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Monoclonal Antibodies to Infectious Pancreatic Necrosis Virus: Analysis of Viral Epitopes and Comparison of Different Isolates

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SUMMARY

A panel of five monoclonal antibodies (MAbs) produced against the West Buxton isolate of infectious pancreatic necrosis virus was used to investigate viral antigens and to compare different aquatic birnavirus isolates antigenically. Reciprocal blocking ELISA and neutralization assays indicated that these MAbs identified four, and possibly five, structurally and/or functionally different epitopes on the virion. Western immunoblot analysis demonstrated that one epitope was present on VP2, the large (51000 mol. wt., 51K) capsid protein, and another epitope was located on the two smallest structural proteins, VP3 (32K) and VP4 (30K). Three MAbs did not react with any of the solubilized viral proteins; the epitopes recognized by these MAbs may have been altered when the virion was solubilized with SDS. Comparison of reactivity patterns of the five MAbs with various aquatic birnaviruses in ELISA and neutralization tests demonstrated that 14 isolates tested from four serotypes represented a minimum of nine antigenically distinct viruses; i.e., distinct patterns of reactivity were shown among several viruses within the same serotype. Two MAbs identified different epitopes that were highly conserved among, and largely restricted to, members of the West Buxton (U.S.A.) serotype, whereas two other MAbs recognized an epitope(s) present only on some members of this serotype. The other MAb defined an epitope that was more widely distributed among the aquatic birnaviruses and found on all representatives tested from two serotypes.

INTRODUCTION

Infectious pancreatic necrosis virus (IPNV), together with the avian infectious bursal disease virus (IBDV) and Drosophila X virus of insects (DXV), is a member of the newly recognized family designated *Birnaviridae* (Dobos *et al.*, 1979). The birnaviruses are medium sized (approximately 60 nm in diam.), unenveloped, icosahedral viruses with bi-segmented, double-stranded RNA genomes. IPN virus is the aetiological agent of an economically important disease of salmonid fishes (Wolf *et al.*, 1960). Viruses similar or identical to IPNV have been isolated from various other fish species and marine molluscs in North America, Europe and Asia (Hill, 1976*a*; Sano, 1977; Sonstegaard *et al.*, 1972; Stephens *et al.*, 1980; Underwood *et al.*, 1977; Wolf & Mann, 1980).

Serological studies of the aquatic birnaviruses have indicated that most cross-react to some extent but significant antigenic differences occur among the numerous isolates. Based on the relative degree of cross-neutralization by rabbit antisera, at least three serotypes have been identified (Hill, 1976*b*; Macdonald & Gower, 1981; Okamoto *et al.*, 1983). Recently, Hill & Way (1983) have examined by cross-neutralization with polyclonal rabbit antisera 175 virus isolates from 44 fish and shellfish species from 11 countries and demonstrated six additional serotypes which together with the three serotypes originally described represent a major serogroup. Four additional isolates were identified that represented a second unrelated serogroup. All of the virus isolates from the United States represented a distinct serotype originally designated VR299 but which Hill & Way designated as West Buxton. Previous investigations of the kinetics of IPNV

neutralization by polyclonal antisera have indicated that significant antigenic differences exist among individual members of the West Buxton (U.S.A.) serotype (Nicholson & Pochebit, 1981).

In an effort to investigate the antigenic relationships among these viruses in greater depth and to provide more specific and standardized reference antisera, we have generated monoclonal antibodies (MAbs) against the West Buxton (U.S.A.) isolate of IPNV. This report describes the production of these MAbs and their use in the identification of specific viral antigenic determinants and the comparison of different virus isolates.

METHODS

Viruses. Stock preparations of 14 isolates of IPNV and other aquatic birnaviruses from North America, Europe and Asia were cloned by endpoint dilution and propagated in CHSE-214 cell cultures (Lannan *et al.*, 1984) at 20 °C in Eagle's MEM supplemented with 10% foetal bovine serum as described previously (Nicholson *et al.*, 1979). Viral isolates from trout and Atlantic salmon in North America and Europe have been maintained in our laboratory for many years and included West Buxton (Maine), Dry Mills (Maine), Berlin (New Hampshire), Gilbert (Massachusetts), American Type Culture Collection strain VR299 (West Virginia), Reno (Nevada), Buhl (Idaho), AS (Quebec, Canada), d'Honnincthun (France), Sp (Denmark) and Ab (Denmark). An isolate from menhaden (Stephens *et al.*, 1980) was kindly provided by F. Hetrick, University of Maryland, College Park, Md., U.S.A. Also included was a virus from oysters (Hill, 1976*a*) isolated by B. Hill, MAFF Fish Diseases Laboratory, Weymouth, U.K. and one isolated from trout in Taiwan kindly provided by J. Wu, Academia Sinica, Taipei, Republic of China.

Virus purification. For immunization of mice, the West Buxton IPNV isolate (WB-IPNV) was concentrated by polyethylene glycol (PEG), extracted with freon and purified by isopycnic CsCl gradient centrifugation as described by Macdonald & Yamamoto (1977).

