Dual-Viral Vector Approach Induced Strong and Long-Lasting Protective Immunity against Very Virulent Infectious Bursal Disease Virus

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To induce strong protective immunity against very virulent infectious bursal disease virus (vvIBDV) in chickens, two viral vector systems, Marek's disease and Fowlpox viruses expressing the vvIBDV host-protective antigen VP2 (rMDV, rFPV), were used. Most of chickens vaccinated with the rFPV or rMDV alone, or vaccinated simultaneously with both at their hatch (rMDV-rFPV14d), were protected against developing clinical signs and mortality; however, only zero to 14% of the chickens were protected against gross lesions. In contrast, gross lesions were protected in 67% of chickens vaccinated primarily with the rMDV followed by boosting with the rFPV 2 weeks later (rMDV-rFPV14d). Protection against the severe histopathological lesions of rFPV, rMDV, rMDV-rFPV14d, and rMDV-rFPV144 vaccine groups were 33, 42, 53, and 73%, respectively. Geometric mean antibody titers to VP2 of chickens vaccinated with the rFPV, rMDV, rMDV-rFPV14d, and rMDV-rFPV144 before the challenge were 110, 202, 254, and 611, respectively. Persistent infection of the rMDV in chickens after the booster vaccination with rFPV was suggested by detection of the rMDV genes from peripheral blood lymphocyte DNA at 28 weeks of age. These results indicate that the dual-viral vector approach is useful for quickly and safely inducing strong and long-lasting protective immunity against vvIBDV in chickens. © 2000 Academic Press

INTRODUCTION

Infectious bursal disease virus (IBDV) causes considerable economic loss in the poultry industry by inducing severe clinical signs, immunosuppression, and high mortality (≥50%) in chickens (Lukert and Saif, 1997). B-lymphocytes of the bursa of Fabricius (BF) are target cells for IBDV infection. 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 et al., 1987), and segment A encodes two proteins, precursor polyprotein and a small protein (VP6). The precursor polyprotein is processed to three mature viral proteins, VP2, VP3, and VP4. VP2 is a conformational host-protective antigen (Azad et al., 1991; Bechet et al., 1988; Fahey et al., 1989; Kibenge et al., 1988) and also an apoptotic inducer (Fernandez-Arias et al., 1997). VP3 is a group-specific antigen; however, the protective role has not yet been discovered (Opplinger et al., 1991). VP4 may be a viral protease. VP5 is not essential for viral replication (Mundt et al., 1997), and the VP5-inactivated mutant virus induced a high level of protective immunity in chickens despite an absence of BF lesions (Yao et al., 1998).

IBD has been controlled by live IBDV vaccines (Ismail and Saif, 1991); however, it is difficult to protect field chickens with the maternal antibodies by the live vaccines (Ismail and Saif, 1991; Lukert and Saif, 1997; Tsukamoto et al., 1995b). In addition, live vaccines induce moderate bursal atrophy (Muskett et al., 1985), and the antigenic or pathogenic characters are not stable. Passages of live IBDV vaccines to chickens have been shown to increase the virulence (Muskett et al., 1985). Thus, as a new generation of vaccine, safer and more efficacious IBD vaccines must be studied.

There are several viral vector systems including retrovirus, poxvirus, herpesvirus, adenovirus, and adeno-associate virus, which are useful for gene therapy and recombinant vaccines, as well as in vitro expression systems. Research on the viral vector-based polyvalent vaccines is especially required for livestock to control several important infectious diseases. Three live vaccine-based viral vectors of chickens lead this research field: Marek's disease virus (MDV) (Cantello et al., 1991), herpesvirus of turkey (HVT) (Morgan et al., 1992), and fowlpox virus (FPV) (Bayliss et al., 1991). Both MDV and HVT vectors are developed for the induction of long-term protective immunity in chickens because both vectors are herpesviruses, whereas the FPV vector is used to quickly induce protective immunity in chickens.
viral vector-based recombinant vaccines are safe for chickens and have no risk of producing antigenic/pathogenic variants because of subunit-type vaccines. Their low vaccine efficacy, however, is a hindrance for practical use in the field when compared to commercial live vaccines (Ismail and Saif, 1991; Tsukamoto et al., 1995b).

