Characterization by Western Blotting of the Immunogens of Infectious Bursal Disease Virus

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SUMMARY

The Australian isolate of infectious bursal disease (IBD) virus (002/73) was purified from infected bursae by rate-zonal and density-equilibrium centrifugation and characterized by polyacrylamide gel electrophoresis. Two major polypeptides having approximate mol. wt. of 32000 (32K) and 37K and three other polypeptides of approximate mol. wt. 29K, 41.5K and 91.5K were present in all preparations of virus having a buoyant density of 1.33 g/ml. Western blotting of the polypeptides of IBD virus showed that the initial antibody response of chickens infected with live virus or injected with an inactivated oil-emulsion vaccine was directed primarily towards the 32K polypeptide. Only sera obtained late in the response to live virus or following hyperimmunization contained antibodies recognizing the 29K, 37K and 41.5K polypeptides. An antibody response to the 91.5K polypeptide was not detected routinely by this technique. It was concluded that the 32K polypeptide is a major immunogen of IBD virus.

INTRODUCTION

Infectious bursal disease (IBD) virus causes a highly contagious immunodepressive disease of young chickens which is characterized by a marked necrosis of lymphoid cells in the bursa of Fabricius. Humoral antibody protects against infection, and maternal antibody transmitted to chickens via the egg yolk protects them through the critical first 4 to 5 weeks after hatching (Wyeth & Chettle, 1982). Injecting breeding hens, previously exposed to the live IBD virus, with an inactivated oil-emulsion whole virus vaccine prior to the point of lay has proved an important means of elevating the levels of maternal antibody to IBD virus in chickens and improving the performance of commercial flocks (Wyeth et al., 1981). As a first step towards the development of a safe, inexpensive and effective subunit vaccine to control this economically important disease, it was necessary to identify the structural proteins of the virus which are immunogenic in chickens.

Overseas isolates of IBD virus have been found to have four structural proteins with mol. wt. in the regions of 28000 (28K), 32K, 40K and 90K (Nick et al., 1976; Dobos, 1979; Todd & McNulty, 1979; Müller & Becht, 1982) with a precursor protein of 47K to 49K being present in some preparations (Dobos, 1979; Müller & Becht, 1982). The genome of IBD virus has been shown to consist of two segments of dsRNA with mol. wt. of $2.5 \times 10^6$ and $2.2 \times 10^6$ (Müller et al., 1979).

The present paper reports the purification for the first time of an Australian isolate of IBD virus and the characterization of its structural polypeptides. A direct comparison with exotic strains of this virus was not possible as these cannot be imported into Australia. The specificities of the antibodies produced during the primary responses of chickens to an experimental infection with IBD virus or the injection of a commercial inactivated whole virus vaccine were analysed by Western blotting, as were the specificities of the antibodies that arise following hyperimmunization.
Growth and purification of IBD virus. The isolate 002/73 of IBD virus was originally obtained in Australia by Firth (1974) from commercial poultry with varying degrees of bursitis and identified serologically as type 1 IBD virus at the Central Veterinary Laboratory, Weybridge, U.K. Following propagation at a limiting dilution of infectivity, the virus was routinely propagated by intraocular inoculation of 4- to 6-week-old specific-pathogen-free (SPF) white Leghorn chickens (CSIRO SPF Poultry Unit, Maribyrnong, Victoria, Australia). Homogenates of infected bursae of Fabricius were prepared as 10% (w/v) suspensions in phosphate-buffered saline (PBS) and stored at −80°C. The IBD virus stocks appeared to be free of contamination by other poultry viruses on electron microscopic examination and did not cross-react in the agar gel precipitation test with antisera to avian reovirus.

The virus was purified by a modification of the method of Todd & McNulty (1979). An equal weight of chilled PBS was added to the freshly harvested bursae which were homogenized in an ice bath by three 20 s bursts of a Polytron (PT-10-OD; Kinematica, Luzern, Switzerland) on setting 5. The homogenate was frozen to −80°C and thawed rapidly before an equal volume of the fluorocarbon Arkline (Wertheim Laboratories, Melbourne, Australia) was added and the mixture rehomogenized. After centrifugation at 10000 g for 30 min at 5°C, the aqueous phase (approx. 7 ml) was collected and layered onto chilled preformed 20% to 40% (w/v) CsCl gradients (10 ml) prepared in 0.1 M-NaCl, 0.01 M-Tris-HCl buffer pH 7.6 (NaCl-Tris buffer). After centrifugation at 30000 r.p.m. for 1.5 h in a Beckman SW28 rotor at 5°C, the gradients were harvested from the bottom in 1 ml fractions. These were then examined by gel electrophoresis, Western blotting with hyperimmune sera, ELISA and by an assay for dsRNA in order to determine the position of complete or incomplete particles of virus and soluble proteins of the virus. (In later preparations the virus was collected from the interface of a step gradient prepared by overlaying 10 ml of 40% sucrose on 5 ml of 60% sucrose.) Fractions containing complete (intact) virus were pooled and layered onto chilled preformed 20% to 40% (w/v) CsCl gradients (10 ml) which were centrifuged at 30000 r.p.m. for 18 h at 5°C in a Beckman SW40 Ti rotor and the band of IBD virus was harvested through the side of the tube. The virus was dialysed against NaCl-Tris buffer to remove CsCl and then against NaCl-Tris buffer containing 0.05% (w/v) sodium azide before being stored at 4°C or else made 50% (v/v) in glycerol and stored at −20°C.