For coating wells of microtitration plates used in enzyme-linked immunosorbent assays (ELISA), each virus isolate was partially purified by layering on a 30 to 60% (w/w) sucrose step gradient in TNE buffer (0·025 M-Tris, 0·1 M-NaCl, 1 mM-EDTA, pH 7·3) and centrifuging at 95000 *g* in a SW28 rotor in a Beckman L5-65 ultracentrifuge for 2 h at 4°C. The virus was removed from the interface between the 30% and 60% sucrose and dialysed against saline for 16 h.

Production of hybridomas. Eight-week-old BALB/c mice (Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.) were immunized with purified WB-IPNV ($4{\cdot}6 \times 10^8$ $TCID_{50}$, approx. 0·8 μg protein) at approximately 1 month intervals. The first injection was administered intraperitoneally (i.p.) with complete Freund's adjuvant, the second i.p. but without adjuvant. Approximately 1 month later, mice were given a boosting dose of $6{\cdot}3 \times 10^8$ $TCID_{50}$ of virus (approx. 1·0 μg protein) intravenously (i.v.) in the tail vein. Three days after the last injection, serum samples from each mouse were taken and assayed for IPNV antibody in an ELISA (Nicholson & Caswell, 1982). Mice with high antibody titres were used as a source of spleen cells on the 4th day after the last injection.

The fusion protocol was a modification of that of Kwan *et al.* (1980). Spleen cells were fused with Sp2/0-Ag-14 mouse myeloma cells (Schulman *et al.*, 1978) at a ratio of 10 : 1 with PEG (mol. wt. 1500) in serum-free growth medium consisting of Dulbecco's modified Eagle's MEM with D-glucose (4·5 mg/ml), NCTC 109 medium (10%), bovine pancreatic insulin (0·2 units/ml), oxaloacetic acid (1 mM) and pyruvic acid (0·45 mM). The fused cells were resuspended at a concentration of $2{\cdot}5 \times 10^5$ myeloma cells/ml in growth medium (GM) supplemented with 15% foetal bovine serum, hypoxanthine (0·1 mM), aminopterin (4×10^{-4} mM) and thymidine ($1{\cdot}6 \times 10^{-2}$ mM) (GM-HAT). Aliquots of 0·1 ml of cell suspension were added to each well of 96-well microtitre plates. The cultures were maintained by feeding every 5 to 7 days with GM-HAT for 3 weeks at which time they were adapted to HAT-free GM. The supernatants were tested periodically for WB-IPNV-specific antibody using an ELISA (Nicholson & Caswell, 1982). Hybridomas producing specific antibody were expanded, cloned at least twice, and frozen in liquid nitrogen. The MAbs used in this investigation were obtained from hybridoma culture supernatants or mouse ascites. Ascitic fluids containing MAbs were obtained from syngeneic mice inoculated i.p. with 5×10^5 hybridoma cells 2 weeks after i.p. inoculation of Pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemicals, Milwaukee, Wis., U.S.A.).

Determination of immunoglobulin isotype. Monoclonal antibody isotypes were determined in an ELISA using heavy and light chain class- and subclass-specific reagents (Zymed Laboratories, Burlingame, Ca., U.S.A. or Boehringer Mannheim Biochemicals, Indianapolis, Ind., U.S.A.).

MAb blocking assays. The ability of one MAb to block the subsequent binding of another MAb in ELISA was tested by the additivity method of Friguet *et al.* (1983). This method requires that the antigen be saturated with each antibody tested. To ensure that excess antibody was available and that antigen saturation was achieved, curves of the saturation of each antigen by each antibody (obtained from ascites) were determined. Also, these experiments were repeated with twice the amount of enzyme-linked tracer to be certain that the plateaus were reached because of antigen saturation and not because the tracer was exhausted. Each MAb was used in the assay

at concentrations 25- to 100-fold in excess of the minimum concentration required for antigen saturation. A negative control antibody (WO) obtained from ascitic fluid produced by an IPNV antibody-negative hybridoma was used at dilutions (e.g., 1:500) comparable to the lowest-titred MAb. Immulon II microtitration plates (Dynatech Laboratories, Alexandria, Va., U.S.A.) were coated with 2×10^9 $TCID_{50}$/ml of virus (approx. 3 μg protein/ml) in coating buffer (0·5 M-carbonate–bicarbonate, pH 9·6) at 20 °C for 24 h. The plates were washed and sites not binding virus were blocked by adding a 1% solution of bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0·05% Tween-20, for 1 h at room temperature. After washing, the first IPNV MAb (MAb1) at antibody excess or negative control antibodies were added, incubated for 2 h at room temperature and washed. The second MAb (MAb2) or negative control antibodies were then added and similarly incubated and washed. Rabbit anti-mouse Ig–alkaline phosphatase (Ig–ALP) (Miles Scientific, Naperville, Pa., U.S.A.) was added, incubated for 2 h, and washed. Finally, substrate was added, incubated for 30 min, and the reaction was stopped by the addition of 1 M-NaOH. The absorbances were read at 405 nm and additivity indices (AI) were calculated as follows:

$$AI = 100 \times [(2 \times A \text{ of MAb1 and MAb2 together})/(A \text{ of MAb1 alone}) + (A \text{ of MAb2 alone})] - 1).$$