For example, viral vector-based recombinant vaccines hardly protected against the formation of gross BF lesions when challenged with very virulent IBDV (vvIBDV), although they provided protection against the development of clinical signs and mortality (Bayliss et al., 1991; Darteil et al., 1995; Tsukamoto et al., 1999). Further studies are required not only to develop highly efficacious recombinant vaccines but also to know how to use them effectively.

In this study, we compared the efficacy of two viral vectors, rMDV and rFPV, against vvIBDV, and tested a new vaccine regimen using them to induce a higher level protective immunity. This study demonstrated that the MDV vector system induced a higher level protective immunity than the FPV vector system, and the rMDV-induced protective immunity was significantly enhanced by subsequent vaccination with the rFPV. The anti-VP2 antibody kinetic curve of the rMDV-rFPV group was higher than that of the rMDV alone and almost comparable with that of live IBDV vaccine for 28 weeks. The dual-viral vector approach is a new experimental method for inducing strong and long-lasting protective immunity against a wide range of infectious diseases of animals. The approach may be applicable to other viral vector combinations.

RESULTS

Analysis of constructed rFPV. Insertion of the VP2 expression cassette into the genome of FPV was determined by PCR amplification of the VP2 gene. As shown in Fig. 1A, the expected size of the DNA fragment (1.3 kbp) was detected from DNA prepared from the culture fluid of the rFPV-infected CEF culture, whereas no band was detected in the wt FPV-infected cell culture fluid. The VP2 DNA size was identical to that of the rMDV (Fig. 1A).

Western blotting analysis (Fig. 1B) confirmed synthesis of the VP2 protein in CEF cells infected with rFPV. The size of VP2 expressed by the rFPV was about 42 kDa, which was identical to that of VP2 expressed by the rMDV. These results indicated the expected construction of the rFPV.

Protection against clinical signs and mortality. All of the chickens vaccinated with the rFPV, rMDV, rMDV-rFPV1d, or rMDV-rFPV14d were challenged with vvIBDV at 30 days of age. As a result, each recombinant vaccine or each combination group conferred protection against clinical signs and mortality to all of the vaccinated chickens except for a chicken vaccinated with the rMDV alone, which exhibited clinical signs and died of IBD. Live vaccine IBDV-A conferred full protection against the challenge. All of the chickens in the challenge control groups succumbed to the infection; the morbidity and mortality were 100 and 60%, respectively.

Protection against gross lesions. As shown in Fig. 2A, the severe gross BF lesions were protected at a low frequency in chickens vaccinated with the rFPV (0%, 0/15), rMDV (14%, 2/14), or rMDV-rFPV15 (13%, 2/15) after the challenge. In contrast, 67% (10/15) of the chickens vaccinated with the rMDV-rFPV14d were protected against gross BF lesions. Ten percent or 100% of chickens vaccinated with the commercial vaccine IBDV-A or IBDV-C had mild BF gross lesions, respectively; the mild gross lesions might be side effects of the vaccine strain itself.
(Tsukamoto et al., 1995b). All chickens in the challenge control group had severe gross lesions (Fig. 2A).

**Protection against histopathological lesions.** As shown in Fig. 2B, the average histopathological BF lesion score of chickens vaccinated with the rFPV, rMDV, or rMDV-rFPV\(^{1d}\) was approximately 4 (4.3, 3.6, and 3.7, respectively). In contrast, the score of chickens vaccinated with the rMDV-rFPV\(^{14d}\) was 1.7, which was close to that of the commercial vaccine IBDV-A (1.2). Commercial vaccine IBDV-C and the challenge control groups had BF lesion scores of 4.0 and 5.0, respectively.

Our protection criteria mean that chickens with BF lesion scores of 0–4 were considered protected histopathologically (Tsukamoto et al., 1999). According to this criteria, protective efficacy against the histopathological BF lesions of the rFPV, rMDV, rMDV-rFPV\(^{1d}\), and rMDV-rFPV\(^{14d}\) vaccine groups was 33, 42, 53, and 73%, respectively, whereas that of the commercial vaccine IBDV-A was 100% (Fig. 2A).