Chicken antisera to IBD virus. SPF chickens were infected intraocuarily with IBD virus and bled 0, 3, 5, 7, 10 and 14 days later, then every 2 weeks. Chickens injected intramuscularly with 0.5 ml of commercial inactivated oil-emulsion vaccine (Arthur Webster Pty Ltd., Northmead, New South Wales, Australia) were bled fortnightly, as were chickens given a second intraocular infection with IBD virus and previously infected chickens given an intramuscular injection of commercial inactivated vaccine. The sera were collected by centrifugation at 400 g for 15 min and stored at −20°C.

Enzyme-linked immunosorbent assay (ELISA). The ELISA method used to assess the presence of IBD viral antigen in various gradient fractions or chicken antibody to IBD virus was essentially that described by York et al. (1983), except that the microtitre trays (Nunc Immunoplate I) were coated with rabbit anti-IBD virus IgG prepared by hyperimmunizing rabbits with 002/73. To titrate antibodies to IBD virus, dilutions of chicken sera were added to the wells that had first been coated with rabbit antibody followed by a standardized concentration of an extract of infected bursae. The amount of chicken antibody binding to the viral antigen in each assay was then quantified by adding sheep IgG anti-chicken IgG-horseradish peroxidase conjugate, followed by the substrate 5-aminosalicylic acid. To detect viral antigens in the gradient, dilutions of each of the fractions were added to the coated wells, which were then treated with a 1:500 dilution of chicken antiserum to IBD virus which was capable of producing a maximum A450 of 1.0 following the addition of the conjugated antisera and substrate. The trays were shaken for 30 min and the A450 was then read immediately on a Titertek Multiskan (Flow Laboratories).

Polyacrylamide gelelectrophoresis (PAGE) and Western blotting. Aliquots (40 μl) of each sucrose gradient fraction were dried under vacuum, resuspended in 20 μl of the sample buffer described by Laemmli (1970) which contained SDS and a trace of bromophenol blue dye, then heated for 3 min in a boiling water-bath. These samples were examined by discontinuous PAGE (Laemmli, 1970) with Coomassie Brilliant Blue staining. The structural proteins of the virus, separated by SDS-PAGE, were also examined by the Western blotting procedure described by Burnette (1981). The viral proteins were transferred to nitrocellulose membrane filters (Schleicher & Schüll, BA-83 0.2 μm) and probed with chicken antisera diluted 1:500 in 1% (w/v) gelatin in NaCl-Tris buffer. The chicken antibodies binding to viral polypeptides were identified with rabbit IgG anti-chicken IgG (Cappel Laboratories) diluted 1:1000 in NaCl-Tris-gelatin buffer followed by 1 μCi of 125I-Protein A (Amersham) in the same buffer. An autoradiograph of the nitrocellulose membrane was prepared using Fuji Rx Medical X-ray film and Ilford Fast Tungstate intensifying screens for 16 to 24 h at −70°C.

Detection of IBD virus by the RNA content of various fractions. Sucrose gradient fractions were diluted to 1:4 with 10 mM-Tris–HCl, 50 mM-NaCl, 0.2% SDS buffer, pH 7.5 and treated with 0.5 mg/ml ribonuclease-free Pronase ( Worthington) for 1 h at 37°C. The solutions were made 0.3 M with respect to NaCl and the nucleic acids were extracted with phenol and chloroform then ethanol-precipitated (Azad et al., 1985). Samples of RNA were electrophoresed under non-denaturing conditions in 1% (w/v) agarose slab gels in 20 mM-phosphate buffer pH 6.8,
together with λ DNA standards (Boehringer). When the gels were stained with acridine orange and illuminated with u.v. light, the dsDNA or dsRNA appeared as green bands while ssRNA appeared as red bands (McMaster & Carmichael, 1977).