Analysis of MAb binding sites by Western immunoblot. Purified WB-IPNV (2×10^{10} $TCID_{50}$, approx. 33 μg protein) was solubilized, applied onto 7·5% polyacrylamide–SDS gels (Laemmli, 1970) and electrophoresed at 250 V for approximately 3 h in a 14·4 mM-Tris–glycine running buffer pH 8·8. Fully reduced proteins were prepared by boiling in sample buffer containing 5% 2-mercaptoethanol and 2% SDS. Unreduced proteins were prepared without boiling or mercaptoethanol treatment. All gels and running buffers contained SDS. Electrophoretically separated proteins were transferred electrophoretically to nitrocellulose at 400 mA for approximately 4 h using a transfer buffer of 25 mM-Tris, 192 mM-glycine, 20% methanol, pH 8·3. Strips of nitrocellulose were removed and stained directly with amido black. The remainder of the nitrocellulose sheets were washed overnight in PBS with 0·5% BSA and cut into 6 mm-wide strips. After being saturated with protein by soaking in PBS with 3% BSA and 10% calf serum (CS) for 1 h at room temperature, the strips were probed with the appropriate MAb from an appropriate dilution of mouse ascites fluid in PBS, 3% BSA, 10% CS for 4 h at room temperature. Following washing, the strips were incubated in a 1:500 dilution of goat anti-mouse IgA, IgM, IgG conjugated with alkaline phosphatase (Cappel Laboratories, Cochranville, Pa., U.S.A.) for 2 h at room temperature. Finally, the strips were washed and incubated in BCIP/NBT phosphatase substrate, consisting of 5-bromo-4-chloro-3-indolyl phosphate disodium salt 0·15 mg/ml *p*-nitro blue tetrazolium chloride, 0·30 mg/ml in carbonate buffer (0·1 M-$NaHCO_3$, 1·0 mM-$MgCl_2$, pH 9·8) (Kirkegaard and Perry Laboratories, Gaithersburg, Md., U.S.A.) for 15 min at room temperature. The reaction was stopped by washing in distilled water.

Neutralization tests. Aliquots of fourfold dilutions of hybridoma supernatants were mixed with an equal volume of growth medium containing 2×10^3 $TCID_{50}$/ml of virus. The virus–antibody mixtures were incubated for 30 min at room temperature and 0·1 ml of each reaction mixture was added to four wells of a 96-well microtitration plate with monolayer cultures of CHSE-214 cells in 0·1 ml of growth medium. Cytopathic effects were determined after incubation at 20 °C for 7 days and the 50% neutralizing dose (ND_{50}) was calculated by the method of Kärber (1931).

Cross-reactivity in ELISA of WB-IPNV MAbs with other aquatic birnaviruses. The cross-reactivity of WB-IPNV MAbs with other birnaviruses was tested in an indirect ELISA modified from the method of Nicholson & Caswell (1982). Wells of Immulon II plates were coated directly with partially purified preparations of each virus (approx. 10^8 $TCID_{50}$/ml or 0·2 μg protein/ml) in coating buffer, incubated for 16 h at 4 °C or 48 h at 20 °C, and washed with PBS–Tween. This concentration of virus was 30- to 100-fold greater than the minimum required for a positive result in ELISA with homologous virus and antibody (Nicholson & Caswell, 1982 and unpublished data). The MAbs from hybridoma culture supernatants were standardized to contain at least 1000 to 2000 times the minimum amount required to give a positive ELISA with homologous virus, added to plates, incubated for 2 h, and the plates were washed. Rabbit anti-mouse IgG–ALP then was added, incubated for 2 h, and washed. Finally, substrate was added, incubated for 45 min, and the reaction stopped with 2 M-NaOH. The absorbances were read at 405 nm. A reaction was considered positive when the mean A_{405} with specific antibody was equal to or greater than twice the A_{405} with negative control antibody, i.e. a positive to negative (P/N) ratio $\geqslant 2$. Occasionally, the method of Nicholson & Caswell (1982) was used with isolates which were known to react with polyclonal guinea-pig WB-IPNV antiserum. Immulon II plates were first coated with guinea-pig antiserum followed by the addition of unpurified virus. Subsequent steps for comparison of reactivity with MAbs were identical to those using virus bound directly to the plastic.