The chickens vaccinated with the rMDV-rFPV\(^{14d}\) were divided into two groups based on the BF lesion scores: about 1/3 of the chickens had no or mild BF lesions (scores = 0–2), whereas 2/3 of the chickens still had severe BF lesions (score = 5) (Fig. 2B).

**Antibody responses against VP2 before the challenge.** The geometric mean (GM) EIA antibody titers against VP2 in chickens vaccinated with the rFPV, rMDV, or rMDV-rFPV\(^{14d}\) were 110, 202, or 254, respectively (Fig. 3A).
In contrast, chickens vaccinated with the rMDV-rFPV\textsuperscript{14d} had the higher GM antibody titer (EIA antibody titer = 611), which was close to the protection level (EIA antibody titer = 640) as described below. However, two chickens in the rMDV-rFPV\textsuperscript{14d} group still had low antibody titers (40 or 160). The commercial IBDV-A vaccine produced a higher level of anti-IBDV antibodies ($\geq 1280$). The AGP test results were similar to the EIA test results. The AGP antibody-positive chickens were detected at a higher frequency (80%, 12/15) in the rMDV-rFPV\textsuperscript{14d} group than the other three vaccine groups: 0% (0/15), 21% (3/14), and 27% (4/15) in the rFPV, rMDV, and rMDV-rFPV\textsuperscript{1d} groups, respectively. The AGP antibodies were detected in all of the chickens vaccinated with the IBDV-A vaccine (10/10).

Since both rMDV and rFPV expressed VP2, the four recombinant vaccine groups were considered in the same criteria to determine a relationship between serum antibody levels to VP2 and protection against histopathological BF lesions. As shown in Fig. 3B, there is a clear correlation between the two. The EIA antibody titers of 320 or below did not protect chickens efficiently against gross (5%, 2/42) and histopathological BF lesions (33%, 14/42). In contrast, the antibody titers of 640 or higher conferred protection at a high frequency against gross (70%, 12/17) and histopathological BF lesions (94%, 16/17). The presence of the AGP antibody to VP2 was also associated with approximately 70 and 90% protection against gross and histopathological BF lesions, respectively (Fig. 3C).

**Persistence of the humoral immunity in chickens.** The humoral immunity level was determined to know how long the protective level of the AGP antibody to VP2 was maintained in chickens after vaccination with the IBDV-A, rMDV alone, or rMDV-rFPV\textsuperscript{14d}. The AGP antibody was detected at a higher frequency at 4 or 8 weeks of age.

FIG. 3. Humoral immune responses to VP2 of each vaccine group. The chickens were vaccinated as described under Materials and Methods, and the sera were collected at 30 days of age before the challenge. See the legend to Fig. 2 for details. (A) Comparison of antibody titers to VP2 among vaccine groups. Geometric mean EIA antibody titers to VP2 of each vaccine group and percentage of chickens in each group that were positive in the AGP test are shown. (B) Correlation between the EIA antibody titers to VP2 and the protection against gross and histopathological BF lesions. All of the sera collected from chickens in each vaccine group dealt with one group since both recombinant vaccines expressed the same VP2 antigen. (C) Percentage of chickens that were protected against gross and histopathological BF lesions in the AGP-antibody positive or negative chicken groups.
than other periods in all three vaccine groups. The AGP antibody to VP2 was detected from 4 to 20 weeks of age in chickens vaccinated with the rMDV-rFPV 14d with a range of 20–80%, whereas it was detected only from 4 to 8 weeks of age in the rMDV group (20%). The AGP antibody kinetic curve of the rMDV-rFPV 14d group was similar to that of the current live vaccine group IBDV-A, which induced the AGP antibody from 4 to 16 weeks of age with a range of 20–100% (Fig. 4A). In contrast, there was no significant difference in EIA anti-MDV antibody titers between the two recombinant vaccine groups (Fig. 4B). The EIA anti-MDV antibody reached a peak level at 8–12 weeks of age, and the antibody level was maintained throughout the experiment in both vaccine groups. No anti-MDV antibody was detected in chickens vaccinated with IBDV-A throughout the experiment.

