as a herpesvirus infects chickens persistently, and so the vaccine immunity may also continue to the end of the chickens' lives. (ii) In practice, only MDV vaccines can be inoculated into day-old field chicks that have high titers of maternal antibodies because the degree of growth interference of MDV *in vivo* by the maternal antibodies is less than that of some other virus vaccines. The MDV vaccines are virus-infected cell vaccines and spread by a cell-to-cell transmission mechanism in chickens. In contrast, other virus vaccines are susceptible to maternal antibodies. (iii) The MDV genome is large enough for multiple foreign genes to be inserted in its genome. Five foreign gene-insertion sites in the unique short (US) region of serotype 1 MDV (Cantello *et al.,* 1991; Parcells *et al.,* 1994; Sakaguchi *et al.,* 1993, 1994; Sonoda *et al.,* 1996) and the two gene-insertion sites in the US region of serotype 3 MDV, herpesvirus of turkey (HVT) (Darteil *et al.,* 1995; Morgan *et al.,* 1992), were identified. A recombinant HVT (rHVT) expressing IBDV VP2 at the *gI* site was constructed but *in vivo* replication of the rHVT was not efficient; the rHVT induced some protection against virulent IBDV (60%) and low protection against MDV (10% protection) (Darteil *et al.,* 1995). Therefore, further studies are required to develop a more efficacious herpesvirus-based recombinant IBD vaccine that causes persistent infection in chickens.

In this study, we demonstrated that an effective and safe herpesvirus-based IBD vaccine could be constructed by expressing IBDV VP2 antigen at the *US2* site of serotype 1 MDV vaccine: effective protection against very virulent IBDV (vvIBDV) and full protection against vvMDV were achieved. Persistent infection of the rMDV in chickens or continuous stimulation of the host immune systems by the VP2 antigen was suggested, because antibody levels against IBDV and MDV following the vaccination increased continuously for at least 10 weeks. Variation in the immune responses to VP2 was found.

RESULTS

Analysis of rMDV

Polymerase chain reaction (PCR) analysis of DNA prepared from chicken embryo fibroblasts (CEFs) infected with the rMDV with primer pair P-MD1 and P-MD2 indicated that the rMDV was isolated without contamination of its parental CVI-988 (Figs. 1B and 1C). The findings that the PCR product prepared from the rMDV was hybridized with both IBDV VP2 and MDV US2 probes indicate that the VP2 expression cassette was inserted into the expected *US2* site of the CVI-988 genome by homologous recombination (Figs. 1C and 1D).

Expression of IBDV VP2 protein in the rMDV was clearly demonstrated by the immunostaining of the plaques with anti-IBDV rabbit antibodies (Fig. 2A). Western blot analysis of the cell lysate from the rMDV-infected CEFs with anti-IBDV rabbit antibodies indicated that the molecular mass of the expressed VP2 was approximately 42 kDa (Fig. 2B). Several IBDV protein bands were detected in the IBDV J1 strain-infected CEF lysates, whereas there were no clear bands in the CVI-988-infected CEF lysates.

To determine the cellular localization of the expressed VP2, the rMDV-infected CEFs were incubated with anti-IBDV rabbit serum followed by horseradish peroxidase (HRPO)-conjugated goat anti-rabbit IgG before or after the CEF cells were fixed with cold acetone. As a result, the rMDV plaques were barely stained before the cell fixation but were clearly stained after the fixation (Fig. 2A). In addition, larger amounts of VP2 antigens were detected in cell extracts from the rMDV-infected CEFs than in the culture fluid using IBDV antigen-capture enzyme-linked immunosorbent assay (ELISA) (Tsukamoto *et al.,* 1995a) (Fig. 3). These results indicate that the expressed VP2 was not present on the cell surface but accumulated in the cells.

Protection against vvIBDV

For determination of efficacy of the rMDV against vvIBDV, twenty 1-day-old SPF chicks were vaccinated with the rMDV and challenged 6 weeks later with 10^5 EID_{50} of vvIBDV Ehime/91 strain. All of the chickens vaccinated with the rMDV or live IBDV vaccine, IBDV-A, were free of clinical signs and mortality after the challenge (Table 1). Of the chickens vaccinated with rMDV or IBDV-A, 65 or 40%, respectively, had gross lesions after the challenge. The gross lesions of the chickens vaccinated with the commercial vaccine IBDV-A might be caused by the vaccine itself. In contrast, all of the chickens vaccinated with CVI-988 and those in the challenge control group showed severe clinical signs and high mortality (70–75%).

Histopathological examinations of BFs showed that there were some variations in the BF lesion scores in chickens vaccinated with rMDV after the challenge; some chickens did not have any lesions (score 0, 4/20), whereas other chickens had mild (scores 1–4, 7/20) or severe (score 5, 9/20) BF lesions (Table 2). The rMDV conferred 55% protection against vvIBDV in chickens (11/20) when no or mild BF lesions (scores 0–4) were considered protected. In contrast, the IBDV-A vaccine conferred full protection against vvIBDV; all of the chickens had BF lesion scores of less than 4 (20/20). Both the challenge control chickens and the chickens vaccinated with CVI-988 had severe BF lesions (score 5, 20/20) (Table 1).

The association between enzyme immunoassay (EIA) antibody titers to IBDV before the challenge and the BF lesion scores in the rMDV-vaccinated chickens was determined. There was a tendency for chickens with the low antibody titers (20 or 40) to IBDV to have a severe BF lesion score (score 5). Although all of the chickens pro-

