Comparative Studies on Structural and Antigenic Properties of Two Serotypes of Infectious Bursal Disease Virus

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SUMMARY

The electrophoretic mobilities of the two genome segments and the structural polypeptides of the chicken strain Cu-1 (serotype I) and the turkey isolate 23/82 (serotype II) of infectious bursal disease virus were compared. There is a close antigenic relationship between the smaller of the two major structural proteins (32K) of both strains. Neutralizing monoclonal antibodies are induced by the larger protein (40K in Cu-1) which differentiates between the two serotypes. The 40K structural protein also has epitopes which do not induce neutralizing antibodies and which are common to both strains. There is evidence that the antigenic region responsible for the production of neutralizing antibodies is highly conformation-dependent. Passively administered neutralizing antibodies directed against the 40K structural polypeptide of Cu-1 confer protective immunity to susceptible chickens, whereas antibodies directed against the 32K structural protein do not have any protective effect.

INTRODUCTION

The basic structure of the infectious bursal disease virus (IBDV), the aetiological agent of a disease of chickens which results in the destruction of the bursa of Fabricius, has been elucidated (Nick et al., 1976; Müller et al., 1979) and has been shown to exhibit the structural characteristics of the Birnaviridae (Dobos et al., 1979; Brown, 1986). During the past few years isolates of IBDV from turkeys have been reported to be essentially identical to the chicken strains, but the turkey isolates were not pathogenic and could be distinguished by neutralization (McNulty et al., 1979; Jackwood et al., 1982); a second serotype was therefore established (McFerran et al., 1980).

In order to localize the antigenic determinants on the structural proteins of IBDV which are responsible for the induction of neutralizing antibodies, a more detailed comparative analysis of the two types of viruses was necessary. Attempts were therefore made to obtain more precise information about the size of the two genomic segments of dsRNA and the structural proteins of the turkey strain in relation to the known elements of the chicken virus. The antigenic sites which both types of viruses have in common and those which permit their distinction were traced with monoclonal antibodies. The need for a more precise analysis of the antigenic structure of IBDV was underlined by the attempts of other authors to describe antigenic regions which would lend themselves to the production of a vaccine in Escherichia coli or by peptide synthesis (Fahey et al., 1985a,b; Azad et al., 1986; Hudson et al., 1986).

METHODS

Viruses and cells. The chicken isolate Cu-1 (Nick et al., 1976) of IBDV represented serotype I. IBDV strain 23/82 isolated from a turkey (Chettle et al., 1985) was kindly supplied by N. E. Reed (Central Veterinary Laboratory, New Haw, Weybridge, U.K.). Both strains were propagated in chicken embryo (CE) cells at 39 °C, and were plaque-purified several times as previously described (Müller et al., 1986).


**Purification of virus.** IBDV grown in CE cells was concentrated from the culture medium by ultracentrifugation as described in detail elsewhere (Müller et al., 1986).

**Polyacrylamide gel electrophoresis.** Nucleic acids released from the purified virus particles by treatment with protease K in the presence of SDS (Müller et al., 1979) were analysed in 2.8% polyacrylamide, 6 M-urea slab gels as described elsewhere (Spies et al., 1987). Proteins were separated by SDS-PAGE in 15% gels using a discontinuous buffer system (Laemmli, 1970).

**Preparation of antisera.** Antibodies against whole virus were prepared by emulsifying concentrated, purified virus suspensions containing IBDV particles with bovine serum albumin in PBS for 2 h at 4 °C. The membranes were then incubated with monoclonal antibodies for 14 h at 4 °C. Detection was achieved using a biotin-streptavidin system with horseshad peroxidase as substrate. After each step the strips were washed extensively with PBS containing 0.05% Tween 20.

**Serological procedures.** For neutralization assays the virus plaque reduction neutralization test on CE cells was performed as previously described (Müller et al., 1987). Proteins were separated by SDS-PAGE in 15% gels using a discontinuous buffer system (Laemmli, 1970).

**Radiochemical labelling and immunoprecipitation of intracellular and structural IBDV polypeptides.** Labelling of proteins with [35S]methionine and immunoprecipitations were carried out as previously described (Müller & Becht, 1982). After a pulse with [35S]methionine for 12 h, labelled virus particles were sedimented from the culture medium and resuspended in the particle-dissociating RIPA buffer (10 mM-phosphate buffer pH 7.2 containing 10 mM-EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 2% Trasylol). The labelled infected or uninfected CE cells were lysed in the same buffer. Immunoprecipitates were analysed by SDS-PAGE.