To determine whether the rMDV persisted in vaccinated chickens after the booster vaccination, both first and nested PCR analyses to detect IBDV VP2 and MDV US2 genes were performed on peripheral blood lymphocyte DNA at 28 weeks of age. DNA samples collected from chickens vaccinated with the rMDV alone were used as the control. As shown in Table 1 and Fig. 5, the IBDV VP2 gene was detected in 0% (0/5) and 80% (4/5) of chickens in the rMDV-rFPV 14d group by first and nested PCR analyses. Chicken No. 10 in the rMDV-rFPV 14d group was positive in the subsequent nested PCR analysis. The VP2 gene was also detected in 80% (4/5) and 100% (5/5) of chickens in the rMDV group by the first and nested PCR analyses, respectively. PCR analysis data for the MDV US2 gene were almost the same as those of the IBDV VP2. These results indicated that the rMDV infected persistently in chickens for at least 26 weeks after the booster vaccination.

**DISCUSSION**

Recombinant herpesvirus or poxvirus vaccines offer great hope for improved immunization against a wide range of pathogens because both vectors are safe, stable, and can express multiple antigens. However, by themselves they can elicit only a low level of protective immunity so far. All of the recombinant viral vector vaccines expressing IBDV VP2 (rFPV, rHVT, and rMDV) failed to protect the development of gross lesions when challenged with vvIBDV even though they were able to protect chickens against developing clinical signs and mortality (Bayliss et al., 1991; Darteil et al., 1995; Heine and Boyle, 1993; Tsukamoto et al., 1999). So, we have focused on the development of a new vaccine regimen based on both herpesvirus and poxvirus vector systems, rMDV and

![FIG. 4. Persistence of humoral immune responses in chickens for 28 weeks after vaccination with IBDV-A, rMDV, or rMDV-rFPV 14d. (A) Persistence of the AGP antibody to VP2 in these chickens. Percentage of chickens that were positive in the AGP test is shown. (B) Persistence of the EIA antibody to MDV in these chickens. The geometric mean antibody titer to MDV is shown. Each serum antibody titer is expressed as the reciprocal of serum dilution which showed positive reaction.](image-url)
rFPV. To our knowledge, this is the first report describing a dual-viral vector approach in order to generate a high level of protective immunity in animals. Our study showed that a single administration of both viral vectors was sufficient to induce a high level of the protective immunity against vvIBDV. Eleven of 15 chickens in the rMDV-rFPV group were histopathologically protected, and 10 of them were almost protected against the vvIBDV infection because their BF lesion scores were zero or 1. This high efficacy was not achieved by the simultaneous vaccination with the rMDV and the rFPV at their hatch, but achieved by inoculation with the rFPV 2 weeks later after the primary vaccination with the rMDV. This result suggested that this high efficacy might be due to a booster phenomenon. This is different from herpes-primed/herpes-boosted or pox-primed/pox-boosted vaccine regimens. Two times vaccination with the rFPV expressing VP2 did not protect from BF gross lesions (Bayliss et al., 1991). Also, it is unlikely that the primary immune responses were enhanced by readministration with the rMDV because the rMDV vaccination inhibited replication of even vvMDV in chickens (Tsukamoto et al., 1999). Only consecutive immunization strategy involving priming with one viral vector and boosting with the other viral vector is a key to triggering a strong booster phenomenon in animals. Recently, consecutive immunization strategies have been reported in human immunodeficiency virus (HIV). Immunization involving priming with DNA (two administrations) followed by boosting with rFPV expressing HIV antigens induced greater HIV-specific humoral and cellular immunity in macaques than either vaccine alone (Kent et al., 1998). DNA-priming and recombinant protein-boosting vaccine regimens also elicited neutralizing antibodies to HIV in rabbits (Richmond et al., 1998). Our herpesvirus-primed and poxvirus-boosted vaccine regimen is theoretically advantageous to these strategies because of quick and long-lasting induction of a high level of protective immunity in animals.

