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Infectious bursal disease virus structural protein VP 2 expressed by a fowlpox virus recombinant confers protection against disease in chickens

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Summary. Two fowlpox virus recombinants were constructed which expressed the host-protective antigen, VP 2, of infectious bursal disease virus (IBDV). Recombinant FPV-VP 2.4.3 contained the gene for the VP 2-VP 4-VP 3 polyprotein under the control of the vaccinia virus late promoter P.L 11 inserted within the thymidine kinase (*TK*) gene of FPV. In infected chicken embryo skin (CES) cells VP 2 and VP 3 proteins were correctly processed from the polyprotein precursor molecule. Recombinant FPV-VP 2 contained only the VP 2 encoding region under the control of the fowlpox early/late promoter P.E/L inserted immediately downstream of the *TK* gene. The expression level of VP 2 from FPV-VP 2 was approximately 5 times higher than from FPV-VP 2.4.3. Wing web inoculation of birds resulted in the development of typical fowlpox lesions and the development of antibodies to FPV with either of the recombinants, but only birds vaccinated with FPV-VP 2 developed antibodies to IBDV. When challenged with IBDV (strain 002-73), a significant level of protection was provided by FPV-VP 2 vaccination, although the level was lower than the protection provided by an oil adjuvanted inactivated whole IBDV vaccine. Birds vaccinated with FPV-VP 2.4.3 were not protected from infection as assessed by ELISA for the presence of IBD virus in bursae.

Introduction

Infectious bursal disease virus (IBDV) is a member of the family *Birnaviridae* [11] for which the genome consists of two segments of dsRNA [15]. IBDV is the aetiological agent of infectious bursal disease (IBD) (Gumboro disease) which causes significant losses to the poultry industries either by causing high mortality in an acute disease or as a consequence of immunodepression in young birds provoked by the destruction of the developing B lymphocytes in the bursa of Fabricius [13, 24]. Adequate control of IBD is only possible by vaccination

as IBD is a highly contagious disease and IBDV is a very stable virus which can persist in poultry houses even after thorough cleaning and disinfection [27]. The structural protein VP 2 (approx. 494 amino acid residues long) has been identified as the major host-protective antigen of IBDV [2, 6, 18]. VP 2 is encoded on the large genome segment A (3.2 kb) and is produced by self-processing of a 115 kDa VP 2-VP 4-VP 3 precursor polyprotein [1, 2, 21, 23]. The amino acid sequence of VP 2 is highly conserved between different Standard type 1 strains and antigenic variations have been identified only in the central region (residues 206 to 350) of VP 2 [4, 20, 25] which defines the conformational discontinuous host protective epitope [2]. The emergence of new, highly virulent variant strains of IBDV in several countries [22, 33, 34] has led to IBDV becoming a major problem in the intensive poultry industries worldwide and renewing interest in the development of new vaccine strategies. A recombinant IBDV vaccine based on antigen expression in yeast has been reported [28] to provide protective levels of IBDV maternal antibodies to day old chickens following vaccination of the parent birds. In this study we have evaluated the use of recombinant fowlpox virus (rFPV) as vector for an IBDV vaccine.

FPV has a dsDNA genome of about 300 kb [14] and a host range limited to avian species. A number of nonessential regions of the FPV genome have been identified and used for the insertion of foreign DNA [7]. Attenuated FPV strains are already accepted vaccines in poultry. The efficacy of rFPVs as vaccines has been demonstrated for recombinants expressing the fusion and hemagglutinin-neuraminidase genes of Newcastle disease virus [31, 35] and the glycoprotein B gene of Marek's disease virus [30]. Vaccination with a rFPV containing the IBDV VP 2 antigen gene fused to the β-galactosidase gene has been shown to lead to a reduction in mortality from IBDV infection, but did not protect against damage to the bursa of Fabricius [5]. In this report we describe the construction of two rFPVs containing IBDV antigen genes, their expression in cell culture and demonstrate in vaccination and challenge experiments in SPF birds, that vaccination with rFPV can protect chickens from IBDV infection and bursal damage.

Materials and methods

Cells and viruses

FPV-M 3 is a tissue culture passage strain derived from the mild vaccine strain FPV-Web (Arthur Webster Pty Ltd; Australia) and was grown in chicken embryo skin (CES) cell monolayers as described previously [8].

Antibodies

Murine monoclonal antibodies (MAb) specific for the VP 2 protein or the VP 3 protein of IBDV have been described [19]. Horseradish peroxidase-conjugated and fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin were obtained from Silenus Laboratories (Hawthorn, Australia). Polyclonal antibodies against FPV-M 3 were raised in SPF chickens by wing web vaccination.

DNA manipulation and sequencing

The general techniques required for manipulation of recombinant DNA were carried out by using standard procedures [32]. Polymerase chain reaction (PCR) was carried out using AmpliTaq kit (Perkin-Elmer Cetus). dsDNA templates were sequenced using the TaqTrack sequencing system (Promega). Primer P 330,
5′-TAGGTATAGACGAGGC-3′ was homologous to FPV *TK* gene, and P 348,
5′-ATCGAACTCCATTCCG-3′ was complimentary to the open reading frame (ORF) 3′ of the *TK* gene. P 402,
5′-ATTAGGATCCATGACAAACCTGTCAGATCAAACCCAG-3′ was homologous to the 5′ end of the IBDV VP 2 gene [21] and contained a *Bam* HI restriction site to facilitate the subcloning of PCR fragments. P 403,
5′-GTACAAGCTTCATGCTTTTCCTGACGCGGCTCGAGCAG-3′ was complimentary to the 3′ end of the IBDV VP 2 gene and contained a *Hind* III site for subcloning and an in frame translation stop codon. P 426,
5′-CTAGCCATTTAGTATCC-3′ was homologous to a region within FPV P.E/L promoter and was used for DNA sequencing and PCR analysis of rFPV genome arrangements.

