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Fowl adenovirus recombinant expressing VP2 of infectious bursal disease virus induces protective immunity against bursal disease*

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Summary. The right hand end *Nde* I fragment 3 (90.8–100 map units) of the fowl adenovirus serotype 10 (FAV-10) was characterised so as to allow the location of an insertion site for recombinant vector construction. Infectious bursal disease virus (IBDV) VP2 gene from the Australian classical strain 002/73, under the control of the FAV-10 major late promoter/leader sequence (MLP/LS) was inserted into a unique *Not* I site that was generated at 99.5 map units. This recombinant virus was produced without deletion of any portion of the FAV-10 genome. When administered to specific pathogen free (SPF) chickens intravenously, intraperitoneally, subcutaneously or intramuscularly, it was shown that the FAV-10/VP2 recombinant induced a serum VP2 antibody response and protected chickens against challenge with IBDV V877, an intermediate virulent classical strain. Birds were not protected when the recombinant was delivered via the conjunctival sac.

Introduction

Infectious bursal disease virus (IBDV) induces an immunosupressive disease of chickens. The primary effect of this disease is the destruction of B-lymphocytes [27] and consequently the severe impairing of the chicken to develop antibodies to other avian pathogens or vaccines [42]. Vaccination of breeding hens sensitised by natural exposure, by live vaccine, or inactivated oil-emulsion vaccine, produces a long lasting high serum antibody response [36, 53]. Maternal antibodies are

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then transferred to the progeny chickens via the yolk sac providing protection for the first few critical weeks after hatching [53]. In recent times an emerging problem of bursal disease in the poultry industry has been with antigenic variants in the USA [46, 49, 50] and the very virulent strains in Europe [51]. These new antigenic strains of IBDV are able to escape from maternally derived antibodies induced by the classical strains. The emergence of these new strains has resulted in changes to vaccination regimes, with broilers being vaccinated in ovo or at 2–3 weeks of age when maternal antibody has declined, with more virulent vaccines. More recently there has been the successful use of an antibody-virus complex vaccine [52].

IBDV is a member of the family *Birnaviridae*, and has two segments of double-stranded RNA [13]. A precursor polyprotein VP2-VP4-VP3, encoded on the larger genomic A segment is processed by autoproteolysis to produce viral proteins VP2, VP3 and VP4 [2, 3, 30, 31]. The amino acid sequence is highly conserved between the classical strains and antigenic variations are confined to the central region of the protein between residues 206 to 350 [5, 25, 34], the conformational discontinuous host protective epitope [3]. Virus neutralizing (VN) mouse monoclonal antibodies are primarily directed against VP2 [3, 7, 18]. This protein has been the focus of attempts to produce new vaccines by recombinant DNA technology. VP2 expressed in *E. coli* reacted with a range of VN monoclonal antibodies, although the protein had been injected in milligram quantity [4]. Yeast expressed and purified VP2 was incorporated into an oil emulsion vaccine that was able to induce both virus neutralizing and ELISA antibodies in SPF chickens [19]. These antibodies were passed on to the progeny and gave protection when challenged with virulent IBDV. VP2 was also expressed in a fowlpox virus recombinant [6]. Significant levels of protection were provided by vaccination with this recombinant, although the level of protection was lower than that provided by an inactivated oil emulsion vaccine containing whole IBDV [26]. Finally, a herpesvirus of turkeys (HVT) recombinant expressing VP2 induced protection against virulent IBDV challenge [12].

Adenoviruses have a number of advantages as vectors including, stable DNA that is readily manipulated, the virus is relatively easily propagated, many of the serotypes have low pathogenicity in man or animals and the virus can induce cellular, humoral or mucosal immune responses. Human adenovirus (HAV) vectors have been successfully used to express a variety of viral and cellular genes. The first immunogenic protein expressed by a HAV vector was the hepatitis B surface antigen [40]. Since that time, HAV vectors have been constructed that express a wide variety of foreign immunogens including the herpes simplex virus glycoprotein B [22, 23, 32, 38], respiratory syncytial virus F and G genes [29], human immunodeficiency virus gp 120 [35, 41], rabies virus glycoprotein gene [10, 33, 37] pseudorabies virus genes [44], Epstein-Barr virus genes [44, 45], porcine respiratory coronavirus spike antigen [9], and expression of luciferase in an bovine adenovirus vetor [39].

Fowl adenoviruses (FAV) have a number of features that make them attractive to the poultry industry as a vector for vaccine delivery. Included in these features

are (i) the ease of propagation, (ii) the high titres achievable (10^9–10^{10} pfu/ml), (iii) stability of its genome, (iv) the ease of administration (water, aerosol or injection), (v) a large range of serotypes that vary in virulence [14–16] and (vi) a genome that is relatively easy to manipulate and can tolerate insertion and expression of foreign DNA. In this report we describe the construction of a recombinant FAV serotype 10 containing the VP2 gene from IBDV and demonstrate in vaccination via various routes that this recombinant can protect SPF chickens from IBDV infection and bursal damage.

