Infectious bursal disease virus (IBDV) is a pathogen of major economic importance to the world's poultry industries (reviewed in Kibenge et al., 1988). It causes severe immunodeficiency in young chickens by destroying the precursors of antibody-producing B cells in the bursa of Fabricius (Nick et al., 1976). IBDV is a member of the Birnaviridae family and its genome consists of two segments of dsRNA (Dobos et al., 1979). The smaller segment B encodes VP1, the putative dsRNA polymerase (Azad et al., 1985; Morgan et al., 1988; Müller & Nitschke, 1987; Spies et al., 1987), whereas the larger segment A encodes a precursor polyprotein that is processed into mature VP2, VP3 and VP4 (Hudson et al., 1986). VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of IBDV, and contains the antigenic regions responsible for the induction of neutralizing antibodies (Azad et al., 1987; Becht et al., 1988; Fahey et al., 1989; Heine et al., 1991). VP3 contains the structural group-specific antigens (Becht et al., 1988), whereas VP4 is a protease involved in the processing of the precursor protein (Azad et al., 1987; Jagadish et al., 1988).

The principal method of controlling IBDV infection in young chickens is by vaccination with an avirulent strain of IBDV or by the transfer of high levels of maternal antibody induced by the administration of live and killed IBDV vaccines to breeder hens (Wyeth & Cullen, 1976). In recent years, virulent strains of IBDV (Delaware and GLS variants) have been isolated from vaccinated flocks on the Delmarva peninsula in the U.S.A. (Rosenberger et al., 1985; Snyder et al., 1988b, c). These variant strains are antigenically different from the classic strains of IBDV isolated before 1985, and lack epitope(s) defined by neutralizing monoclonal antibodies (MAbs) B69 and R63. As a result of this antigenic shift, chickens vaccinated with classic strains of IBDV have only partial protection against variant virus infection (Snyder et al., 1992).

In an effort to develop a recombinant vaccine for IBDV in chickens, a number of investigators have expressed the VP2 antigen of IBDV in yeast (Macreadie et al., 1990) as well as in a recombinant fowlpox virus (Bayliss et al., 1991). The yeast-derived antigen afforded only passive protection in chickens against IBDV infection, whereas the fowlpox virus-vector antigen afforded protection against mortality, but not against damage to the bursa of Fabricius. In this communication, we describe the synthesis of the structural proteins of virulent IBDV strain GLS-5 in a baculovirus expression system and evaluation of the protective properties of the recombinant proteins in chickens.

The virulent GLS-5 strain of IBDV was isolated from the bursae of infected specific-pathogen-free (SPF) chickens as described by Snyder et al. (1988a). The genomic RNA was extracted from purified virus (bursa-derived) and was subjected to 1% low melting tem-
temperature agarose gel electrophoresis. The large RNA segment (approx. 3.2 kb) was recovered from the gel and used for cloning. Complementary DNA segments of IBDV were synthesized by the method of Gubler & Hoffman (1983), using a cDNA synthesis kit (Promega), and specific primers complementary to the 3' end of the VP2 gene sequence (Hudson et al., 1986) or to the 3' end of the large genomic segment (Bayliss et al., 1990). The cDNA produced was ligated with EcoRI adaptors and inserted into the unique EcoRI site of the pGEM-7Zf(+) vector (Promega). Four overlapping cDNA clones, pGLS-1, -2, -3 and -4, were selected which spanned the whole coding region of the genome segment A of IBDV strain GLS-5. The complete nucleotide sequence of these clones will be described elsewhere. A full-length cDNA clone, pGLS-5, was constructed by digesting DNA of the above plasmids with appropriate restriction enzymes, and ligating the cDNA fragments (see Fig. 1).