Construction of plasmid insertion vectors

Plasmid pAF09 has a pBS(+) backbone (Stratagene) and was constructed for the cloning of vaccine antigen genes behind the FPV P.E/L promoter and to facilitate their insertion into the FPV genome immediately downstream of the *TK* gene. This plasmid contains the bivalent FPV P.E/L promoter [26] with a multiple cloning region for the insertion of vaccine antigen genes immediately downstream of the ATG initiation codon of the P.E/L promoter portion (Fig. 1 A) and the *E. coli lac* Z gene under the control of the P.L promoter portion. Plasmid pAF09 also contains the *E. coli gpt* selectable marker under the control of the vaccinia P.7.5 promoter. This gene insertion and selection/screening cassette is flanked by the FPV *TK* gene and the promoter and ORF of the gene downstream of the *TK* gene. Recombinant FPVs are constructed on the basis of amplification in MXHAT medium using the *gpt* gene [9], and plaque purification on the basis of β-galactosidase expression. This plasmid (pAF09) allowed rapid construction of FPV recombinants based upon *gpt* selection for amplification and β-galactosidase screening for plaque purification.

Plasmid pFN01 is based on pAF09 and contains the full length VP 2 gene of the Australian IBDV strain 002-73 inserted as a 1.5 kb *Bam* HI-*Hind* III fragment behind the FPV P.E/L promoter. The VP 2 gene was amplified by PCR from plasmid p 611 [29] using PCR primers P 402 and P 403. The ATG initiation codon of native VP 2 is preceded by four codons from the vector including the ATG initiation codon which is part of the P.E/L promoter (Fig. 1 A). DNA sequencing of pFN01 revealed the presence of a single nucleotide substitution from the published VP 2 sequence near the N terminus of VP 2, causing an amino acid change at position 12 in VP 2 from Val (GTT) to Ile (ATT). This mutation was already present in the template p 611. The antigenic and immunogenic properties of VP 2 are not likely to be affected by this mutation as it lies outside the virus-neutralising epitope and the biochemical properties (hydrophobicity and size) of Val and Ile are very similar.

Plasmid pDB 25 contains the large genome segment (VP 2-VP 4-VP 3) of the Australian IBDV strain 002-73 behind the vaccinia virus promoter P.L 11 as an insertion in the FPV *TK* gene. The IBDV polyprotein was cloned from p0 [2] without the first five amino acids of the native VP 2 and fused in frame with the ATG of the P.L 11 vaccinia virus promoter in pDB 22 [10]. The codons for the first five amino acid residues of VP 2 had been replaced by vector sequences encoding five different amino acid residues (Fig. 1 A).

