Mapping of neutralization epitopes on infectious pancreatic necrosis viruses

Petter Frost,1,* Leiv Sigve Håvarstein,2† Bjarte Lygren,4 Stefan Ståhl,3 Curt Endresen4 and Karen Elina Christie1

1 Intervet Norbio A/S, Thormøhlensgate 55, N-5008 Bergen, Norway, 2 Center of Biotechnology, University of Bergen, N-5020 Bergen, Norway, 3 Department of Biochemistry and Biotechnology, Royal Institute of Technology, KTH, S-100 44 Stockholm, Sweden and 4 Department of Fisheries and Marine Biology, University of Bergen, N-5020 Bergen, Norway

We have characterized and mapped variable and conserved neutralization epitopes of serogroup A strains of aquatic birnaviruses. Epitope mapping using monoclonal antibodies (MAbs) and Escherichia coli-expressed deletion fragments of VP2 of the N1 strain of infectious pancreatic necrosis virus (IPNV) demonstrated that two variable epitopes, H8 and B9, depend on the variable region between amino acid 204-330. A conserved neutralization epitope, F2, was shown to depend on the same region as epitopes H8 and B9 but was additionally dependent on amino acids between 153-203. The neutralization epitopes H8, B9 and F2 were also shown to overlap by a competitive binding assay. One conserved neutralization epitope, AS-1, was not exposed on any of the recombinant VP2 deletion fragments and was therefore not possible to map. However, the MAbs AS-1 and F2 were partly competitive indicating that these epitopes are overlapping. All neutralization epitopes were independent of a conserved non-neutralization epitope, E4. Our results demonstrate that the central third of VP2 contains several partly overlapping neutralization epitopes, both variable and conserved among serogroup A strains of IPNV.

Introduction

Infectious pancreatic necrosis viruses (IPNV) are aquatic birnaviruses responsible for infectious pancreatic necrosis (IPN) in various fish species. Since 1986, acute IPN with considerable losses has frequently been diagnosed among farmed Atlantic salmon (Salmo salar) in Norway (Christie et al., 1990; Melby et al., 1994). Mortality in salmonids is believed to be highest in fry at first feeding and to become negligible by approximately 6 months of age (Frantsi & Savan, 1971). However, recently an increasing number of IPN outbreaks with high mortalities have been reported among post-smolt of Atlantic salmon following transfer to seawater (Smail et al., 1992; Melby et al., 1994). Developing an effective IPN vaccine is a major priority.

The birnavirus genus, family Birnaviridae, contains animal viruses with double-stranded bisegmented (segments A and B) RNA genomes contained within a non-enveloped icosahedral capsid. Segment A encodes three known gene products within one large open reading frame (Duncan & Dobos, 1986; Håvarstein et al., 1990), and segment B encodes a RNA-dependent RNA polymerase (VP1) (Duncan et al., 1991). The segment A-encoded polyprotein, pVP2–NS–VP3, is autocatalytically cleaved by the endoprotease (NS) to produce the structural proteins VP2 and VP3 (Duncan et al., 1987). Variations in the polypeptide patterns of different virus strains have been reported (Macdonald et al., 1982; Christie et al., 1988). The classification of aquatic birnaviruses is disputed and varies depending on the method used (Caswell-Reno et al., 1986, 1989; Christie et al., 1988; Heppell et al., 1993). Based on serological analyses, the aquatic birnaviruses have been divided into two serogroups (A and B) (Hill & Way, 1988). The predominant serogroup A is found globally and includes most serotypes known to be pathogenic to fish.

The major neutralization epitopes of aquatic birnaviruses are localized within VP2 (Caswell-Reno et al., 1986; Christie et al., 1990; Tarrab et al., 1993), but neutralization epitopes have also been suggested for VP3 (Tarrab et al., 1993).

An internal variable region located in the central part
of VP2 has been identified (Hávarstein et al., 1990) and was suggested to contain serotype-specific epitopes of birnaviruses. A VP2-specific monoclonal antibody (MAB), AS-1, neutralizing all serogroup A strains of IPNV has been reported (Caswell-Reno et al., 1989; Lipipun et al., 1991), indicating the presence of a conserved neutralization epitope on VP2. Due to the antigenic variability of aquatic birnaviruses a subunit vaccine against IPN should be based on neutralization epitopes known to be conserved among the relevant virus strains.

