Arch Virol (1991) 120: 193–205

Archives of Virology



# A recombinant fowlpox virus that expresses the VP2 antigen of infectious bursal disease virus induces protection against mortality caused by the virus

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Accepted February 28, 1991

**Summary.** The coding sequences of VP2 from a virulent strain, 52/70, of infectious bursal disease virus (IBDV) were excised from a cDNA clone and inserted into a fowlpox plasmid insertion vector. The resulting plasmid, pIBD 1, was used to construct a recombinant fowlpox virus, fpIBD 1, which expressed VP2 as a β-galactosidase fusion protein. Chickens vaccinated with fpIBD 1 at 1 and 14 days of age, were challenged at 28 days with either IBDV strain 52/70 or the highly virulent strain CS89. These chickens were protected against mortality, but not against damage to the bursa of Fabricius. The protection achieved by the use of fpIBD 1 shows that VP2 is a host protective antigen.

## Introduction

Infectious bursal disease virus (IBDV) causes an acute disease, infectious bursal disease (IBD), in chickens usually between two and eight weeks of age. IBDV also infects chickens under two weeks of age producing few clinical signs but causing a severe immunodepression of the B cell response by destruction of lymphocytes in the bursa of Fabricius [for review, see 21]. The recent appearance of new, highly virulent strains of IBDV in Europe and the U.K. [12], has led to IBDV becoming a major problem in the poultry industry and the need for development of new vaccines has become critical [10]. One possible approach is the development of recombinant vaccines based on, for example, fowlpox virus (FPV) as a vector. That such an approach is possible in principle is indicated by the recent reports [8, 9, 33] that the F or HN genes of Newcastle

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disease virus (NDV) expressed in FPV give excellent protection against NDV challenge.

IBDV is a member of the family *Birnaviridae*, genus *Birnavirus,* naked icosahedral viral particles with a genome of two segments of double-stranded RNA [15, 18, 24]. The properties of the viral proteins have been reviewed previously [18]. Comparisons of the sequences of the structural proteins encoded by segment A of both four [5] and two [19] IBDV strains have been reported. Thus the data necessary to begin construction of recombinant viruses is available.

VP 2 a/b (henceforward referred to as VP 2) and VP 3 have already been shown to be protective antigens. VP 3, purified by electro-elution from SDS-PAGE gels and injected into young chickens, induced neutralising antibodies which protected passively against infection [16]. This was confirmed by the report [1] that cloned VP 3, expressed as a β-galactosidase fusion protein in *E. coli*, could be used to immunise chickens. One of these chickens produced low levels of neutralising antibodies which protected passively. Similar preparations of VP 2 did not induce neutralising antibodies. It was then found that neutralising monoclonal antibodies (MAbs), raised against virus particles, recognise VP 2 but only under non-denaturing conditions [2, 3, 6]. VP 2 has been separated from the other viral proteins by passaging preparations of viral particles through an adsorption column containing a MAb specific for VP 3 [17]. This preparation of non-denatured VP 2 elicited virus neutralising antibodies in chickens which also protected passively. These results indicate that VP 2 is the major antigen responsible for protection against IBDV.

In this paper we report the expression of cloned VP 2 in FPV and show that this recombinant produced VP 2 in a conformation recognisable by an IBDV convalescent serum. It was able to protect susceptible chickens against mortality resulting from challenge with IBDV.

## Materials and methods

### *Virus strains*

Two virulent IBDV strains, 52/70 [11] and a recently isolated very virulent strain, CS 89, were supplied by the Central Veterinary Laboratory, Weybridge, U.K. The IBDV vaccine strain, PBG 98, was supplied by W. Baxendale, Intervet, Houghton, U.K. The FPV strain, FP 9, was a plaque-purified isolate of a tissue-culture adapted strain of FPV HP 444 [22]. The β-galactosidase FPV recombinant virus, fpEFL 2, has been described previously [8].

### *Construction of the plasmid insertion vector*

pEFL 18 was created by digestion of the FP insertion vector pEFL 2 [8] with SphI and NcoI followed by repair and re-ligation. The coding sequences of VP 2 and IBDV strain 52/70 were excised with PvuI and HincII from a clone (F 10) of this virus which was made by insertion of cDNA into the PstI site of pBR 322 [4, 5]. This digest was ligated to pEFL 18 DNA which had been digested with HindIII and repaired with DNA polymerase I (Klenow). The resulting plasmid was designated pIBD 1.