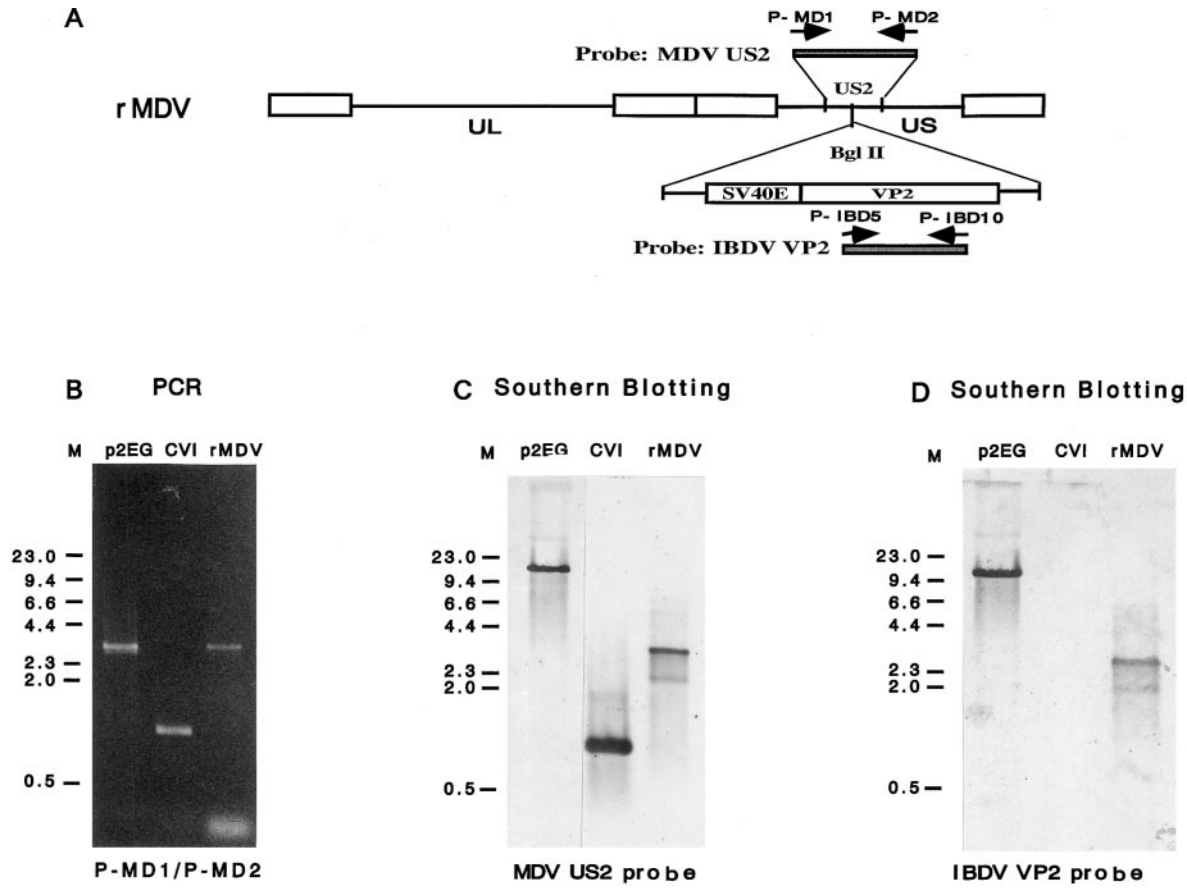

FIG. 1. (A) Gene structure of the rMDV. Both MDV US2 and IBDV VP2 probes and the primer pairs to synthesize the probes are shown. (B) Amplification of MDV US2 gene by PCR. The US2 gene was amplified from DNAs prepared from the p2EG plasmid, the rMDV-infected CEF cells, or CVI-988-infected CEF cells. The reaction products were submitted to agarose gel electrophoresis and stained with ethidium bromide. (C) The US2 gene was amplified from DNAs prepared from CVI-988-infected CEF cells, or the rMDV-infected CEF cells, and processed for Southern blot analysis with the MDV US2 probe. p2EG directly underwent Southern blot analysis without PCR amplification. (D) The same DNAs were processed for Southern blot analysis with the IBDV VP2 probe.

duced the anti-IBDV antibodies 6 weeks after the rMDV vaccination, the anti-IBDV antibody titers of the rMDV-vaccinated chickens were much lower than those of the chickens vaccinated with the live IBDV-A vaccine (Table 2). The geometric mean antibody titer of the rMDV-vaccinated chickens was approximately 1/30 of that of the live IBDV-A vaccinated chickens when IBDV-infected CEF cells were used as the antigen. In four chickens vaccinated with the rMDV, a low antibody level against VP2 (titer 40 or 80) was sufficient to protect the chickens against vvIBDV challenge.

Protection against vvMDV

To determine the efficacy of the rMDV vaccine against vvMDV, the RB1B strain, the vaccinated chickens were challenged 7 days after the vaccination and examined for clinical MD signs and tumor developments for another 6 weeks. The chickens vaccinated with the rMDV, as well as those vaccinated with CVI-988, did not show any clinical signs and had no gross/histopathological tumors (Table 3), indicating that the rMDV could confer full protection to chickens against vvMDV. Ninety percent (18/20) of the chickens in the unvaccinated challenge control group died of MD, and the remaining 2 chickens had histopathological MD lesions at the necropsy.

Antibody responses and contact transmission

Antibody responses of chickens vaccinated with the rMDV were tested for 10 weeks by the EIA tests. As a result, some variations in anti-IBDV antibody titers among the chickens vaccinated with the rMDV were also revealed; most of the chickens (8/9) produced anti-IBDV antibodies up to 6 weeks after the vaccination, but one chicken did not produce the antibodies up to 10 weeks following the vaccination (Table 4). It was shown that the antibody titers to IBDV increased gradually in the remaining 8 chickens after the vaccination with rMDV, for at least 10 weeks.

Antibody titers against MDV were also measured by

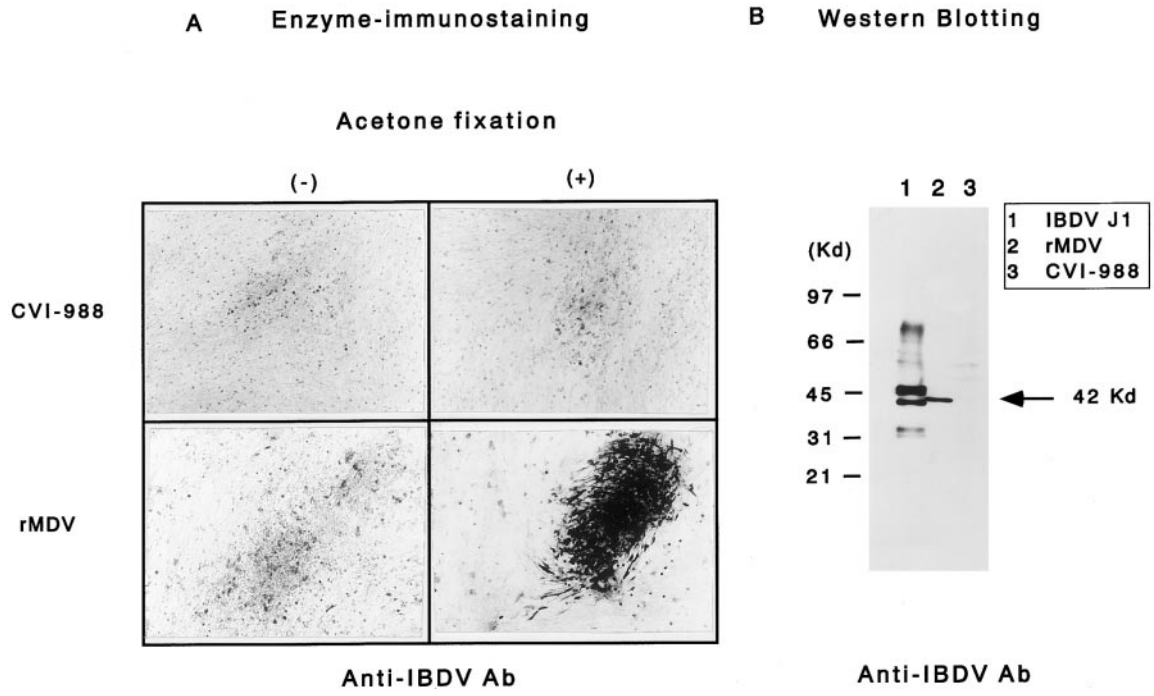

FIG. 2. (A) Immunological staining of the CVI-988 or rMDV plaques with rabbit anti-IBDV antibodies with or without acetone fixation of the MDV-infected cells. The plaques on the left were not fixed with acetone (Acetone (−)) but were incubated with anti-IBDV antibodies, HRPO-conjugated goat anti-rabbit IgG antibodies, and a DAB substrate. The plaques on the right were stained after acetone fixation (Acetone (+)). (B) CEF lysates prepared from the rMDV-infected CEF cells were subjected to SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blotted membrane was blocked and reacted with rabbit anti-IBDV antibodies followed by HRPO-conjugated goat anti-rabbit IgG antibodies and ECL Western blotting detection reagents.

the EIA tests to investigate the persistent infection by the rMDV in chickens. The data summarized in Table 4 clearly indicate that the anti-MDV antibody titers gradually increased and reached a level similar to those of chickens vaccinated with CVI-988. These serological data suggest persistent infection by the rMDV in chickens or continuous stimulation of the immune systems by the VP2 antigen.