FIG. 5. Detection of rMDV genes by PCR from peripheral blood lymphocyte DNA collected at 28 weeks of age from chickens vaccinated with the rMDV alone or rMDV-rFPV14d. (A) Position of IBDV VP2 and MDV US2 genes amplified by first and nested PCR analyses. (B) Agarose gel electrophoresis analysis of PCR products. Plasmid p2EG containing MDV US2 and IBDV VP2 genes was used as the control template. The expected PCR products are indicated by arrows.
The presence of low responders is a weak aspect of subunit-type vaccines. The low responders against VP2 were confirmed in the rMDV group as previously described (Tsukamoto et al., 1999). One chicken in the rMDV group succumbed to the infection after showing clinical IBD. It was not determined whether such low responders resulted from low growth efficiency of the rMDV in chickens, weak immunological responses to the subunit antigen VP2, or high susceptibility to IBDV (Bumstead et al., 1993). At least, it is significant that some low responders were promoted to the well responders by the booster vaccination with the rFPV; protection efficacy against gross and histopathological lesions and antibody titers against VP2 increased to 4.8, 1.7, and 3.0 times as high as those by the rMDV alone, respectively (Table 2). This study also shows that the efficacy of the rFPV was lower than that of the rMDV (Table 2). In addition, the rFPV may be a more powerful booster vaccine than live IBDV vaccines because live IBDV vaccines are highly sensitive to neutralizing antibody to IBDV (Tsukamoto et al., 1995b). Therefore, it is preferable to use the rFPV as a booster vaccine. Scabbing was observed in chickens vaccinated with rFPV at the injection site 6 days after the vaccination, which was evidence of a successful vaccination against fowlpox (FP). It was previously shown that the rMDV vaccination completely suppressed tumor development caused by vvMDV in chickens (Tsukamoto et al., 1999). Therefore, it is likely that our vaccine regimen should be effective for MD, FP, and IBD, and other infectious diseases could be added to the dual-viral vector approach. Both MDV and FPV vector vaccines have been used for layers and parent chickens for more than 20 years worldwide. Both rMDV and rFPV may be more resistant to the maternal antibodies to IBDV than the current IBDV live vaccines. Thus theoretically, there is no major obstacle to applying this approach to layers and parent chickens. Although further studies are required to improve the efficacy of both vaccines to achieve full protection, the goal may not be far.

Lifetime persistence of protective immunity in animals is a key theme for the herpesvirus vectors; however, the study is limited. An MDV-based recombinant using an SV40 late promoter maintained a protective level of antibodies to NDV F protein for more than 24 weeks (Sakaguchi et al., 1998). The protective level of AGP antibodies to VP2 was detected in 80% of chickens vaccinated with the rMDV-rFPV$^{14d}$, but the antibody gradually declined and had not been detected since 24 weeks of age. One possible explanation of this antibody decline is that the rFPV-booster vaccination quickly enhanced the primary immune responses; however, the immunity level then gradually declined to a level equal to that induced by the rMDV alone. This antibody decline may not be disappointing because the decline pattern was also observed in chickens vaccinated with IBDV-A; the chickens were protective throughout their lives (Fig. 4A). In addition, adult chickens (more than 20 weeks of age) are resistant to IBDV because of atrophy of the BF, a target organ for IBDV. It is encouraging that both the IBDV VP2 and the MDV US2 genes of the rMDV were detected from peripheral blood lymphocyte DNA of chickens vaccinated with rMDV-rFPV$^{14d}$ at 28 weeks of age by nested PCR analyses (Table 1, Fig. 5). We did not attempt to reisolate the rMDV from the chickens. It is likely that despite the booster vaccination with the rFPV at 2 weeks of age, the rMDV might persist in the vaccinated chickens for at least 180 days. The rMDV DNA level, however, was lower in the rMDV-rFPV$^{14d}$ group than in the rMDV group, and the AGP antibody to VP2 had not been detected from 24 weeks of age. The rMDV must be improved to persist more efficiently in chickens after the booster vaccination. Glycoprotein promoters derived from herpesviruses are believed to help persistent infection of the recombinant herpesvirus vaccines in animals. Pseudorabies virus recombinant based on a gG promoter or MDV recombinant based on a gB promoter to express a foreign antigen induced highly efficient protection in animals (Sonoda et al., 1996; Van Zijl et al., 1991).