*Production and analysis of recombinants*

The recombination of the VP2 sequences of IBDV from pIBD1 into FPV FP9, the identification of recombinant plaques by detection of β-galactosidase activity, and the purification of the recombinant, fpIBD1, were carried out by methods described previously [8]. The DNA of fpIBD1 was analysed after its extraction from the recombinant and parent viruses by probing with labelled FPV DNA [7] and with the labelled insert of the original IBDV clone.

FPV-infected chick embryo fibroblasts (CEFs), IBDV PBG 98-infected CEFs or IBDV 52/70 particles prepared as described previously [5] were boiled for 2 min in SDS-PAGE sample buffer [20] containing 2% w/v SDS and 0.1 M dithiothreitol. Samples were electrophoresed on 10% polyacrylamide gels. Western blotting and antibody probing were carried out as described previously [29] except that a phosphatase conjugated rabbit anti-chicken antibody (Zymed) was used to detect chicken antibody.

Immunoprecipitation was carried out by a method based on one described previously [32].

*Protection experiments*

Two experiments were performed to assess the ability of the FPV-recombinants to protect against challenge with virulent IBDV strains. In the first, chicks were challenged with the classical 52/70 strain, the strain used to construct fpIBD1, and in the second with a recent very virulent isolate (CS 89). Groups of one-day-old Houghton Laboratory specified pathogen-free Rhode Island red chicks were inoculated via the wing-web with either the FPV/IBDV recombinant strain, fpIBD1 or a β-galactosidase/FPV recombinant, fpEFL2. Each chicken received $1 \times 10^7$ pfu of FPV in 50 µl of 10 mM Tris buffer, pH 9.0. Chicks were revaccinated 14 days later with $1 \times 10^7$ pfu (experiment 1) or $1 \times 10^8$ pfu (experiment 2) of FPV. At 28 days of age these groups, together with an unvaccinated group, were challenged intranasally (0.1 ml) with either the 52/70 strain of IBDV (experiment 1) or the CS 89 strain (experiment 2) as a bursal homogenate from infected chickens. In both experiments, a fourth group served as unvaccinated, unchallenged controls. The number of chicks in each group is shown in Table 1.

Chickens were killed 7 or 21 days post infection (p.i.) and necropsied. Due to the high mortality in two of the groups in experiment 2, none of the chickens in those groups were killed at day 7. The ratios of bursa of Fabricius to body weight were calculated. Bursal lesion scores were assessed on haematoxylin and eosin stained sections of bursae, as described previously [26]. Briefly: 0, no damage; 1, mild necrosis in isolated follicles; 2, moderate generalised lymphocyte depletion or isolated follicles with severe depletion, 3, over 50% of follicles with severe lymphocyte depletion; 4, only outline of follicles remaining with few lymphocytes and increase in connective tissue, cysts, and thickened, corrugated epithelium; 5, loss of all follicular architecture with fibroplasia.

*Serology*

Sera collected from the chickens immediately prior to, and 21 days after, challenge were tested for IBDV antibodies by an ELISA [31] using the PBG 98 IBDV strain grown in Vero cells as the antigen and a rabbit anti-chicken IgG phosphatase conjugate (Zymed) diluted 1:4000 to detect bound antibody. Sera were also tested for FPV antibodies by ELISA [23].

The sera were tested for IBDV neutralising antibodies against 100 $TCID_{50}$ of the PBG 98 IBDV strain using CEFs as indicator cells. Titres were calculated by the method of Reed and Muench [28].

## Results

### *Construction of the recombinant*

The PvuI/HincII fragment of the IBDV clone F 10 contained the sequences of segment A of IBDV strain 52/70 from 125 bp to 1727 bp, which covers amino acids 1 to 532 of the polyprotein encoded by segment A and the whole of the coding region of VP 2 [5]. The sequence probably also includes 29 to 80 amino acids of VP 4, the cleavage site between VP 2 and VP 4 being unknown. Insertion in the correct orientation produced the plasmid pIBD 1 and resulted in the initiation codon of VP 2 being the first ATG after the mRNA start site of the p 7.5 vaccinia promoter (checked by plasmid sequencing). The construct was also designed so that the ORF would be maintained between the VP 2 and the β-galactosidase genes such that a fusion protein would be produced (Fig. 1).