To insert IBDV structural genes into the baculovirus transfer vector pBlueBacI (Invitrogen), plasmid pCRGLS was constructed as follows. Oligonucleotides containing the NheI site and having the sequence CGATCGCTAGCGATGACAAAC (upstream primer) and AGACTCCCAGCTAGCTCATTCAA (downstream primer) were synthesized and used to amplify the IBDV segment from plasmid pGLS-5 by PCR. Amplification by PCR was carried out according to the protocol of the supplier (Perkin Elmer-Cetus), except that the final concentration of each dNTP was 400 µM. Thirty cycles (95 °C for 1.5 min, 37 °C for 2 min, and 72 °C for 5 min) were employed for the amplification of the above segment. Approximately 150 ng of amplified DNA product was ligated into the vector pCR1000, supplied in a PCR cloning kit (Invitrogen). A recombinant plasmid, pCRGLS, encoding the structural protein genes of IBDV was selected. The integrity of the pGLS-5 and pCRGLS constructs was tested by in vitro transcription (using T7 RNA polymerase) and translation. In both cases, the expected IBDV-specific products were observed after immunoprecipitation with polyclonal antisera and gel electrophoresis (results not shown). A 3.05 kb NheI fragment containing the large open reading frame sequence was excised from plasmid pCRGLS and inserted at the unique NheI site of pBlueBacI to form pGLSBacI. Recombinant virus vIBD-7 was obtained by cotransfecting pGLSBacI and Autographa californica nuclear polyhedrosis virus (AcNPV) DNA into Spodoptera frugiperda (Sf9) cells, and plaque-purifying the

Table 1. Reactivities of MAbs with various IBDVs and baculovirus-expressed proteins

<table>
<thead>
<tr>
<th>MAb</th>
<th>Eliciting IBDV strain</th>
<th>VN tests*</th>
<th>AC-ELISA†</th>
<th>Expessed proteins‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IBDV</td>
<td>GLS-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Classic</td>
<td>E/Del</td>
<td></td>
</tr>
<tr>
<td>B29</td>
<td>STC (Classic)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>GLS-5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>GLS-5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>179</td>
<td>GLS-5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B69</td>
<td>STC (Classic)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BK9</td>
<td>E/Del</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>57</td>
<td>GLS-5</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* A positive result indicates that the MAb neutralized IBDV in VN tests.
† IBDV antigens derived from bursal homogenates (1:40 dilutions) were evaluated with various MAbs in AC-ELISA (Snyte et al., 1992). IBDV strains used were STC (Classic), Delaware variant E (E/Del) and GLS-5.
‡ Expressed proteins derived from the recombinant virus (vIBD-7) or the wild-type baculovirus (AcNPV)-infected cell lysates (1:150 dilutions) were evaluated with various MAbs in AC-ELISA.
recombinant virus as described (Summers & Smith, 1987; Vialard et al., 1990). AcNPV and recombinant virus stocks were propagated in Sf9 cells using Hink's TNM-FH medium (JRH Biosciences) supplemented with 10% fetal calf serum in either tissue culture flasks or in spinner flasks at 28 °C (Summers & Smith, 1987). Cells grown in spinner flasks contained 0.1% polyethylene polypropylene glycol (‘Pluronic F-68’, JRH Biosciences) in addition to the above supplemented medium.

In order to characterize the IBDV proteins expressed in insect cells, Sf9 cells were infected with either the wild-type baculovirus or the recombinant virus (vIBD-7) and harvested at 72 h post-infection (p.i.). The cells were washed three times in PBS and lysed in a buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.5 M-NaCl, 0.5 M-Tris–HCl pH 7.2, 0.01 M-EDTA and 0.1% SDS. A portion of the cell lysate was immunoprecipitated with GLS-5 strain-specific MAbs 8, 10, 57 and 179 (see Table 1) and a polyclonal rabbit anti-IBDV serum. Immunoprecipitated proteins were resolved on a 12.5% SDS–polyacrylamide gel and detected immunologically following Western blotting with polyvalent chicken anti-IBDV serum (Becth et al., 1988). The results are shown in Fig. 2. Cells infected with recombinant virus expressing IBDV proteins were correctly processed (lane 5) and comigrated with the marker proteins VPX/VP2, VP3 and VP4, derived from strain GLS-5-infected chicken embryo fibroblast (CEF) lysates (lane 1; the mobility of the VP2 protein is slightly different as the lane was overloaded). A protein band between VP3 and VP4 was often seen and is derived from VP3 (Fahey et al., 1989). In addition, MAbs 8, 10, 57 and 179 immunoprecipitated IBDV structural proteins VPX/VP2 and VP4 (lanes 6 to 9, respectively). IBDV-specific proteins could be detected in neither uninfected CEFs, uninfected Sf9 cells nor AcNPV-infected Sf9 cells (lanes 2 to 4, respectively). In previous studies, the precursor protein of IBDV was correctly processed by VP4 when the entire cloned IBDV segment A was expressed in Escherichia coli (Azad et al., 1987; Jagadish et al., 1988) and in yeast (Macreadie et al., 1990). Similarly, bacterial expression of the large genomic segment of infectious pancreatic necrosis virus (IPNV), a prototype of the Birnaviridae family, also yielded IPNV proteins that were cleaved by the non-structural protein (Manning et al., 1990; Manning & Leong, 1990).

