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Antigenic and In Vitro Host Range Variety in Populations of Aquatic Birnaviruses Demonstrated by Selection for Neutralization Resistance to Monoclonal Antibodies

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Way-Shyan Wang and Bruce L. Nicholson (1996) Antigenic and in vitro host range variety in populations of aquatic birnaviruses demonstrated by selection for neutralization resistance to monoclonal antibodies. *Zoological Studies* **35**(2): 93-104. Variation in populations of aquatic birnaviruses was investigated by propagating several virus strains in cell cultures in the presence of neutralizing monoclonal antibodies (MAbs) to inhibit replication of the parental antigenic type in the virus population and to isolate MAb neutralization-resistant viruses representing one or more subpopulations of viruses. Selected MAb neutralization-resistant viruses were plaque purified and characterized in regard to antigenic and biological properties. In addition to the MAb used for selection, some variants failed to react in immunoassays with one or more other MAbs that reacted with the parental virus type. Other viruses, although completely resistant to neutralization, reacted with the selecting MAb. Several viruses selected for resistance to neutralization with a given MAb reacted in immunobinding and neutralization assays with one or more MAbs that did not react with the parental virus type. Also, several of the selected viruses replicated to higher titers and produced larger-sized plaques in susceptible cell cultures. Three MAb-resistant viruses were also found to be in vitro host range variants; two were no longer capable of infecting fat head minnow (FHM) cell cultures, whereas one, in contrast to the parental virus population, replicated to high titers in FHM cells. Comparison of the molecular weights of virion proteins of the MAb-selected viruses and the parental virus type revealed little or no alterations of virion proteins VP1 and VP3. However, differences in size were demonstrated between VP2 proteins of all MAb-selected viruses. In some cases, VP2 and VP2 precursor proteins of the MAb-resistant viruses were larger whereas in other cases they were smaller in size.

Key words: Infectious pancreatic necrosis virus, Antigenic, Neutralization-resistant variants.

The aquatic birnaviruses comprise the largest and most diverse group of viruses within the family Birnaviridae and include a variety of viruses from numerous species of fish and marine invertebrates (Wolf 1988). Many of these viruses, such as infectious pancreatic necrosis virus (IPNV), have been proven or implicated as the etiological agents of diseases in a variety of species worldwide used in fish farming and aquaculture. Furthermore, aquatic birnaviruses have been isolated from a variety of apparently healthy aquatic and marine species. All of the aquatic birnaviruses are similar in structure (Dobos et al. 1979) and most are closely related antigenically (Hill and Way 1983, Caswell-Reno et al. 1989, Lipipun et al. 1989).

Aquatic birnavirus virions contain a bi-segmented dsRNA genome within a nonenveloped, icosahedral capsid approximately 60 nm in diameter (Wolf 1988). The smaller genome segment B encodes a single protein (VP1, 90-110 kDa), the virion-associated transcriptase. The larger genome segment A (approximately 3 000 bp) contains one large open reading frame that encodes a precursor polyprotein (100 kDa) which is subsequently cleaved to form three viral proteins (pVP2, 63 kDa; NS, 29 kDa; and VP3, 29-31 kDa)

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by protease activity associated with the NS protein (Duncan et al. 1987, Manning and Leong 1990, Manning et al. 1990). The gene order is 5'-pVP2-NS-VP3-3'. The pVP2 protein is further processed to yield the major capsid protein VP2 (50-55 kDa).

The vast majority of individual aquatic birnavirus isolates, regardless of host species or geographic origin, are closely related antigenically (Dobos et al. 1979, Hill and Way 1983). Based on reciprocal cross neutralization tests with polyclonal antisera, Hill and Way (1983) demonstrated that the majority of aquatic birnavirus isolates worldwide comprise a major serogroup (Serogroup A) which includes nine related but distinct serotypes: West Buxton (WB) or VR-299, Spjarup (Sp), Abild (Ab), Hecht (He), Tellina (Te), Canada 1 (C1), Canada 2 (C2), Canada 3 (C3), and Jasper (Ja). Four individual isolates were antigenically unrelated to the majority of aquatic birnaviruses and represented a second, minor serogroup (Serogroup B). Recently, Christie et al. (1990) have presented evidence for a tenth serotype (N1) within Serogroup A. Most isolates from the United States are members of the WB serotype whereas most isolates from Canada fall into serotypes C1, C2, C3, or Ja. European isolates represent either Sp, Ab, Te, He, or N1 serotypes with Sp, Ab, and, more recently, N1 predominating. Serotype Ab, Sp, and WB viruses have been identified in Asia with Ab being the most common.

