Antigenic characterization of serogroup ‘A’ of infectious pancreatic necrosis virus with three panels of monoclonal antibodies

Esther Tarrab, Laurent Berthiaume, Joël Heppell, Maximilien Arella and Jacqueline Lecomte*

Institut Armand-Frappier, Centre de Recherche en Virologie, 531 Boulevard des Prairies, Laval, Québec, Canada H7N 4Z3

Monoclonal antibodies (MAbs) were produced against three serotypes of infectious pancreatic necrosis virus (IPNV): A1 (LWVRT 60-1, U.S.A.), A2 (d’Honninc-thun, France) and A9 (Jasper, Canada). Each panel of MAbs (identified as LW, HF and JA) was analysed by ELISA with the 10 proposed serotypes of IPNV and their specificity defined by immunoprecipitation and Western immunoblotting analysis. A first group of MAbs, directed against the outer capsid protein VP2, reacted with linear or conformational epitopes. A second group of MAbs, directed against the internal protein VP3, reacted with linear epitopes. There was no relationship between the neutralizing property of anti-VP2 MAb and the configuration of the epitope that it recognized. The MAbs were used for antigenic characterization of serogroup A. Each panel of MAbs showed a characteristic pattern of reactivity. The European HF series was predominantly cross-reactive and detected conserved epitopes among the 10 serotypes for both VP2 and VP3. The North American LW and JA series identified a group of conserved epitopes on VP3 and new specific epitopes on VP2 and VP3. The higher variability observed for VP2 in comparison with VP3 is one example of how external pressures may promote natural selection of those epitopes required for virus survival. Our results are consistent with an ancestral relationship of the European to the North American strains, the latter having developed new antigenic determinants upon evolution in their new geographical location.

Infectious pancreatic necrosis virus (IPNV), a common viral pathogen in brook and rainbow trout hatcheries, was first isolated by Wolf et al. (1960). Since then, similar viruses have been isolated from different fish and molluscs in North and South America, Europe and Asia. The IPNV, together with the avian infectious bursal disease virus (IBDV) and the virus X of the drosophil fly, are part of the Birnaviridae family (Brown, 1986). These viruses are characterized by icosahedral non-enveloped particles with two segments, A and B, of double-stranded RNA. Three structural proteins are present in IPNV (Dobos & Roberts, 1982). VP1, of Mr 105K, is the RNA polymerase and is derived from monocistronic translation of the small RNA B segment. VP2, of 54K, is the outer capsid protein and VP3, of 31K and 29K, has been described as the internal (Dobos et al., 1977; Dobos & Mertens, 1983; Duncan et al., 1987) or capsid protein (Duncan & Dobos, 1986). In vitro, both VP2 and VP3 proteins are derived from proteolytic cleavage of a polyprotein precursor encoded by the large RNA A segment (Duncan et al., 1987).

Antigenic and functional analyses of these structural proteins from IPNV and IBDV have shown important homologies. It has been demonstrated that VP2 elicits neutralizing antibodies and carries serotype-specific antigenic determinants (Caswell-Reno et al., 1986; Christie et al., 1990; Jagadish & Azad, 1991; Oppling et al., 1991a). IPNV and IBDV neutralizable epitopes are localized in a hydrophilic internal hypervariable segment of VP2 and have been found to be highly conformation-dependent (Håvarstein et al., 1990; Heine et al., 1991; Lana et al., 1992) although some authors reported some hydrophobic character of this protein (Azad et al., 1987). Besides this region, a linear epitope on the IBDV VP2 protein was shown to be recognized by cross-reacting monoclonal antibodies (MAbs) (Oppling et al., 1991a). Recently, linear neutralizable epitopes have been described for IBDV (Fahey et al., 1991). The biological function of the VP3 protein has not yet been established. Because it is immunodominant and highly immunogenic, specific antibodies have easily been obtained for IBDV (Azad et al., 1986; Fahey et al., 1989) with no conclusive results about their protective capacity (Fahey et al., 1985; Azad et al., 1986). Indeed, it was suggested that sequential epitopes on VP3 could contribute to the folding of VP2 (Fahey et al., 1989). Little information has been obtained for VP2 because neutralization epitopes are rapidly lost during denaturation, and
for VP3 because no biological function has yet been assigned to this protein.