No evidence of contact transmission of the rMDV from nine rMDV-vaccinated chickens to their two SPF penmates was detected during the experimental period by the serological tests (Table 4). This was confirmed by histopathological examinations of the penmates; no histopathological changes were detected. Two SPF penmates reared with chickens vaccinated with CVI-988 produced anti-MDV antibodies from 8 weeks of age, and the histopathological changes in infection were detected at necropsy (Table 4). These results indicate that contact transmission of the rMDV was not detected in this experiment, although horizontal transmission of the parent virus was observed. It is possible that contact transmission of the rMDV may occur but at a level below that detectable in this assay (two birds), and it seems reasonable that contact transmission of the rMDV does not exceed that of its parent virus CVI-988.

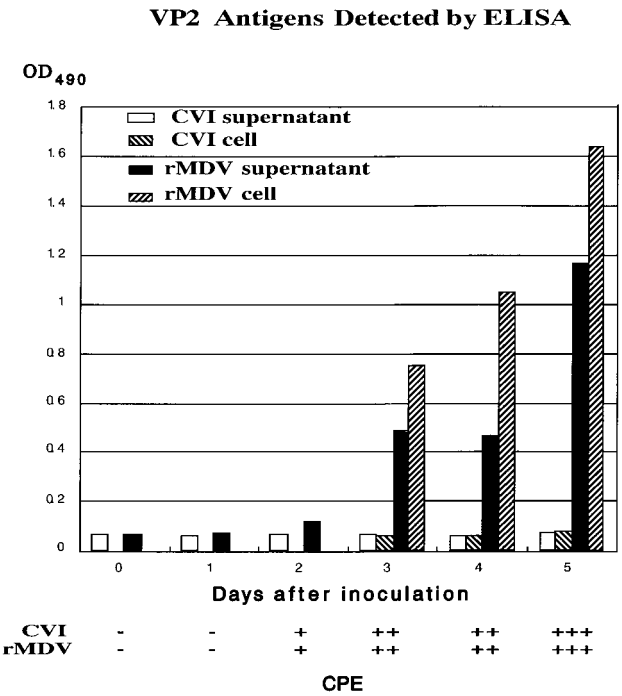

FIG. 3. Detection of the IBDV VP2 antigen produced in the culture fluids and cell lysates from the rMDV-infected CEF cells. These samples prepared from the CVI-988-infected CEF cells were used as a control. The VP2 antigens were detected by antigen-capture ELISA.

TABLE 1

Efficacy of the rMDV, IBDV-A, and CVI-988 Vaccines against vvIBDV Ehime/91 Strain in SPF Chickens

Vaccine	Challenged with	Clinical signs	Mortality	Gross BF lesions	Average histopathology BF lesion scores	Protection
rMDV	Ehime/91	−	0% (0/20)	65% (13/20)	3.3	55% (11/20)[a]
IBDV-A	Ehime/91	−	0% (0/20)	40% (8/20)	1.6	100% (20/20)
CVI-988	Ehime/91	+	70% (14/20)	100% (20/20)	5.0	0% (0/20)
—	Ehime/91	+	75% (15/20)	100% (20/20)	5.0	
—	—	−	0% (0/10)	0% (0/10)	0.0	

Note. One-day-old SPF chickens were vaccinated with the rMDV or CVI-988 strains and challenged with vvIBDV Ehime/91 strain 6 weeks later. IBDV-A (live IBDV vaccine) was inoculated into 20-day-old SPF chickens, which were challenged at 6 weeks of age as above.

[a] Number of chickens with no or mild BF lesion scores (0 to 4)/number tested.

Vaccine safety

Histopathological examinations of the rMDV-vaccinated chickens were done 10 weeks after the vaccination to determine the safety of the rMDV vaccine in chickens. No lesions were found in any of the tissues tested from the rMDV-vaccinated chickens as well as from those vaccinated with the parent virus CVI-988. These data clearly demonstrate the safety of the rMDV for chickens.

DISCUSSION

rHVT/rMDV vaccines have been developed for Newcastle disease virus (NDV), an avian paramyxovirus, which causes a highly contagious and fatal respiratory disease in birds (Heckert *et al.,* 1996; Morgan *et al.,* 1992; Reddy *et al.,* 1996; Sakaguchi *et al.,* 1998). The rHVT expressing NDV fusion (F) envelope glycoprotein at the *US10* site offered 90% protection against an intramuscular challenge with velogenic NDV (Morgan *et al.,* 1992). More recently, rHVT expressing both hemagglutinin–neuraminidase (HN) and F envelope glycoproteins protected all of the chickens tested against velogenic NDV (Heckert *et al.,* 1996; Reddy *et al.,* 1996). It was shown that both rHVT and rMDV expressing NDV were applicable to field chickens with maternal antibodies (Morgan *et al.,* 1993; Sakaguchi *et al.,* 1998). However, only one paper reported construction of rHVT expressing IBDV VP2, which conferred partial protection against IBDV (60%) and poor protection against vvMDV (10%), and the *in vivo* replication was inefficient (Darteil *et al.,* 1995).

In this study, we constructed an rMDV expressing the IBDV VP2 antigen, which conferred partial protection against vvIBDV (55%) and full protection against vvMDV (Tables 1, 2, and 3). The criteria that the no/mild BF lesions are considered protected means that the rMDV suppressed the damage caused by vvIBDV to a level equal to or milder than those caused by IBDV vaccine strains. Both the rMDV and the rHVT expressing VP2 showed partial protection against IBDV; however, the rMDV may be more efficacious because the rMDV was evaluated at a higher dose ($10^{5.0}$ EID_{50}/chicken) of very virulent IBDV (70% mortality to SPF chickens), whereas the rHVT was evaluated at a lower dose ($10^{2.5}$ EID_{50}/chicken) of virulent IBDV (vIBDV) (mild clinical signs). The rMDV conferred full protection against vvMDV (Table 3),

TABLE 2

Relationship between Serum Antibody Titers to IBDV and Histopathological BF Lesion Scores in Chickens Vaccinated with the rMDV and Challenged with vvIBDV Ehime/91 Strain

			Histopathological BF lesion scores						
Vaccine	Ab titers to IBDV	Gross BF lesions	0	1	2	3	4	5	Average
rMDV	20[a]	6/6					1	5	4.8
	40	4/10	3	2		1		4	2.5
	80	2/3	1			1	1		2.3
	160	1/1					1		4.0
	Average 37	Total 13/20	4	2		2	3	9	3.3
IBDV-A	Average 1151	8/20	2	13	1		4		1.6

Note. One-day-old SPF chickens were vaccinated with the rMDV and challenged 6 weeks later. Twenty-day-old SPF chickens were vaccinated with IBDV-A vaccine and challenged at 6 weeks of age. The Ehime/91 strain of vvIBDV was used as a challenge strain.