A number of investigators have used monoclonal antibodies (MAbs) to investigate the antigenic characteristics of these viruses in more detail (Anderson 1986, Caswell-Reno et al. 1986, Wolski et al. 1986, Lipipun 1988, Caswell-Reno et al. 1989, Lipipun et al. 1989, Christie et al. 1990). Some of these Mabs recognize epitopes that have been highly conserved among aquatic birnaviruses whereas other MAbs recognize epitopes that are restricted to one or more serotypes or even individual strains. For example, the neutralizing MAb AS-1 was found to react with all Serotype A aquatic birnaviruses tested worldwide (Caswell-Reno et al. 1989) suggesting that the epitope recognized by this MAb is universally conserved by these viruses. However, another neutralizing MAb (E3) recognizes an epitope restricted to only certain strains of viruses of the Ab serotype.

Although identical in structure and closely related antigenically, individual aquatic birnaviruses differ markedly in a variety of biological properties including pathogenicity, host range and optimum temperature of replication (Wolf 1988). Previous studies have demonstrated that the ability of some strains of aquatic birnaviruses to replicate in various cell cultures results from the presence of in vitro host range variants in the virus population (Nicholson et al. 1979). The mechanisms involved in the generation and selection of such variants, as well as the relationship of antigenic properties with biological properties, are unknown. However, the selection of such variant strains of aquatic birnaviruses in the natural environment may be the basis by which these viruses have become so ubiquitous and are able to infect different host species and apparently cause different diseases. In previous studies with monoclonal antibodies, we have shown that the ability of certain MAbs to neutralize infectivity varies among various aquatic birnavirus isolates even in cases where the MAb is capable of binding to the virion. Furthermore, we have shown that at least one in vitro host variant, in comparison to the parental virus population, is altered in a specific epitope identified by one MAb (Lipipun 1988, Lipipun et al. 1991a).

In this study, diversity in populations of aquatic birnaviruses was investigated by propagating several virus strains in cell cultures in the presence of neutralizing MAbs to inhibit replication of the parental antigenic type in the virus population and to isolate MAb neutralization-resistant viruses representing one or more subpopulations of viruses. Selected MAb neutralization-resistant viruses were plaque purified and characterized in regard to antigenic and biological properties.

MATERIALS AND METHODS

Cell cultures

CHSE-214 (Chinook salmon embryo), BB (brown bullhead), FHM (fathead minnow), BF-2 (bluegill fry), and RTG-2 (rainbow trout gonad) cell lines were cultured in Eagle's Minimum Essential Medium (MEM) with 10% (V/V) fetal bovine serum (FBS) in a humidified 7% CO_2-in-air atmosphere incubator at 20 °C as described previously (Nicholson 1989).

Viruses

The four viruses used in this investigation and their origins are listed in Table 1. Three viruses (ASV, WBV, and EEV) had been maintained in our laboratory for several years. The FV virus was a

new isolate propagated only two to three times in cell culture. Stock preparations of viruses were propagated in CHSE-214 cell cultures as described previously (Nicholson 1989).

Monoclonal antibodies (MAbs)

Hybridoma cell lines for production of MAbs (Table 2) and the Sp2/0-Ag14 mouse myeloma cell line (Shulman et al. 1978) were propagated as described by Caswell-Reno et al. (1989).

Virus infectivity assay

Viral infectivity was determined by inoculating wells of microtiter plates containing monolayer cell cultures with 0.1 ml of serial 10-fold dilutions of virus. After incubation at 20 °C for 7 d, the wells were observed for CPE and the 50% tissue culture infectious dose ($TCID_{50}$) determined by the method of Reed and Muench (1938).