RESULTS

Production of hybridomas

A number of successful fusions were achieved using spleen cells from mice immunized with purified WB-IPNV as described under Methods. Approximately 25% of the wells seeded yielded hybridomas of which approximately 10% produced sustained levels of WB-IPNV-

Table 1. *Reciprocal blocking of WB-IPNV monoclonal antibodies in ELISA measured by additivity index*

	MAb2†					
MAb1*	W1	W2	W3	W4	W5	WO‡
W1	−1·0§	−6·4	20·8	69·0	37·8	86·2
W2	17·0	7·4	32·6	65·0	46·3	102·9
W3	22·2	46·6	5·3	57·9	54·6	87·2
W4	62·8	64·7	41·5	40·5	42·8	71·2
W5	50·0	46·8	51·8	58·4	−10·2	87·0
WO	102·8	117·8	99·5	145·6	69·6	ND‖

* MAb1, Monoclonal antibody applied first.
† MAb2, Monoclonal antibody applied second.
‡ WO, Mouse ascites from hybridoma producing antibody not reactive with WB-IPNV.
§ Additivity index (see Methods). Values are average of two or three experiments.
‖ ND, Not done.

specific antibodies detected by ELISA screening. Ultimately five hybridomas designated W1, W2, W3, W4 and W5 were selected for further characterization, cloned, and used in this investigation. Four different isotypes were represented among the five MAbs: W1, IgG2b; W2, IgG2a; W3, IgG2b; W4, IgG3; W5, IgG1. All MAbs contained κ light chains.

MAb blocking assays

The epitope specificities of the five MAbs were analysed using a reciprocal blocking ELISA as described under Methods. All possible pairs of antibodies at concentrations in excess of that required for antigen saturation were tested to determine whether the binding of one MAb blocked the subsequent binding of any other. This method enabled the determination of an AI for each antibody combination which compared the amount of two MAbs binding together to virus-coated plates with the sum of the amount of each MAb binding separately. Theoretically, the AI for two MAbs that bind independently at different sites should equal 100, whereas two MAbs that bind at the same site and/or completely block each other should yield an AI of 0. AI values between 0 and 100, therefore, reflect the relative degree to which one MAb interferes with the binding of a second MAb.

As shown in Table 1, all five WB-IPNV MAbs interfered to some degree with each other in the ELISA. However, from the relatively high reciprocal AIs of each combination it appeared that MAbs W3, W4 and W5 recognized epitopes that were different from each other and different from the epitope(s) recognized by MAbs W1 and W2. W1 completely blocked the subsequent binding of W2; however, addition of W2 first did not completely block the subsequent binding of W1. Because some interference in binding was demonstrated for all MAb combinations, the ability of each MAb to block the binding of the other antibodies in an ELISA was also tested by taking advantage of the differences in immunoglobulin isotype. The amount of a single antibody binding alone was compared to that antibody binding in the presence of another antibody using specific, enzyme-labelled anti-isotype antibodies (data not shown). In this case, no competition was detected between W3, W4 and W5. However, as with the previous test, there was a non-reciprocal blocking observed with W1 and W2; i.e., W1 effectively blocked the binding of W2 but W2 did not block W1.

Virus polypeptide specificity of monoclonal antibodies

Western immunoblot analysis was employed in an effort to determine which IPNV polypeptides contained the epitopes delineated by the five MAbs. Purified WB-IPN virions were solubilized, the proteins were separated by SDS–PAGE, transferred to nitrocellulose paper and stained directly or probed with the appropriate antibody as described under Methods. The results are shown in Fig. 1. Lane (*a*) shows the four viral proteins on an amido black-stained blot: VP1, mol. wt. 91 000 (91K); VP2, 51K; VP3, 32K; VP4, 30K; the latter is produced by cleavage

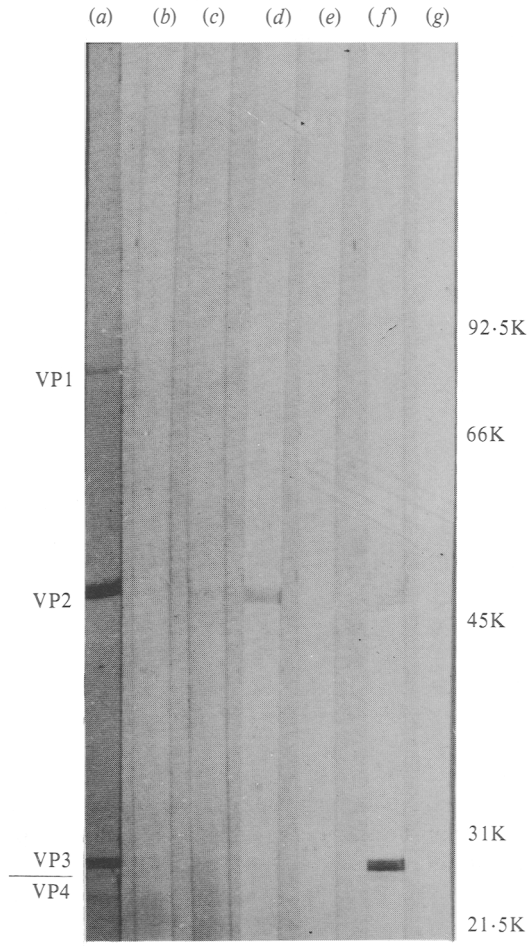

Fig. 1. Western immunoblot of reaction of five WB-IPNV MAbs with viral proteins. (*a*) Amido black stain of solubilized viral proteins. (*b* to *f*) Separated viral proteins reacted with MAbs W1, W2, W3, W4 and W5, respectively. (*g*) Blotting with MAb WO which does not react with WB-IPNV.