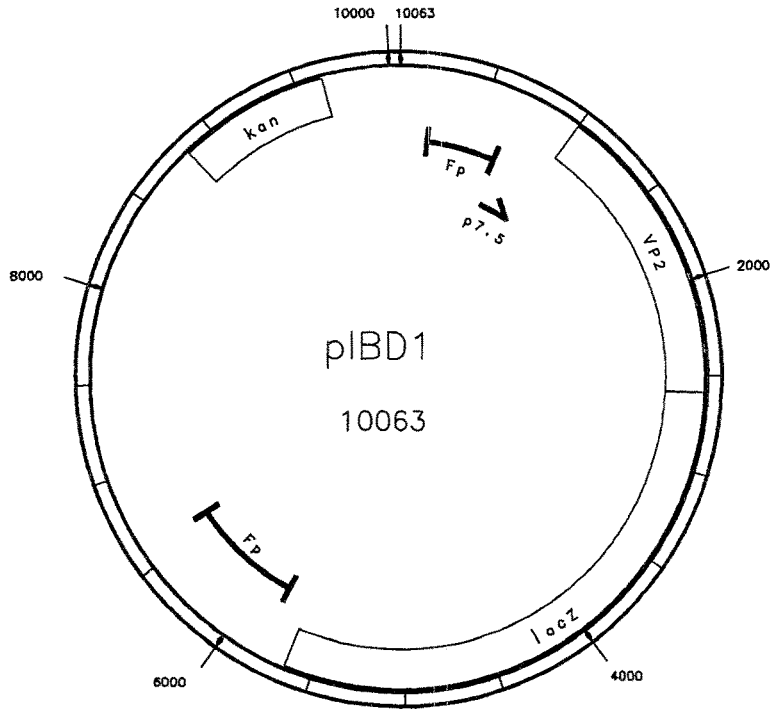
The plasmid pIBD 1 was then recombined into the FP 9 strain of FPV, recombinant viruses being identified as blue plaques. These viruses were plaque purified three times. Analysis of the DNA from one of these recombinants, termed fpIBD 1, showed that the VP 2 sequences were present and that recombination had taken place in both terminal inverted repeats of the FPV [4].

### *Analysis of the proteins of the recombinant*

Western blot analysis of the proteins of fpIBD 1, fpEFL 2 (a β-galactosidase FPV recombinant) and FP 9 (the parental FPV), probed with a mouse anti-β-galactosidase serum indicated that fpIBD 1 contained a β-galactosidase fusion protein whose size was close to the expected size of the fusion protein encoded by pIBD 1, estimated at 172 kDa (data not shown). Western blots were also probed with an IBDV 52/70 convalescent antiserum. No reaction was observed between this serum and either the fusion protein or the VP 2 protein of 52/70, suggesting that this serum only recognises conformational epitopes of VP 2. This IBDV convalescent serum, together with an anti β-galactosidase serum and normal chicken serum, were then used for immunoprecipitation of the viral proteins of fpIBD 1 (Fig. 2 A) and of fpEFL 2 (Fig. 2 B). The normal chicken serum did not precipitate any proteins (Fig. 2, track 1) while the anti-β-galactosidase serum specifically precipitated the β-galactosidase protein of fpEFL 2 (Fig. 2 B, track 3) and the fusion protein of fpIBD 1 (Fig. 2 A, track 3). The convalescent serum specifically immunoprecipitated the fusion protein of fpIBD 1 but not any proteins of fpEFL 2 (Fig. 2, track 2).

### *Protection experiments*

The results summarised in Table 1 show that the chicks vaccinated with the IBDV-FPV recombinant (fpIBD 1) were protected against the mortality resulting from challenge with virulent IBDV observed in the unvaccinated challenge group and the group vaccinated with FPV expressing β-galactosidase. This was particularly noticeable in the second experiment, challenged with a




```
          --------------- 7.5kD Promoter ---------------------
.........ACCCATAAATAATAAATACAATAATTAATTTCTCGTAAAAGTAGAAAATATA
              --LATE----
-------------------------------------------------------->      <-
TTCTAATTTATTGCACGGTAAGGAAGTAGAATCATAAAGAACGTGACGGATCGAAGCTCG
          -EARLY-

--------------------------- VP2 ----------------------------
CAGCGATGACAAACCTGCAAGATCAAACCCAACAGATTGTTCCGTTCATA..........
      M  T  N  L  Q  D  Q  T  Q  Q  I  V  P  F  I

--------------->          <----- Lac Z (B-galactosidase) --
...AATCCCGTAGTTAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAA...
    N  P  V  V  S  L  A  L  A  V  V  L  Q  R  R  D  W  E
```