Materials and methods

Cells and viruses

FAV serotype 10 (FAV-10) and was grown in chicken kidney (CK) cells derived from 3 week old specific pathogen free chickens (SPF Unit, Maribyrnong, Victoria). The IBDV challenge virus was V877, an intermediate virulent classical strain, was supplied by Arthur Websters (Castle Hill, Australia).

Construction of the expression cassette

An expression cassette was constructed for insertion into the FAV-10 genome. The FAV-10 major late promoter and splice leader sequences (MLP/LS) ([47]; GenBank accession number AF007577) were inserted into pUC18 along with a multiple cloning site (MCS) and an SV40 polyA recognition sequence (Fig. 3a). The expression cassette was flanked by *Not* I sites to allow subsequent insertion into the *Not* I site engineered into the FAV-10 *Nde* I fragment 3.

Construction of a FAV-10 vector

The right hand end *Nde* I fragment 3 of FAV-10 was cloned (Fig. 2) and sequenced (GenBank accession number AF007578). A unique *Not* I site was inserted into the *Bam* HI site (99.5 map units). CK cells were transfected with the *Nde* I fragment 3 containing the *Not* I site together with the *Spe* I fragment 1 which contained nearly the entire FAV-10 genome except for the right hand end 4.1 kb and overlapped with the *Nde* I fragment 3 by 150 bp. Homologous recombination between these two DNA fragments resulted in a complete and viable FAV-10 but with a unique *Not* I site in the genome (Fig. 2).

Construction of a FAV-10/IBDV VP2 recombinant

The IBDV VP2 gene from Australian strain 002/73 [25] was inserted into the MCS of the expression cassette (Fig. 3b) Restriction enzyme digestion and DNA sequencing confirmed the construct. The expression cassette was isolated as a *Not* I fragment and cloned into the *Not* I site of the cloned FAV-10 *Nde*I fragment 3. The plasmid containing the VP2 gene was linearized and transfected with the FAV-10 *Spe* I fragment 1 into CK cells. The resultant recombinant virus designated as rFAV-10/VP2 (Fig. 3b), was plaque purifed three times. Genomic DNA was prepared and checked by restriction enzyme analysis and Southern blotting to confirm the presence of the expression cassette and VP2 gene within the *Not* I site of FAV-10 *Nde* I fragment 3 (data not shown). The immunogenicity of the expressed VP2 was confirmed in an antigen ELISA using the neutralising monoclonal antibody 17–82 [20].

Chicken inoculations and challenge

Experiment 1

Three week old SPF birds were divided into two groups. One group was vaccinated with rFAV-10/VP2 intravenously (i.v.) at a dose of 10^7 pfu in approximately 200 μl of inoculum. A second group was vaccinated i.v. with FAV-10 at a dose of 10^4 pfu [15]. Birds were bled two weeks post-vaccination (p.v.) and then every week until six weeks p.v. Antibodies to FAV and VP2 were assessed by ELISAs.

Experiment 2

Three week old SPF birds were divided into five groups. One group was vaccinated with rFAV/VP2 i.v. at a dose of 10^7 pfu in approximately 200 μl of inoculum. A second group was vaccinated i.v. with FAV-10 at a dose of 10^4 pfu. For comparative purposes, a commercially available inactivated oil emulsion IBDV 002/73 vaccine (Bursavac K, Websters) was included in the trial. This vaccine was administered in a 0.5 ml volume by the subcutaneous (s.c.) route, on the inside of the thigh muscle. A fourth group was unvaccinated. Birds in Groups 1–4 were challenged with IBDV strain V877 instilled via the conjunctival sac in 100 μl of inoculum. Group 5, unvaccinated, were not challenged. All birds were bled weekly for three weeks prior to challenge. At 4 days post-challenge, all birds were euthanised, the bursa removed and divided, half used for immunological testing (antigen ELISA) and other half for immunohistology.

Experiment 3

In order to further explore possible vaccination strategies, one day old SPF chickens vaccinated either intraperitoneally (i.p.), intramuscularly (i.m.), s.c. or instilled via the conjunctival sac with either FAV-10 or rFAV-10/VP2. Eight groups of day old SPF chicks, 10 per group, were vaccinated with rFAV/VP2 via conjunctiva 10^7 pfu (Group 1), i.p. 10^7 pfu (Group 2), i.p. 10^5 pfu (Group 3), i.p. 10^3 pfu (Group 4), i.m. 10^7 pfu (Group 5), s.c. 10^7 pfu (Group 6) or with FAV-10 10^4 pfu via conjunctiva (Group 7) or unvaccinated (Group 8). Birds in groups 1–8 were challenged with IBDV V877 instilled via the conjunctival sac in 100 μl of inoculum. All birds were bled weekly for three weeks prior to challenge with IBDV V877. At 4 days post-challenge, all birds were euthanised and bursa removed for immunological testing in an antigen ELISA and immunohistology.