In order to develop an effective subunit vaccine the epitopes responsible for protective immunity have to be identified. In this communication we report the characterization and mapping of variable and conserved neutralization epitopes on serogroup A strains of aquatic birnaviruses using MAbs and Escherichia coli-expressed deletion fragments of VP2.

### Methods

**Cells and viruses.** The VR-299 (A1) and West Buxton (A1) virus strains were obtained from Dr J. C. Leong (Oregon State University, USA), the Sp (A2) and Ab (A3) virus strains were obtained from Dr P. E. V. Jergensen (The State Veterinary Serum Laboratory, Århus, Denmark), the Hecht (A4) virus strain from Dr W. Ahne (University of Munich, Germany), the Tellina (A5) virus strain from Dr D. A. Small (Agriculture and Fisheries Department Marine Laboratory, Aberdeen, Scotland), the Canada-1, -2 and -3 (A6, A7 and A8) virus strains from Dr B. L. Nicholson (University of Maine, USA) and the Jasper-Dobos virus strain (A9) from Dr P. Dobos (University of Guelph, Canada).

Isolation of the N1 virus strain (A10) from Norwegian Atlantic salmon (Salmo salar) has been described elsewhere (Christie et al., 1988). All viruses were propagated in Chinook salmon embryo (CHSE-214) cells grown by standard methods. During virus propagation no serum was added to the cell growth medium. Virus used in sandwich ELISA or neutralization assay was either taken directly from the master seed or propagated once in CHSE-214 cells. Virus for SDS–PAGE and Western blotting was purified according to Christie et al. (1988).

**Monoclonal antibodies.** The VP2-specific MAbs F2 and E4 were produced against the N1 (A10) strain of IPNV as described elsewhere for the VP2-specific MAbs H8, B9 and for the VP3-specific MAB C12 (Christie et al., 1990). MAB AS-1 (Caswell-Reno et al., 1989), was kindly provided by Dr B. L. Nicholson (University of Maine, USA). MAbs H8, B9 and F2 were purified by the MAbTrap G procedure (Pharmacia) and labelled with biotin by standard methods using 100 μg N-hydroxysuccinimide biotin/mg immunoglobulin.

**Isolation and subcloning of cDNA encoding VP2.** A cDNA encoding VP2 was isolated from the lambda gt10 cDNA library previously used for sequencing segment A of the N1 virus strain (Hávarstein et al., 1990). A VP2 cDNA of approximately 14 kbp was subcloned into the expression vector pVABBmp8 encoding a 25 kDa serum albumin binding domain (BB) of protein G of Streptococcus G148, 5′ to the polylinker.