It has been shown that group- and serotype-specific epitopes can be demonstrated on IPNV (Wolski et al., 1986; Lecomte et al., 1992) and IBDV VP3 proteins (Öppling et al., 1991b). Antigenic diversity has also been reported for both viruses (Jackwood & Saif, 1987; Hill & Way, 1988). Hill & Way (1988) proposed two antigenically unrelated serogroups, A and B, comprising nine and six serotypes respectively. The majority of aquatic birnavirus isolates worldwide belong to serogroup A. Most of the strains isolated in North America are similar and included in the A1 serotype, except for a few Canadian strains which were grouped in the A6 to A9 serotypes. European strains are mainly included in the A2 and A3 serotypes, with some strains corresponding to the A4, A5 and A10 serotypes. The A10 serotype was recently isolated in Norway by Christie et al. (1988). These viruses form a complex group and different patterns of reactivity exist between members of the same or different serotypes. MAbs have been useful in the antigenic analysis and serotyping of IPNV, enabling a fine discrimination between strains (Caswell-Reno et al., 1986, 1989; Wolski et al., 1986; Lipipun et al., 1989; Christie et al., 1990; Dominguez et al., 1990, 1991). However, a certain confusion still persists because either a limited number of MAbs have been employed or some but not all of the serotypes were analysed. As new viruses are obtained, it will be necessary to compare them with the 10 proposed serotypes and to monitor any antigenic changes.

We have prepared MAbs against the A1, A2 and A9 IPNV serotypes since they represent three geographical areas of interest and a comparative analysis of all the known serotypes of group A viruses could give a better idea of their antigenic diversity. Chinook salmon embryo cells (CHSE-214), rainbow trout gonad tissue cells (RTG-2) and IPNV strains of serotypes A1 [LWVRT 60-1 (U.S.A.)], A2 [d'Honnincuthun (France)], A3 [Ab (Denmark)], A4 [Hecht (Germany)], A5 [Tellina-2 (England)], A6 (Canada-1), A7 (Canada-2), A8 (Canada-3) and A9 [Jasper (Canada)] were obtained from the American Type Culture Collection. The A10 serotype (N1, Norway) was kindly provided by Dr K. E. Christie (University of Bergen, Norway). LWVRT, d'Honnincuthun and Jasper strains were plaque-purified before use. Cell cultures were grown at 20 °C in 1:1 Hanks’ 199-MEM with Earle’s salts, supplemented with 10% fetal bovine serum (FBS) as described previously (Heppell et al., 1992). Suspensions of stock viruses were prepared from clarified supernatants (10000 g, 15 min) obtained after three cycles of freezing and thawing.

For production of MAbs, BALB/c mice received intraperitoneally 1 to 4 μg of concentrated virus (LWVRT, d'Honnincuthun or Jasper strains) emulsified in Freund's complete adjuvant, according to the previously published schedule (Lecomte et al., 1992). Spleen cells were fused with the P3X63Ag.8.653 murine myeloma cells (Lecomte, 1985). Hybridoma secretion was monitored by ELISA (Lecomte et al., 1992).

Radiolabelling of virus was done in CHSE-214 cells infected at high m.o.i. with the d'Honnincuthun, LWVRT or Jasper strains according to the method of Dobos & Rowe (1977). One h later, the inoculum was replaced with growth medium containing 2% dialysed FBS and 1% HEPES. Infection was continued for another 8 h (16 h for Jasper) and for 1 h a methionine-free growth medium was substituted. Infected cultures were u.v.-irradiated and labelled with 50 μCi/ml of [35S]methionine (1079 Ci/mmol, ICN). After 2 h, cells were washed with fresh Earle's salt solution and lysed in cold RIPA buffer (154 mM-NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1% aprotinin, 1 mM-PMSF, 10 mM-Tris–HC1 pH 7-4). Uninfected cells were similarly processed and used as controls.