IBDV antibody and antigen detection ELISA

Antibody and antigen-capture ELISA for IBDV has been described previously [20]. Briefly, for the detection of antibodies to IBDV the microtitre plates were coated with a standardised amount of purified virus, followed by serial dilutions of the chicken sera. Affinity purified goat anti-chicken IgG (H + L) peroxidase labelled antibody (KPL, Australia) was added, followed by the substrate 2,2′-azino-bis(3-ethylbenzthiazoline) sulfonic acid (ABTS). Serum antibody titres were expressed as the reciprocal of dilutions giving an OD_{405nm} of 0.2. The IBDV antigen capture ELISA was performed by preparing a 10% (w/v) bursal homogenate and applying this undiluted or serial dilutions to microtitre wells coated with monoclonal antibody 17–82 [20] followed by conjugated rabbit anti-chicken IgG HRP. Bursae were considered negative for infection when no antigen was detectable in the undiluted 10% (w/v) bursal homogenate.

Adenovirus antibody test

For the detection of FAV-10 antibodies, microtitre plates were coated with a standardised amount of purified FAV-10, followed by serial dilutions of chicken sera. Affinity purified

goat anti-chicken IgG (H + L) peroxidase labelled antibody was added, followed by substrate ABTS. Plates were read at OD_{405nm}. Pooled negative sera determined the cut off at 0.1 OD_{405nm}.

Immunohistology

Immunoperoxidase staining of thin sections of bursa were performed. Briefly, paraffin embedded tissue sections were prepared on glass slides. Sections were treated with trypsin, blocked with 1% horse serum and then incubated with monoclonal antibody 17–82 [20]. A biotinylated horse anti-mouse antibody was applied followed by vectastain (Immunodiagnostics, Australia), then peroxidase substrate (DAB) and counterstained with haematoxylin.

Results

Construction of the recombinant FAV-10 expressing VP2 of IBDV

The *Nde* I fragment 3 of 4.25 kb at the very right hand end of the FAV-10 genome was cloned (Fig. 1a) and sequenced in both directions (GenBank submission AF007578), revealing a number of open ending frames (ORFs) in both right hand and left hand directions (Fig. 1b). A total of nine open reading frames of at least 200 bp in length were identified, including two open reading frames that were 870 bp (ORF2) and 1 404 bp (ORF3) in length, followed by a putative polyA (AATAAA) recognition sequence. This appeared to signal the most 3′ transcriptionally active region of the FAV-10 genome. However, one of the small ORF's (ORF4) of 252 bp located 5′ of the *Bam* HI site was flanked by a potential

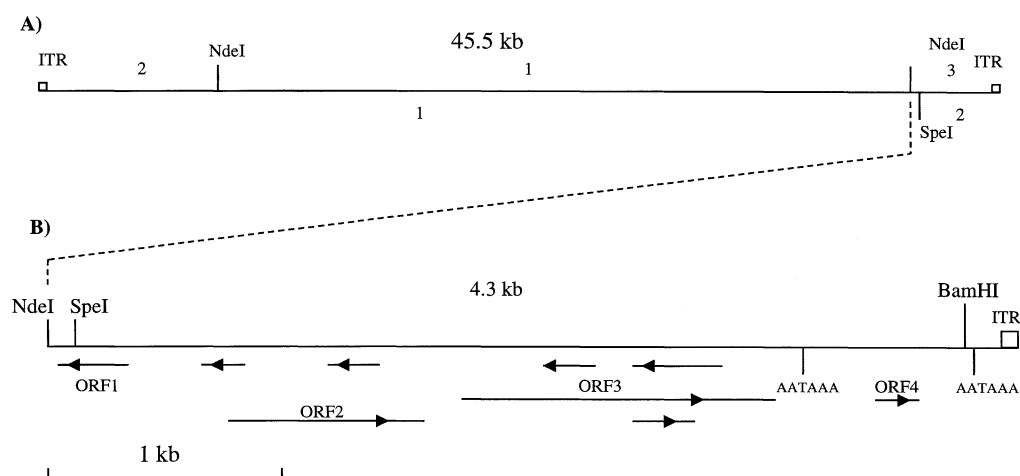

Fig. 1. A Restriction map of the FAV-10 genome showing *Nde* I and *Spe* I sites. The inverted terminal repeats (ITR) are shown as open boxes at the ends of the genome. **B** shows the expanded *Nde* I fragment 3. This fragment was cloned and sequenced. Open reading frames (ORF) of 200 base pairs or greater are indicated below the map, with the direction of the putative transcriptional units shown by arrows. The large ORFs of 870 and 1 404 bp are indicated by ORF2 and ORF3, respectively. Polyadenylation signals, AATAAA, are indicated. Restriction sites *Nde* I, *Spe* I and *Bam* HI are shown