**Monoclonal antibodies.** To produce monoclonal antibodies (Köhler & Milstein, 1975), BALB/c mice were injected intraperitoneally twice at an interval of 3 weeks with whole purified virus mixed with an equal volume of aluminium hydroxide (Alu-S; Serva, Heidelberg, F.R.G.). Four days after the last inoculation spleens were removed and cells were fused with P3-X63-Ag8 myeloma cells by polyethylene glycol 4000 following published protocols (Fazekas de St. Groth & Scheidegger, 1980). Hybridomas secreting virus-specific antibodies were selected by screening the culture medium of wells containing colonies of fused cells by virus neutralization and ELISA. Hybridoma cells from positive cultures were cloned. For the production of large amounts of antibodies the hybridomas were isolated by passing the culture medium, which had been clarified by centrifugation, through the column of Sepharose conjugated with anti-mouse Ig as described above.

**Immunoblotting.** Proteins separated by SDS-PAGE were electrophoretically transferred from the gel to blotting membranes (Durapore; pore size 0.22 µm; Millipore) by use of a 'semi-dry' horizontal electrophoretic apparatus (Khyse-Andersen, 1984). Non-specific protein binding sites were blocked by treatment with 10% horse serum, 1% bovine serum albumin, 0.05% Tween 20 in PBS for 2 h at 4 °C. The membranes were then incubated with monoclonal antibodies for 14 h at 4 °C. Detection was achieved using a biotin–streptavidin system with biotinylated anti-mouse Ig, streptavidin-conjugated horse radish peroxidase (Amersham Buchler), and 4-chloro-1-naphthol as substrate. After each step the strips were washed extensively with PBS containing 0.05% Tween 20.
RESULTS

Structural analysis of IBDV strains Cu-1 and 23/82

The polyacrylamide gel in Fig. 1 (a) shows that segment A of the genomic RNA of the turkey strain 23/82 is smaller than the corresponding genome segment of the chicken strain Cu-1. By comparing the migration distances with values obtained previously by electrophoresis under fully denaturing conditions and from direct length measurements under the electron microscope (Müller & Nitschke, 1987), the difference in size can be estimated to be approximately 70 bp. There is a slight but definite difference in the size of segment B which amounts to about 20 bp according to the same criteria. This slightly faster migration rate of segment B in strain 23/82 became evident only after long electrophoretic separation for 80 h.

One of the two major structural polypeptides, with an estimated $M_r$ of about 32000 (32K) is almost identical in size in both strains; polypeptides which form the second prominent group, however, are considerably larger in the turkey isolate than the 40K polypeptide (p40) and its precursors p49/48 (Müller & Becht, 1982) of strain Cu-1 (Fig. 1b). In the high $M_r$ class, polypeptides with $M_r$s of about 90K are almost identical in size in both IBDV strains. There is relatively less 90K polypeptide in all preparations of strain 23/82 than strain Cu-1 preparations; this cannot be seen in Fig. 1(b), but is clearly visible in the autoradiographs in Fig. 2.

Serological cross-reactions between the two IBDV strains

Rabbit hyperimmune sera prepared by injecting purified whole undenatured virus particles together with incomplete particles (Müller et al., 1986) revealed cross-reactions between both IBDV isolates. Immunofluorescence with both antisera demonstrated common antigens in CE cells infected with either virus strain (not shown), and both antisera precipitated IBDV-specific proteins from lysed CE cells, regardless of the virus strain used for infection. Immunoprecipitated polypeptides obtained with any combination of hyperimmune serum and infected cell lysates (not shown) belonged to the three size classes of structural proteins of IBDV outlined in Fig. 1. The same was true when $^{35}$S-labelled virus particles which had been concentrated from the culture medium of infected CE cells and dissociated with RIPA buffer reacted with either of the rabbit sera (Fig. 2). The relative proportions of structural proteins in Fig. 1(b) and Fig. 2 cannot be compared directly, since polypeptides from purified virus particles have been stained in Fig. 1(b), whereas labelled virus particles pelleted from the culture medium had been dissociated for cross-precipitations in Fig. 2. Such crude virus preparations have been shown to contain incomplete particles the polypeptide composition of which is different from the pattern obtained with whole virus particles (buoyant density 1.325 g/ml; Müller et al., 1986).