of VP3 during maturation. Lane (*d*) which was overlaid with MAb W3 followed by enzyme-conjugated anti-mouse IgG serum showed strong staining of VP2. Lane (*f*), which was treated with MAb W5, showed staining of the two smallest proteins VP3 and VP4. MAbs W1 and W2, although used in concentrations with ELISA endpoint titres equal to MAb W3 and MAb W5, failed to stain any of the blotted viral proteins (lanes *b*, *c*). Also, MAb W4 (lane *e*) failed to stain any of the viral proteins, but the amount of antibody in this preparation was approximately fivefold less (in terms of ELISA endpoint titre) than that of the other MAbs. All MAbs were present in concentrations sufficient to stain unsolubilized virus in a nitrocellulose immunodot assay (unpublished results). Similar analyses of blots with proteins obtained by treatment of virus with SDS alone also failed to demonstrate immunostaining with W1, W2 and W4 antibodies.

Neutralization

The ability of each MAb to neutralize WB-IPNV was tested. MAbs W3 and W4 demonstrated high levels of neutralization (mean $\log_{10}$ ND_{50} 4·6 and 4·0 respectively). MAbs W1, W2 and W5 did not exhibit detectable neutralizing activity, indicating that the five MAbs identify at least two different functional types of epitopes.

Table 2. *Cross-reaction of WB-IPNV monoclonal antibodies with aquatic birnavirus isolates in an indirect ELISA*

		MAb									
		W1		W2		W3		W4		W5	
Isolate	Serotype	P/N*	Result†	P/N	Result	P/N	Result	P/N	Result	P/N	Result
West Buxton	West Buxton	2·61	+	2·51	+	2·16	+	8·57	+	9·48	+
Dry Mills	West Buxton	3·88	+	3·99	+	3·89	+	10·43	+	10·26	+
Taiwan	West Buxton	3·72	+	3·56	+	3·64	+	9·28	+	8·92	+
Gilbert	West Buxton	2·21	+	1·54	−‡	1·57	−‡	6·28	+	6·14	+
Berlin	West Buxton	1·00	−	1·14	−	1·65	−‡	10·57	+	12·52	+
VR299	West Buxton	1·19	−	1·19	−	1·68	−‡	8·86	+	9·50	+
Reno	West Buxton	0·99	−	0·90	−	1·24	−‡	5·71	+	6·60	+
Buhl	West Buxton	1·06	−	1·30	−	2·02	+	7·43	+	8·59	+
Menhaden virus	West Buxton	0·86	−	0·92	−	1·36	−	6·71	+	7·70	+
d'Honnincthun	Sp	0·86	−	0·93	−	2·96	+	0·86	−	0·77	−
Oyster virus-2	Sp	1·00	−	1·14	−	3·96	+	1·14	−	1·07	−
Sp	Sp	1·00	−	1·07	−	2·64	+	1·19	−	1·00	−
Ab	Ab	2·27	+	1·07	−	0·98	−	0·71	−	0·83	−
AS	Canada 1	0·84	−	0·98	−	0·98	−	1·00	−	0·77	−

* Average of two or three experiments.
† For a positive result, P/N $\geqslant$ 2·0.
‡ Result was positive in an indirect double antibody sandwich ELISA.

Table 3. *Cross-reactions of WB-IPNV monoclonal antibodies W2 and W3 with aquatic birnavirus isolates in an indirect double antibody sandwich ELISA*

	MAb			
	W2		W3	
Isolate	P/N*	Result†	P/N	Result
West Buxton	7·36	+	7·42	+
Gilbert	3·00	+	6·50	+
Berlin	0·50	−	5·94	+
VR299	1·54	−	5·54	+
Reno	1·54	−	4·60	+

* Each value represents the average of two replicate experiments.
† See Table 2.

Reactivity in ELISA of WB-IPNV MAbs with heterologous aquatic birnaviruses

There are numerous isolates of IPNV and other aquatic birnaviruses that differ in geographical area of isolation, host range and pathogenicity. Most cross-react to some degree in neutralization tests with rabbit antisera; however, at least nine serotypes can be identified based on the relative degree of cross-reaction (Hill & Way, 1983). In this investigation, 14 aquatic birnavirus isolates that represent several of the most studied serotypes from North America, Europe and Asia were selected for comparison with MAbs to WB-IPNV. The cross-reactivity in ELISA of each WB-IPNV MAb with these other birnavirus isolates is shown in Table 2. Four different patterns of cross-reactivity with the 14 viruses were observed. The MAbs W4 and W5 displayed identical patterns of cross-reactivity; these MAbs appeared to be West Buxton serotype-specific, reacting exclusively with all members of the West Buxton serotype.