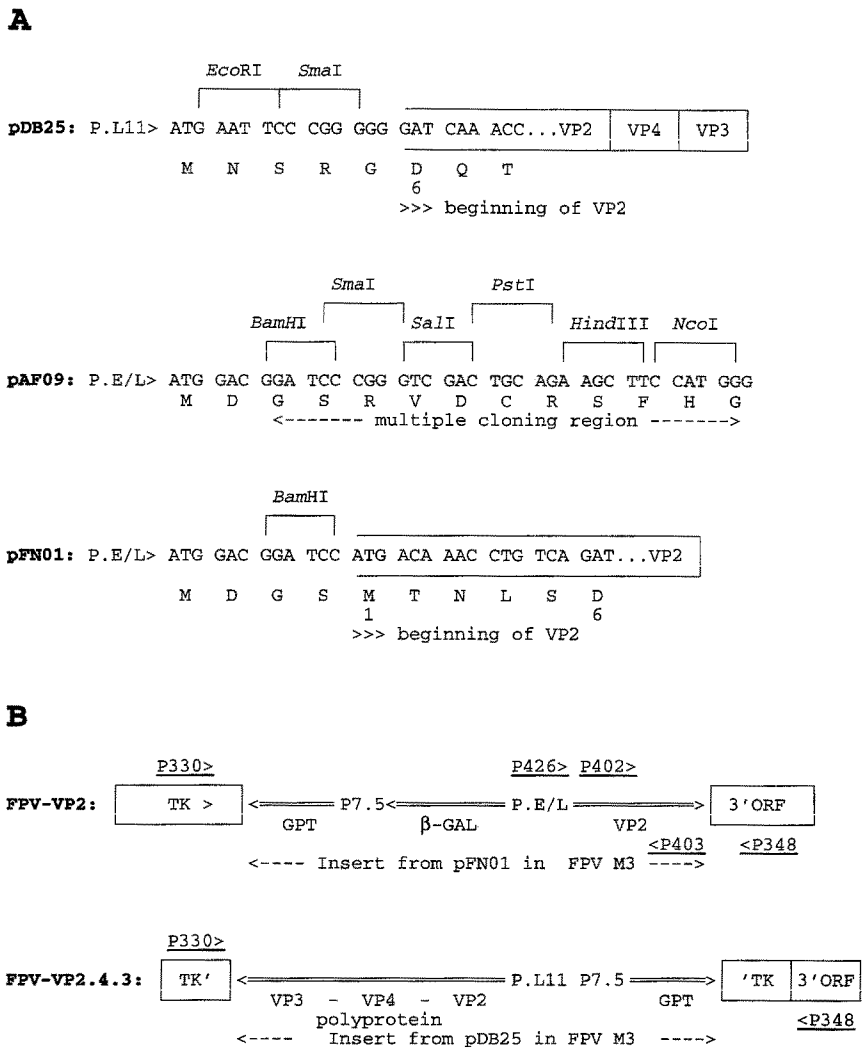

Fig. 1. FPV recombinants: Plasmid constructs in the area of the promoter - VP 2 fusion (**A**) and genome arrangements of rFPVs in *TK* region (**B**). Positions of PCR primers (Pxyz, underlined) are indicated

Isolation of recombinant viruses

FPV recombinants were constructed as described previously [10] using the co-expressed *gpt* gene for recombinant virus amplification and selection. Recombinant virus FPV-015 (in this paper referred to as FPV-VP 2) expressing β-galactosidase was screened by plaquing under non-selective conditions and staining of plaques with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Recombinant virus FPV-pDB 25 (in this paper referred to as FPV-VP 2.4.3) was screened by plaque hybridisation with p0 probe specific for the IBDV VP 2-VP 4-VP 3 gene fragment [2]. Positive plaques were picked and purified by 3-4 cycles of plaquing under non-selective conditions. DNA from recombinant viruses was checked by restriction enzyme digestion and Southern blot hybridisation and by PCR analysis to confirm the predicted structure of the genome and absence of wildtype FPV. Virus stocks were prepared in CES monolayers and titrated by plaque assay.

Extraction of virus DNA and PCR analysis of genome structure

For DNA preparation 100 µl aliquots of virus stock were digested with 500 µg/ml proteinase K in 10 mM Tris-HCl, pH 7.6, 50 mM 2-mercaptoethanol, 100 mM NaCl, 10 mM EDTA, 1% sarkosyl, 26% sucrose for 1 h at 56 °C, then extracted with phenol/chloroform and precipitated with ethanol. The DNA pellet was redissolved in 50 µl TE containing 20 µg/ml RNase and denatured for 10 min at 100 °C. 1–5 µl of this DNA was then used in 50 µl PCR reaction with primers at 0.4 µM. Primers were homologous to FPV sequences flanking the expected DNA insertion sites and/or homologous to the opposite strand of the inserted DNA. The PCR reaction was carried out as follows 94 °C/1 min, 60 °C/2 min, 72 °C/5 min for 30 cycles with each cycle extended by 20 seconds. 10 µl from the 50 µl reaction was analysed by agarose gel electrophoresis and photographed.

Immunofluorescence assay (IFA)

CES cells were grown on 8 well Polystyrene chamber well slides (Nunc) and infected with rFPV at a multiplicity of infection (m.o.i.) of 2.5–10. Two or 3 days post infection cells were fixed in cold methanol and appropriate dilutions of MAb in PBS, 4% bovine serum albumin (BSA) were added to the slides and incubated at 37 °C for 30 min. Remaining MAb was removed by washing three times in PBS, 4% BSA. Sheep anti-mouse IgG coupled to fluorescein isothiocyanate (FITC) (Silenus) was then added at a dilution of 1 : 100 and incubated for 30 min at 37 °C. Slides were washed three times and examined microscopically.

Dot blot immunoassay (DBIA)

CES cells infected with rFPV were harvested by scraping into the growth medium 2 days post-infection. Cells were lysed by one freeze-thaw cycle followed by sonication, and the lysates were cleared by low speed centrifugation. For DBIA serial dilutions (1 : 5) of the cleared lysate (the most concentrated sample corresponding to 3×10^4 cells) were loaded onto 0.2 µm nitrocellulose (Schleicher & Schuell) using a 96 well manifold microfiltration apparatus (Bio-Rad). Membranes were incubated in blocking solution (5% nonfat dry milk in PBS), and were exposed to appropriate dilutions of MAb followed by horseradish peroxidase (HRP) labelled sheep anti-mouse immunoglobulin (Silenus). Labeled protein was visualised with 4-chloro-1-naphtol (Sigma) as the substrate. For the time course experiment cells were infected at a m.o.i. of 10 pfu/cell and grown either in the presence or absence of 50 µg/ml cytosine arabinoside (Ara C) to determine early and late expression of products.

Animal inoculation

Specific pathogen free (SPF) poultry 1 day or 3–4 weeks of age were pre-bleed at the day of vaccination (day 0) and vaccinated via the wing web with FPV-M 3 (titer 2.5×10^7 pfu/ml) and FPV-VP 2 (titer 5×10^7 pfu/ml) using commercial bifurcated wing web stab inoculators, or by intra muscular inoculation with 0.5 ml per bird of oil adjuvanted killed vaccine based on the Australian IBDV isolate 002-73. One group of birds remained unvaccinated. Wing web thickness was measured 5 or 6 days post primary vaccination, and wing web lesions were swabbed for reisolation of recombinants and analysis of viral genome by PCR. At day 21 post primary vaccination each bird was challenged via conjunctiva with IBDV 002-73. Three to 4 days later bursae were removed for histology and antigen detection by ELISA. Sera for ELISA antibodies to IBDV and FPV were collected at days 0, 14, and 20 or 21. Further details of individual experiments are provided in figure and table legends.