---

### Table 1. Plasmid constructs for expression of VP2 fragments of the N1 strain of IPNV in Escherichia coli

<table>
<thead>
<tr>
<th>Name of plasmid</th>
<th>Origin*</th>
<th>VP2 codons encoded</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET11-VP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET11-VP2 ΔXmnI</td>
<td>Complete digestion of the BamHI VP2 fragment with XmnI. Ligation of Ncol linker 5′CAGCCATGGCTG 3′ to the blunt end, complete digestion with Ncol and BamHI, and insertion into Ncol/BamHI-linearized pET11d</td>
<td>153–453</td>
</tr>
<tr>
<td>pET11-VP2 ΔHincII</td>
<td>Complete digestion of the BamHI VP2 fragment with HincII. Ligation of Ncol linker 5′AGCCATGGCTG 3′ to the blunt end, complete digestion with Ncol and BamHI, and insertion into Ncol/BamHI-linearized pET11d</td>
<td>234–453</td>
</tr>
<tr>
<td>pET11-VP2 ΔMscI</td>
<td>Complete digestion of the BamHI VP2 fragment with MscI. Ligation of Ncol linker 5′CAGCCATGGCTG 3′ to the blunt end, complete digestion with Ncol and BamHI, and insertion into Ncol/BamHI-linearized pET11d</td>
<td>280–453</td>
</tr>
<tr>
<td>pET11-VP2 ΔAatII</td>
<td>Partial digestion of pET11-VP2 with AatII and religation</td>
<td>3–269</td>
</tr>
<tr>
<td>pET11-VP2 ΔSacIIΔSmaI</td>
<td>Complete digestion of pET11-VP2 with SacII and blunt ended with Klenow 3′–5′</td>
<td>3–201</td>
</tr>
<tr>
<td>pET11-VP2 ΔBsrBI</td>
<td>Complete digestion of pET11-VP2 with BsrBI and religation</td>
<td>3–127</td>
</tr>
<tr>
<td>pET11-VP2 ΔAatII</td>
<td>Complete digestion of pET11-VP2 with AatII and religation</td>
<td>3–86</td>
</tr>
<tr>
<td>pET11-VP2 ΔApHincIIΔAatII</td>
<td>Complete digestion of pET11-VP2 ΔHincII with XbaI and BamHI. Complete digestion of the XbaI/BamHI fragment with AvaiI, blunt end repair with T4 DNA polymerase. Complete digestion with Ncol and insertion into Ncol/BamHI-linearized pET11d</td>
<td>153–300</td>
</tr>
<tr>
<td>pET11-VP2 ΔApHincIIΔAapAvaiII</td>
<td>Complete digestion of pET11-VP2 ΔHincII with XbaI and BamHI. Partial digestion of the XbaI/BamHI fragment with AvaiI and blunt end repair with T4 DNA polymerase. Complete digestion with Ncol and insertion into Ncol/SmaI-linearized pET11d</td>
<td>153–424</td>
</tr>
<tr>
<td>pET11-VP2 ΔpHincIIΔSacIIΔAatII</td>
<td>Complete digestion of pET11-VP2 ΔHincII with SacII followed by partial digest with AatII, blunt end repair with T4 DNA polymerase and religation</td>
<td>153–201 &amp; 270–453</td>
</tr>
<tr>
<td>pVABB-dVP2</td>
<td>Complete digestion of pET11-VP2 ΔHincII with SacII followed by partial digest with AatII, blunt end repair with T4 DNA polymerase and religation</td>
<td>204–331</td>
</tr>
</tbody>
</table>

* The location of the restriction sites used to construct the pET11-VP2 derived deletion mutants are shown in Fig. 1.

† Expression vector pVABBmp8 encodes a 25 kDa serum albumin binding domain (BB) of protein G of Streptococcus G148, 5′ to the polylinker.
sterile distilled water prior to SDS-PAGE analysis and lyophilization. For gel filtration, lyophilized samples were solubilized in ice-cold 6 M guanidine.HCl, 16 mm-Tris–HCl pH 7.4, to a final concentration of 10–20 mg/ml. Undissolved material was removed by 0.22 μm filtration or by centrifugation (12000 g for 15 min) and samples of 200 μl applied on a Superose 12 HR 10/30 gel filtration column (Pharmacia) using the liquid chromatography (FPLC) system (Pharmacia) according to the manufacturer’s instructions. Eluted fractions were monitored at 280 nm and analysed by SDS-PAGE. Relevant fractions were pooled and dialysed overnight against ice-cold 2 M-guanidine.HCl, 16 mm-Tris–HCl pH 7.4. The protein concentrations were determined according to the A280, and all samples were diluted in additional 2 M-guanidine.HCl to a final protein concentration of approximately 100 μg/ml.

*E. coli* strain RR1AM15 (Rüther, 1982) was used for expression of the pVABBmp8 constructions. *E. coli* cells harbouring the pVABB-dVP2 or the pVABBmp8 vector were grown at 30 °C in tryptic soy broth medium (Difco; 30 mg/ml) with addition of yeast extract (Difco; 5 mg/ml) and ampicillin (Astra; 100 μg/ml). Induction of gene expression with indole acrylic acid was performed as described by Köhler et al. (1991) at OD529 of 1.5. Cells were harvested by centrifugation (3000 g for 20 min) at OD529 of 6 and resuspended in ice-cold TN-Tween buffer (25 mm-Tris–HCl pH 7.4, 150 mm-NaCl, 0.05% Tween-80). The intracellular proteins were released by pulsed sonication for 6 min and the BB-dVP2 and BB proteins purified on a HSA–Sepharose affinity chromatography column as described by Nygren et al. (1988). Eluted fractions were collected according to A280 and analysed by SDS-PAGE. Relevant fractions were pooled, the protein concentration determined by A280 analysis, lyophilized and resuspended in 2 M-guanidine.HCl to a final protein concentration of approximately 100 μg/ml.