MAb W3 displayed the most extensive pattern of cross-reaction. As described under Methods, a P/N ratio equal to or greater than 2 was considered positive; however, W3 frequently displayed P/N values for some West Buxton serotype viruses that were relatively high but less than 2 (Table 2). The ability of polyclonal guinea-pig WB-IPNV antiserum to bind to these viruses was utilized to compare the reaction of W3 in an indirect double antibody sandwich ELISA using the guinea-pig antiserum as capture antibody. MAb W3 reacted with all of the West Buxton serotype viruses assayed in this way (Table 3). Thus, MAb W3 reacted with all members of the West Buxton serotype except menhaden; in addition, W3 also reacted with all three SP serotype viruses tested.

MAb W2 recognized an epitope on some, but not all, of the West Buxton serotype viruses and failed to react with members of any other serotype. As with W3 and some viruses, the reaction of W2 with one West Buxton serotype virus (Gilbert) was equivocal in the ELISA with virus adsorbed directly to plastic, but was positive in the indirect double antibody sandwich ELISA (Table 3). W1 displayed a pattern of cross-reaction similar to that of W2 except that it also reacted with the Ab isolate, the only one of these MAbs to do so. None of the MAbs reacted with the AS isolate.

Reactivity in neutralization tests of WB-IPNV MAbs with heterologous aquatic birnaviruses

The ability of the two neutralizing WB-IPNV MAbs (W3 and W4) to neutralize other birnaviruses was tested (Table 4). In general, W3 displayed a pattern of cross-reaction in neutralization tests similar to that observed in the ELISA, i.e. W3 neutralized all isolates for which positive ELISA reactions were obtained except for Gilbert, Buhl and Reno which were weakly neutralized or not neutralized. Another exception was the menhaden virus which was neutralized in half the trials, albeit at relatively low titres, by W3 even though W3 did not react with this virus in the ELISA. As with W3, MAb W4 exhibited high neutralization titres for some isolates and lower titres for others. The pattern of cross-reaction in neutralization tests with the heterologous birnaviruses again was similar to that obtained in the ELISA with two exceptions:

Table 4. *Cross-neutralization of aquatic birnavirus isolates by WB-IPNV monoclonal antibodies W3 and W4*

Isolate	W3 Positive trials/ total trials	W3 mean ND_{50}	W3 Result	W4 Positive trials/ total trials	W4 mean ND_{50}	W4 Result
West Buxton	8/8	4·8*	+++†	7/7	4·2	+++
Dry Mills	2/2	3·8	+++	2/2	4·2	+++
Taiwan	4/4	3·6	+++	2/2	3·8	+++
Gilbert	1/5	2·4	+	2/4	1·3	+
Berlin	2/2	2·8	++	2/2	4·0	+++
VR299	4/4	2·8	++	3/3	3·1	++
Reno	0/2	<1·4	−	2/2	2·6	++
Buhl	1/4	1·2	+	0/3	<1·4	−
Menhaden	3/6	2·0	+	3/5	3·1	++
d'Honnincthun	4/4	⩾4·5	+++	3/5	1·9	+
Oyster virus-2	4/4	4·2	+++	0/4	<1·4	−
Sp	3/3	⩾4·2	+++	0/2	<1·4	−
Ab	0/2	<1·4	−	0/2	<1·4	−
AS	0/2	<1·4	−	0/2	<1·4	−

* $\log_{10}$ Geometric mean of the neutralizing dose, 50% endpoint.
† +++, $\log_{10}$ Mean neutralizing dose (ND_{50}) ⩾ 3·5; ++, $\log_{10}$ ND_{50} ⩾ 2·5; +, $\log_{10}$ ND_{50} ⩾ 1·2; −, no detectable neutralization.

Table 5. *Serological groups of aquatic birnavirus isolates based on ELISA and neutralization reactions with WB-IPNV monoclonal antibodies*

	ELISA results					Neutralization results*	
Isolate	W1	W2	W3	W4	W5	W3	W4
West Buxton serotype							
1. West Buxton	+	+	+	+	+	+++	+++
Taiwan	+	+	+	+	+	+++	+++
Dry Mills	+	+	+	+	+	+++	+++
Gilbert	+	+	+	+	+	+	+
2. Berlin	−	−	+	+	+	++	+++
VR299	−	−	+	+	+	++	++
3. Reno	−	−	+	+	+	−	++
4. Buhl	−	−	+	+	+	+	−
5. Menhaden virus	−	−	−	+	+	+	++
Sp serotype							
6. d'Honnincthun	−	−	+	−	−	+++	+
7. Oyster virus-2	−	−	+	−	−	+++	−
Sp	−	−	+	−	−	+++	−
Ab serotype							
8. Ab	+	−	−	−	−	−	−
Canada 1 serotype							
9. AS	−	−	−	−	−	−	−

* +++, $\log_{10}$ Mean neutralizing dose (ND_{50}) $\geqslant$ 3·5; ++, $\log_{10}$ ND_{50} $\geqslant$ 2·5; +, $\log_{10}$ ND_{50} $\geqslant$ 1·4; −, no detectable neutralization.

although Buhl was positive in ELISA it was not neutralized by W4; d'Honnincthun, which was negative in the ELISA with W4, was occasionally, but weakly, neutralized by this MAb.