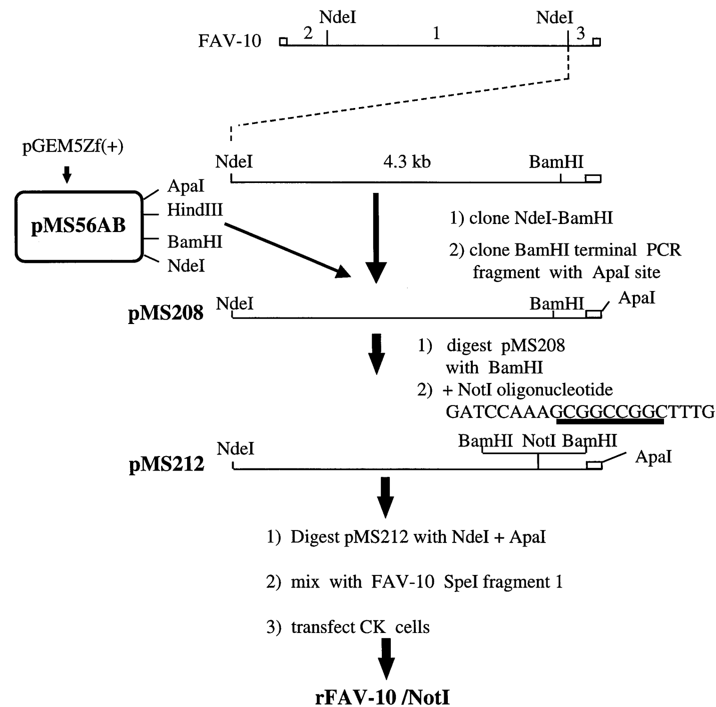

Fig. 2. Construction of the recombinant viruses. The *Nde* I fragment 3 was cloned as follows: The plasmid pGEM5Zf(+) MCS was modified to contain *Hin*d III and *Bam* HI sites (pMS56AB). The left portion of *Nde* I fragment 3 was cloned as a *Nde* I-*Bam* HI fragment into pMS56AB. The terminal *Bam* HI fragment was generated by polymerase chain reaction, with an *Apa* I site 3′ of the ITR, and cloned into pMS56AB already containing the *Nde* 1-*Bam* HI fragment resulting in plasmid pMS208. A *Not* I site was inserted into the *Bam* HI site of pMS208, resulting in plasmid pMS212. The recombinant rFAV-10/*Not* I was generated by digesting pMS212 with *Nde* I-*Apa* I and mixing with FAV-10 *Spe* I fragment 1 using a calcium chloride transfection procedure to transfect CK cells. Resulting plaques were plaque purified and tested for the presence of a unique *Not* I site

AATAAA sequence downstrem from this restriction site. It is not known whether this region contains an actual coding or non-coding sequence. If this small putative ORF is not transcribed, then a relatively large region of up to 824 bp (not including the ITR) could possibly be deleted, allowing extra room for insertion of foreign DNA.

In the first construction, the *Bam* HI site in the FAV-10 *Nde* I fragment 3 was replaced with a *Not* I site and the resulting plasmid transfected with *Spe* I fragment 1 to produce a recombinant FAV-10 containing a unique *Not* I site (rFAV-10/*Not*I) (Fig. 2). The recombinant was plaque purified three times and genomic DNA analysed by restriction digestion and Southern blot comparison to parental FAV-10 DNA (data not shown). In order to confirm that the insertion of a *Not* I site had not interrupted any functional transcription unit, day old SPF chickens were vaccinated via the conjunctival sac with 10^7 pfu of rFAV-10/*Not* I. At day 7 and