**SDS-PAGE and Western blotting.** SDS-PAGE was performed by standard methods using 4% stacking gels and 12% resolving gels. Purified virus (10 μg/cm gel) or *E. coli* containing recombinant VP2 fragments was solubilized by boiling for 2 min in sample buffer (62.5 mm-Tris–HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue). Samples containing guanidine.HCl (6 M or 2 M) were diluted 10-fold in pre-heated (98 °C) sample buffer, boiled for 2 min and loaded onto a pre-heated polyacrylamide gel (100 V for 10 min at room temperature). The gels were stained by standard methods using Coomassie brilliant blue or silver nitrate, or soaked for 30 min in electroblotting buffer (0.25 M-Tris–HCl pH 8.3, 0.192 M-glycine, 20 % methanol) and electroblotted onto nitrocellulose (NC) sheets (0.145 μm; Schleicher & Schuell). Following post-coating of the NC sheets with 3% (w/v) dry milk (Nestlé non-fat instant milk) in TBS-Tween (25 mm-Tris–HCl, pH 7.4, 0.1% Tween-20) for 60 min at room temperature, the NC sheets were incubated overnight at 4 °C with hybridoma cell culture supernatant. For comparison of virus strains, biotin-labelled goat anti-mouse secondary antibodies (Amersham), diluted 1:200 were incubated at room temperature for 60 min before addition of peroxidase-conjugated streptavidin (Amersham) diluted 1:3000, and a further 30 min incubation. The NC sheets were developed for 10 min with peroxidase-conjugated biotin (Bio-Rad) diluted 1:2000. Sandwich ELISA and competitive binding assay. Sandwich ELISA experiments with MAb AS-1 were performed as described by Melby &
Table 2. Analysis of epitopes on aquatic birnaviruses with VP2 specific monoclonal antibodies by Western blotting (WB), virus neutralization assay* (Neut) and ELISA†

<table>
<thead>
<tr>
<th>Virus strain‡</th>
<th>H8</th>
<th>B9</th>
<th>F2</th>
<th>AS-1</th>
<th>E4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
<td>WB</td>
<td>Neut</td>
<td>ELISA</td>
<td>WB</td>
</tr>
<tr>
<td>VR-299 (A1)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>West Buxton (A1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sp (A2)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ab (A3)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hecht (A4)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tellina (A5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Canada-1 (A6)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Canada-2 (A7)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Canada-3 (A8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jasper-Dobos (A9)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>N1 (A10)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* The maximum dilutions giving 50% reduction in plaque forming units were > 1:1000 (+ + +), between 1:100 and 1:1000 (+ + ) or < 1:100 (+).
† The ELISA results except for MAb AS-1 are based on Melby & Christie (1994). The relative recognition of virus was quantified to be more than eight times (+ + +), between four and eight times (+ + ), between two and four times (+) or less than two times the control value.
‡ The numbers in the parentheses correspond to the proposed serotyping of aquatic birnaviruses.
ND, Not done.

Christie (1994) using the N1 strain of IPNV as antigen. For competitive binding analysis of the MAbs excess of an unlabelled MAb (hybridoma medium) was incubated overnight at 4 °C, followed by a 3 h incubation at room temperature with a sub-saturating concentration (approximately 3 μg/ml) of a purified biotin-labelled MAb, and a 30 min incubation with peroxidase-conjugated streptavidin (Amersham) diluted 1:3.000. The positive controls were incubated with 100 μl PBS-Tween (10 mM-Na2HPO4, 1.75 mM-KH2PO4, 150 mM-NaCl, 0.05% Tween-20) containing 1% dry milk instead of excess of unlabelled competitive MAbs.

Neutralization plaque reduction assay. The dilution of the individual MAbs giving 50% plaque reduction after 1 h incubation with virus was determined. The concentration of infectious virus (p.f.u.) was estimated by a plaque assay using agarose with a gelling temperature less than 28 °C (SeaPlaque; FMC-BioProducts). Virus suspension (100 μl) diluted in Eagle’s MEM (Flow) was placed centrally on a monolayer of CHSE-214 cells in a 26 x 33 mm tissue culture plate well (Nunc) and incubated at 20 °C with 0.5 % CO2. After 1 h, 5 ml growth medium with reduced fetal bovine serum concentration (2% v/v) containing 0.5% (w/v) solubilized agarose and equilibrated to 22 °C, was added. Following a further 36 h incubation, the cells were stained with 2 ml 0.001% neutral red in 0.15 M-NaCl for 2 h, and the plaques were counted.