Serological grouping of aquatic birnaviruses with WB-IPNV MAbs

The results of the cross-reactions of the five WB-IPNV MAbs with the 14 representative aquatic birnaviruses in ELISA and neutralization tests are summarized in Table 5. Using this panel of MAbs, it was possible to demonstrate that 14 isolates represented at least nine antigenically distinct viruses. Moreover, the MAb panel demonstrated antigenic differences among members of the same serotype. Whereas West Buxton, Dry Mills, Gilbert and Taiwan were indistinguishable in these tests, other members of the West Buxton serotype exhibited different patterns of reaction with the MAbs; the Berlin and VR299 isolates appeared identical but were distinct from the former group and from Reno, Buhl and menhaden, each of which exhibited unique patterns of reactivity. Also, oyster virus-2 and Sp, which belong to the Sp serotype, were alike but distinguished from another Sp virus (d'Honnincthun) and from the other isolates.

DISCUSSION

In previous serological studies of the aquatic birnaviruses using polyclonal antisera, a minimum of three distinct but related serotypes of isolates have been clearly defined (Hill, 1976*b*; Macdonald & Gower, 1981; Okamoto *et al.*, 1983). With the isolation of these viruses from wider geographical and host ranges, analysis of over 175 isolates has indicated there are at least nine serotypes within one serogroup and four other isolates of a single serotype within a second serogroup (Hill & Way, 1983). Consistently, isolates from the U.S.A. have been described in these studies as members of a single serotype referred to as West Buxton or VR299 (Hill, 1976*b*; Hill & Way, 1983; Macdonald & Gower, 1981; Okamoto *et al.*, 1983). More detailed antigenic analysis of these viruses as well as the improvement of methods of diagnosis and virus identification require more specific, sensitive and standardized antibodies.

In this investigation, five MAbs were produced against the West Buxton isolate of IPNV.

Reciprocal blocking assays, neutralization tests, immunoblots and patterns of MAb cross-reactivity with heterologous aquatic birnaviruses indicated that these five MAbs recognized four, and possibly five, structurally and/or functionally different epitopes. MAbs W3, W4 and W5 define epitopes that are different from each other and from the epitope(s) delineated by antibodies W1 and W2. Furthermore, these epitopes represent at least two functional types. Two MAbs (W3 and W4) neutralized viral infectivity, whereas three MAbs (W1, W2 and W5) were able to bind to virus but did not neutralize.

In two types of antibody blocking assays, binding of W2 was effectively blocked by prior treatment with W1; however, W2 did not completely interfere with the binding of W1. This competition for binding sites on the virus by MAbs W1 and W2 could indicate the following: (i) the two antibodies bind to the same epitope, but with different avidities, (ii) the two antibodies bind to totally different epitopes whose proximity creates steric hindrance between antibodies (Friguet *et al.*, 1983), or (iii) the attachment of one antibody produced allosteric alterations of the second antigenic determinant (Lubeck & Gerhard, 1981). It should be noted that in the AI blocking assay unusually high values were obtained with MAb W4, particularly when tested with itself. Since conditions of antibody excess were ensured from antigen saturation curves, this did not result from a failure to saturate all antibody-binding sites during the first application of the antibody. However, this result would be expected, under the washing and incubation conditions of this assay, if this antibody binds with low avidity. It is also interesting that, although some values with the negative control antibody in this assay were occasionally lower than expected, the consistently lower AI values with each MAb combination indicated that all five antibodies interfered with each other to some extent. This suggests that these determinants may be partially overlapping in an operational sense and may, therefore, represent an operational antigenic site. Additional investigations should be pursued to elucidate the relationships of these epitopes in more detail and determine if, in fact, these epitopes are partially overlapping.

Western immunoblot analysis of each MAb demonstrated that W3 recognized a determinant on VP2, the 51K viral protein. This antibody also possessed neutralizing activity and, thereby, defines a determinant on VP2 that is important in infectivity. The epitope defined by W5 is present on the two smallest viral proteins, VP3 and VP4. The demonstration of the presence of a common epitope on both proteins was not unexpected in that VP4 is produced by the cleavage of VP3 during maturation (Dobos & Roberts, 1982). Reaction of MAb W5 in an ELISA with an epitope present on VP3 and VP4 is particularly interesting in that these proteins have been claimed to be internal proteins (Dobos *et al.*, 1977). The results presented here suggest that at least portions of one or both proteins may be exposed and accessible to antibody under the conditions of these assays, i.e. virus bound to plastic and high pH. The other three MAbs failed to react with the viral proteins in immunoblots. MAbs W1 and W2 were used in concentrations equal to those of W3 and W5; therefore, the failure to observe a positive reaction was not the result of insufficient concentrations of these antibodies. Somewhat lower concentrations of W4 were used; however, the concentration of this antibody was sufficient to stain unsolubilized virus in nitrocellulose immunodot assays (unpublished results). Therefore, it is unlikely, but possible, that staining of one or more solubilized proteins would have been observed with increased concentrations of this antibody. The failure of three MAbs to react with individual viral proteins suggests that these three determinants are either discontinuous and present only on the intact virion or that they are altered antigenically when solubilized.