[a] Sera were collected before the challenge, and the antibody titers against IBDV were measured by indirect EIA tests.

TABLE 3

Efficacy of the rMDV and CVI-988 Vaccines against vvMDV RB1B Strain in SPF Chickens

Vaccines	Challenged with	Clinical signs	Mortality	Gross MD lesions	Histopathological MD lesions	Protection
rMDV	RB1B	−	0% (0/18)	0% (0/18)	0% (0/18)	100%
CVI-988	RB1B	−	0% (0/20)	0% (0/20)	0% (0/20)	100%
—	RB1B	+	90% (18/20)	95% (19/20)	100% (20/20)	
—	—	−	0% (0/10)	0% (0/10)	0% (0/10)	

Note. One-day-old SPF chickens were vaccinated with the rMDV or CVI-988 vaccines and challenged 7 days later with vvMDV RB1B strain. Clinical signs and mortality were then observed for 6 weeks, and both dead chickens and the surviving chickens necropsied were subjected to gross and histopathological examinations. Chickens with gross tumor lesions were considered positive for histopathological lesions.

whereas the rHVT offered poor protection (10% protection). Serological data suggest that the rMDV should infect chickens persistently or stimulate host-immune systems continuously (Table 4). In contrast, *in vivo* replication of the rHVT was poor or absent (Darteil *et al.,* 1995). It was shown that the *gI* site, which was used to express the VP2 in the rHVT, plays an important role in cell-to-cell transmission of HSV-1 *in vivo* (Balan *et al.,* 1994). Recombinant fowlpox virus (rFPV)-based IBD vaccines have also been reported; however, their efficacy was not satisfactory (Bayliss *et al.,* 1991; Heine and Boyle, 1993). Chickens vaccinated with rFPV at 1 and 14 days of age were not protected against the gross BF lesions after challenge with vvIBDV (Bayliss *et al.,* 1991). No lesions in chickens vaccinated with the rMDV and no contact transmission of the rMDV to SPF penmates were confirmed (Table 4). These results indicate that a more efficacious and safer herpesvirus-based IBD vaccine was constructed by expressing the VP2 gene at the *US2* site of serotype 1 MDV, CVI-988. Nevertheless, the efficacy is lower than that of conventional IBDV live vaccines (Tables 1 and 2) (Ismail and Saif, 1991; Tsukamoto *et al.,* 1995b). Further studies are required to improve the efficacy of the rMDV.

There are several factors affecting efficacy of the recombinant herpesvirus vaccines: vector virus strains, insertion sites in the vector virus genome, promoters, and host-protective antigens. We used the CVI-988 vaccine strain as a vector, *US2* as a gene insertion site, and an SV40 early promoter to construct the rMDV, whereas Darteil *et al.* (1995) used an HVT as a vector, the *gI (UL40)* as a gene insertion site, and a CMV promoter. There are several reasons for our construction. (i) The CVI-988 is the most effective MD vaccine obtained thus far and may replicate efficiently in chickens (Witter *et al.,* 1995). (ii) It was shown that insertion of a marker gene at the US2 site did not affect the viral replication of the recombinant virus in chickens (Cantello *et al.,* 1991; Parcells *et al.,* 1994). Antibody responses against IBDV and MDV following the vaccination increased gradually until the end of this experiment (10 weeks) (Table 4), and the antibody titers to MDV of chickens vaccinated with the rMDV were comparable to those of chickens vaccinated with the parental CVI-988 (Table 4). These data suggest that the rMDV may persistently infect chickens as efficiently as the parent virus CVI-988. From these viewpoints, our strategy may be useful for constructing efficacious rMDV vaccines for other infectious diseases.

TABLE 4

Geometric Mean Serum Antibody Titers against IBDV and MDV of Chickens Vaccinated with the rMDV or CVI-988 Vaccines and Those of Each of Two SPF Penmates

			Weeks after vaccination				
Vaccine	Antibodies to	No. of chickens	2	4	6	8	10
rMDV	IBDV	9	0 (0/9)[a]	5 (5/9)	17 (8/9)	31 (8/9)	45 (8/9)
		2 penmates	0	0	0	0	0
	MDV	9	7 (4/9)	200 (9/9)	341 (9/9)	584 (9/9)	858 (9/9)
		2 penmates	0	0	0	0	0
CVI-988	IBDV	7	0	0	0	0	0
		2 penmates	0	0	0	0	0
	MDV	7	24 (7/7)	316 (7/7)	631 (7/7)	848 (7/7)	848 (7/7)
		2 penmates	0	0	0	79 (2/2)	224 (2/2)

Note. One-day-old SPF chickens were vaccinated with the rMDV or CVI-988 vaccines and reared for 10 weeks with each of two SPF penmates in an isolater. Sera were collected at 2-week intervals.

[a] Serum antibody titers were measured by indirect EIA tests. No. positive/number tested.

It is known that the VP2 is a conformational antigen (Becht *et al.,* 1988; Fahey *et al.,* 1989). Denatured VP2 does not induce protection in chickens (Fahey *et al.,* 1989), and denatured and renatured VP2 also lost the ability to induce neutralizing antibodies in chickens (Öppling *et al.,* 1991b). Multimeric forms of VP2 were highly immunogenic, whereas the monomeric forms were nonimmunogenic (Azad *et al.,* 1991). Therefore, the rMDV should express structurally native VP2 as well as other vector systems such as yeast (Fahey *et al.,* 1991a; Macreadie *et al.,* 1990), baculovirus (Snyder *et al.,* 1994; Vakharia *et al.,* 1993, 1994), FPV (Bayliss *et al.,* 1991; Heine and Boyle, 1993; Heine *et al.,* 1994), and HVT (Darteil *et al.,* 1995). The VP2 expressed by rMDV was hardly detected on the cell surface unless the rMDV-infected CEF cells were fixed with acetone (Fig. 2A), indicating that the expressed VP2 was not present on the cell surface but accumulated in the cells. Intracellular localization of VP2 corresponds to the previous study, in which VP2 expressed by rFPV was nonglycosylated and absent on the cell surface (Heine and Boyle, 1993). It was shown that modifications of VP2 to cell surface localization by adding a transmembrane domain or a secreted form of VP2 by adding secretion signal peptides failed to enhance immunogenicity (Heine *et al.,* 1994). These data also suggest that intracellular localization of VP2 may be critical to preserve the native conformational structure of VP2.