For immunoprecipitation, supernatants of precleared (90000 g, 5 min, 20% sucrose cushion, Beckman airfuge) labelled cell lysates were reacted with undiluted MAbs as hybridoma supernatants or rabbit antiserum diluted 1:100 (1 h at 4 °C). Protein A–Sepharose (10% in RIPA buffer) was added, incubated for 1 h at 4 °C and immune complexes were collected by centrifugation (12800 g). Beads were extensively washed in the RIPA buffer, eluted in the electrophoresis sample buffer (ESB; Dobos & Rowe, 1977) and centrifuged at 12800 g. Supernatants were heated for 10 min at 96 °C and electrophoresed on 10% polyacrylamide gels (Laemmli, 1970). The gels were fixed in 10% acetic acid 30% methanol, soaked in En3Hance (New England Nuclear), rinsed in distilled water and dried at 80 °C. They were exposed to X-Omat film at −70 °C for 1 to 5 days. Western immunoblotting was carried out as described previously (Lecomte et al., 1992).

Each series of MAbs (HF, LW and JA, for d'Honnincuthun, LWVRT and Jasper, respectively), were selected by ELISA against the homologous strain. Specific reactions (A > 0.5) were obtained using 1:100 dilutions of concentrated virus. MAbs showed negligible values with concentrated non-infected cells (A < 0.05). Supplementary controls included rabbit antiserum, normal serum and irrelevant MAbs. In this way, nine MAbs from the A2 serotype (HF MAbs), eight from the A1 serotype (LW MAbs) and five from the A9 serotype (JA MAbs) have been retained for further characterization (Table 1). The isotype analysis indicated that, except for LW8, which is an IgM (data not shown), all the MAbs are of the IgG class (subclasses 1, 2a, 2b and 3) with light chains...
Table 1. Characterization of MAbs HF, LW and JA

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>ELISA*</th>
<th>WB~</th>
<th>IP</th>
<th>NEUT.§</th>
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<tr>
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</tr>
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<td>0.1</td>
<td>0.1</td>
<td>VP3</td>
</tr>
<tr>
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<td>--</td>
<td>VP2</td>
</tr>
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<td>IgG2 (\kappa)</td>
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<td>0.7</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
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<td>1.0</td>
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</tr>
<tr>
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<td>IgG1 (\kappa)</td>
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<td>--</td>
<td>--</td>
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</tr>
<tr>
<td>HF7</td>
<td>IgG1 (\kappa)</td>
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<td>--</td>
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<tr>
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<td>0.6</td>
<td>--</td>
<td>VP3</td>
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<tr>
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<td>0.8</td>
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<tr>
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<td>1.4</td>
<td>0.8</td>
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<td>1.6</td>
<td>0.9</td>
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<tr>
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<tr>
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<td>--</td>
<td>0.9</td>
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</tr>
<tr>
<td>JA7</td>
<td>IgG1 (\kappa)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>VP2</td>
</tr>
</tbody>
</table>

* ELISA values represent the absorbance at 492 nm with undiluted hybridoma supernatants (– represents < 0.1).
† IP: Protein specificity in immunoprecipitation (ND, not done).
‡ WB: Protein specificity in Western blots (–, antibody failed to react).
§ NEUT.: Plaque neutralization assays of 100 to 200 p.f.u./ml with undiluted hybridoma supernatants (+, complete neutralization; +/–, partial neutralization; –, absence of neutralization).