IBDV antibody and antigen detection ELISA

Antibody and antigen-capture ELISA for IBDV have been described previously [17]. Briefly, for the detection of antibodies to IBDV the microtiter plates were coated with rabbit anti IBDV IgG (prepared by hyperimmunising rabbits with purified IBDV 002-73) and then a standardised amount of a soluble extract of homogenised IBDV infected bursae or purified virus was added. Serial dilutions of the chicken sera were added to the wells followed by sheep anti-chicken IgG-alkaline phosphatase conjugate. Serum antibody titers were expressed as the reciprocal of the dilutions giving an OD_{405nm} of 0.2. The IBDV antigen capture ELISA was performed by adding a 10% (w/v) bursal homogenate (or serial dilutions) to rabbit anti-IBDV coated plates. Bound virus antigen was detected by the addition of MAb9-6 (anti-VP 2), followed by sheep anti-mouse IgG-HRP conjugate. Bursae were considered negative for infection when no antigen was detectable in the undiluted 10% (w/v) bursal homogenate.

Results

Construction of plasmid vectors and FPV recombinants

The construction of plasmid vectors has been described in Materials and methods, and the genetic organisation of the promoter region for antigen expression is illustrated in Fig. 1 A. Recombinant FPVs were isolated from FPV-M 3 infected CES cells transfected with the plasmid insertion vectors pDB 25 or pFN01. The genome arrangement of isolated and purified rFPVs was confirmed by PCR and Southern blot analysis. The recombinant FPV-VP 2.4.3 derived from pDB 25 contained the genes for the VP 2-VP 4-VP 3 polyprotein of IBDV behind the vaccinia virus promoter P.L 11 inserted in the *TK* gene of FPV-M 3 (Fig. 1 B). The recombinant FPV-VP 2 derived from pFN 01 contained the VP 2 gene without the VP 4 and VP 3 portions of the IBDV polyprotein under the control of the FPV promoter P.E/L inserted in FPV genome immediately downstream of the FPV *TK* gene (Fig. 1 B).

Expression and gene products in cell culture

A panel of MAbs raised against the Australian IBDV strain 002-73 [19] was used to analyse in DBIA (Table 1) the antigenic properties of VP 2 expressed by the rFPVs. Only two of the seven VP 2 specific MAbs (39 A and 3-1) failed to react with the VP 2 protein expressed from either recombinant. The MAbs reacted stronger with FPV-VP 2 than with FPV-VP 2.4.3 expression products. The levels of VP 2 protein synthesis from both rFPVs were compared in a time course experiment with the virus infected cells grown either in the presence or absence of AraC. Samples were taken at various times post infection and analysed by DBIA (Fig. 2) and antigen detection ELISA (data not shown) using MAb 9-6 for VP 2 detection. VP 2 expressed from the FPV P.E/L promoter (FPV-VP 2) could be detected much earlier and in approximately five times higher amounts than VP 2 expressed from the vaccinia virus P.L 11 promoter (FPV-VP 2.4.3) (Fig. 2). In FPV-VP 2 infected cells VP 2 protein could be detected as early as 4 h after infection, whereas in FPV-VP 2.4.3 infected cells VP 2

Table 1. Immuno dot blot analysis of VP 2 expression from rFPVs

MAb[a]	IBDV 002-73[b]	FPV-M3[c]	FPV-VP 2.4.3[c]	FPV-VP 2[c]
39 A (VP 2)	+ + +	−	−	−
3-1 (VP 2)	+ + +	−	−	−
32-3 (VP 2)	+ + +	−	(+)	+
33-10 (VP 2)	+ + +	−	+ +	+ + +
9-6 (VP 2)	+ + +	−	+ + +	+ + + +
17-82 (VP 2)	+ + +	−	+ + +	+ + + +
44-18 (VP 2)	+ + +	−	+ +	+ + +
17-80 (VP 3)	+ +	−	+ +	−

[a] Characterisation and specificity (in brackets) of MAbs published in [19]

[b] Purified viral proteins

[c] CES cells infected with rFVP were lysed and serial dilutions of the lysates assayed by DBIA

Reactivity with MAb is indicated (− no reaction; + to + + + + increasing reactivity)

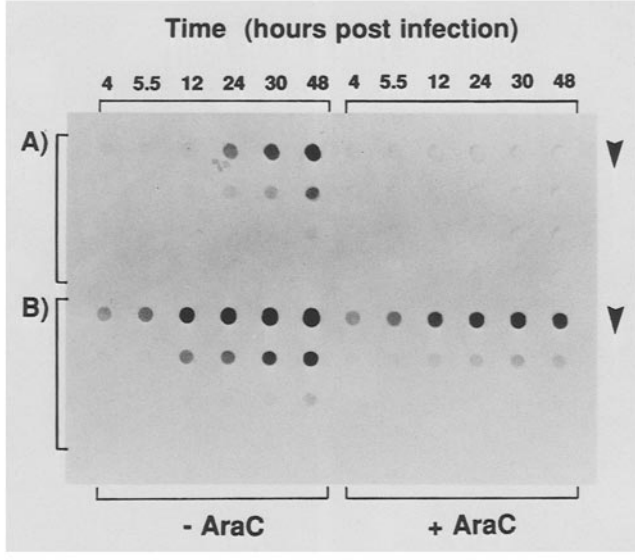

Fig. 2. Time course of VP 2 expression from infected CES cells analysed by DBIA. CES cells infected with FPV-VP 2.4.3 (**A**) or FPV-VP 2 (**B**) at a m.o.i. of 10 pfu/cell were grown either with or without 50 µg/ml of AraC. At the indicated times post infection, monolayers were harvested and serial dilutions of the lysates equivalent to 3×10^4, 6×10^3, 1.2×10^3 and 240 cells were loaded onto nitrocellulose membrane for immuno dot blot assay with MAb 9-6, and stained with HRP