B

**Fig. 1.** A diagram showing pIBD 1, the fowlpox plasmid insertion vector which was used to insert the VP2 gene of IBDV into the fowlpox genome. **A** A plasmid map of pIBD 1 on which are marked the non-essential fowlpox sequences (*Fp*), the vaccina 7.5 kDa promoter (*p 7.5*), the IBDV VP2 gene (*VP 2*), and the β-galactosidase gene (*lacZ*). The kanamycin resistance gene, which was used to select for the plasmid, is also shown (*kan*). The figures are the number of base pairs. **B** The nucleotide and amino acid sequences at either end of the IBDV VP2 gene. The early and late mRNA start sites of the vaccinia promoter [13], are also marked

very virulent IBDV strain, where 74% and 70% mortality were recorded in the groups not given the IBDV recombinant.

In each experiment, mortality occurred within 5 days of challenge. During this time chicks were observed daily for clinical signs of IBDV infection, such as ruffled feathers, prostration and diarrhoea. In experiment 1 such signs were observed in two of the chicks vaccinated with fpIBDV, whereas all the chicks

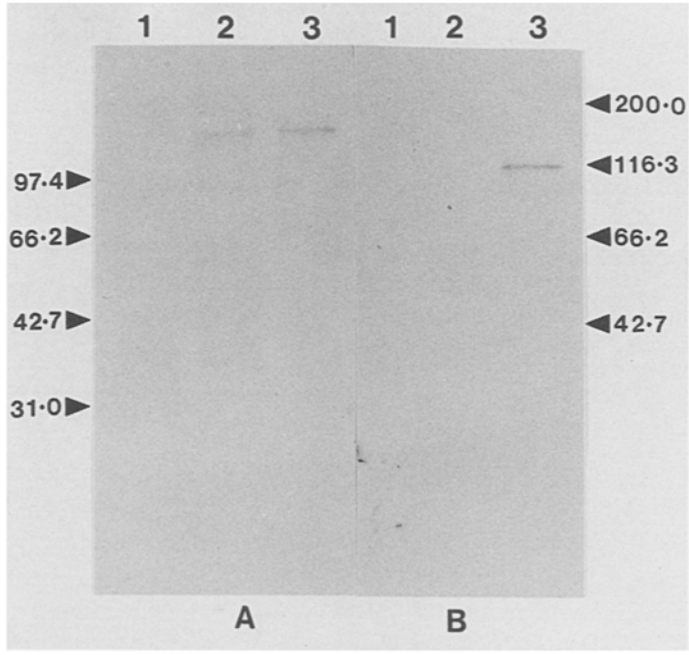



**Fig. 2.** Autoradiograph of labelled proteins of fpIBD 1 (**A**) and fpEFL 2 (**B**) immunoprecipitated with various sera. *1* Normal chicken serum, *2* IBDV 52/70 convalescent serum, and *3* mouse anti-β-galactosidase serum. Markers are low and high molecular weight SDS-PAGE markers (Biorad)

**Table 1.** Mortality following IBDV challenge of chicks vaccinated at 1 and 14 days of age with an FPV/IBDV recombinant expressing VP 2 (fpIBD 1), or an FPV/β-galactosidase recombinant (fpEFL 2)

| Vaccination | Mortality following challenge at 28-day-old with | | | |
|---|---|---|---|---|
| | IBDV strain 52/70 | | IBDV strain CS 89 | |
| | no. dead | % | no. dead | % |
| fpIBD 1 | 0/23[a] | 0 | 1/20 | 5 |
| fpEFL 2 | 5/22 | 23 | 14/19 | 74 |
| Not vaccinated/challenged | 4/17 | 24 | 14/20 | 70 |
| Not vaccinated/not challenged | 0/6 | 0 | 0/14 | 0 |

[a] Number dead/number in group

in the other two groups showed clinical signs from 2 days post challenge. In experiment 2, one chick in the fpIBDV-vaccinated group was prostrate on day 2 post challenge; this chick died on day 3. The other chicks in the group remained

lively throughout. All chicks in the other two challenge control groups appeared depressed from 2 days post challenge.

Bursal to body weight ratios are shown in Table 2. At 7 days post challenge the mean values and ranges indicate that, in both experiments, some of the chicks were partially protected against challenge, but by 21–26 days post challenge, severe bursal atrophy was evident. The bursal lesion scores (Table 3) indicate that, although a moderate amount of damage had occurred in the fpIBD 1-vaccinated group, their scores were generally lower than in the challenge controls and the group vaccinated with fpEFL 2.