Plaque assay

Serial 10-fold dilutions of virus were made in serum-free MEM and 0.1 ml of each dilution was added to each of three wells of six-well plates containing monolayers of CHSE-214 cells for 1 h at 20 °C. Three milliliters of agarose-MEM overlay were added to each well with the plates chilled on ice. After 4-d incubation at 20 °C, the cell monolayers were fixed overnight with 10% formalin (V/V). The agarose overlays were removed and the cells stained with 2% (W/W) crystal violet solution, 1% (V/V) ammonium oxalate, and 95% ethyl alcohol for 10 min. The numbers of virus plaques at each dilution were counted, and the virus titer calculated as plaque forming units (PFU) per ml.

Table 1. Aquatic birnaviruses used in this study

Virus	Country of origin	Animal of origin	Serotype[a]
Atlantic salmon virus (ASV)	Canada	Atlantic salmon	C1
West Buxton virus (WBV)	USA	trout	WB
FV	USA	trout	WB
European eel virus (EEV)	Japan	eel	Ab

[a]Hill and Way 1983, Caswell-Reno et al. 1989.

Table 2. Monoclonal antibodies used in this study

	Virion protein (VP) specificity[a,b]	Aquatic birnavirus serotype specificity[a,c]	MAb Ig subclass[a,b]
AS-1[c]	VP2	All serotypes in Serogroup A	IgG_1
W1[a]	VP2	Ab, WB, and Ja	IgG_{2b}
W2[a]	VP2	Ab, WB, and Ja	IgG_{2a}
W3[a]	VP2	Sp, WB, and Ja	IgG_{2b}
W4[a]	VP2	WB and Ja	IgG_3
W5[a]	VP3	WB and Ja	IgG_1
E1[c]	VP3	Sp, Ab, WB, Te, C1, and Ja	IgG_{2a}
E3[c]	VP2	Ab (EEV only)	IgG_{2b}
E5[c]	VP3	All serotypes in Serogroup A	IgG_1
E6[c]	VP3	Ab, WB, Te, C1, C2, and C3	IgG_1
E0[d]	—[d]	—[d]	—[e]

[a]Caswell-Reno et al. 1986.
[b]Lipipun et al. 1992a.
[c]Caswell-Reno et al. 1989.
[d]non-birnavirus MAb.
[e]unclassified.

Selection of MAb neutralization-resistant viruses

Neutralization-resistant viruses were selected using a modification of the method of Blondel et al. (1986). Equal volumes of virus (2 000 $TCID_{50}$) and MAb (200 times the ND_{50} concentration) were mixed and incubated at 20 °C for 1 h. Each virus-MAb mixture was added to one well of a 12-well microtiter plate with CHSE-214 cells. After adsorption for 30 min at 20 °C, the monolayers were washed twice with MEM, the plates chilled on ice for 5 min, and 1.5 ml of agarose (0.8%)-MEM with 150 μl undiluted MAb were added to each well. After incubation for 4-5 d, individual plaques were picked, treated by ultrasonication and centrifuged (1000 rpm for 10 min). Supernatants (0.5 ml) were mixed with 0.5 ml of undiluted MAb for 1 h at 20 °C and inoculated onto monolayer cultures of CHSE-214 cells in 25 cm^2 flasks. After adsorption for 30 min, the flasks were washed twice with MEM and 5 ml of growth medium with 10% (V/V) undiluted MAb were added. After incubation at 20 °C for 4-5 d, MAb neutralization-resistant viruses were harvested, plaque purified, and stored at −80 °C.

Neutralization assays

Serial 2-fold dilutions of each MAb were reacted with an equal volume of virus (2 000 $TCID_{50}$/ml) for 1 h at 20 °C. The mixtures were then inoculated onto 96-well microtiter plates with CHSE-214 cell monolayers and incubated at 20 °C for 7 d. The 50% neutralizing dose (ND_{50}) was calculated by the method of Reed and Muench (1938). Alternatively, in some experiments, equal volumes of undiluted MAb and virus (2 000 $TCID_{50}$/ml) were mixed, incubated, and inoculated onto four wells of a 96-well plate containing CHSE-214 cell cultures. After incubation at 20 °C for 7 d, evidence of neutralization was determined by the absence of CPE in inoculated wells (neutralization titers were not determined in these cases).