Serological tests are a good indicator to determine the efficacy of the rMDV, because serum neutralizing antibodies to VP2 play an important role in IBDV protection (Fahey *et al.,* 1991b; Lukert and Saif, 1997). There was a tendency for the chickens with lower anti-IBDV antibody titers before the challenge (EIA antibody titer of 20 or 40) to have severe BF lesions (score 5), whereas chickens with higher antibody titers (40–160) had no or mild BF lesions (score 0–4) (Table 2). We confirmed that the antiserum had virus-neutralizing activity against IBDV (data not shown). However, unexpectedly, a small amount of antibodies against VP2 was sufficient to protect chickens against clinical IBD (Table 2). In particular, despite the low level of the antibodies, four chickens were protected completely (no BF lesions). Higher virus-neutralizing antibody titers (approximately 10^3) are usually required to protect chickens against IBDV when purified VP2 is injected into the chickens (Macreadie *et al.,* 1990; Vakharia *et al.,* 1993, 1994). These data suggest that the rMDV might induce cellular immunity to VP2, which might work effectively for IBDV protection. Although cellular immunity to IBDV has not been demonstrated, it is speculated that rFPV-based IBD vaccines might protect chickens via the stimulation of cell-mediated immunity (Bayliss *et al.,* 1991). Further studies are required to determine which is more effective in protecting chickens against IBDV infection, humoral immunity or cell-mediated immunity.

Although live IBDV vaccines proved highly immunogenic and effective in chickens (Ismail and Saif, 1991; Tsukamoto *et al.,* 1995b), there are several advantages to the rMDV vaccine developed here. (i) This rMDV is a subunit vaccine. Therefore, it does not induce side effects in the BF, and the intact BF may work effectively for other disease vaccinations. In contrast, live IBDV vaccines induce atrophy of the BF (Mazariegos *et al.,* 1990; Tsukamoto *et al.,* 1995b). (ii) Variant IBDV strains do not come from the rMDV, whereas live IBDV vaccines may produce pathogenically or antigenically variant strains (Muskett *et al.,* 1985). Antigenic variant IBDV strains, which are difficult to control by classical types of live IBDV vaccines (Jackwood and Saif, 1987; Sharma *et al.,* 1989), are suspected to be derived from live IBDV vaccines (Giambrone and Closser, 1990). It may be possible that both classical and variant types of IBDV could be controlled by an rMDV expressing both classic and variant types of VP2 antigens. (iii) It is expected that the protective immunity to IBDV will continue for an extended period of time, because the vaccine is a herpesvirus. (iv) It was shown in this study that contact transmission of the rMDV to the SPF penmates did not occur (Table 4), whereas live IBDV vaccines are transmitted from chicken to chicken (Tsukamoto *et al.,* 1995b).

MATERIALS AND METHODS

Viruses, cell culture, and chickens. The CVI-988 vaccine strain of serotype 1 MDV was used as a parent virus for construction of an rMDV. MDVs were propagated in CEFs prepared from 10-day-old embryonated eggs obtained from our SPF chicken flock, line PDL-1 (Furuta *et al.,* 1980), using growth medium consisting of Dulbecco's MEM (Nissui, Tokyo, Japan), 5% fetal calf serum, 10% tryptose phosphate broth, and antibiotics. The Ehime/91 strain of vvIBDV (Tsukamoto *et al.,* 1992) was used to clone the host-protective antigen gene of the VP2. The Ehime/91 and vvMDV RB1B strains (Schat *et al.,* 1982) were used as challenge viruses.

All white leghorn SPF chickens were purchased from SPAFAS (Norwich, CT) and reared in negative-pressure isolators set in a negative-pressure chicken house.

Detection of antibodies to IBDV and MDV. Antibodies against IBDV VP2 and MDV were detected by an EIA. CEF-adapted IBDV $J1^{CEF72}$ strain (3×10^6 PFU) (Tsukamoto *et al.,* 1995a) was inoculated into 10 ml of primary CEF cells (8×10^6 cells) in a 96-well plate (Falcon, Lincoln Park, NJ) and the cells were cultured for 24 h. After removal of the medium and subsequent rinsing of the cells twice with phosphate-buffered saline (PBS), the cells were fixed with a fixation solution (70% acetone in water) for 5 min at room temperature. After removal of the fixation solution, the antigen-coated plates were stored at −80°C before use. For preparation of MDV-infected cells, 3% of the CVI-988-infected CEFs were cocultivated

with primary CEF cells (3×10^4 cells) for 4 days in 96-well plates, fixed, and stored as described above. Serum samples were diluted with a diluent (5% skim milk powder/PBS), and the diluted samples were added to wells coated with IBDV- or MDV-infected cells. After incubation for 1 h at 37°C and a subsequent washing of the cells, the plates were incubated with HRPO-conjugated goat anti-chicken IgG (Fc) (1:500 dilution with the diluent; Bethyl Laboratories, Inc., Montgomery, TX). After incubation at 37°C for 30 min and washing, the substrate (3,3′-diaminobenzidine tetrahydrochloride) was added for color development. After incubation for 10 min, the enzyme reaction was stopped by replacing the substrate solution with water, and the cells were observed under a light microscope.

Western blotting analysis. Lysates of CEFs infected with rMDV, CVI-988, or IBDV $J1^{CEF72}$ strains were subjected to a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel electrophoresis (PAGE) followed by a Western blotting analysis. CEF cells infected with IBDV $J1^{CEF72}$ strain were harvested 20 h after the virus inoculation, and the cell lysate was used as the positive control. Anti-IBDV rabbit antiserum (Tsukamoto *et al.,* 1995a) and enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Buckinghamshire, England) were used.

Detection of IBDV VP2 antigens by ELISA. The IBDV VP2 antigens produced from the rMDV were measured by an antigen-capture ELISA as described previously (Tsukamoto *et al.,* 1995a). A CEF monolayer in a 6-well tissue culture plate was inoculated with 1000 PFU of the rMDV and cultured for 5 days. The CVI-988 was used as a control. The cytopathic effects were observed, and then both the culture supernatant and the cells were harvested from 1 well per day and stored at −80°C. The supernatants were mixed with an equal amount of ELISA diluent, and the cell lysates were homogenized in an equal volume of the same ELISA diluent. These samples were subjected to the measurement of the VP2 antigen by ELISA.