Dot blot analysis with MAbs and deletion fragments of VP2. Purified E. coli-expressed VP2 fragments 1 μl (approx. 100 ng) and BB control antigen were dotted onto NC strips, air dried for 30 min and washed with TBS for 10 min at room temperature. CHSE-214 culture medium (1 μl) with propagated N1 virus strain diluted 1:3 in 6 mM guanidine-HCl (denatured) or in 16 mM-TRIS-HCl pH 7.4 (native), were dotted onto the NC plates as positive control antigens. Protein immobilization was demonstrated using Colloidal Gold Total Protein Stain (Bio-Rad) according to the supplier's instructions. Post-coating, incubation with hybridoma cell culture supernatant, AP conjugate incubation and colour development were performed as described for Western blotting.

Sequence analysis. Jameson-Wolf antigenicity index analysis was performed using GCG package software (Madison, WI, USA).

Results

Characterization of epitopes on aquatic birnavirus strains

Table 2 shows the results of Western blotting and virus neutralization analyses of selected virus strains representing the ten proposed serogroup A serotypes of aquatic birnaviruses, with MAbs. Table 2 also shows the ELISA results with MAb AS-1 and for comparison the ELISA results with MAbs H8, B9, F2 and E4 previously reported elsewhere (Melby & Christie, 1994).

The neutralizing MAb AS-1 reported to react with all tested serogroup A virus strains by dot blot (Caswell-Reno et al., 1989) recognized all tested virus strains except Hecht (A4) and Tellina (A5) by ELISA, and neutralized all virus strains except Hecht (A4). MAb F2 both neutralized and reacted by Western blotting with all virus strains except Tellina (A5), although it did give a weak reaction with this virus strain by ELISA. On the other hand, although MAb F2 neutralized Canada-2 and -3 (A7 and A8), these virus strains were not reactive by ELISA.
Table 3. Percentage binding inhibition of labelled VP2 specific MAb s to the N1 strain of IPNV by unlabelled VP2-specific MAb s in competitive ELISA*

<table>
<thead>
<tr>
<th>Competitive MAb</th>
<th>Labelled MAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>H8</td>
<td>95</td>
</tr>
<tr>
<td>B9</td>
<td>32</td>
</tr>
<tr>
<td>F2</td>
<td>38</td>
</tr>
<tr>
<td>AS-1</td>
<td>13</td>
</tr>
<tr>
<td>E4</td>
<td>4</td>
</tr>
</tbody>
</table>

* Based on three experiments.

MAbs H8 and B9 neutralized all virus strains recognized by ELISA. MAb H8 neutralized the virus strains West Buxton (A1), Sp (A2), Jasper-Dobos (A9) and N1 (A10), while MAb B9 only neutralized the Sp (A2) and the N1 (A10) virus strains. However, in Western blotting MAb H8 recognized all virus strains except Tellina (A5) and MAb B9 recognized all virus strains except Hecht (A4) and Tellina (A5). The non-neutralizing VP2-specific MAb E4, which recognized all tested serogroup A strains by ELISA, did not recognize the Sp (A2), Tellina (A5), Canada-2 (A7) and Canada-3 (A8) virus strains by Western blotting.

Competitive binding of virus neutralizing MAb s

Table 3 shows the results of competitive binding ELISA performed to determine whether the virus neutralizing MAb s were independent of each other and of the VP2-specific non-neutralizing MAb E4. Pre-incubation with MAb H8 showed a very limited effect on the binding of the labelled MAb s B9 (16%) and F2 (17%) to the N1 (A10) virus strain. However, MAb B9 partly blocked the binding of both MAb H8 (32%) and F2 (48%), and MAb F2 blocked the binding of MAb B9 (62%) very effectively relative to the blocking effect of MAb F2 on itself (76%). Furthermore, pre-incubation with MAb F2 partly blocked the binding of H8 (38%) to the virus. MAb AS-1 partly blocked the binding of MAb F2 (37%), but had little effect on MAb B9 (10%) and MAb H8 (13%). Pre-incubation with the non-neutralizing MAb E4 was not effective in blocking the binding of any of the labelled MAb s.