was not detected before 12 h post infection. Expression of VP 2 by the late vaccinia virus promoter P.L 11 (FPV-VP 2.4.3) was completely inhibited in the presence of AraC, whereas in FPV-VP 2 infected cells only the late expression was inhibited by AraC, and the early expression was unaffected. The polyprotein

from FPV-VP 2.4.3 appeared to be correctly processed since only a specific 32 kDa protein band corresponding to the native processed VP 3 protein was detected in Western blots with MAb 17–80 (anti-VP 3). VP 2 was detected from both rFPVs as a 41 kDa band in Western blots with MAb 9-6 (anti-VP 2), although the band from FPV-VP 2.4.3 was very weak (data not shown). Expression and processing of VP 4 was not analysed due to the lack of a specific MAb. The cellular location of VP 2 and VP 3 proteins was analysed by IFA of infected CES cells (Fig. 3). The VP 2 products from both rFPVs were detected throughout the cytoplasm but not in the nucleus, and the VP 2 expression level from FPV-VP 2 (Fig. 3 c) was higher than from FPV-VP 2.4.3 (Fig. 3 a). Different cell

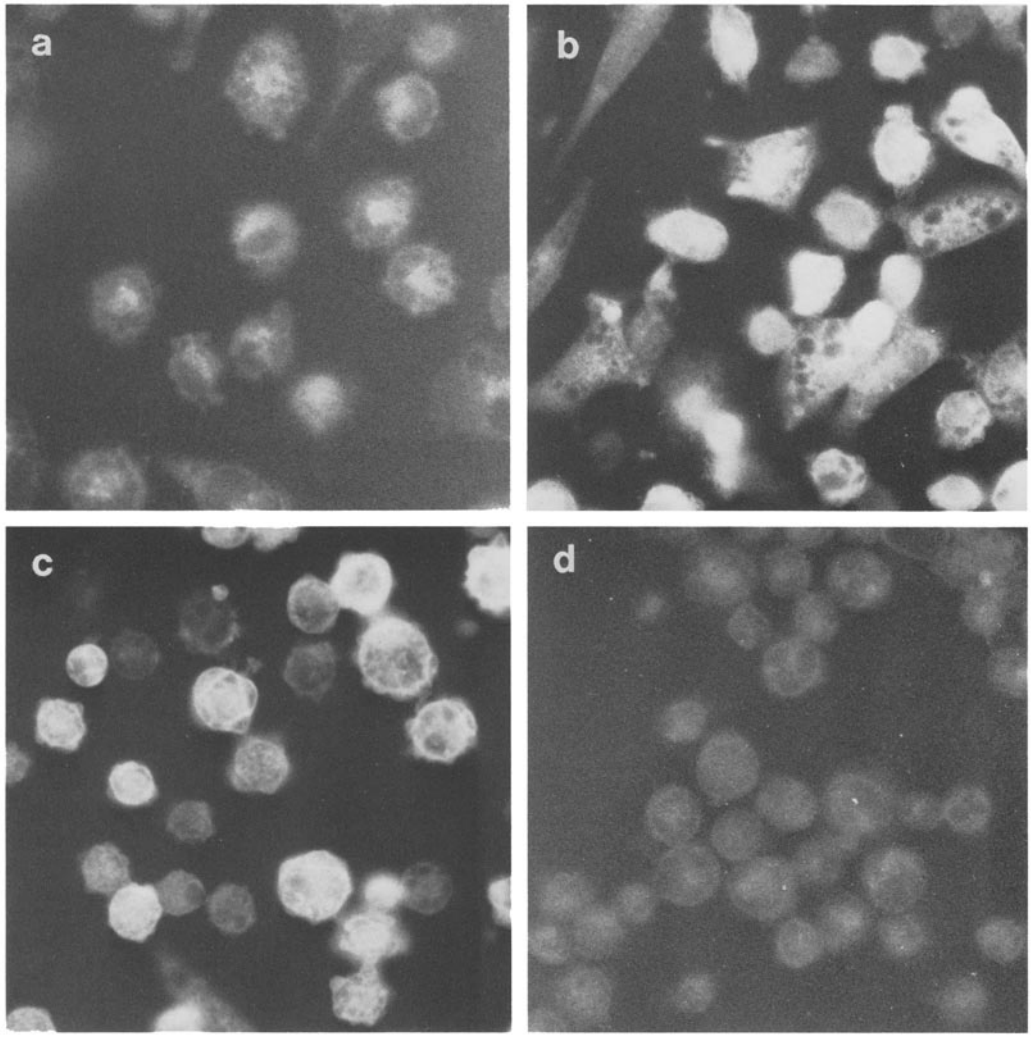

Fig. 3. Immunofluorescence assay of CES cells infected with rFPVs. Monolayers of CES cells grown on plastic chamber well slides were infected with FPV-VP 2.4.3 (**a, b**) or with FPV-VP 2 (**c, d**). Three days post infection, cells were fixed in methanol and reacted with MAb 9-6 (anti-VP 2) (**a, c**) or MAb 17–80 (anti-VP 3) (**b, d**) followed by FITC conjugate