Generation of expression cassette. Total MDV DNA was prepared from the CVI-988-infected CEFs as described previously (Morgan *et al.,* 1990) and used as a template to amplify a 6.5-kb MDV DNA fragment covering the *US2* region with two primers, P-MD9 (5′-ACCATGGAAACCCGATGTCG-3′) and P-MD10 (5′-ATGCCCGTCTTGATGGACGA-3′). These primers were made according to the *US* region sequences of MDV GA strain (Brunovskis and Velicer, 1995). The 6.5-kb fragment was cloned into a plasmid vector, pCRII (Invitrogen Corp., San Diego, CA), according to the manufacturer's recommendations. Part of the *US2* region was sequenced with two primers, P-MD1 (5′-GGTTGTAGCGATGCACCTAAT-3′) and P-MD2 (5′-TAGTTTTAACCCTGTTTCATA-3′). The construct was digested with *Sal*I–*Bam*HI and the *Sal*I–*Bam*HI fragment (6.5 kb) was recloned into a vector pUC18, and the plasmid was designated pUS2 (Sambrook *et al.,* 1989). The entire putative VP2 gene (from nucleotides 1 to 1359 of segment A open reading frame) was amplified from viral RNA prepared from the Ehime/91-infected bursal homogenates by using an RNA PCR Kit (Takara Shuzo Co., Shiga, Japan) and two primers, P-IBD1 (5′-ATAAGAATGCGGCCGCATGACAAACCTGCAAGATCAAACCCA-3′) and P-IBD2 (5′-ATAGTTTAGCGGCCGCTTACCTCCTTATAGCCCGGATTATGT-3′) (Brown *et al.,* 1994; Brown and Skinner, 1996). The VP2 gene was cloned into a plasmid vector, pSVβ (Clontech Laboratories, Palo Alto, CA), by replacing the β-galactosidase gene, and plasmid pSV-VP2 was obtained. The pSV-VP2 plasmid was digested with *Eco*RI/*Hin*dIII, and the resulting 2.0-kb DNA fragment that contained SV40 early promoter, the putative entire VP2 gene, and the SV40 polyadenylation signal was cloned into the *Bgl*II site in the *US2* region of the transfer vector pUS2 after the ends were blunted. The expression cassette was designated p2EG.

Construction of rMDV. Transfection of the expression cassette p2EG into CEFs was done as described previously (Sakaguchi *et al.,* 1993). Briefly, CEFs (8×10^6 cells) were cultivated with the CVI-988-infected CEFs (5×10^5 cells) for 4 h, and then the cells were trypsinized. The cell suspensions were mixed with 40 μg of p2EG in 0.7 ml of PBS and electroporated in an electric field (1.5 kV/cm) at room temperature for 0.5 ms using gene pulser (Bio-Rad Laboratories, Hercules, CA). After 3 days of cultivation in a tissue culture dish, the cells were transferred into 96-well tissue culture plates and cultured until the plaques were visible. Duplicates of the culture were made, and one of the plates was stained with anti-IBDV rabbit IgG followed by HRPO-conjugated anti-rabbit IgG (goat IgG) by the EIA. Antigen-positive wells of the other plate were subcultured. The cloning process was repeated until most of the plaques became immunologically positive. Then the infected cells were sonicated and the resulting supernatant was inoculated onto CEFs to obtain pure rMDV.

PCR and Southern blot analysis. To determine whether the rMDV was contaminated with wild-type CVI-988, a PCR amplification that covered the US2 region with two primers, P-MD1 (5′-GGTTGTAGCGATGCACCTAAT-3′) and P-MD2 (5′-TAGTTTTAACCCTGTTTCATA-3′), was performed against high-molecular-weight DNA prepared from MDV-infected CEFs. The amplified PCR products were hybridized with MDV US2 or IBDV VP2 probes. The US2 and VP2 probes were labeled with digoxigenin (DIG) according to the manufacturer's manual (Boehringer Mannheim, Mannheim, Germany) using primer pairs P-MD1/P-MD2 and P-IBD5 (5′-CCATAAACGCCGTGACCTTC-3′)/P-IBD10 (5′-ATCCTGTTGCCACTCTTTCG-3′) (Brown *et al.,* 1994; Brown and Skinner, 1996), respectively. The DIG-labeled hybridized DNA was detected by enzyme-linked immunoassay using anti-DIG antibodies conjugated with alka-

line phosphatase and subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium salt.

Protection against vvIBDV. A total of 90 SPF chickens were used in this experiment. Each of twenty 1-day-old SPF chickens was vaccinated with the rMDV (10^4 PFU/chick) or the CVI-988 (5×10^3 PFU/chick) subcutaneously (sc). Twenty 20-day-old SPF chickens were inoculated orally with 1 dose of a conventional live IBDV vaccine, IBDV-A. All of the chickens were bled at 6 weeks of age, and the sera were tested for antibody titers against IBDV J1 strain (Tsukamoto *et al.,* 1995a) by the EIA. The chickens were then challenged orally with the vvIBDV Ehime/91 strain (10^5 50% of egg infective dose (EID_{50})/chick). Twenty unvaccinated challenged SPF chickens and 10 unvaccinated unchallenged SPF chickens were used as challenge and negative controls, respectively. The chickens were examined for clinical signs and mortality for 7 days, and both dead chickens and surviving chickens that were sacrificed were subjected to gross and histopathological examinations for BF lesions. BFs were fixed in 10% buffered formalin solution and subjected to the conventional paraffin-embedding procedure for hematoxylin and eosin staining. The histopathological BF lesions were classified into five categories (1–5) as described previously (Tanimura *et al.,* 1995). The extent of histologic lesions was scored on the basis of lymphoid necrosis and/or depletion: a score of 0 = <5% affected follicles; a score of 1 = 5–25% affected follicles; a score of 2 = 25–50% affected follicles; a score of 3 = 50–75% affected follicles; a score of 4 = >75% affected follicles; and a score of 5 = nearly 100% affected follicles with acute inflammatory infiltration. The scores of 1–4 are a consequence of the infection with attenuated live IBDV vaccines, whereas a score of 5 is the result of the virulent or very virulent IBDV infection (Tsukamoto *et al.,* 1995b). Thus, our criterion for protection is a score of 0–4 after the challenge.

Protection against vvMDV. A total of 68 SPF chickens were used in this experiment. Each of twenty 1-day-old SPF chickens was vaccinated with the rMDV or the CVI-988 as above and challenged 7 days later with the vvMDV RB1B strain (500 PFU/chick) intraperitoneally. Twenty unvaccinated challenged SPF chickens and 10 unvaccinated unchallenged SPF chickens were used as challenge and negative controls, respectively. The chickens were examined for clinical signs and mortality for 6 weeks after the challenge. Both dead chickens and surviving chickens that were sacrificed were subjected to gross and histopathological observations for MD lesions in the liver, kidneys, spleen, nerves, and skin.

Monitoring of antibody responses, safety, and contact transmission to chickens. A total of 20 SPF chickens were used for determination of the antibody responses to the rMDV as well as the safety of the rMDV for chickens. Nine 1-day-old SPF chickens were inoculated with rMDV. Seven 1-day-old SPF chickens were inoculated with CVI-988 and used as the control. The sera were collected at 2-week intervals until the chickens reached 10 weeks of age. The serum antibody titers against IBDV and MDV were measured by the EIA. Ten weeks after the vaccination, these chickens were necropsied for histopathological examination in major tissues (cerebrum, optic lobe, cerebellum, brain stem, lumbosacral enlargement, siatic nerve, skin, skeletal muscle, BF, thymus, spleen, cecal tonsil, proventriculus, duodenum, pancreas, heart, lungs, liver, kidney, and ovary/testis).