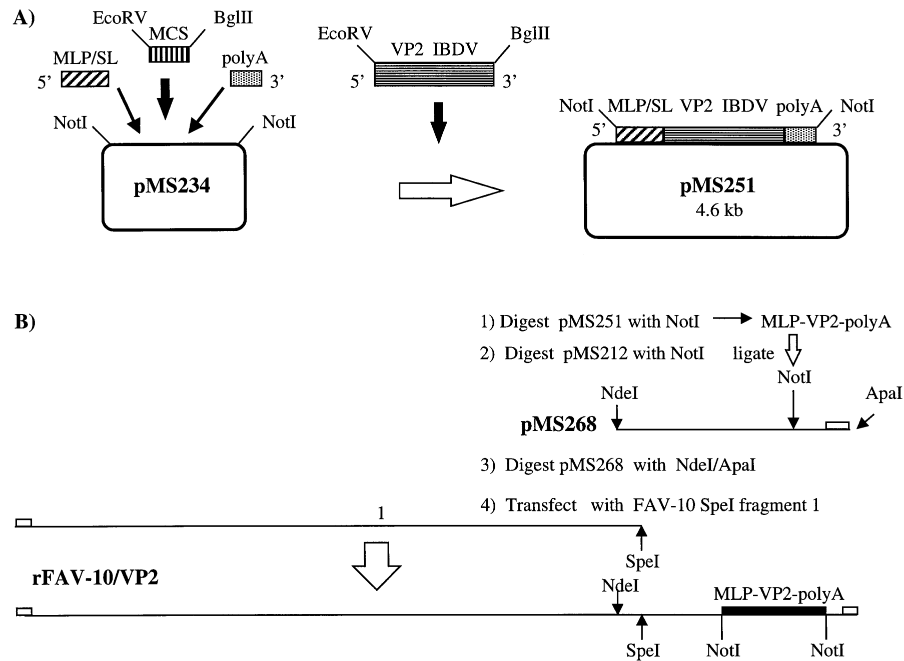

Fig. 3. **A** Construction of the expression cassette: pUC18 multiple cloning site (MCS) was altered for the cloning of the major late promoter/splice leader sequences (MLP/SL) of FAV-10. The SV40 polyadenylation signal was cloned 3′ of an *Eco* RV-*Bgl* II MCS. The entire MLP/SL-MCS-polyA could be removed by cutting resulting plasmid (pMS234) with *Not* I. The VP2 gene from IBDV was cloned as an *Eco* RV-*Bgl* II fragment into the MCS of pMS234 resulting in pMS251. The direction of the transcriptional unit is indicated 5′ to 3′. **B** The recombinant FAV-10/VP2 was generated as follows: The plasmid pMS212 (*Nde* I fragment 3/*Not* I) was digested with *Not* I and the *Not* I fragment containing the MLP/SL-VP2-poly A expression cassette from pMS251 ligated in, resulting in plasmid pMS268. The plasmid pMS268 was digested with *Nde* I-*Apa* I and mixed with FAV-10 *Spe* I fragment 1 and transfected into CK cells using a calcium chloride procedure. Virus was plaque purified and screened for the presence of the VP2 gene

day 10 post vaccination, kidneys were removed, homogenised and plated onto CK cell monolayers. Cultures were passaged 2–3 times until cytopathic effect (c.p.e.) was observed. Genomic DNA was prepared, restriction digested with *Not* I, Southern blotted and probed with FAV-10 *Nde* I fragment 3. The results (not shown) confirmed that rFAV-10/*Not* I replicated in vivo and that no functional transcription unit was interrupted.

We then constructed a second recombinant, not deleting any of the FAV-10 genome but took advantage of the 105% genome packaging availability of the virus capsid [8, 24]. The VP2 gene from the Australian strain 002/73 of IBDV [25] was inserted into the major late promoter expression cassette (Fig. 3b). The VP2 expression cassette was then cloned into the *Not* I site of *Nde* I fragment 3 in plasmid pMS212. The resulting recombination expression cassette plasmid

pMS268 was transfected FAV-10 *Spe* I fragment 1, a recombinant virus, rFAV-10/VP2 isolated and plaque purified three times (Fig. 3b). The recombinant was analysed by restriction digestion and Southern blots, probed with IBDV VP2 DNA or FAV-10 *Nde* I fragment 3 (data not shown). The immunogenicity of the VP2 expressed by the recombinant in cell cultures was further analysed in an antigen capture ELISA. The recombinant expressed VP2 was shown to react strongly with the neutralising monoclonal antibody 17–82 [21].

Vaccination of chickens with rFAV-10/VP2

Experiment 1

In order to confirm the expression of the IBDV VP2 by the recombinant in vivo, three-week-old SPF chickens were inoculated with either 10^4 pfu of FAV-10 or 10^7 pfu of rFAV-10/VP2 i.v. All chickens were pre-bled, then at two weeks p.v., and then weekly until week six p.v. The sera was tested by ELISA for antibody to IBDV (VP2). Table 1 shows that detectable antibodies to VP2 were present 14 days p.v. in birds vaccinated with rFAV-10/VP2. Generally, VP2 antibodies

Table 1. FAV or VP2 antibody titers from three-week-old birds injected i.v. with either FAV-10 or rFAV-10/VP2

a FAV antibody titres[c]

Bird	FAV-10 injected i.v. Time post vaccination (weeks)						Bird	rFAV-10/VP2 injected i.v.					
	P.B.[a]	2[b]	3	4	5	6		PB	2	3	4	5	6
M39	–	800	1 600	3 200	1 600	1 600	R38	–	3 200	1 600	1 600	3 200	800
M40	–	400	800	1 600	3 200	1 600	R46	–	3 200	1 600	>12 800	>12 800	6 400
M41	–	800	200	200	1 600	800	R47	–	100	800	400	1 600	800
M42	–	400	800	1 600	1 600	400	R48	–	200	200	200	200	400
M43	–	1 600	3 200	800	6 400	>12 800	R49	–	200	400	800	400	800

b VP2 antibody titres[c]

Bird	FAV-10 injected i.v.						Bird	rFAV-10/VP2 injected i.v.					
	P.B.	2	3	4	5	6		PB	2	3	4	5	6
M39	–	–	50	–	–	50	R38	–	400	3 200	6 400	800	1 600
M40	–	–	–	–	–	–	R46	–	1 600	>6 400	6 400	6 400	1 600
M41	–	–	–	–	–	–	R47	–	50	800	1 600	6 400	400
M42	–	–	–	–	–	–	R48	–	50	200	100	200	100
M43	–	50	–	–	–	50	R49	–	3 200	3 200	>6 400	1 600	1 600