Immunodot assay

The immunodot assaay used was a modification of the procedure of Caswell-Reno et al. (1989). Briefly, nitrocellulose paper previously immersed in TBS (Tris buffered saline) was placed in a BIO-DOT 96-well plate. One microliter of virus (10^5-10^6 $TCID_{50}$) was added to each well. After incubation for 2 h, 100 μl of blocking solution (5% BSA and 10% FBS in TBS) were added for 2 h at room temperature or overnight at 4 °C. After washing four times with TBS, 100 μl of MAb were added to each well and incubated for 2 h. Following four washes with TBS, 100 μl of an appropriate dilution of goat anti-mouse IgG conjugated with horseradish peroxidase were added to each well and incubation continued for 2 h. Following four washes with TBS, 100 μl of substrate (4-chloronapthol plus 1% H_2O_2) were added to each well. Reactions were stopped by rinsing three times with distilled water. Positive reactions were indicated by the appearance of a dark purple spot.

Radioisotope labeling of viruses

Labeling with ^{35}S-methionine was performed by a modification of the method of Dobos et al. (1977a). Six-well plates containing monolayers of CHSE-214 cell cultures were washed three times with methionine-free MEM medium. Before virus inoculation, all cells were incubated with methionine-free MEM-1% FBS medium for 4 h. Viruses were inoculated onto the plates at an M.O.I. of 10 to 100. After 2 h absorption, the cells were washed twice with methionine-free MEM-1% FBS medium. Methioninee-free MEM-1% FBS medium containing ^{35}S-methionine (100 μCi/ml) (DuPont NEN Research Products, Boston, MA; ICN Biomedicals, Inc, Costa Mesa, CA) was added to the wells. When complete CPE was observed, labeled virus was harvested and stored at −80 °C.

Polyacrylamide-SDS gel electrophoresis

This assay was performed according to the protocol of Laemmli (1970). The polyacrylamide (Bio-Rad Lab., Richmond, CA) concentrations of the stacking gel and separating gel were 4.5% and 10%, respectively. Radioactively labeled, immunoprecipitated virion proteins or purified viruses were mixed with sample buffer (5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 0.125 M Tris buffer, and 0.025% bromophenol blue) at a ratio of 1:2 and boiled in a water bath for 3 min. ^{14}C-labeled molecular weight standards (DuPont NEN Research Products, Boston, MA) were prepared in a similar manner. Fifty microliters of each sample and 15 μl of molecular weight standards mixture were loaded into wells of the gel. A constant current of 25 mA was applied for running the stacking gel. For running the separating gel, a constant current of 30 mA was used. After electrophoresis for 2 to 3 h, the gel was removed and soaked with 10% acetic acid and 10% methanol in distilled water for 15 min with gentle shaking. After washing twice with distilled water, the gel was soaked in autoradiography enhancer (Enlightning, DuPont NEN Research Products, Boston, MA) for 30 min with gentle shaking. The gel then was placed on a piece of chromatography paper (3MM Chr; Whatman International Ltd. Maidstone, England) and dried on a gel dryer at 80 °C for 1 to 1.5 h. Finally, an autoradiogram was made be exposing the gel to a Kodak XAR-5 film (Sigma Chemical Co., St Louis, MO) for 2 to 3 d. In some cases, the amount of isotope incorporation into virion proteins was determined by cutting chromatography paper containing a dried gel into small strips (each sample lane was cut into 20 small strips) and the radioactivity of each strip was counted by liquid scintillation spectrometer (Beckman, LS 3801; Beckman Instruments, Inc., Fullerton, CA).