Serum-neutralizing antibodies correlate with the protection in IBDV infection (Lukert and Saif, 1997). This study showed a high degree of correlation between the serum antibody level to VP2 and the protective efficacy against vvIBDV when compared with our previous study (Figs. 3B and 3C) (Tsukamoto et al., 1999). The high level of the EIA (≥1:640) or AGP antibodies to VP2 correlated with approximately 70% protection against the gross lesions and 90% protection against the histopathological lesions. The high level of the antibodies may play a role

### TABLE 2

Comparison of Vaccine Efficacy against vvIBDV of Four Vaccine Groups with That of rMDV

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in prevention against vvIBDV infection. The reason for the higher level of the antibodies in this study than in our previous study is not known; however, this difference might be due to a different SPF chicken line used between the experiments. In addition, as previously described (Bayliss et al., 1991; Tsukamoto et al., 1999), a vital role of the cellular immunity to VP2 in vvIBDV protection was suggested. Protection against mortality and gross lesions was achieved in some chickens despite the low level of the antibodies to VP2 (Fig. 3B). It remains to be determined to what degree the cellular immunity was enhanced by the booster vaccination with the rFPV, or how long the enhanced cellular immunity is maintained in chickens.

This study clearly indicates that strong and long-lasting protective immunity could be easily generated by the dual-viral vector approach through a booster phenomenon. Since the immunity was much quicker and stronger than that induced by other immunization approaches, the dual-viral vector approach has the following merits. First, it is useful not only in controlling multiple infectious diseases but also for analyzing the protective immunity against antigens. Expression of immunomodulating cytokines of Th1 or Th2 types along with protective antigens in both viral vectors may provide insights into the roles of cytokines in protective immunity. Second, since replication of the booster vaccine was scarcely interfered by the primary vaccination, the strategy may be applicable to other vector combinations. For example, herpesvirus-primed and adenovirus-boosted immunization regimens may generate strong mucosal immunity against gastroenteric pathogens. These studies may allow us to design targeting induction of local protective immunity in animals. Third, this approach is advantageous for inducing strong protective immunity in large animals such as chimpanzees, pigs, and cattle. It is especially interesting to apply this approach to a study of HIV vaccination.

In summary, this study shows that the herpesvirus-primed and poxvirus-boosted immunization strategy is an efficient means of safely inducing strong and long-lasting protective immunity against a wide range of infectious diseases in animals. The dual-vector approach is also useful for analyzing host-protective immunity, and other viral vector combinations may be applicable. Our rMDV-primed and rFPV-boosted vaccine regimen is a new experimental approach for studying the induction of strong and long-lasting protective immunity against fetal infectious diseases in chickens.

MATERIALS AND METHODS

Cells and viruses. Chick embryo fibroblast (CEF) cultures were prepared from 10-day-old embryos of white Leghorn line M (Nippon Institute of Biological Science, Tokyo, Japan) and grown in Dulbecco’s MEM (Nissui, Tokyo, Japan), 5% fetal calf serum, 10% tryptose phosphate broth, and antibiotics.

A large-plaque variant of FPV (Nazerian et al., 1989), originally derived from a vaccine strain (CEVA Laboratory, Overland Park, KS), was used for the generation of rFPV. Recombinant MDV expressing IBDV host-protective antigen VP2 (from 1 to 453 aa of Seg. A ORF) (rMDV) (Tsukamoto et al., 1999) and the parental MDV CVI-988 strains were used. These FPV, rFPV, MDV, and rMDV were propagated in CEFs. The vvIBDV Ehime/91 strain (Tsukamoto et al., 1992) was used as a challenge virus. Two commercial live IBDV vaccines, IBDV-A and IBDV-C, were used in a challenge experiment.

Transfer plasmid vector for rFPV. The VP2 gene (from 1 to 453 aa of Seg. A ORF) of the vvIBDV Ehime/91 strain (GenBank Accession No. AB024076) was amplified by PCR using primers with unique restriction sites (5'-GGATCCCCGGACCGATGACGAACCTGCAAGATC-3' and 5'-GTCGACTCACCTCCTTAGGGCCCGAATTATG-3') and subcloned into a pNZ1729R digested with BamHI and SalI under the control of an FPV synthetic promoter (Yanagida et al., 1992). The resulting plasmid, pNZ29R/IBDVP2, which had a nonessential FPV gene for replication and a lacZ reporter gene, was used as a transfer vector.