[a] *P.B.* Pre-bleed
[b] Weeks post vaccination
[c] FAV or VP2 titres of each bird expressed as reciprocal dilutions

Table 2. Serological responses induced by inoculation of three week old SPF birds with rFAV-10/VP2, FAV-10 by intravenous (i.v.) route or inactivated IBDV vaccine

	Route/vaccine	Dose pfu	VP2 Ab titre[a]	FAV-10 Ab titre[b]
Group 1	i.v. rFAV-VP2	10^7	4 000	360
Group 2	i.v. FAV-10	10^4	<10	390
Group 3	s.c. Bursavac K[c]	0.5 ml[c]	6 400	<10
Group 4	unvac/chall[d]	–	<10	<10
Group 5	unvac/unchall[e]	–	<10	<10

[a] VP2 antibody titres are the mean geometric titres at 21 days post vaccination
[b] FAV-10 antibody titres are the mean geometric titres at 21 days post vaccination
[c] Bursavac K; inactivated oil-emulsion vaccine (Webstters); dose as recommended by the manufacturer
[d] *Unvac/chall* Unvaccinated birds received tissue culture media containing no virus and were challenged at 3 weeks with live IBDV. Bursavac K (killed IBDV) was delivered s.c. to the inner thigh
[e] *Unvac/unchall* Unvaccinated birds were vaccinated with media containing no virus and were not challenged

increased and peaked 21–28 days p.v. and was still detectable 42 days p.v. High VP2 antibody levels correlated with high FAV-10 antibodies, indicating that dose was important.

Experiment 2

In order to assess the efficacy of the rFAV-10/VP2, three-week-old SPF chickens were inoculated with 10^7 pfu of rFAV-10/VP2 i.v. (Group 1), 10^4 pfu of FAV-10 i.v. (Group 2), or with a killed commercial IBDV vaccine K s.c. (Group 3). Two other groups were unvaccinated (Groups 4 and 5). All chickens were prebled, then bled weekly for three weeks post vaccination. The sera were tested by ELISA for antibody to IBDV. Table 2 shows the results of experiment 2. As was shown in experiment 1, detectable antibodies to VP2 were present 14 days post vaccination with rFAV-10/VP2 (not shown) and increased by 21 days post inoculation. High VP2 antibody levels correlated with high FAV-10 antibodies in the recombinant vaccinated group 1. To examine the efficacy of the recombinant vaccine, groups 1–4 were challenged with IBDV strain V877. Group 5 birds (unvaccinated) were not challenged. Chickens vaccinated at 3 weeks i.v. with rFAV-10/VP2 were protected from challenge with IBDV V877. In contrast, birds vaccinated with FAV-10 or unvaccinated birds were not protected from challenge as shown in Table 3. All chickens vaccinated with either Bursavac K or rFAV-10/VP2 had detectable levels of VP2 antibodies and unvaccinated chickens or chickens vaccinated with FAV-10 only, had no detectable VP2 antibodies (not shown). At 4 days post challenge, the bursa from all chickens were extracted and tested for the presence of IBDV antigen by ELISA. The presence of IBDV antigen in the bursa indicates the failure to protect against IBDV challenge. The results

Table 3. Examination the effects of vaccination with rFAV-10/VP2 virus upon challenge with Bursavac S (live), and determining levels of protection at 4 days post-challenge using a IBDV(VP2) antigen ELISA

Bird #[a]	VP2 Ag	VP2 Ab[b]	FAV-10 Ab[c]	Bird#	VP2 Ag	VP2 Ab[b]	FAV-10 Ab[c]
M380	32	–[d]	800	R391	±[e]	6 400	200
M381	>128	–	400	R392	–	6 400	200
M382	>128	–	400	R393	±	3 200	200
M383	>128	–	100	R394	±	>6 400	800
M384	>128	–	400	R395	–	3 200	200
M386	+[f]	–	200	R396	–	3 200	200
M387	2	–	400	R397	–	3 200	200
M388	>128	–	400	R398	–	>6 400	400
M389	>128	–	400	4399	–	800	400
M390	>128	–	400	R400	–	800	200

[a]Bird # M380-M390 are Group 2, FAV-10 vaccinated; Bird # R391–R400 are Group 1, rFAV-10/VP2 vaccinated. Group 3 birds (Bursavac K vaccinated-challenged) were negative for VP2 antigen (Ag), negative for FAV-10 Ag and positive for VP2 antibodies (Ab) (results not shown). Group 4 (unvaccinated-challenged) were negative for VP2 Ab, negative FAV-10 Ab at day 21 pre-challenge (results not shown). Group 5 birds (unvaccinated – no challenge) were all negative for VP2 Ag and Ab ELISAs (results not shown)

[b] VP2 antibody titres at day 21 pre-challenge

[c] FAV-10 antibody titres at day 21 pre-challenge

[d] –; undetectable in undiluted bursa homogenates

[e]±; negative using titrated bursa homogenates, weakly positive using undiluted bursa homogenates