fixation conditions, either 100% methanol, 100% acetone, 70% ethanol, or 4% paraformaldehyde, did not significantly change the VP 2 staining pattern (data not shown). With MAb 17–80 (anti-VP 3) only FPV-VP 2.4.3 infected cells but not FPV-VP 2 infected cells were stained as expected (Fig. 3 b, d), and the location of the VP 3 product was also cytoplasmic. In order to detect possible cell surface expression, infected cells were reacted with MAb and fixed before staining with FITC conjugate. In this experiment no cells reacted with MAb 9-6, indicating that VP 2 was not expressed on the cell surface. In a control using antiserum against FPV instead of MAb 9-6, distinct cell surface staining was achieved (data not shown).

Vaccination and challenge experiments in SPF poultry

The efficacy of the rFPVs as vaccines against IBDV infection was determined in vaccination and challenge experiments in SPF poultry. Different doses of challenge virus were used to determine the level of protection afforded by the recombinant in comparison with an oil adjuvanted killed IBDV vaccine. The biological characteristics of the rFPVs were compared with parent virus FPV-M 3 and assessed with regard to wing web thickness and inoculation dose.

Replication and stability of rFPV

Wing web thickness at day 5 post primary vaccination was normally used as a measure of the biological character of the recombinants. Wing web thickness increased after infection and was highest 5 days post primary vaccination with high doses (10^{-1} dilution) of virus, but reached a maximum at day 7 when lower doses (10^{-3} to 10^{-5} dilution) of virus were used for vaccination (Fig. 4). The wing web thickness of birds vaccinated with FPV-VP 2 (Fig. 4 B) was slightly lower than with the parent virus FPV-M 3 (Fig. 4 A). For FPV-VP 2.4.3 the wing web thickness was lower than with FPV-VP 2 (data not shown). FPV was isolated from the wing web swabs taken at day 5 post vaccination and the DNA analysed by PCR. From the analysis of the PCR products all virus genomes appeared to be stable upon passage in birds as they maintained the inserted gene in the correct arrangement within the *TK* region of the FPV genome. There was no apparent reversion to wild type.

Immunogenicity of rFPV

FPV ELISA antibodies were detected in all birds vaccinated with the rFPVs. The levels in the FPV-M 3 vaccinated birds were higher than the levels in the birds vaccinated with the FPV recombinants, of which FPV-VP 2 had slightly higher levels compared to FPV-VP 2.4.3 (data not shown). ELISA antibodies to IBDV were measured at 2 and 3 weeks post vaccination in 1 day old (Table 3 B) or 3 week old birds (Tables 2, 3 A) vaccinated with either recombinant FPV-VP 2 or the inactivated vaccine. In 3 week old chickens the IBDV antibody responses at 2 and 3 weeks post vaccination were higher in killed vaccine

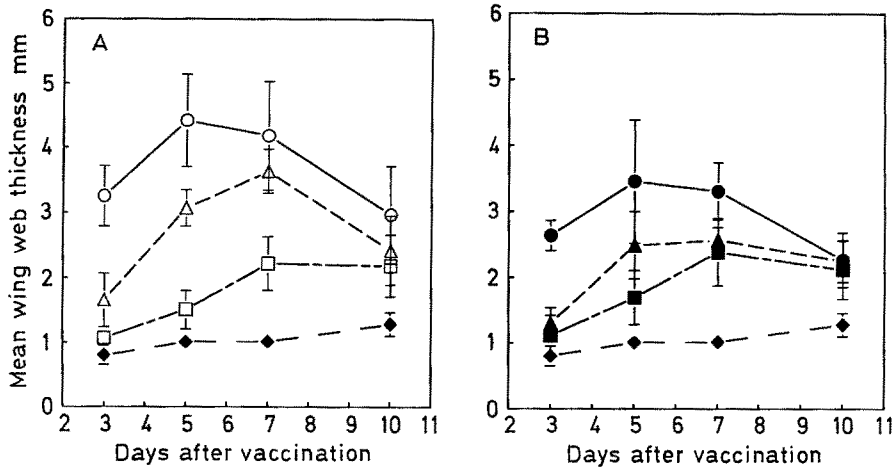

Fig. 4. Development of wing web lesions in response to vaccination with different dilutions (10^{-1}, circle; 10^{-3}, triangle; 10^{-5}, square symbols) of virus stock of FPV-M 3 (**A**; open symbols) or FPV-VP 2 (**B**; filled symbols) as described in Materials and methods. Unvaccinated controls represented by filled diamond. Standard deviations are indicated by error bars. Six birds were measured in each group

vaccinated birds than in FPV-VP 2 vaccinated birds. No antibodies to IBDV were detected in FPV-VP 2.4.3 vaccinated birds at any time (Table 2 A). In chickens vaccinated at the age of 1 day IBDV responses were detected at 21 days but not at 10 days post vaccination, and the responses were lower than in the older birds. Birds vaccinated with FPV-VP 2 had only very low serum neutralising (SN) antibody titers, whereas birds vaccinated with inactivated IBDV vaccine had high SN antibody responses (data not shown).