Radioimmune precipitation assay (RIP)

The method performed was a modification of that of Lipipun et al. (1992a). Virus-infected cells (about 10^6 to 10^7 $TCID_{50}$) and uninfected control cells were treated with extraction buffer (1% Triton-

X and 0.1 mM phenylmethylsulfonyl fluoride in PBS, pH 8.0) in 300 μl volumes. Twenty-five microliters of normal mouse serum (NMS) (Organon Teknika Corp., West Chester, PA) were added into a micro centrifuge tube containing 300 μl of ^{35}S-methionine labeled cellular extracts. After reaction at 4 °C for 90 min, 50 μl of preabsorbed protein A-sepharose 6MB (Pharmacia Fine Chemicals, Sweden) which had been washed twice with 1% BSA in PBS and finally suspended in 1% BSA in PBS were added to the mixture and incubated for 2 h at 4 °C. The complex was centrifuged in a microcentrifuge at 14 000 rpm for 5 min, then the clarified supernatant was removed to other tubes and reacted with 50 μl of a mixture of two monoclonal antibodies (25 μl of MAb AS-1 and 25 μl of MAb E1) overnight at 4 °C. After the MAb-viral protein reaction complex formed, 150 μl of goat anti mouse IgG, approximately 15 μg, (Sigma Chemical Co., St Louis, MO) were added. After 2-h reaction at 4 °C, 300 μl of pretreated protein A-Sepharose 6MB (For pretreatment, the protein was washed once and resuspended in 1% BSA in PBS, then absorbed with a double volume of unlabeled, uninfected CHSE-214 monolayer cell lysate for 2 h at 4 °C, and centrifuged, and finally the treated beads were washed three times with 1% BSA in PBS and finally suspended in 1% BSA in PBS) were added and incubated for 2 h at 4 °C. Protein A-Sepharose 6MB beads binding with the MAb-virion protein complexes were pelleted by centrifugation in a microcentrifuge at 14 000 rpm for 5 min. The beads were washed twice with extraction buffer and twice with 0.05% Tween-20 in PBS. The beads were then removed to a new microcentrifuge tube and washed.

RESULTS

Selection of MAb neutralization-resistant viruses

Four neutralizing MAbs (AS-1, W3, W4, and E3) were used to select specific MAb neutralization-resistant viruses. Since no single strain of virus reacts with all four neutralizing MAbs, neutralization-resistant viruses were selected using several different virus strains. In addition to the MAb used for selection, each variant virus also was tested for neutralization by the other three MAbs. The results are shown in Table 3.

A variant selected from the ASV population for neutralization resistance to MAb AS-1, in contrast to the parental virus type, was neutralized at high titers by MAbs W3 and W4. Interestingly, a MAb AS-1 neutralization-resistant virus isolated from preparations of EEV was not altered in its inability to be neutralized by MAbs W3 and W4. Indeed, this virus was resistant to neutralization by all four MAbs. A variant selected from the EEV population that was completely resistant to neutralization by MAb E3 also displayed increased (but not complete) resistance to neutralization by MAb AS-1. Also, as with the ASV/AS-1 variant, the MAb E3 neutralization-resistant virus selected from EEV was neutralized by MAbs W3 and W4, MAbs which did not neutralize the parental EEV strain. A virus selected for resistance to MAb W3 from WBV also was resistant to neutralization by MAb W4. Moreover, these MAb W4 neutralization-resistant viruses were also resistant to neutralization by MAb W3. Variants selected from the population of FV virus, a recent isolate and a member of the WB serotype, exhibited characteristics similar to variants isolated from the WBV population; that is, FV variants selected for resistance to neutralization by either MAb W3 or W4 were either completely or highly resistant to neutralization by both MAbs.

Immunodot assay

Each MAb neutralization-resistant virus also was compared with the respective parental virus type in immunodot assays using a panel of 10

Table 3. MAb neutralization of parental virus populations and MAb neutralization-resistant virus populations

	MAb (ND_{50}/ml[a])			
Virus	AS-1	W3	W4	E3
ASV[b]	238	—[d]	—	—
ASV/AS-1 var[c]	—	1 094	2 727	—
EEV[b]	1 007	—	—	9 201
EEV/E3 var	70	1 291	16 127	—
EEV/AS-1 var	—	—	—	—
WBV[b]	158	2 733	14 367	—
WBV/W3 var	282	—	—	—
WBV/W4 var	282	—	—	—
FV[b]	282	124	1 270	—
FV/W3 var	154	—	31	—
FV/W4 var	64	—	—	—

[a]ND_{50}/ml = 50% neutralization dose/ml.
[b]Original stock virus population.
[c]var = MAb variant (e.g., AS-1 var = neutralization-resistant variant selected with MAb AS-1).
[d]— = no detectable neutralization.