In addition, for determination of the contact transmission of the rMDV, two SPF chickens were reared for 10 weeks in the same isolator with the nine chickens vaccinated with the rMDV. As a control, two SPF chickens were also reared with the seven chickens vaccinated with the CVI-988. Sera were collected at 2-week intervals from their penmates and measured for the presence of antibodies against IBDV and MDV by the EIA tests. Then the penmates were also necropsied and the tissues (liver, kidney, spleen, nerve, and skin) were subjected to the histopathological examinations to determine the contact transmission of the rMDV.

REFERENCES

Azad, A. A., Mckem, N. M., Macreadie, I. G., Failla, P., Heine, H. G., Chapman, A., and Fahey, K. J. (1991). Physiochemical and immunological characterization of recombinant host-protective antigen (VP2) of infectious bursal disease virus. *Vaccine* **9,** 715–722.

Balan, P., Davis-Pointer, N., Bell, S., Atkinson, H., Browne, H., and Minson, T. (1994). An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *J. Gen. Virol.* **75,** 1245–1258.

Bayliss, C. D., Petres, R. W., Cook, J. K., Reece, R. L., Howes, K., Binns, M. M., and Boursnell, M. E. (1991). A recombinant fowlpox virus that expresses the VP2 antigen of infectious bursal disease virus induces protection against mortality caused by the virus. *Arch. Virol.* **120,** 193–205.

Becht, H., Müller, H., and Müller, H. K. (1988). Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. *J. Gen. Virol.* **69,** 631–640.

Bottcher, B., Kiselev, N. A., Stel'Mashchuk, V. Y., Perevozchikova, N. A., Borisov, A. V., and Crowther, R. A. (1997). Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. *J. Virol.* **71,** 325–330.

Brown, M. D., Green, P., and Skinner, M. A. (1994). VP2 sequences of recent European 'very virulent' isolates of infectious bursal disease virus are closely related to each other but are distinct from those of 'classical' strain. *J. Gen. Virol.* **75,** 675–680.

Brown, M. D., and Skinner, M. A. (1996). Coding sequences of both genome segments of an European 'very virulent' infectious bursal disease virus. *Virus Res.* **40,** 1–15.

Brunovskis, P., and Velicer, L. F. (1995). The Marek's disease virus (MDV) unique short region: Alphaherpesvirus-homologous, fowlpox virus-homologous, and MDV-specific genes. *Virology* **206,** 324–338.

Calnek, B. W., and Witter, R. L. (1997). Marek's disease. *In* "Diseases of Poultry" (B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif, Eds.), 10th ed., pp. 369–413. Iowa State Univ. Press, Ames, IA.

Cantello, J. L., Anderson, A. S., Francesconi, A., and Morgan, R. W.

(1991). Isolation of a Marek's disease virus (MDV) recombinant containing the *lacZ* gene of *Escherichia coli* stably inserted within the MDV US2 gene. *J. Virol.* **65,** 1584–1588.

Darteil, R., Bublot, M., Laplace, E., Bouquet, J.-F., Audonnet, J.-C., and Riviere, M. (1995). Herpesvirus of turkey recombinant viruses expressing infectious bursal disease virus (IBDV) VP2 immunogen induce protection against an IBDV virulent challenge in chickens. *Virology* **211,** 481–490.

Fahey, K. J., Erny, K., and Crooks, J. (1989). A conformational immunogen on VP-2 of infectious bursal disease virus that induces virus-neutralizing antibodies that passively protect chickens. *J. Gen. Virol.* **70,** 1473–1481.

Fahey, K. J., Chapman, A. J., MaCreadie, I. G., Vaughan, P. R., McKern, N. M., Ward, C. W., and Azad, A. A. (1991a). A recombinant subunit vaccine that protects progeny chickens from infectious bursal disease. *Avian Pathol.* **20,** 447–460.

Fahey, K. J., McWaters, P., Brown, M. A., Erny, K., Murphy, V. J., and Hewish, D. R. (1991b). Virus-neutralizing and passively protective monoclonal antibodies to infectious bursal disease virus of chickens. *Avian Dis.* **35,** 365–373.

Fernandez-Arias, A., Martinez, S., and Rodriguez, J. F. (1997). The major antigenic protein of infectious bursal disease virus, VP2, is an apoptotic inducer. *J. Virol.* **71,** 8014–8018.

Furuta, K., Ohashi, H., Obana, J., and Sato, S. (1980). Performance of three successive generations of specific-pathogen-free chickens maintained as a closed flock. *Lab. Anim.* **14,** 107–112.

Giambrone, J. J., and Closser, J. (1990). Efficacy of live vaccines against serologic subtypes of infectious bursal disease virus. *Avian Dis.* **34,** 7–11.

Heckert, R. A., Riva, J., Cook, S., McMillen, J., and Schwartz, R. D. (1996). Onset of protective immunity in chicks after vaccination with a recombinant herpesvirus of turkeys vaccine expressing Newcastle disease virus fusion and hemagglutinin-neuraminidase antigens. *Avian Dis.* **40,** 770–777.

Heine, H. G., and Boyle, D. B. (1993). Infectious bursal disease virus structural protein VP2 expressed by a fowlpox virus recombinant confers protection against disease in chickens. *Arch. Virol.* **131,** 277–292.

Heine, H. G., Hyatt, A. D., and Boyle, D. B. (1994). Modification of infectious bursal disease virus antigen VP2 for cell surface location fails to enhance immunogenicity. *Virus Res.* **32,** 313–328.

Ismail, N. M., and Saif, Y. M. (1991). Immunogenicity of infectious bursal disease viruses in chickens. *Avian Dis.* **35,** 460–469.

Jackwood, D. H., and Saif, Y. M. (1987). Antigenic diversity of infectious bursal disease viruses. *Avian Dis.* **31,** 766–770.

Käufer, I., and Weiss, E. (1980). Significance of bursa of Fabricius as target organ in infectious bursal disease of chickens. *Infect. Immun.* **27,** 364–367.

Kibenge, F. S. B., Dhillon, A. S., and Russell, R. G. (1988). Biochemistry and immunology of infectious bursal disease virus. *J. Gen. Virol.* **69,** 1757–1775.

Lukert, P. D., and Saif, Y. M. (1997). Infectious bursal disease. *In* "Diseases of Poultry" (B. W. Calnek, B. W. Barnes, C. W. Beard, L. R. McDougald, and Y. M. Saif, Eds.), 10th ed., pp. 721–738. Iowa State Univ. Press, Ames, IA.

Macreadie, J. G., Vaughan, P. R., Chapman, A. J., McKern, N. M., Jagadish, M. N., Heine, H. G., Ward, C. W., Fahey, K. J., and Azad, A. A. (1990). Passive protection against infectious bursal disease virus by viral VP2 expressed in yeast. *Vaccine* **8,** 549–552.