All five MAbs were used in ELISA and MAbs W3 and W4 were used in neutralization tests to define further the antigenic relationships among a number of aquatic birnaviruses representing several of the most studied serotypes from North America, Europe and Asia. The results provided the first information about the basis of the antigenic differences and similarities of these viruses both within and among serotypes. The determinants delineated by antibodies W4 and W5 are highly conserved among members of the West Buxton (U.S.A.) serotype as evidenced by reaction with all of the representatives of this group that were tested. Furthermore, with one exception (inconsistent and weak neutralization of d'Honnincthun), these epitopes were not detected on any other isolates tested from three other serotypes. Additional isolates

representing these and other serotypes are currently being tested to confirm that these determinants are specific for the West Buxton serotype.

Antibodies W1 and W2 recognized determinants that were present on approximately half of the West Buxton serotype isolates tested. With the exception of Ab (W1 only), these epitopes were not identified in the other serotypes.

MAb W3 displayed the most extensive degree of cross-reactivity with the various birnaviruses, indicating that this epitope is more widely dispersed among these viruses. The epitope was identified on all isolates of the West Buxton serotype and on all three representatives of the Sp serotype, but not on Ab or AS.

The distribution of these epitopes, particularly those recognized by W3 and W4 which function in infectivity, provides some evidence to explain the fact that all aquatic birnaviruses cross-react to some extent with polyclonal antisera in neutralization tests but can be divided into discrete serotypes based on the relative degree of cross-neutralization. From the data presented here, West Buxton and Sp serotype viruses share epitope W3; therefore, antisera produced against one will also neutralize the other. However, the fact that West Buxton serotype viruses share epitope W4 which is not present on Sp viruses would explain, in part, why antisera to West Buxton viruses exhibit higher neutralization titres against other members of this serotype in comparison to Sp viruses. Presumably, other widely dispersed as well as serotype-specific epitopes exist among these and the other aquatic birnaviruses.

In general, analysis of neutralization of the various isolates by the MAbs W3 and W4 yielded patterns of cross-reaction similar to those in the ELISA but with some interesting exceptions. The Reno isolate reacted with W3 and the Buhl isolate reacted with W4 in the ELISA but neither was neutralized by the respective antibody. The basis for this apparent anomaly is unknown. However, a similar situation has been reported for poliovirus where one MAb prepared against the type 1 Sabin strain was able to bind to both the Mahoney and Sabin strains but was capable of neutralizing only the Sabin strain (Emini *et al.*, 1983). Also, some birnavirus isolates which did not react in the ELISA were neutralized as in the cases of the menhaden virus with W3 and the d'Honnincthun isolate with W4. In most of these cases, however, the neutralization was inconsistent and relatively weak. Perhaps, because of low antibody avidity or partial differences in epitope specificities, these determinants are only detected by the neutralization assay which may require fewer antigen–antibody reactions than the ELISA for a demonstrable positive result. Alternatively, binding of the virus to plastic in ELISA may alter the conformation of the virus, thereby altering the specificity of an epitope(s) or limiting access to antibody. Such conformational changes in a virus when bound to plastic have been documented for tobacco mosaic virus by Al Moudallal *et al.* (1984). That this may occur with the birnaviruses is suggested by the results in Table 4 demonstrating that MAbs W3 and W4 which gave marginal or equivocal results with some isolates in an ELISA when virus was adsorbed directly to plastic reacted much more strongly when virus was attached by an intermediary layer of antibody. Also preliminary results of investigations in our laboratory (unpublished data) indicate that the manner in which epitopes are presented (e.g. attachment of virus to polystyrene versus nitrocellulose substrates) can markedly affect the binding of certain MAbs.

Comparison of the patterns of cross-reactivity of the five MAbs with various aquatic birnaviruses in both ELISA and neutralization tests demonstrated that the 14 isolates tested represented at least nine antigenically distinct viruses. Furthermore, distinct differences in several antigenic determinants were shown among several viruses within the same serotype.

In summary, we have reported the development of five MAbs for IPNV which recognize four, and possibly five, structurally and/or functionally different epitopes. These MAbs can be used for more detailed antigenic studies and as relatively inexpensive, specific and standardized reference reagents for screening and identification of IPNV and other aquatic birnaviruses. For example, a combination of MAbs W1, W3 and W5 in an ELISA will identify all but one of the viruses tested. Furthermore, the use of individual MAbs will permit the specific identification of several of the viruses, even within a single serotype.

We are currently producing additional MAbs for WB-IPNV as well as several aquatic birnavirus serotypes that will be used with the MAbs described here in more detailed studies of

the mechanisms of neutralization, comparison of the antigenic relationships of isolates worldwide, and monitoring antigenic changes among these viruses.

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