The two strains could be clearly distinguished in plaque reduction neutralization tests where cross-reactions in either direction could be observed only in very low serum dilutions (Table 1).

Definition of the antigenic region responsible for the production of neutralizing antibodies using monoclonal antibodies

Table 1 shows that monoclonal antibodies 1/A6 and 14/B5 raised against Cu-1 reacted with the homologous strain but did not neutralize the turkey virus. However, concentrated 14/B5 cross-neutralized whereas 1/A6 concentrated to 10 mg/ml neutralized only the homologous strain Cu-1. The monoclonal antibodies 41/A1 and 44/A2 raised against 23/82 (Table 1) showed some cross-neutralization with Cu-1 at low dilutions. All of these neutralizing antibodies bound to the medium size class of structural proteins with $M_r$s of about 40K. In immunoprecipitates formed with dissociated Cu-1 virus particles, p40 and its precursor polypeptides p49/48 (Müller & Becht, 1982) are clearly visible in electropherograms (lane 1 in Fig. 3). Cross-precipitations, however, were either faint or totally absent. This was true for monoclonal antibody 1/A6 where not even a trace of precipitation was visible above background levels (lane 4 in Fig. 3). Essentially the same pattern was obtained with the turkey strain. Immunoprecipitates obtained by reacting virus-infected cell lysates with a monoclonal antibody prepared against 23/82 contained the precursor protein of the p40 analogue (Fig. 6, lanes 5 and 9) because IBDV-infected cells do not contain the final structural polypeptide of mature virus particles (Müller &
Fig. 1. Structural analysis of IBDV isolates by gel electrophoresis. (a) Analysis of dsRNA in a 2·8 % polyacrylamide gel: lane 1, chicken strain Cu-1 (segment A, Mr, 2·2 × 10⁶; segment B, Mr, 1·9 × 10⁶; Müller & Nitschke, 1987); lane 2, turkey strain 23/82; a mixture of both virus strains was applied to lane 3. Silver stain was used. (b) Structural proteins analysed in a 15 % SDS–polyacrylamide gel: lane 1, chicken strain Cu-1 (polypeptides are labelled on the left of the figure with the notation used by Müller & Becht, 1982); lane 2, turkey strain 23/82. Stained with Coomassie Brilliant Blue.

Fig. 2. Autoradiograph of ³⁵S-labelled polypeptides from dissociated IBDV particles, immunoprecipitated with rabbit hyperimmune sera prepared against strain Cu-1 (lanes 1 to 3), or strain 23/82 (lanes 4 to 6), and analysed by SDS–PAGE. Virus particles were concentrated from the culture medium of CE cells infected with strain Cu-1 (lanes 1 and 4), or 23/82 (lanes 2 and 5), and were lysed with RIPA buffer. Analogously treated medium of mock-infected CE cells is represented by lanes 3 and 6. The arrowhead marks the start of the gel.
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Fig. 3. Autoradiograph of 35S-labelled polypeptides from IBDV particles (lanes 1 to 3, strain Cu-1; lanes 4 to 6, strain 23/82) dissociated as in Fig. 2 and immunoprecipitated in this experiment with monoclonal antibodies. Lanes 1 and 4, neutralizing monoclonal antibody 1/A6, prepared against strain Cu-1; lanes 2 and 5, non-neutralizing monoclonal antibody 6/A2, prepared against Cu-1; lanes 3 and 6, non-neutralizing monoclonal antibody 30/D10, prepared against polypeptide p40 of strain Cu-1 isolated by SDS–PAGE. Designation of Cu-1 polypeptides is as in Fig. 1. Arrowhead as in Fig. 2. The film was heavily overexposed to the gels to demonstrate traces of cross-precipitation.