RESULTS

Purification of IBD virus from infected bursae

Following centrifugation of the clarified bursal homogenates in 25 to 50% continuous sucrose gradients, a major band of material was visible approximately three-fifths of the way down the gradient. SDS-PAGE analysis of the gradient fractions indicated that the highest concentration of viral proteins was in the visible band, while fractions immediately above and below the major band contained lower amounts of the viral proteins. Similarly, the ELISA for viral antigen revealed peaks of IBD viral antigen throughout the gradient, although the visible band again contained the highest relative concentration.

The electrophoretic profiles of total RNA from different sucrose gradient fractions located two segments of viral RNA in two regions of the gradient, one corresponding to the major visible band low in the gradient and the other near the top of the gradient. The colour reaction with acridine orange showed that the two viral RNA segments were double-stranded. Electron microscopic examination of the fractions revealed that complete virus particles were only present in the lower region of the gradient. When electrophoresed under non-denaturing conditions with λ dsDNA as standards, the two viral RNA segments appeared to have mol. wt. of 2.5 × 10^6 and 2.2 × 10^6 respectively.

CsCl density equilibrium centrifugation of complete virus from the continuous sucrose gradients revealed one major band which was visible under reflected light and had a mean buoyant density of 1.33 g/ml. When the crude virus from the interface of a 40 to 60% sucrose step gradient was further purified on CsCl, a second, less dense, band was frequently seen which appeared by electron microscopy to contain a high proportion of 'core' particles.

Effect of the duration of infection on the yield of virus

No band of IBD virus was visible in CsCl gradients of virus from bursae harvested from chickens 2 days after infection. A distinct band of virus was visible in CsCl gradients of bursae from chickens infected for 3 days, but was more diffuse when the virus was purified from bursae harvested 4 days after infection. An ELISA for IBD viral antigen also found maximum titres of antigen in bursal homogenates obtained 3 or 4 days after infection. Consequently, virus was routinely prepared from bursae that were collected 3 days after infection.

Amino acid analysis of an acid hydrolysate, assuming a mean amino acid residue mol. wt. of 110, showed that up to 250 μg of viral protein could be obtained from a single bursa.

Coomassie Brilliant Blue staining after SDS–PAGE of purified virus

As shown in Fig. 1, purified preparations of intact virus contained two major polypeptides with approximate mol. wt. of 37K and 32K, and three other components (arrows in Fig. 1) of approximate mol. wt. 91.5K, 41.5K and 29K. Although the polypeptide of mol. wt. 32K was a major component of all preparations of virus, densitometer tracings from polyacrylamide gels of different preparations of virus revealed that the relative amounts of the other polypeptides, particularly the 41.5K polypeptide, varied between preparations.

Kinetics and specificity of the primary antibody response of chickens infected with IBD virus

The appearance of serum antibody to IBD virus, as determined by ELISA, was followed in six SPF chickens infected intraocularly at 6 weeks of age with isolate 002/73. Antibody was first detected on day 5 (mean titre 250), rising quickly to a mean titre of 10000 on day 10 and 17000 on day 14. The analyses by Western blotting of the sera obtained from one of these chickens is shown in Fig. 2. Antibody binding to the 32K polypeptide of IBD virus was detected on day 5, with the intensity of the bands increasing with time after infection. The antibodies present in the circulation of this chicken remained relatively specific for the 32K polypeptide, at least until day
Fig. 1. (a) PAGE of purified IBD virus in a 12.5% gel. The gel was stained with Coomassie Brilliant Blue (a) to reveal two major bands of approximate mol. wt. 37K and 32K and three others (arrowed) of approximate mol. wt. 91.5K, 41.5K and 29K. (b) Autoradiograph of a Western blot of virus preparation from (a) after probing with serum from a chicken experimentally infected with live IBD virus 2 months previously. Mol. wt. standards are on the left-hand side of each gel [(a) Pharmacia standards and (b) Amersham 14C standards; mol. wt. × 10^{-3}].

14 of the response. Western blots of the antibody present in sera obtained 14 days after infection from all six chickens are shown in Fig. 3 (lanes 1 to 6). The early antibody response of all chickens was predominantly to the 32K polypeptide and the response of five of the six chickens appeared to be monospecific (Fig. 3, lanes 2 to 6), even when the autoradiographs were exposed for 7 days. By 28 days after a primary infection the chickens had produced antibodies to all IBD virus polypeptides, except the 91.5K polypeptide (Fig. 3, lane 7).