Fig. 2. Dot blot analysis of IPNV and purified recombinant deletion fragments of VP2 with MAb s against the N1 strain of IPNV. The VP3-specific MAb C12 was used as a negative control antibody and E. coli-expressed BB domain of streptococcal protein G as negative control antigen. All recombinant polypeptides were solubilized in 2 M-guanidine.HCl. N1 virus-infected CHSE-214 medium in 2 M-guanidine.HCl (top) or in buffer (bottom) were used as positive control antigens. Immobilization of the individual antigens on nitrocellulose was demonstrated by total protein staining. The numbers indicate the N- and C-terminal VP2 amino acids in the E. coli-expressed VP2 fragments (black bars), and correspond to the amino acid numbers of VP2 of the N1 virus strain. The dotted line in antigen VP2ΔpHincIIΔSacIIΔpAatII indicates the internal VP2 deletion made in this VP2 fragment.
MAb E4 showed no competitive binding for any of the virus-neutralizing MAbs tested.

Expression of VP2 fragments in Escherichia coli

The VP2-encoding cDNAs of the expression plasmid constructs described in Table 1 were successfully over-expressed in E. coli. With exception of the deletion fragment VP2ΔpAatII, which showed the lowest level of expression in E. coli, protein purification resulted in approximately 70–95% pure deletion fragments of VP2, determined by SDS-PAGE and silver staining (data not shown).

Mapping of MAb specificity by reaction with deletion fragments of VP2

The recognition of recombinant deletion fragments of VP2 by MAbs was analysed by Western blotting and dot blot analysis. There were no differences in the qualitative reaction pattern of the individual virus-neutralizing MAbs with the VP2 fragments in the Western blotting (data not shown) compared to the dot blot analysis (Fig. 2). Following deletion of amino acids (aa) 153–234 at the N-terminal part (antigen VP2ΔHincII) or aa 270–453 at the C-terminal part (antigen VP2ΔpAatII) of VP2, the virus-neutralizing MAbs H8, B9 and F2 were all unable to bind these deletion fragments. Internal deletion of aa 201–270 (antigen VP2ΔpHincIIΔSacIIΔpAatII) also abolished the binding of the virus neutralizing MAbs H8, B9 and F2.

The smallest VP2 fragment recognized by the MAbs H8 and B9 was antigen BB-dVP2 (aa 204–331), and the smallest VP2 fragment recognized by the MAb F2 was antigen VP2ΔpHincIIΔ4vaII (aa 153–330). The weaker binding of MAb F2 in the dot blot analysis compared to MAbs H8 and B9 could not be detected by Western blotting (data not shown). The binding of the MAbs F2 and E4 to denatured virus was very weak compared to binding to native virus (Fig. 2). MAb E4 was unable to bind any recombinant VP2 fragments in the dot blot analysis but recognized antigen VP2 (aa 3–453) in Western blotting (data not shown). The conserved virus-neutralizing MAb AS-1 was only able to bind to native virus in the dot blot analysis. No binding of any MAb to the control antigen (BB) or binding of the VP3-specific control MAb C12 to any VP2 deletion fragments were detected.

Amino acid sequence analysis

Eleven regions with a theoretical Jameson–Wolf antigenicity index of 1.5 or more were identified within the VP2 amino acid sequence (aa 1–500) of the N1 virus strain (Fig. 3). All 11 regions were located between aa 149 and 390.

Discussion

Our results demonstrate that the central third of VP2 of the serogroup A strains of IPNV contains at least three partly overlapping neutralization epitopes, two variable and one conserved. The variable epitopes B9 and H8 depend on amino acids between 204 and 330 which correspond to almost the entire variable region of VP2 identified by Høivarstein et al. (1990) (Fig. 3). The conserved neutralization epitope F2 depends not only on the region between amino acid 204 and 330 but also on some amino acids between 153 and 203. Deletion of amino acids 153 to 234 or 270 to 330 hindered binding of the MAbs H8, B9 and F2 to the deletion fragments of VP2 (Fig. 2), demonstrating the conformation-dependent nature of these epitopes. However, since all these MAbs recognized denatured VP2, the epitopes must easily renature to a form recognizable by the MAbs. The conserved and conformation-dependent epitopes AS-1 and E4 were not possible to map since no binding to VP2 deletion fragments could be detected. However, MAb AS-1 showed some competitive binding with the F2 MAb indicating that the F2 and the AS-1 epitope or parts of them merge into each other. The competitive binding assay further indicated that the neutralization epitopes H8, B9 and F2 are overlapping and that they are independent of the conserved non-neutralization epitope E4.
The results of the Jameson–Wolf antigenicity index sequence analysis also indicate that the central third of VP2 contains the dominant epitopes of VP2. Of the 11 regions within VP2 with a theoretical Jameson–Wolf antigenicity index of 1.5 or more, seven were located within the F2 epitope region which consists of approximately 35% of the VP2 sequence.