Table 1. Comparison of antibodies to IBDV isolates Cu-1 and 23/82 in neutralization tests

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Cu-1</th>
<th>23/82</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-1</td>
<td>Polyclonal (rabbit)</td>
<td>32000</td>
<td>128</td>
</tr>
<tr>
<td>23/82</td>
<td>Polyclonal (rabbit)</td>
<td>128</td>
<td>16000</td>
</tr>
<tr>
<td>Cu-1</td>
<td>Monoclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1/A6</td>
<td>2048</td>
<td>&lt;8</td>
</tr>
<tr>
<td></td>
<td>14/B5</td>
<td>256</td>
<td>&lt;8</td>
</tr>
<tr>
<td>23/82</td>
<td>Monoclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41/A2</td>
<td>8</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>44/A1</td>
<td>8</td>
<td>512</td>
</tr>
</tbody>
</table>

* Reciprocal dilution of antibody that reduced plaque counts by more than 80%.

Becht, 1982). Here again, only a faint precipitate appeared in the heterologous Cu-1 lysate (Fig. 6, lane 5). These results indicate that the formation of immunoprecipitates followed the neutralization pattern of the monoclonal antibodies listed in Table 1.

In addition to these neutralizing anti-p40 antibodies there is a group of monoclonal antibodies that recognize antigenic structures common to p40 in Cu-1 and its counterpart in strain 23/82. Monoclonal antibodies 6/A2 and 30/D10 produced comparable immunoprecipitates with antigens of the homologous and heterologous strains (Fig. 3, lanes 2, 5 and 3, 6). However, these
two antibodies did not produce the same precipitation patterns, and they did not bind equally well to the precursor and product proteins (immunoblots in Fig. 4, lanes 2, 3, 5, and 6). None of these antibodies had any neutralizing activity.

Evidence for conformation-dependence of the neutralizing epitope

In immunoblots prepared with purified virus, bands of the medium size class polypeptides were produced with the non-neutralizing antibodies described in the previous section (Fig. 4, lanes 2, 5, and 3, 6), confirming the data shown in Fig. 3. However, these structural components appeared only faintly or were totally absent when neutralizing antibodies were used to develop
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the bands, in spite of the fact that both types of antibodies are directed against the same structural protein. Fig. 4, lane 1, exemplifies this type of reaction where an immunoblot developed with the efficiently neutralizing monoclonal antibody 1A6 indicated the location of p40 only faintly, or had no staining at this location at all. The crucial role of the native state of p40 in Cu-1 for the induction of neutralizing antibodies and their reaction with this undenatured protein was underlined when rabbit antibodies were produced against denatured p40 which had been separated by SDS-PAGE. This polyclonal anti-p40 serum did not neutralize viral infectivity but formed precipitates with radiolabelled structural proteins (Fig. 5) and bands in immunoblots (not shown). When the same immunogen was used to produce monoclonal antibody 30/D10 neutralization was absent again in spite of the antibody’s specificity for the medium-sized polypeptides (Fig. 3, lanes 3 and 6; Fig. 4, lanes 3 and 6).

32K structural polypeptides carry a common antigen

A series of 40 hybridomas originating from mice immunized with strain Cu-1 or 23/82 produced antibodies that reacted equally well with the 32K structural polypeptide of either strain. Three examples of this type of reaction are presented in Fig. 6 where all precipitates are found in the position of 32K, and the heterologous bands (lanes 2 to 4) appear at least as intense as the homologous precipitates (lanes 6 to 8). The bands which could be developed in immunoblots had identical positions (not shown). In no case, however, was there any trace of neutralizing activity with monoclonal antibodies directed against these polypeptides.
Table 2. Protective effect of monoclonal antibodies against IBDV strain Cu-1 major structural proteins

<table>
<thead>
<tr>
<th>Antibody designation</th>
<th>Directed against structural protein†</th>
<th>Animals treated with antibody sick or dead‡</th>
<th>Untreated controls, sick or dead‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/A6 neutralizing</td>
<td>p40</td>
<td>0/6</td>
<td>4/4</td>
</tr>
<tr>
<td>27/B1 non-neutralizing</td>
<td>p32</td>
<td>4/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* See Methods.  
† Structural polypeptides are designated according to Müller & Becht (1982).  
‡ Numbers refer to animals with clinical signs/animals tested.