Response of SPF chickens to vaccination with an inactivated oil-emulsion IBD vaccine

Six chickens, when 5 weeks of age, were injected intramuscularly with 1 ml of a commercial inactivated whole virus vaccine. The sera from the two chickens with the highest ELISA titres at 8 weeks post-vaccination (titres of 25600 and 51200 respectively) were analysed by Western bloting. At 4 and 8 weeks after vaccination the primary antibody response of both chickens to the inactivated vaccine was relatively specific for the 32K polypeptide of IBD virus (Fig. 4a, b). By 4 weeks after a second intramuscular injection of inactivated vaccine at 13 weeks of age, however, both chickens had produced serum antibodies which reacted with at least three of the structural proteins (Fig. 4c).

Response of sensitized chickens to an inactivated oil-emulsion IBD vaccine

Chickens that had been given live IBD virus at 5 weeks of age were injected at 25 weeks of age with a commercial inactivated vaccine. Sera were obtained 4 weeks and 20 weeks after the primary infection and then 4 and 8 weeks following revaccination. Analysis by Western blotting showed that initially there had been a response to four of the structural proteins, which had
Fig. 2. Specificity of the serum antibody response of a 6-week-old chicken to live IBD virus, as assessed by reacting 1:500 dilutions of serum collected 3, 5, 7, 10 and 14 days after infection, with Western blots of the viral polypeptides separated by SDS–PAGE. $^{14}$C mol. wt. standards are on the left-hand side.

Initially, difficulties were encountered in purifying intact virus which seemed to relate to the titre of the virus inoculum, the time after infection that the bursae were harvested, the storage of bursal homogenates at $-80^\circ$C for extended periods of time prior to purification and finally the detection of intact virus in the sucrose gradients. SDS–PAGE and ELISA identified viral proteins and antigens respectively, but did not discriminate between intact virus and soluble viral proteins. The analysis of dsRNA readily located the intact virus in the gradient; the position was confirmed subsequently by electron microscopy.

Studies on the Cu-1 isolate of IBD virus grown in vitro (Dobos, 1979) or in vivo (Müller & Becht, 1982) detected two major structural proteins with mol. wt. ranging from 40K to 41K (VP-2) and 32K to 35K (VP-3) respectively, although Todd & McNulty (1979), studying an isolate from Alabama, U.S.A., reported three major polypeptides of mol. wt. 50K, 45K and 30K respectively. When the Australian isolate of IBD virus (002/73) was purified from infected bursae by rate-zonal and density equilibrium centrifugation and analysed by polyacrylamide gel
electrophoresis in reducing or non-reducing buffers, two major structural proteins with approximate mol. wt. of 37K and 32K and three other proteins of approximate mol. wt. 91·5K, 41·5K and 29K were present in all preparations of purified virus having a buoyant density of 1·33 g/ml. While the major 32K structural polypeptide of the Australian isolate is comparable in size to VP-3 described for overseas isolates of IBD virus (Dobos, 1979; Todd & McNulty, 1979; Müller & Becht, 1982), the major 37K polypeptide of the Australian virus is smaller than the VP-2 of overseas isolates and the 47K to 48K VP-X described by Dobos (1979) and Müller & Becht (1982) was not detected in preparations of intact Australian virus. Preliminary results from HPLC analysis of trypsin digests of the structural polypeptides of the Australian virus indicate that the 37K polypeptide is cleaved from the 41·5K protein (N. M. McKern et al., unpublished data). Although direct comparison with Cu-1 or other exotic isolates of IBD virus is not possible in Australia, the Australian isolate has been serologically confirmed as type 1 IBD virus and the virus has the same size, dsRNA genome, tissue specificity and buoyant density as those described for overseas isolates. Variations in techniques, particularly the percentage of polyacrylamide in the gels (10 to 15%), could explain these apparent differences in mol. wt., although the near universality of the major 32K polypeptide suggests that the 37K and its 41·5K precursor polypeptides may be unique to the Australian virus. The relationship, if any, between
Immunogens of IBD virus

Chicken number

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Fig. 4. Specificity of the primary antibody response of two SPF chickens (1 and 2) injected at 5 weeks of age with a commercial inactivated oil-emulsion vaccine. Sera obtained from the chickens 4 weeks (a) and 8 weeks (b) after primary vaccination are compared with serum obtained 4 weeks (c) after a second injection of inactivated vaccine at 13 weeks of age. $^{14}$C mol. wt. standards are on the left-hand side.

the size of VP-2 of the Australian virus and its relatively low pathogenicity in susceptible chickens (compared with overseas reports) awaits resolution.