Mazariegos, L. A., Lukert, P. D., and Brown, J. (1990). Pathogenicity and immunosuppressive properties of infectious bursal disease "intermediate" strain. *Avian Dis.* **34,** 203–208.

Morgan, R. W., Cantello, J. L., and McDermott, C. H. (1990). Transfection of chicken embryo fibroblasts with Marek's disease virus DNA. *Avian Dis.* **34,** 345–351.

Morgan, R. W., Gelb, J., Jr., Schreurs, C. S., Lütticken, D., Rosenberger, J. K., and Sondermeijer, P. J. (1992). Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. *Avian Dis.* **36,** 858–870.

Morgan, R. W., Gelb, J., Jr., Pope, C. R., and Sondermeijer, P. J. (1993). Efficacy in chickens of a herpesvirus of turkeys recombinant vaccine containing the fusion gene of Newcastle disease virus: Onset of protection and effect of maternal antibodies. *Avian Dis.* **37,** 1032–1040.

Mundt, E., Kollner, B., and Kretzschmar, D. (1997). VP5 of infectious bursal disease virus is not essential for viral replication in cell culture. *J. Virol.* **71,** 5647–5651.

Muskett, J. C., Reed, N. E., and Thornton, D. H. (1985). Increased virulence of an infectious bursal disease live virus vaccine after passage in chicks. *Vaccine* **3,** 309–312.

Öppling, V., Müller, H., and Becht, H. (1991a). The structural polypeptide VP3 of infectious bursal disease virus carries group- and serotype-specific epitopes. *J. Gen. Virol.* **72,** 2275–2278.

Öppling, V., Müller, H., and Becht, H. (1991b). Heterogeneity of the antigenic site responsible for the induction of neutralizing antibodies in infectious bursal disease virus. *Arch. Virol.* **119,** 211–223.

Parcells, M. S., Anderson, A. S., Cantello, J. L., and Morgan, R. W. (1994). Characterization of Marek's disease virus insertion and deletion mutants that lack US1 (ICP22 homolog), US10, and/or US2 and neighboring short-component open reading frames. *J. Virol.* **68,** 8239–8253.

Reddy, S. K., Silim, A., and Ratcliffe, M. J. (1992). Biological roles of the major capsid proteins and relationships between two existing serotypes of infectious bursal disease virus. *Arch. Virol.* **127,** 209–222.

Reddy, S. K., Sharma, J. M., Ahmad, J., Reddy, D. N., McMillen, J. K., Cook, S. M., Wild, M. A., and Schwartz, R. D. (1996). Protective efficacy of a recombinant herpesvirus of turkeys as an *in ovo* vaccine against Newcastle and Marek's diseases in specific-pathogen-free chickens. *Vaccine* **14,** 469–477.

Sakaguchi, M., Hirayama, Y., Maeda, H., Matsuo, K., Yamamoto, M., and Hirai, K. (1994). Construction of recombinant Marek's disease virus type 1 (MDV1) expressing the *Escherichia coli lacZ* gene as a possible live vaccine vector: The US10 gene of MDV1 as a stable insertion site. *Vaccine* **12,** 953–957.

Sakaguchi, M., Urakawa, T., Hirayama, Y., Miki, N., Yamamoto, M., and Hirai, K. (1993). Marek's disease virus protein kinase gene identified within the short unique region of the viral genome is not essential for viral replication in cell culture and vaccine-induced immunity in chickens. *Virology* **195,** 140–148.

Sakaguchi, M., Nakamura, H., Sonoda, K., Okamura, K., Yokogawa, H., Matsuo, K., and Hirai, K. (1998). Protection of chickens with or without maternal antibodies against both Marek's and Newcastle diseases by one-time vaccination with recombinant vaccine of Marek's disease virus type 1. *Vaccine* **16,** 472–479.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schat, K. A., Calnek, B. W., and Fabricant, J. (1982). Characterization of two highly oncogenic strains of Marek's disease virus. *Avian Pathol.* **11,** 593–605.

Sharma, J. M., Dohms, J. E., and Metz, A. L. (1989). Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and their effect on humoral and cellular immune competence of specific-pathogen-free chickens. *Avian Dis.* **33,** 112–124.

Snyder, D. B., Vakharia, V. N., Mengel-Whereat, S. A., Edwards, G. H., Savage, P. K., Lütticken, D., and Goodwin, M. A. (1994). Active cross-protection induced by a recombinant baculovirus expressing chimeric infectious bursal disease virus structural proteins. *Avian Dis.* **38,** 701–707.

Sonoda, K., Sakaguchi, M., Matsuno, K., Zhu, G.-S., and Hirai, K. (1996). Asymmetric deletion of the junction between the short unique region and the inverted repeat does not affect viral growth in culture and

vaccine-induced immunity against Marek's disease. *Vaccine* **14,** 277–284.

Spies, U., and Müller, H. (1990). Demonstration of enzyme activities required for cap structure formation in infectious bursal disease virus, a member of the birnavirus group. *J. Gen. Virol.* **71,** 977–981.

Spies, U., Müller, H., and Becht, H. (1987). Properties of RNA polymerase activity associated with infectious bursal disease virus and characterization of its reaction products. *Virus Res.* **8,** 127–140.

Tanimura, N., Tsukamoto, K., Nakamura, K., Narita, M., and Maeda, M. (1995). Association between pathogenicity of infectious bursal disease virus and viral antigen distribution detected by immunohistochemistry. *Avian Dis.* **39,** 9–20.

Tsukamoto, K., Tanimura, N., Hihara, H., Shirai, J., Imai, K., Nakamura, K., and Maeda, M. (1992). Isolation of virulent infectious bursal disease virus from field outbreaks with high mortality in Japan. *J. Vet. Med. Sci.* **54,** 153–155.

Tsukamoto, K., Matsumura, T., Mase, M., and Imai, K. (1995a). A highly sensitive, broad-spectrum infectivity assay for infectious bursal disease virus. *Avian Dis.* **39,** 575–586.

Tsukamoto, K., Tanimura, N., Kakita, S., Ota, K., Mase, M., Imai, K., and Hihara, H. (1995b). Efficacy of three live vaccines against highly virulent infectious bursal disease virus and the optimum vaccination time. *Avian Dis.* **39,** 218–229.

Vakharia, V. N., Snyder, D. B., He, J., Edwards, G. H., Savage, P. K., and Mengel-Whereat, S. A. (1993). Infectious bursal disease virus structural proteins expressed in a baculovirus recombinant confer protection in chickens. *J. Gen. Virol.* **74,** 1201–1206.

Vakharia, V. N., Snyder, D. B., Lütticken, D., Mengel-Whereat, S. A., Savage, P. K., Edwards, G. H., and Goodwin, M. A. (1994). Active and passive protection against variant and classic infectious bursal disease virus strains induced by baculovirus-expressed structural proteins. *Vaccine* **12,** 452–456.

Witter, R. L., Lee, L. F., and Fadly, A. M. (1995). Characteristics of CVI988/Rispens and R2/23, two prototype vaccine strains of serotype 1 Marek's disease virus. *Avian Dis.* **39,** 269–284.