Type kappa. The immunoglobulin concentrations of the hybridoma supernatants were 30 to 75 µg/ml. Antigenic specificities of the selected MAbs, as determined by immunoprecipitation and Western immunoblotting (Fig. 1, Table 1), showed that proportionally anti-VP3 antibodies were more frequent in the HF series and that anti-VP2 MAbs were predominant in the LW and JA panels. Whereas anti-VP3 MAbs reacted exclusively with linear epitopes (HF1, 2, 3, 7, 8, 9; LW1, 3; JA2), anti-VP2 MAbs exhibited two different binding patterns: one group reacted only in immunoprecipitation (HF4, 5; LW9, 10; JA1, 3, 7) and the other group also reacted in Western immunoblotting (HF6; LW4, 6, 7; JA5). These observations strongly suggest the existence of conformational and linear epitopes on VP2. Furthermore, both types of VP2 epitopes could be either implicated or not in virus neutralization. For example, neutralizing MAbs HF4, LW9, 10, JA1, 3 and non-neutralizing MAbs HF5 and JA7 recognized conformational epitopes, whereas neutralizing MAbs LW4, 6, 7, JA5 and non-neutralizing MAb HF6 recognized linear epitopes. On the other hand, none of the anti-VP3 MAbs were found to have neutralizing properties, except MAb LW3 which constitutes a singular case because it neutralized 100% of infectious virus when undiluted but failed to neutralize upon dilution. The degree of cross-reactivity, as determined by ELISA, was characteristic of individual panels. The HF MAbs were predominantly cross-reactive, but the LW and JA MAbs were more restricted in their specificity. Anti-VP3 MAbs recognized heterologous strains with one exception (HF3) and anti-VP2 MAbs recognized homologous strains with some exceptions (HF4, 5, 6, 7).

Antigenic relationships of the 10 IPNV serotypes were analysed by ELISA with the three series of MAbs. As shown in Fig. 2, IPNV strains appeared highly related when HF MAbs were used to study their cross-reactivities. The HF panel showed a high antigenic homogeneity among the European strains and a close relationship between some of them and the North American strains. Indeed, the 10 serotypes exhibited common epitopes on VP3 and VP2 with MAbs HF1, 2, 5 and 9. On the other hand HF4, the only neutralizing anti-VP2 MAb of this panel, cross-reacted with all the European strains except Hecht but it did not recognize any of the North American strains, whereas HF6, a less specific non-neutralizing anti-VP2 antibody, did not recognize LWVRT and Jasper. In both cases Hecht was different from the other European strains. Among the anti-VP3 MAbs, only HF3 exhibited a restricted pattern since it did not react with the North American strains except Hecht but it did not recognize any of the North American strains, whereas HF6, a less specific non-neutralizing anti-VP2 antibody, did not recognize LWVRT and Jasper. In both cases Hecht was different from the other European strains. Among the anti-VP3 MAbs, only HF3 exhibited a restricted pattern since it did not react with the North American strains (except for a weak reaction with Canada-1) and with the European strain Hecht.

LW MAbs prepared against the A1 serotype showed a limited pattern of reactivity against the 10 serotypes.
None of these antibodies reacted with Ab, Tellina-2 and Canada-1 which are probably different at the VP2 and VP3 levels. Anti-VP3 LW MAbs were significantly more cross-reactive than anti-VP2 MAbs. Among the first ones, LW2 and LW3 showed that LWVRT, Jasper, Canada-2, d’Honnincthun, N1 and probably Canada-3 and Hecht share a similar epitope. LW1 MAb was more restricted since it did not react with any of the European strains and recognized only two North American strains, LWVRT and Jasper. The anti-VP2 MAbs constitute a relatively specific group. Beside the homologous strains, weak cross-reactivities occurred with Jasper (LW4, 6, 7) and N1 (LW6, 7, 9, 10). MAbs of the LW group demonstrated a close relationship between LWVRT and Jasper at both the VP3 and VP2 levels. The European strain N1 was also recognized by some MAbs against VP2 and VP3. These three strains are probably antigenically related.

The JA MAbs include one anti-VP3 antibody (JA2) which cross-reacted with the 10 serotypes and four highly specific anti-VP2 antibodies (JA1, 3, 5, 7). Three were specific for Jasper only and the other (JA3) reacted also with Canada-1. As was observed with the HF and LW MAbs, IPNV strains share VP3 epitopes but are more variable in their VP2 epitopes (Fig. 2).