[f] Positive result using undiluted bursa homogenate

presented in Table 3 show birds vaccinated with FAV-10 produced detectable levels of IBDV (VP2) antigen present in the bursa. All these birds were negative for VP2 antibody in pre-challenge sera. All recombinant vaccinated birds had high levels of VP2 antibodies in pre-challenge serum and either showed no detectable IBDV antigen in the bursa or in three cases, only detectable in undiluted bursa homogenates. This indicates that in these three cases some IBDV was getting into the bursa. Birds that were vaccinated with the recombinant FAV-10/VP2 and tested negative for IBDV antigen by ELISA were also negative for IBDV antigen by immunohistology.

Experiment 3

To further investigate the efficacy of the rFAV-10/VP2, groups of day old SPF birds were vaccinated via the conjunctival sac, i.p., i.m. or s.c. Table 4 shows the results of the experiment. Birds vaccinated by the i.p. route developed antibodies to FAV-10 and VP2 in a dose dependent manner, as shown by groups 2, 3 and 4. All routes, except via conjunctiva were successful in establishing an immune response sufficient to protect birds from challenge with IBDV V877. Unvaccinated birds were not protected. All birds protected showed no evidence by immunohistology of IBDV antigen in the bursae 4 days post challenge, further indicating that the rFAV-10/VP2 was successful in eliciting a protective immune response.

Table 4. Serological response and protection induced by inoculation of day old SPF birds with rFAV-10/VP2 vaccine via various routes

	Route	Virus/dose	VP2 Ab titres[a]	FAV-10 Ab titres[b]	VP2 Ag titres	Protection[d]
Group 1	conjunctiva	rec[c] 10^7	<10	<10	>128	0/10
Group 2	i.p.	rec 10^7	1 600	1 200	4	9/9
Group 3	i.p.	rec 10^5	250	190	>128	3/15
Group 4	i.p.	rec 10^3	<50	10	>128	0/15
Group 5	i.m.	rec 10^7	1 000	540	4	10/10
Group 6	s.c.	rec 10^7	2 900	85	4	9/9
Group 7	conjunctiva	FAV-10 10^4	<10	<10	>128	0/10
Group 8	unvaccinated	none	<10	<10	>128	0/10

[a]Geometric mean VP2 antibody titres at day 21 pre-challenge

[b]Geometric mean FAV-10 antibody titres at day 21 pre-challenge

[c]rec, rFAV-10/VP2; all birds were bled before vaccination and tested in VP2 and FAV-10 antibody ELISA. None contained detectable antibodies and the results are not shown. Birds were bled at 21 days post vaccination and antibody titres to VP2 and FAV-10 determined. Groups 1 to 8 were challenged at day 21 with live IBDV (Bursavac S). Protection assessed by the number of birds with VP2 antigen titres of less than 8, negative for immunoperoxidase staining and showing no bursal depletion in histopathology

[d] Protection assessed by negative antigen ELISA and no antigen detected by immunohistology

Discussion

Since the early 1980s adenoviruses, herpesviruses and poxviruses have been investigated as candidates for use as live vectors for the delivery of vaccines. Generally, when constructing an adenovirus vector, either the E3 region (replication competent) or the E1 region (replication defective) is deleted and a foreign gene is inserted [9, 10, 22, 23, 25, 29, 32, 33, 37–41, 44, 45]. We chose to avoid both of these regions and instead inserted the IBDV VP2 gene into a non-coding region that was identified in the right hand end of the genome just upstream of the inverted terminal repeat. This site was chosen as it did not appear to disrupt any FAV gene expression. The FAV major late promoter and leader sequences [47] were chosen to express the IBDV VP2 gene as opposed to a heterologous promoter. Packaging ability and stability of recombinant human adenoviruses have been shown to be limited to approximately 105% of the original genome [8, 24]. The FAV-10 genome is some 10 kb larger than the HAV genome. Based on these criteria, it is possible that a foreign insert of up to 2.3 kb could be integrated into the FAV-10 genome without requiring deletion of any part of genome. The VP2 expression cassette consisting of FAV-10 MLP/LS VP2 coding region and SV40 polyA region totaled 2.1 kb and it was decided to insert this expression cassette without deleting any of the FAV-10 genome.

The *Nde* I fragment 3 (90.8 to 100 map units) was chosen as a potential region for the possible insertion of the expression cassetle. Sequencing of this fragment revealed a number of unidentified open reading frames and a *Bam* HI restriction

site 243 bp from the right hand end of the genome. Sequence analysis suggested that this site was in a transcriptionally active region. A *Not* I site was inserted into the *Bam* HI site as *Not* I does not cleave the FAV-10 genome at any other site or the expression cassette and a recombinant virus was generated which contained a unique *Not* I site. Inoculation of SPF birds, re-isolation in CK monolayers and analysis by restriction digesion demonstrated that this recombinant was stable and viable in vitro and in vivo. It was concluded that this region of FAV-10 was not transcriptionally active and therefore ideal for the insertion of an expression cassette. The MLP/SL-VP2-polyA expression cassette was inserted into the *Not* I site of *Nde* I fragment 3, and the resulting fragment was used to generate a recombinant FAV-10 that contained the IBDV VP2 gene (rFAV-10/VP2) by homologous recombination. The VP2 antigen expressed by the recombinant was immunogenic as tested by its reactivity with the conformational dependent neutralising monoclonal antibody 17–82.