As far as we know regions of VP2 outside the variable region have not yet been shown to be part of any neutralization epitopes of birnaviruses. For infectious bursal disease virus (IBDV), a birnavirus pathogenic to chickens, only variable epitopes have been mapped (Azad et al., 1987; Schnitzler et al., 1993). Conserved epitopes of IBDV, like the AS-1 epitope of IPNV, appear to be highly conformation-dependent (Snyder et al., 1988, 1992) and therefore difficult to map. Recently Vakharia et al. (1994) reported major amino acid variation within the variable region of VP2 between neutralizing MAb escape strains of IBDV. In spite of major sequence variation within the variable region of these virus strains, one conserved neutralization epitope was demonstrated for all virus strains, indicating that regions outside the variable region may be part of this epitope.

Previously we reported that the IPNV-neutralizing MAb H8 and B9 were unable to recognize the Sp (A2) virus strain (Christie et al., 1990). Later other clones of the same passage of the Sp (A2) type strain have shown positive results with MAb H8 and variable results with MAb B9 (Melby & Christie, 1994). This further demonstrates the high variability of epitopes H8 and B9. Although the Sp (A2) and the N1 (A10) virus strains are serologically related, the results from the characterization of epitopes on aquatic birnavirus strains presented in Table 2 demonstrate that they are not identical. Of the European virus strains only the two related virus strains Sp (A2) and N1 (A10) contain the variable H8 epitope. It was therefore surprising that the H8 epitope is present on the Canadian Jasper-Dobos (A9) and the American West Buxton (A1) virus strains. The amino acid sequences of the N1 (A10) and the Jasper-Dobos (A9) virus strains show a high degree of variation within the H8 epitope region (Hävarstein et al., 1990) while the Jasper-Dobos (A9) and the West Buxton (A1) are believed to belong to the same genogroup (Heppel et al., 1993). Furthermore, both MAb H8 and B9 recognized a greater number of virus strains by Western blotting when compared with ELISA and neutralization assays. This indicates that denaturation induces exposure of internal epitopes or change in conformation. Consequently, although located within a highly variable region, the heterogeneity of some epitopes of aquatic birnaviruses appears to depend on conformational variation, which can be induced by minor changes in the amino acid sequence. Major changes in the amino acid sequence are usually a consequence of evolution and would therefore not be as effective a mechanism for viruses to escape neutralizing antibodies (antigenic drift) as conformation variation following minor changes in the amino acid sequence. Recently, Pryde et al. (1993) identified only two amino acid variations within the variable region of a Scottish Sp (A2) virus strain compared to the N1 (A10) virus strain, both located at the periphery of the H8–B9 epitope region. For IBDV, sequence analysis of neutralization escape variants of IBDV has proven that minor amino acid variations may induce neutralization escape variants (Lana et al., 1992; Vakharia et al., 1994).

Virus-neutralizing MAbs that bind VP2 of aquatic birnaviruses by Western blotting have also been reported by others and the epitopes suggested to be of linear nature (Tarrab et al., 1993). However, epitopes which are recognized by MAb following denaturation by, for instance, SDS–PAGE and Western blotting are not necessarily linear. In fact, specific procedures to increase binding of MAbs to conformation-dependent epitopes following Western blotting have been developed (Dunn, 1986). The neutralization epitope defined by MAb AS-1 and common to all serogroup A virus strains except Hecht (A4) appears unable to spontaneously renature, indicating that the conformation of epitope AS-1 on the virus capsid is of a more complex nature than that of epitopes H8, B9 and F2.

With regard to a candidate subunit vaccine against IPN in fish, the F2 epitope should be an important component since it is common to all serogroup A strains of IPNV. The Tellina strain (A5), which is not neutralized by MAb F2, is an aquatic birnavirus isolated from molluscs and is not known to cause IPN in fish. Furthermore, a structure resembling the epitope F2 is present on recombinant VP2.

We are greatly indebted to Signe Ness and Connie Folkestad Husey for excellent technical assistance. We also thank Dr Peter Nilsson and Helena Granér for their help with the BB and BB-dVP2 polypeptides and Dr B. L. Nicholson for providing the monoclonal antibody AS-1. This work was supported by the Norwegian Research Council of Fisheries.

References


(Received 14 October 1994; Accepted 13 December 1994)