These studies allowed the identification of both conformational and linear neutralization epitopes on VP2 and showed that there is no relationship between the neutralizing property of a MAb and the configuration of the epitope it recognizes. On the other hand, all the neutralizing MAbs, when tested with the 10 serotypes by ELISA, reacted strongly against the homologous strains. Cross-reactivities were limited to a few strains (LWVRT, Jasper, Canada-1 or N1) for LW and JA MAbs. The neutralization epitope defined by HF4 is well conserved among the European strains but is absent from the North American strains. In the same way, North American strains developed neutralization sites different from the European ones. From these observations, we can speculate that IPNV strains acquire new neutralization epitope motifs when propagated in different environments. Such an antigenic polymorphism was also observed for VP2 of IBDV. This was explained by minor changes on the amino acid sequence that contributed to the emergence of variant virus (Heine et al., 1991; Lana et al., 1992). On the other hand, antigenic stability of cross-reactive epitopes has also been reported for IPNV and IBDV (Caswell-Reno et al., 1986; Becht et al., 1988).

Of the three non-neutralizing anti-VP2 MAbs, two reacted with conserved epitopes that were conformation-dependent (HF5) or -independent (HF6) and the other (JA7) reacted with a variable conformation-dependent epitope. This is novel evidence that IPNV non-neutralizable epitopes on VP2 could be either conserved (conformational or linear) or variable (conformational). A cross-reactive site recognized by non-neutralizing antibodies to VP2 was previously reported for IBDV (Becht et al., 1988; Öppling et al., 1991a).

The anti-VP3 MAbs have shown particular characteristics. All reacted with linear epitopes in Western blots. It is possible, as previously suggested, that short segments of these epitopes could be exposed at the virion surface (Caswell-Reno et al., 1986). The immunogenicity of this protein and its hydrophilicity profile (data not shown) are supplementary evidence for a possible external domain. Even if VP3 of IPNV has not been implicated in virus neutralization, the observation that MAb LW3 partially neutralizes virus infectivity supports the possibility that short amino sequences of VP3 could be exposed on the outer capsid. The antigenic analysis of IPNV serotypes with anti-VP3 MAbs has shown an important number of well or relatively well conserved epitopes. Hence, strains were indistinguishable (HF1, 2, 3; JA2) or they could be differentiated only when they failed to react with a particular MAb (HF3, 7, 8; LW1, 2, 3). For example, European and North American strains constitute two separate groups if they were analysed with MAb HF3, whereas individual strains could be distinguished with MAbs HF7 and 8 and with the three anti-VP3 LW MAbs. In this case, the analysis
by ELISA and Western blotting of MAbs LW1, 2 and 3 enabled the prediction of at least two distinct epitopes on VP3. One, revealed with LW2 and LW3 MAbs, is conserved among seven serotypes. The other, detected with LW1 MAbs, is present on two related strains, LWVRT and Jasper. The reactivities of these MAbs in Western blots showed the same weak pattern for LW2 and LW3 whereas a strong reaction was observed for LW1. We conclude that LW2 and LW3 are directed toward the same antigenic site, which is different from that recognized by LW1. This was confirmed by competitive and additivity ELISAs (data not shown). Our results show the importance of using different panels of MAbs for characterizing heterogeneous groups of viruses.

The analysis of IPNV serotypes with all the MAbs has shown a greater antigenic uniformity among the European strains than among the North American serotypes. HF MAbs detected conserved epitopes on VP2 and VP3 proteins, and LW and JA MAbs were cross-reactive on VP3 epitopes but highly specific on VP2 epitopes. Consequently, all IPNV strains seemed closely related when examined with the HF panel or with the anti-VP3 panel of LW and JA MAbs. However, IPNV strains were different with anti-VP2 LW and JA MAbs. This suggests that the European strains are ancestral to the North American strains. The IPNV group is heterogeneous and contains a mosaic of antigenic sites, some conserved and others more variable. Genomic analysis of serotype strains also reflects this variability (Heppell et al., 1992). The simultaneous comparison of the 10 serotypes with three panels of MAbs has allowed prediction of a possible antigenic evolution of these viruses in relation to the structural proteins and changes of their epitopes. Some strains, such as Ab and Tellina-2, were indistinguishable but the others could be related on the basis of VP2 and/or VP3 similarities, for example, LWVRT with Jasper and N1, Canada-2 with Canada-3, Hecht with Jasper, Canada-1 with Ab and Tellina-2. These observations suggest that the subdivision of serogroup A into 10 serotypes should be re-evaluated.

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References


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