Protective effect of monoclonal antibodies directed against p40

Six chickens at the highly susceptible age of 3 weeks received purified neutralizing monoclonal antibody against Cu-1 (about 10 mg/animal; neutralization titre \( >1 : 10000 \)), and were challenged the following day by peroral infection with the highly pathogenic wild-type of IBDV strain Cu-1 (Nick et al., 1976; Lange et al., 1987) in a tissue homogenate of the bursa of Fabricius (about \( 5 \times 10^6 \) p.f.u./animal). They all survived the infection without the slightest clinical sign of disease. All control animals died within 2 to 4 days of infection, exhibiting characteristic signs of IBD. When the experiment was repeated by treating chickens with monoclonal antibodies directed against p32 of Cu-1 (Müller & Becht, 1982), all animals had the same severe clinical and pathological manifestations after challenge as the control group. The results of these experiments are summarized in Table 2.

DISCUSSION

Data on the RNAs and polypeptides of the turkey isolate 23/82 of IBDV (Fig. 1) are in agreement with Jackwood et al. (1984). Genome segment B was found to be slightly larger in the chicken strain, but there was considerable difference in the size of segment A, which codes for all but one of the structural proteins (Azad et al., 1985; H. Müller, unpublished results). The smaller of the two major structural polypeptides (Mr about 32K) is virtually identical in both strains, and it is reciprocally recognized by all monoclonal antibodies directed against this component in either strain. The 32K structural polypeptides can therefore be considered to be a group-specific antigen.

There is antigenic variation in the larger group of polypeptides which differ considerably in Mr. One type of neutralizing monoclonal antibody (e.g. 1/A6) exclusively reacts with the homologous strain, while others show some cross-neutralization similar to polyclonal hyperimmune sera. This indicates that there are at least two antigenic sites capable of inducing neutralizing antibodies; one is strictly strain-specific, whereas the other one has some antigenic relatedness between strains. Their topographical distribution has not yet been explored. The absence of appreciable staining by neutralizing antibodies in immunoblots, particularly the strain-specific antibody 1/A6, demonstrates that although there is good precipitation of the native antigen from cell extracts or mildly dissociated virus particles, the antigenic structure recognized by neutralizing antibodies is strongly conformation-dependent. The crucial importance of the native state of this antigen in the induction of neutralizing antibodies was underlined by the finding that polyclonal antibodies which were produced with the denatured form of the p40 polypeptide of Cu-1, isolated from polyacrylamide gels, did not have any neutralizing activity. Contradictory findings have been reported by Fahey et al. (1985b). In their study chickens immunized with p32, prepared by SDS-PAGE, produced antisera that neutralized the virus \textit{in vitro} and passively protected chickens from infection. Since the Australian isolate 002/73 used by these authors has a medium-size polypeptide of only 37K (Fahey et al., 1985a), the discrepancies might be due to structural differences between the strains used, and it is questionable whether the conflicting results can be compared directly.

In addition to these strain-specific sites there are antigenic regions located on the 40K and precursor molecules which do not depend on the native structure; they are recognized by
antibodies raised against both virus strains and by monoclonal antibody 30/D10. This means that there are common, mainly sequence-dependent antigenic sites located on the 40K structural polypeptide. For another type of common epitope the native state of the precursor molecule seems to have some significance; antibody 6/A2 preferentially precipitates the precursor, but it does not attach to the denatured molecule in immunoblots. After processing, the epitope seems to be optimally exposed on the denatured 40K polypeptide.

The appearance of neutralizing antibodies after infection of susceptible chickens is correlated with the establishment of a state of immunity (Cursiefen et al., 1979). The protective role of neutralizing antibodies was underlined in the present study when administration of neutralizing monoclonal antibodies induced immunity, whereas non-neutralizing anti-p32-specific antibodies were not protective. All neutralizing monoclonal antibodies precipitated p49/48 from infected cell extracts. When binding occurred in radioimmunoprecipitations and in immunoblots, this was observed exclusively at the p40 and p49/49 protein sites. However, antibodies with specificity for the 32K polypeptides never showed any neutralization. All of these observations indicate that the antigenic determinant(s) responsible for the induction of neutralizing antibodies, and consequently of protective immunity, reside in the 40K structural protein.

In efforts to produce vaccines by expressing cloned parts of the genomic segment A in E. coli (Azad et al., 1985), the 40K structural polypeptides merit attention. However it must reach a conformation similar to that of the native protein in the virus particle.

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