To characterize further the expressed IBDV proteins, the cell lysates were analysed with a panel of IBDV-specific MAbs in an antigen-capture ELISA (AC-ELISA) as previously described (Snyder et al., 1992). The panel of MAbs used in AC-ELISA is shown in Table 1. The results indicate that the baculovirus-expressed IBDV antigens bind to MAbs 57, 10, 179 and 8, suggesting that the expressed IBDV antigens are GLS-5 strain-specific. Since these neutralizing MAbs are conformation-dependent, the expressed IBDV antigens appear to form conformationally correct structures.

To prepare an inoculum for immunization, Sf9 cells (in a 1 litre flask) were infected with either a wild-type or a recombinant baculovirus at a multiplicity of 5 p.f.u./cell, and then incubated for 3 to 4 days. The infected cells were recovered by centrifugation, washed twice with PBS, and resuspended in 10 ml of PBS. The cell suspension was sonicated three times for 1 min, at 2 min intervals. A portion of each cell lysate was tested by AC-
Table 2. Protection of inoculated chickens against virulent IBDV challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Inoculation</th>
<th>Challenge (CID50 of GLS-5 strain)</th>
<th>IBDV antigen in bursa</th>
<th>Bursal lesions</th>
<th>BBWR (mean ± s.D)</th>
<th>No. of animals infected (BBWR)</th>
<th>Protection (%)</th>
<th>Neutralizing antibody titre after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>–</td>
<td>–</td>
<td>0/14†</td>
<td>0/14†</td>
<td>5.22 ± 1.27†</td>
<td>0</td>
<td>&lt; 8</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>–</td>
<td>100</td>
<td>14/14</td>
<td>14/14</td>
<td>1.36 ± 0.25</td>
<td>14</td>
<td>0 &lt; 8</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>AcNPV</td>
<td>100</td>
<td>14/14</td>
<td>14/14</td>
<td>1.61 ± 0.35</td>
<td>14</td>
<td>0 &lt; 8</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>vIBD-7</td>
<td>100</td>
<td>1/14</td>
<td>5/14</td>
<td>4.87 ± 1.63</td>
<td>3</td>
<td>79§</td>
<td>256–1024</td>
</tr>
</tbody>
</table>

* Bursal homogenates, prepared 4 days post-challenge, were evaluated with a panel of IBDV-specific MAbs in AC-ELISA (see Table 1). The nominator denotes the number of animals positive for IBDV antigen in bursal tissue and the denominator denotes the number of animals inoculated.

† Bursae, obtained 8 days post-challenge, were analysed for the bursal lesions by histopathological examination. The nominator indicates the number of animals positive for bursal lesions and the denominator denotes the number of animals inoculated.

‡ BBWR was calculated for each animal (Lucio & Hitchner, 1979). Any value for individually challenged chickens falling ± 2 s.D. from the average of the negative control group was scored as a positive indicator of IBDV infection. The number of animals infected, as calculated by BBWR method, is shown in the next column.

§ See text.

ELISA to estimate the amount of IBDV proteins present. Total protein content of the recombinant baculovirus-infected cell lysate was about 4 mg/ml of which approximately 12 to 13% represented IBDV products. Fifteen ml of baculovirus- or recombinant baculovirus-infected cell lysate was emulsified with an equal volume of Freund’s incomplete adjuvant and used for inoculation.

To evaluate the protective properties of the recombinant proteins, 2-week-old white leghorn SPF chickens (SPAFAS, Inc.) were pre-bled and divided into four groups of 28 animals each. Chickens of groups I and II received no inoculations and served as negative and positive controls, respectively. Chickens of groups III and IV were inoculated intramuscularly with 0.5 ml of inoculum prepared from wild-type baculovirus- or recombinant baculovirus-infected cell lysates, respectively. At 4 weeks of age, chickens of groups III and IV were boosted with 0.25 ml of the respective inoculum. The immune response to the recombinant IBDV proteins was detected in a virus neutralization (VN) test which was performed as previously described (Snyder et al., 1988a). In addition, the sera were tested for the presence of anti-IBDV antibody using a commercially available IBDV antibody ELISA test kit (Kirkegaard & Perry), and by the agar gel precipitin test (AGPT) using undiluted antisera to diffuse against a 50% (w/v) bursal homogenate that was prepared from chickens infected with IBDV.