Construction of rFPV. The rFPV was constructed as previously described (Yanagida et al., 1992). CEF monolayers (10<sup>5</sup> cells) were infected with FPV at a multiplicity of infections of 0.1 and incubated for 4 h. The cells were trypsinized, washed twice in saline G (0.14 M NaCl, 5 mM KCl, 1.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM MgCl<sub>2</sub>, 0.01% glucose), and resuspended in 0.7 ml of saline G. This cell suspension was mixed with 10 µg of the transfer vector and subjected to electroporation at 1.2 kV, 25 µF, 0.4 ms, using a Cell-Porator apparatus (Bio-Rad Gene Pulser). Transfected cells were plated onto a 6-cm-diameter culture dish and incubated at 37°C. After 3 days, these cells were harvested by scraping and disrupted by freezing and thawing to release progeny viruses. Recombinant FPV was cloned three times by a limiting dilution method by monitoring the lacZ activity with Bluo-gal (1 mg/ml, Gibco/BRL). The resulting virus was named rFPV.

PCR amplification of VP2 gene from rFPV. To determine whether the rFPV contains the VP2 gene, the entire VP2 gene was amplified by a PCR with two primers, P-IBD1 (5'-ATGACAAAACTGCAAGATCAAACCC-3') and P-IBD2 (5'-TTAATCCCTTATAGGCCCCATG-3'). Using the QIAamp blood kit (Qiagen, Germany), rFPV DNA was prepared from the culture fluid of the rFPV-infected CEFs and used as the template for PCR analyses.

Western blotting (WB) analysis. WB analysis was carried out as previously described (Tsukamoto et al., 1999). CEF cells were infected with the wt FPV or rFPV, harvested 24 h later, and stored at −80°C before use. The cells were subjected to a sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE) followed by a transfer of the proteins to a nitrocellulose
membrane. The membrane was incubated with rabbit anti-IBDV antiserum followed by horseradish peroxidase (HRPO)-conjugated goat anti-rabbit IgG. The VP2 band was detected by enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham, Buckinghamshire, England).

Protection against vvIBDV. One-day-old white leghorn SPF chickens were assigned to seven groups of 10 or 15 chickens each and reared in negative-pressure isolator set in a negative-pressure chicken house. Each chicken in Group 1 was vaccinated subcutaneously with the rFPV (10⁸ PFU/chick) at 14 days of age. Group 2 was vaccinated subcutaneously with the rMDV (10⁸ PFU/chick) at 1 day of age. Group 3 was vaccinated with both rMDV and rFPV at 1 day of age (rMDV-rFPV1⁶). Group 4 was vaccinated with the rMDV at 1 day of age followed by the rFPV at 14 days of age (rMDV-rFPV1⁶). Group 5 was vaccinated orally with one dose of a live IBDV vaccine IBDV-A at 20 days of age. The remaining two groups of 10 chickens each were used as challenge control and unvaccinated control. All of the chickens were bled at 30 days of age, and the sera were stored at −20°C. The chickens were then challenged orally with the vvIBDV Ehime/91 strain (10⁵ 50% of egg infective dose (EID₅₀)/chick). Clinical signs and mortality were observed daily for 7 days, and both dead and surviving chickens that were sacrificed were subjected to gross and histopathological examinations of the BF. The histopathological BF lesion scores were classified into five categories [1–5], from mild to severe lesions as previously described (Tsukamoto et al., 1995b). BF lesion scores 0 or 1 mean that the vvIBDV replication is almost completely suppressed; scores 2 to 4 mean that replication of the challenge virus is suppressed to the level equal to that of the vaccine strain replication; score 5 means that the challenge virus replicates considerably. Economic loss in farms is caused only in chickens with lesion score 5. Chickens with BF lesion scores of 0–4, which were also observed in chickens infected with live vaccine strains (Tsukamoto et al., 1995b), were considered protected histopathologically (Tsukamoto et al., 1999).

In a separate experiment, to see the side effects of live vaccine IBDV-C, ten 30-day-old SPF chickens were vaccinated orally with one dose of IBDV-C. At 37 days old, the BF gross lesion and histopathological lesion score were determined as described above.