The Australian virus was found to contain two segments of dsRNA which, by comparison with $\lambda$ DNA standards, had mol. wt. of 2.2K and 2.5K, similar to those reported for Cu-1 by Müller et al. (1979). The dsRNA found near the top of the sucrose gradient could represent either partially completed virus, viral RNA associated with cellular organelles or the 'top component' of Müller & Becht (1982) or the 'empty particles' of Todd & McNulty (1979), both of which are devoid of VP-3.

When Western blots of the polypeptides of the Australian isolate of IBD virus were reacted with antibodies from chickens infected at least 4 weeks previously with 002/73 or hyperimmunized with an inactivated oil-emulsion vaccine, all structural proteins of the Australian isolate were found to be immunogenic except the minor 91.5K polypeptide. The latter observation may be a technical artefact due to variability in the efficiency of Western
Fig. 5. Specificity of antibodies in sera from a chicken infected at 5 weeks of age with live virus was examined 4 and 20 weeks post-infection (a, b). The chicken was then re-immunized with a commercial inactivated whole virus vaccine at 25 weeks of age and the specificity of the response examined 4 and 8 weeks later (c, d). 14C mol. wt. standards are on the left-hand side.

blotting of high mol. wt. proteins, as antibodies to the 91.5K polypeptide were detected occasionally with hyperimmune serum (Fig. 5).

The antibody response to a single injection of inactivated vaccine was directed primarily against the 32K viral polypeptide, as was the early antibody response to the live virus vaccine. After the first 2 weeks post-infection, however, the response to live virus became progressively broader, possibly as a result of the greater antigenic challenge following virus replication in the infected chickens, compared with the limited amount of antigen in a single dose of inactivated vaccine. The relatively specific response of chickens injected with the inactivated vaccine to the 32K polypeptide highlighted the immunogenicity of this polypeptide, compared for example with the 37K polypeptide which was present in comparable amounts in most preparations of virus. The broader specificity of the response to a second injection of inactivated vaccine indicated that the chickens had been successfully primed by the first injection of inactivated vaccine. A single injection of inactivated vaccine also recalled a poly-specific antibody response in chickens previously sensitized with live virus. These hyperimmune sera also recognized a number of other viral antigens with mol. wt. between 50K and 90K, possibly representing precursor forms of the viral proteins, including the 52K translation product detected in vitro by Azad et al. (1985). Interestingly, while all these positive sera contained antibodies that reacted
with the 32K polypeptide, no serum was found that reacted with the 37K but not the 41.5K polypeptide, or vice versa. Antibodies to these structural proteins arose simultaneously, which further supports the view that the 41.5K polypeptide is the precursor of the 37K polypeptide.

An alternative explanation for the reported specificity of the early antibody response for the 32K polypeptide could be that epitopes on the other polypeptides, possibly even ones important in protection, have been destroyed by denaturation during the Western blotting procedure (Cohen et al., 1984). If this were so then these labile epitopes must be the only ones recognized on these polypeptides early in the response, as convalescent serum and hyperimmune serum contain antibodies to stable epitopes on the 37K and 41.5K polypeptides. Since we have not as yet found a way of disrupting the virus using non-denaturing conditions, this possibility has not been investigated. Other possibilities are that early antibodies to the other polypeptides are of too low affinity to be detected by Western blotting or that these antibodies are of the IgM class. That all such antibodies would be too weak to bind to the denatured proteins seems unlikely, since we have now examined the polyclonal response of over 20 chickens and prolonged exposure of the autoradiographs (3 to 7 days, Fig. 3) revealed no non-32K antibody in the majority of early (less than day 14) sera. We know from fractionating immune chicken serum by column chromatography that the chickens are producing both IgM and IgG antibodies early in the response and that the rabbit anti-chicken IgG (H + L) reagent used routinely in our Western blotting analysis primarily detects IgG antibodies (unpublished data). Practically, this is not of particular concern, as it is only the IgG antibodies that are transferred via the yolk to the progeny chickens (Higgins, 1975) and hence responsible for passive immunity. However, reacting Western blots of whole virus with sheep anti-chicken IgM serum did not reveal the presence of antibodies to the other structural polypeptides of IBD virus in putative anti-32K specific sera (unpublished data).

Accepting the possibility that important immunogenic sites on the other structural polypeptides of IBD virus may be destroyed by denaturation, our evidence suggests that the first antibodies produced by the majority of chickens infected with IBD virus or injected with an inactivated vaccine, are specific for the 32K viral polypeptide and only later in the response or following re-vaccination with an inactivated vaccine could antibodies to the other structural proteins be readily detected. The recognition of the 32K polypeptide as a major immunogen of IBD virus has given impetus and direction to the development of a subunit vaccine for IBD by recombinant DNA technology.

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