### *Serology*

No IBDV antibodies were detected by ELISA in sera collected prior to challenge in either experiment and tested at a 1:50 dilution. This result was corroborated in experiment 1 by serum neutralisation tests in which no IBDV neutralising antibodies were detected in the pre-challenge sera tested at a 1:8 dilution. Following challenge, in both experiments each challenged group produced IBDV-specific antibodies as measured by an ELISA. The sera were also tested against protein preparations of IBDV strains 52/70 and PBG 98 in Western blots, but no reaction to VP 2 was observed.

Sera collected in experiment 2 at the time of IBDV challenge were also examined by ELISA for FPV antibodies. No antibodies were detected in the challenge control and uninoculated groups. In the group given fpEFL 2, the mean ELISA titre was 1:700 (range < 1:100–1:1600) and in the group given fpIBD 1 1:300 (range 1:100–1:800).

## Discussion

The recombinant virus, fpIBD 1, protected against mortality resulting from challenge with two virulent IBDV strains, 52/70 and CS 89, the latter causing at least 70% mortality in unvaccinated chickens. This protection did not result from any effect of vaccination with FPV or from the presence of the β-galactosidase enzyme since appreciable mortality was recorded in chicks challenged after vaccination with the FPV/β-galactosidase recombinant. However, protection afforded by fpIBD 1 was only partial and the majority of vaccinates had severe lymphocyte depletion following challenge. Thus, the FPV/IBDV recombinant, in its present form, protected against mortality but not against clinical disease.

Previous work on the protective ability of individual IBDV proteins relied on demonstrating that antibodies raised to proteins, such as VP 2, could protect chickens passively [1, 16, 17]. One problem with this approach is that such proteins might be contaminated with other proteins or with whole virus particles. In the present work, a clone of VP 2, free from other viral proteins except the coding sequences of a small part of VP 4, was expressed in FPV and shown to induce active protection. This showed that VP 2 must be a host protective

**Table 2.** The effect on bursal/body weight ratios of IBDV challenge of 28-day-old chicks previously vaccinated at 1 and 14 days with an FPV/IBDV recombinant (fpIBD 1) or an FPV/β-galactosidase recombinant (fpEFL 2)

| Time post challenge | Bursal/body weight ratios following challenge with | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | IBDV strain 52/70 | | | | IBDV strain CS 89 | | | |
| | fpIBD 1 | fpEFL 2 | challenge control[a] | uninoculated control[b] | fpIBD 1 | fpEFL 2 | challenge control | uninoculated control |
| 7 days | | | | | | | | |
| No. of chicks | 13 | 8 | 3 | 6 | 9 | – | – | 5 |
| Mean | 34[c] | 20 | 18 | 47 | 23 | – | – | 51 |
| Range | 19–73 | 16–23 | 17–20 | 33–56 | 19–36 | – | – | 48–63 |
| 21 days[d] | | | | | | | | |
| No. of chicks | 10 | 10 | 10 | ND | 10 | 5 | 6 | 9 |
| Mean | 18 | 10 | 11 | ND | 7 | 5 | 5 | 49 |
| Range | 8–43 | 7–16 | 9–12 | ND | 3–10 | 4–7 | 4–7 | 9–67 |

[a] Not vaccinated, but challenged
[b] Not vaccinated, not challenged
[c] % bursal: bodyweight ratio × 100
[d] 26 days post challenge in experiment 2
*ND* Not done
– Not done due to high mortality before day 7

**Table 3.** Bursal lesion scores in chicks vaccinated at 1 and 14 days with an FPV/IBDV recombinant (fpIBD 1) or an FPV/β-galactosidase recombinant (fpEFL 2) and challenged with IBDV at 28 days