Protection against IBDV infection

IBDV antigen in the bursae at 3 to 4 days after conjunctival challenge was used as a measure of infection. In an initial vaccination experiment (Table 2 A) where birds were revaccinated after 14 days, and using a low challenge dose (100 CID_{50}) of IBDV, none of the birds vaccinated with FPV-VP 2 (0/10) or killed IBDV vaccine (0/10), but most of the birds vaccinated with FPV-VP 2.4.3 (8/10), FPV-M 3 (7/10) or unvaccinated birds (10/10) were infected. This indicated a high level of vaccine efficacy for the FPV-VP 2 construct which was also achieved by single dose vaccination (Table 2 B). Histopathological examination of the bursae confirmed the antigen detection data. A significant number of birds had detectable lesions in the bursae in the FPV-M 3, FPV-VP 2.4.3 and unvaccinated groups, but none of the birds vaccinated with FPV-VP 2 or killed IBDV vaccine had detectable bursal lesions.

The level of protection afforded by the recombinant FPV-VP 2 in comparison with the killed IBDV vaccine was determined in a vaccination experiment with 3 week old birds (Table 3 A) using graded doses of challenge IBDV virus. FPV-

Table 2. Vaccination of SPF poultry at 21 days of age with FPV-IBDV vaccine and challenge 21 days later with 100 CID_{50} of IBD virus

A. Vaccination and revaccination 14 days post primary vaccination

Vaccine	IBDV antibody titer[a] at given days post primary vaccination			Protection from IBDV infection[b] No. infected/ No. challenged
	0	14	20	
Control	< 100	< 100	< 100	10/10
FPV-M3	< 100	< 100	< 100	7/10
FPV-VP 2.4.3	< 100	< 100	< 100	8/10
FPV-VP 2	< 100	200	430	0/10
Killed vaccine[c]	< 100	700	> 3200	0/10

B. Single dose vaccination

FPV-M 3	< 100	< 100	< 100	9/10
FPV--VP 2	< 100	370	1300	2/10
Killed vaccine[c]	< 100	> 3200	> 3200	0/10

[a] Antibody titer expressed as the reciprocal of the geometric mean of serum dilution giving OD = 0.2 in the ELISA antibody test. Birds were challenged via the conjunctiva with IBDV 002-73 at 21 days post vaccination. CID_{50} = chick infective doses 50%

[b] Bursae were removed 3 to 4 days post challenge and assayed for the presence of IBDV using an antigen capture ELISA test

[c] The killed IBDV vaccine was provided by Arthur Webster Pty Ltd

M 3 vaccination failed to protect any of the birds against challenge with IBDV 002-73, whilst killed IBDV vaccine protected all birds at all challenge doses. FPV-VP 2 protected 1/6, 2/6 and 3/6 birds following challenge with 30 000, 3 000 and 300 CID_{50} of IBDV. Even though the challenge was much more severe than the previous experiment, FPV-VP 2 vaccination still provided protection to some birds although a lower proportion than with the killed IBDV vaccine.

In chickens vaccinated at one day of age and challenged at three weeks with graded doses of IBDV, good protection was observed (Table 3 B). All birds vaccinated with FPV-M 3 were infected following challenge with 6, 60 or 600 CID_{50} of IBDV. FPV-VP 2 vaccinated birds were well protected when challenged with 6 or 60 CID_{50} of IBDV. Even at challenge with 600 CID_{50} of IBDV some birds were protected. Protection was provided even though the antibody levels in vaccinated day old birds at the time of challenge were lower than those observed in birds vaccinated at 3 weeks of age.

Discussion

Two rFPVs expressing IBDV sequences were constructed and analysed in this study. The three main differences between the two recombinants were the IBDV

Table 3. Vaccination of SPF poultry at one or 21 days of age with FPV-IBDV vaccine and challenge 21 days later with graded dose of IBD virus

A. Vaccination at 21 days of age

Vaccine	IBDV antibody titer[a] at given days post vaccination			IBDV challenge[b] CID_{50}	Protection from IBDV infection[c] No. infected/ No. challenged
	0	12	19		
FPV-M 3	16.5	14	24	30	6/6
				300	6/6
				3000	6/6
FPV-VP 2	nd	208	875	300	3/6
				3000	4/6
				30000	5/6
Killed vaccine[d]	nd	1320	> 3200	300	0/6
				3000	0/6
				30000	0/6

B. Vaccination at one day old

Vaccine	IBDV antibody titer[a] at given days post vaccination			IBDV challenge[b] CID_{50}	Protection from IBDV infection[c] No. infected/ No. challenged
	0	10	21		
FPV-M 3	17.7	22	12.5	0.6	0/7
				6	7/7
				60	7/7
				600	7/7
FPV-VP 2	nd	23	283	6	0/5
				60	1/6
				600	4/6

[a] Antibody titer expressed as the reciprocal of the geometric mean of serum dilution giving OD = 0.2 in the ELISA antibody test

[b] Birds were challenged via the conjunctiva with IBDV 002-73 at 21 days post vaccination. CID_{50} = chick infective doses 50%

[c] Bursae were removed 3 to 4 days post challenge and assayed for the presence of IBDV using an antigen capture ELISA test