Our results indicate that the recombinant proteins induced a significant neutralizing antibody response in chickens and immunoprecipitated anti-IBDV antibodies from a convalescent serum in an AGPT. The average VN titre of the vaccinated chickens was 1024 which is comparable to that obtained from the unprimed chickens vaccinated with inactivated virus. However, the VN titre of naturally infected chickens was much higher and ranged from 16384 to 32768. Sera of vaccinated chickens also immunoprecipitated IBDV antigens in an AGPT and had an average ELISA titre of 3 (on a scale of 0 to 9), which correlated with the VN titre (see Table 2). No anti-IBDV response was elicited in the group of chickens inoculated with wild-type baculovirus-infected cell lysates.

To assess the ability of the recombinant IBDV antigens to induce a protective immunity, 7-week-old chickens of groups II, III and IV were challenged with 100 50% chick infective doses (CID50) of virulent GLS-5 strain by the ocular route. Four days post-challenge, 14 chickens from each group were sacrificed and their bursae were removed. Each bursa was processed and analysed for the presence of IBDV antigen by AC-ELISA using a panel of IBDV-specific MAbs (see Table 1). Eight days p.i. the remaining chickens in all groups were sacrificed and weighed. The bursa of Fabricius from each chicken was carefully excised and also weighed. Bursa to body weight ratio (BBWR) was calculated for each chicken as described by Lucio & Hitchner (1979) and each bursa was assessed for lesions by histopathological examination.

The results of the challenge experiment are shown in Table 2. All the non-vaccinated group (positive controls) and AcNPV-vaccinated group of chickens showed the presence of IBDV antigen, gross bursal lesions and a drastic reduction in their BBWR (due to damage of the lymphoid follicles), all indicating IBDV infection (Table 2). However, in the vIBD-7-vaccinated group, only one of 14 animals (7%) showed the presence of IBDV antigen; five of 14 animals (36%) showed mild to moderate bursal lesions; only three animals (21%) had a BBWR lower than 2 s.D. below the average of the unchallenged group, suggesting IBDV infection. No IBDV infection was detected in the animals of the unchallenged group (negative controls).
In recent studies, the host-protective VP2 antigen of IBDV has been expressed in yeast (Macreadie et al., 1990) as well as in a recombinant fowlpox virus (Bayliss et al., 1991). When microgram quantities of yeast-derived antigens were injected into chickens, they induced high titres of virus-neutralizing antibodies that were capable of passively protecting young chickens against IBDV infection. In contrast, when chickens were vaccinated with a recombinant fowlpox virus expressing the VP2 antigen, it actively protected chickens against mortality but not against bursal atrophy. The majority of vaccinates had severe lymphocyte depletion following virulent IBDV challenge. On the basis of AC-ELISA, BBWR and histopathological analysis, our results indicate that 79% of the vaccinated chickens were protected against virulent IBDV challenge (Table 2). Protection was defined as: (i) the absence of IBDV antigen in the bursa as detected by AC-ELISA; (ii) no detectable bursal damage upon histopathological examination; (iii) no decrease in BBWR of 2 s.d. under the average of the unchallenged group. Failure to achieve complete protection may be accounted for by an insufficient amount of IBDV antigens used for immunization (approx. 125 μg) to give complete protection.

In conclusion, expression in SF9 cells of a construct containing the entire coding region of IBDV structural proteins (VP2, VP3 and VP4) resulted in the synthesis and processing of the IBDV precursor protein and its correct processing. The recombinant IBDV proteins were antigenically similar to the native IBDV proteins and were immunoprecipitated with IBDV-specific MAbs and polyclonal antiserum to IBDV. The recombinant IBDV proteins induced a neutralizing antibody response in chickens and actively protected chickens against virulent IBDV challenge. Work is underway to determine the amount of IBDV antigens needed for complete protection in chickens.

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