| Time post challenge | Number of chicks with bursal lesion scores following challenge with | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | IBDV strain 52/70 | | | | IBDV strain CS 89 | | | |
| | fpIBD 1 | fpEFL 2 | challenge control[a] | uninoculated control[b] | fpIBD 1 | fpEFL 2 | challenge control | uninoculated control |
| 7 days | | | | | | | | |
| Score[c] 0 | 2 | 0 | 0 | 6 | 0 | – | – | 5 |
| 1–2 | 1 | 0 | 0 | 0 | 1 | – | – | 0 |
| 3 | 8 | 0 | 0 | 0 | 4 | – | – | 0 |
| 4 | 2 | 8 | 3 | 0 | 4 | – | – | 0 |
| 5 | 0 | 0 | 0 | 0 | 0 | – | – | 0 |
| 21 days[d] | | | | | | | | |
| Score 0 | 1 | 0 | 0 | ND | 0 | 0 | 0 | 9 |
| 1–2 | 3 | 0 | 0 | ND | 0 | 0 | 0 | 0 |
| 3 | 3 | 0 | 2 | ND | 7 | 0 | 0 | 0 |
| 4 | 3 | 3 | 4 | ND | 2 | 1 | 3 | 0 |
| 5 | 0 | 7 | 4 | ND | 1 | 4 | 3 | 0 |

[a] Not vaccinated, but challenged
[b] Not vaccinated, not challenged
[c] Score as in [25]
[d] 26 days post challenge in experiment 2
*ND* Not done
– Not done due to high mortality before day 7

antigen. It is unlikely that the VP4 sequences had any role in protection since VP4 is only a minor component of viral capsids [25].

Protection against mortality was achieved despite the absence of detectable circulating IBDV antibodies, the presence of which correlates with protection [14]. Failure to stimulate high levels of humoral antibody in chickens to genes inserted in FPV has been shown previously [8, 9], using the present FPV vector to express the F or HN genes of NDV. In that work, despite the low levels of humoral antibody to the inserted genes, chickens were protected against challenge with virulent NDV. It is not known whether T cell mediated immunity to IBDV plays any role in protection. However, the demonstration of protection against mortality in the absence of humoral antibody suggests that cell mediated immunity is involved. This aspect merits further investigation.

There are several possible reasons for the failure of fpIBD 1 to stimulate complete protection. One possibility is that only low levels of the fusion protein are produced by this recombinant. This could be due to the use of the heterologous vaccinia promoter and it may be possible to obtain higher levels of expression using a FPV promoter. A second possibility is that the highly attenuated FPV strain, FP9, did not replicate well in the chickens. However, FPV antibodies were produced, suggesting some replication had taken place. A recombinant constructed with a less attenuated FPV strain might give better replication, thereby improving the degree of protection. Such a strain, P × 4.1, was used previously to construct an FPV/NDV recombinant expressing the F gene. This stimulated complete protection against, but only a low antibody response to, NDV [8]. Another approach might be to administer the FPV recombinant intranasally with a view to stimulating local immunity. Such an approach has been found to be successful in the case of a vaccinia recombinant expressing the influenza haemagglutinin gene [30], where intranasal vaccination of mice protected both the nose and lung against homologous challenge, whilst intradermal vaccination protected only the lung. However, since the role of local immunity in the respiratory tract in relation to IBD is not clear, this approach might not be successful here.

Finally, complete protection may not have been afforded by fpIBD 1 because the VP2 antigen was not present in the correct conformation, possibly due to expression of VP2 as a fusion protein with some of VP4 and β-galactosidase. It has been shown previously [27] that a vaccinia-foot and mouth disease virus (FMDV) recombinant expressing a β-galactosidase-FMDV fusion generated a poor antibody response to FMDV, suggesting that β-galactosidase fusions may not be good immunogens. Alternatively, the correct conformation of VP2 might require the presence of other viral proteins, although purified, non-denatured VP2 induces neutralising antibodies [17]. It may be that there are important protective epitopes on the other viral proteins and in particular on VP3. Current work is addressing these questions.

Conventional IBDV vaccines have, until recently, protected completely against IBD. This was achieved by vaccinating breeding hens and relying on

maternally derived antibodies to protect the progeny. One disadvantage of such an approach is the difficulty in then using conventional live-attenuated vaccines in young chicks to supplement immunity. Recently, highly virulent IBDV strains have caused severe disease problems because they can break through maternally derived immunity [10, 12]. It seems likely that a fowlpox-based recombinant IBDV vaccine would not be affected by IBDV maternal antibodies and could be used to vaccinate chicks with maternally derived immunity, provided that a vaccine which protected against disease, i.e., damage to the B lymphocytes, as well as against mortality can be developed. Such work is the object of future studies.

## Acknowledgements

We would like to thank P. F. Green and C. Mason for technical assistance, the Histology department at Compton Laboratory for preparation of samples, and S. Hodgson for preparation of the photographs. We are also grateful to W. Baxendale for providing the PBG 98 strain of IBDV. C. D. Bayliss was funded by an AFRC research studentship.

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Received January 7, 1991