[d] The killed IBDV vaccine was provided by Arthur Webster Pty Ltd

nd Not done

sequences inserted (VP 2 vs VP 2-VP 4-VP 3), the promoters controlling the expression of the IBDV sequences (P.E/L vs. P.L 11), and the insertion site in the FPV genomes (within the *TK* gene vs immediately downstream). FPV-VP 2

contained only DNA encoding VP 2, whereas FPV-VP 2.4.3 contained DNA encoding the whole VP 2-VP 4-VP 3 polyprotein. Immunization with the VP 2 product alone is sufficient to generate a protective immune response [18] and the VP 3 product does not confer protection, although it is a strong immunogen. The host protective epitope in VP 2 is highly conformation dependent and the correct folding is important to generate a protective immune response [3]. A panel of MAbs has been used to assess the antigenic properties of VP 2 expressed from both rFPVs. No differences in the binding of VP 2 from both rFPVs could be detected by immuno dot blot indicating that VP 2 was correctly folded and the VP 2-VP 4-VP 3 polyprotein was correctly processed. Two of the virus-neutralising MAbs (39 A and 3-1) which did not bind VP 2 expressed from the rFPVs are very sensitive to conformational changes in VP 2 as they are negative in Western blot with IBDV protein [19]. MAb 39A and 3-1 are also unable to block chicken immune serum in blocking ELISA and are thought to bind to a region of the conformational epitope different from the other virus-neutralising MAbs [19]. The correct processing of the VP 2-VP 4-VP 3 polyprotein (approx. 115 kDa) was confirmed by Western blotting of infected CES cell proteins. In Western blots with MAb 9-6 (anti-VP 2) and MAb 17-80 (anti-VP 3) only protein fragments corresponding to the sizes of the processed viral proteins were detected in lysates of FPV-VP 2.4.3 infected cells. VP 2 has a calculated molecular weight of 52 kDa from the DNA sequence, but virus derived VP 2 protein of the Australian IBDV strain 002-73 migrates in SDS-PAGE gels as two bands of ca. 41 (VP 2a) and 37 kDa (VP 2b) [16] of which the smaller band VP 2b appears to be derived from the larger band VP 2a [2]. VP 3 has an apparent molecular weight of 32 kDa.

The expression level of VP 2 from FPV-VP 2 as analysed by dot blotting, exceeded that of FPV-VP 2.4.3 by about five times at late stages of infection. The onset of VP 2 expression from FPV-VP 2 could be detected early (4–5 h) after infection, whereas with FPV-VP 2.4.3 no VP 2 protein could be detected in the early stages of infection. Addition of AraC to the growth medium inhibited expression of VP 2 from FPV-VP 2.4.3 completely, whereas only the late expression was inhibited in the FPV-VP 2 construct. These observations are in agreement with data for expression of β-galactosidase under the control of FPV promoter P.E/L or vaccinia virus promoter P.L 11 [26].

FPV-VP 2 contained the foreign genetic material inserted downstream of the *TK* gene, whereas FPV-VP 2.4.3 contained the foreign insert within the *TK* gene. The effects of the different insertion sites, the lower levels of wing web thickness and reduced FPV antibody titers in FPV-VP 2.4.3 vaccinated birds compared to FPV-VP 2 vaccinated birds may indicate a reduction in the virulence character of FPV-VP 2.4.3. With vaccinia virus [12] TK^- recombinants have been shown to be significantly attenuated. Our observations suggest that *TK* rFPVs are also attenuated (data not presented). Restoration of the TK^- phenotype to wt *TK* may lead to enhanced responses to the vaccine antigen by way of improved replication of the rFPV "in vivo".

Vaccination with FPV-VP 2 consistently provided protection against IBDV infection and also against bursal weight loss and histological damage (data not presented) at day 7 post infection in poultry vaccinated at 3 weeks or 1 day of age then challenged 3 weeks later. Although high levels of protection were provided by the recombinant FPV-VP 2, they did not reach those provided by the commercial oil-adjuvanted killed whole virus vaccine. The FPV-VP 2.4.3 vaccine did not protect chickens from infection and even failed to induce an antibody response to IBDV. This could be due to the low level of VP 2 product made and possibly a reduced virulence character of the FPV-VP 2.4.3 construct. The expression of VP 2 as a VP 2-VP 4-VP 3 precursor polyprotein did not seem to change the antigenic properties of VP 2 as it was correctly processed in cells and recognized by conformation dependent MAbs. Bayliss et al. [5] reported the vaccination of chickens with a rFPV expressing a VP 2-β-galactosidase fusion protein from the vaccinia P 7.5 promoter. Although this construct did provide protection in poultry against mortality from IBDV challenge, it did not protect against infection of and damage of the bursa and did not cause seroconversion. The authors indicated that the highly attenuated FPV strain did not replicate well in chickens and speculated that the VP 2 antigen may not have been present in the correct conformation as it was expressed as a fusion protein to β-galactosidase which also contained a portion of VP 4. The IBDV challenge strains 52/70 and CS 89 used in that study are more virulent than the Australian strain 002-73 used in our study. Due to importation restrictions we were not able to use a more virulent heterologous challenge virus. The main reasons for the efficacy of the FPV-VP 2 vaccine reported in this study appear to be the higher level of VP 2 expressed from the strong early/late promoter and possibly improved replication "in vivo".

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