Detection of antibodies to IBDV VP2 and MDV. Antibodies against IBDV VP2 were detected by an enzyme immunoassay (EIA) as previously described (Tsukamoto et al., 1999). For the EIA, CEF-adapted J₁CEF72 strain of IBDV (3 × 10⁸ PFU) was inoculated onto primary CEF cells (8 × 10⁶ cells) in a 96-well plate (Falcon, Lincoln Park, NJ), and the cells were cultured for 20 h. After rinsing the cells with phosphate-buffered saline (PBS), the cells were fixed with a fixation solution (80% acetone in water) for 5 min, and the antigen-coated plates were stored at −80°C before use. Serum samples were diluted with a diluent (5% skim milk powder/PBS) and added to wells coated with acetone-fixed IBDV-infected CEF cells. After incubation for 1 h at 37°C and subsequent washing of the cells, the plates were incubated with HRPO-conjugated goat anti-chicken IgG (Fc) (1:1000 dilution with the diluent; Bethyl Lab., Inc., Montgomery, TX) for 30 min at 37°C. After washing, the substrate (3,3’-diaminobenzidine tetrahydrochloride) was added, and the plate was incubated for 10 min. The stained cells were observed under a light microscope. Agar gel precipitation (AGP) antibodies to VP2 were also detected using BF homogenate antigen prepared from chickens infected with the IBDV E/91 strain as previously described (Tsukamoto et al., 1999).

Antibody titers to MDV were tested by EIA using CEF cells infected with the CVI-988 strain as the antigen as previously described (Tsukamoto et al., 1999).

Persistence of humoral immunity in chickens. To determine how long the vaccine immunity to IBDV VP2 continued in the vaccinated chickens, sera were tested for the presence of the antibodies. One-day-old SPF chickens were assigned to three groups of 5 chickens each and were leg-banded.

Two groups were vaccinated at 1 day old with the rMDV (10⁸ PFU/chick) (rMDV group), and one of the groups was further vaccinated with the rFPV-VP2 (10⁸ PFU/chick) at 2 weeks of age (rMDV-rFPV1⁶ group). The third group of chickens was vaccinated orally with one dose of live vaccine IBDV-A at one week of age (IBDV-A group). Each group of chickens was reared in a negative-pressure isolator for 28 weeks, and the sera were collected every 4 weeks and stored at −20°C. The sera were used for detecting anti-VP2 antibodies by the AGP tests because the AGP antibody was highly correlated with the protection (Fig. 3C). These sera were also tested for anti-MDV antibodies by the EIA test.

Persistence of rMDV in chickens. Both first and nested PCR assays were used to determine the persistence of the rMDV in chickens vaccinated with the rMDV followed by boosting with the rFPV. Chickens vaccinated with the rMDV alone were used as the control. The PCR analyses were performed on IBDV VP2 and MDV US2 genes of the rMDV using chicken lymphocyte DNA as the templates. At 28 weeks of age, peripheral blood lymphocytes were taken, and the cellular DNA was purified with a QiAamp blood kit (Qiagen, Germany) and used as a template for the PCR. The entire VP2 region (1.35 kbp) was amplified with two primers, P-IBD1 (5'-ATGAGAAACCTGCAAGAT-CAAACCCA-3') and P-IBD2 (5'-TTACCTCTTATAGC-CCGGATTATGT-3'), and the internal region (0.45 kbp) was further amplified by the nested PCR with two primers, P-IBD15 (5'-CCCAAGTGCACCACTATA-3') and P-IBD25 (5'-TCCTGTCACCTTCTT-3'). The VP2, the MDV US2 region (0.7 kbp) was amplified with two primers, P-MD1 (5'-GGTTGTAGCGATGGACCTAATT-
3') and P-MD16 (5'-TCTTCGGAACATCGGACC-3'), and the internal region (0.4 kbp) was further amplified by the nested PCR with two primers, P-MD15 (5'-TGATTCGTTAATGTTGC-3') and P-MD6 (5'-ATACGCTCA-CATAGCTTGGG-3'). Both first and nested PCR conditions are as follows: 1 cycle of 94°C for 1 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and 1 cycle of 72°C for 7 min.

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