Vaccination of three week old SPF birds i.v. with 10^7 pfu of the recombinant produced an antibody response to VP2 of IBDV. This experiment clearly demonstrated that a recombinant FAV expressing VP2 was capable of inducing an immune response in SPF chickens to VP2.

The next experiment examined whether the induction of this response to VP2 was sufficient to protect birds from challenge with IBDV. Vaccination with rFAV-10/VP2 protected birds from challenge with live IBDV V877 as no IBDV was detected in bursal homogenates by antigen ELISA and immunoperoxidase staining of tissue section. Thus, the recombinant stimulated an immune response that was able to prevent IBDV from replicating in the bursa. All birds that were naive, that is unvaccinated or FAV-10 vaccinated, were not protected from challenge with IBDV V877. IBDV antigen was detected by the antigen ELISA on bursa homogenates and by immunohistology for the presence of IBDV in tissue sections. Therefore, in these naive birds, there were deficient antibody levels to provide protection against IBDV challenge. Fahey et al. [17] demonstrated that there was a direct correlation between maternal serum antibody levels and protection with titres of 400 or greater providing complete protection against IBDV. Further, it is now known that serum IBDV antibody levels correlate well with serum neutralising antibody levels, implying that the majority of the serum neutralising antibodies are directed against VP2 (Ignjatovic and Sapats, pers. comm.). The fact that the rFAV-10/VP2 reported in this study induced pre-challenge antibody titres averaging greater than 4 000 directed specifically against VP2 (the major protective antigen) demonstrated the use of this recombinant virus as an effective vaccine vector, not only against IBDV, but potentially against other poultry pathogens.

Vaccination with the recombinant via a number of routes was examined. Although the systemic routes used in this report are not what are commercially acceptable for delivery of vaccines, the data demonstrated that a recombinant adenovirus vector could potentially be developed for commercial application. Bird s vaccinated systemically (i.v., i.p., i.m., s.c.) developed antibodies to FAV-10 and VP2. Birds vaccinated by instillation of the conjunctival sac, developed

antibodies to FAV-10 later than that seen for vaccination by systemic routes, but failed to produce any detectable antibodies to VP2. Vaccination of chickens via the conjunctival sac is essentially oral administration, as fluid delivered drains into the naso-pharyngeal cavity and is swallowed by the bird. Virus re-isolation experiments showed that recombinant virus could be found in the caecal tonsils but was less easily isolated from kidney, liver or spleen (2–3 passages in CK cells) compared with systemically vaccinated birds (1 passage in CK cells). Therefore, FAV-10 replication in natural infection is mainly in the gut and has limited systemic spread. The inefficiency of FAV-10 in crossing the gut is probably due to differences in the cell receptor binding regions present on the fiber. It has been shown for some human adenoviruses that a few amino acid changes in the fiber can determine tissue tropism [1]. In HAV type 5, a region has been identified which binds to the MHCI alpha 2 domain found on the surface of epithelial and β-lymphoblastoid cells [28]. No receptor binding regions have been identified for any FAV fiber. It has been demonstrated with FAV serotype 8 [43], that virulence is function of the short fiber, and that swapping the short fiber from a hypervirulent to a mildly virulent strain resulted in the corresponding phenotypic change. Unlike serotype 8, which has a short fiber [43], with the long fiber not yet located, serotype 10 has both a short [48] and long fiber (unpubl.) in similar positions on the genome relative to that of serotype 1 (CELO) [11]. Further, it appears that the long fiber of FAV-10 is of similar amino acid sequence length to the short fiber (unpubl.) It is likely that the lack of virulence of FAV-10, presumably determined by the short fiber, and correlated with tissue tropism, explains why vaccination via conjunctiva faild to induce a serum VP2 antibody response sufficient to protect birds from challenge. However, when delivered by systemic inoculation, FAV-10 has the ability to bind and infect cells of other organs and subsequently replicate.

This report represents the first construction of a fowl adenovirus viral vector, and demonstrated that it could be used to express an antigen (IBDV VP2) that could produce a protective immune response in a challenge model. The sequence of the VP2 gene from 002/73 is identical to that of the challenge strain V877 (Ignjatovic and Sapats, personal communication). V877 is an intermediate virulent classical strain, which is being used as a vaccine against the very virulent strains of Europe and Asia. It will be of great interest to further test this recombinant and examine whether it can protect commercial broilers against all very virulent IBDV strains and the antigenic variant IBDV strains of the USA.

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