MAbs, including the four neutralizing MAbs as well as seven non-neutralizing MAbs (Table 4). In some cases, as would be expected, MAb neutralization-resistant variants did not react in the immunodot assay with the MAb used for selection (e.g., EEV/E3 var, EEV/AS-1 var, WBV/W4 var, and FV/W4 var), indicating an absence of this epitope. Some variants selected with a given MAb also did not react with one or more other MAbs (e.g., EEV/AS-1 var with MAbs W1, W2, and E3; WBV/W4 var with MAbs W1, W2, W3, W5, E5, and E6). In several instances, although the variant was completely resistant to neutralization by the selecting MAb, the virus was not altered in its ability to bind to the MAb in the immunodot assay (e.g., ASV/AS-1 var reacted with MAb AS-1; WBV/W3 var and FV/W3 var reacted with MAb W3). Two of these MAb neutralization-resistant viruses reacted in immunodot assays with one or more MAbs that do not react with the parental virus type. The ASV/AS-1 var, in contrast to the parental virus, reacted with MAbs W1, W2, W3, W4, and W5; similarly, the EEV/E3 var reacted with MAbs W3, W4, and W5.

Neutralization of variants by normally non-neutralizing MAbs

In view of the fact that some MAb selected viruses reacted in the immunobinding assay with MAbs that did not react with the parental virus type, the ability of all 10 MAbs to neutralize all viruses was tested, including those MAbs with no previously demonstrated neutralizing capability. There were several interesting results as shown in Table 5. As demonstrated previously (Table 3), some variants differed in sensitivity to neutralization by neutralizing MAbs other than the specific MAb used for selection. However, in one case (EEV/AS-1 var) a variant selected for resistance to neutralization by one MAb was also neutralized by MAb E1 which had not been capable of neutralizing any of numerous isolates tested in previous studies (Lipipun et al. 1992b). MAb E1 binds to the EEV parental virus type (Table 4) but this binding does not result in neutralization of infectivity. Binding of these two MAbs to the EEV/AS-1 var, however, did result in virus neutralization.

Plaque titer and plaque size of parental virus types and MAb neutralization-resistant viruses

In order to compare the in vitro replication characteristics of each of the neutralization-resistant viruses and the parental virus types, monolayer cultures of CHSE-214 cells of approximately equal cell numbers (approximately 10^6 cells) were infected with each virus at low M.O.I. (approximately 0.1 to 0.01) and incubated until maximum CPE was observed. Each virus was harvested and titered in CHSE-214 cell cultures by plaque assay. The results are shown in Table 6. The yield of several

Table 4. Cross reaction in immunodot assay of MAbs with parental virus populations and MAb neutralization-resistant virus populations

	MAbs									
Virus	AS1	W1	W2	W3	W4	W5	E1	E3	E5	E6
ASV[a]	+	−	−	−	−	−	+	−	+	+
ASV/AS-1 var[b]	+	+	+	+	+	+	+	−	+	+
EEV[a]	+	+	+	−	−	−	+	+	+	+
EEV/E3 var	+	+	+	+	+	+	+	−	+	+
EEV/AS-1 var	−	−	−	−	−	−	+	−	+	+
WBV[a]	+	+	+	+	+	+	+	−	+	+
WBV/W3 var	+	−	−	+	−	−	+	−	−	−
WBV/W4 var	+	−	−	−	−	−	+	−	−	−
FV[a]	+	−	−	+	+	+	+	−	+	+
FV/W3 var	+	−	−	+	−	+	+	−	+	+
FV/W4 var	+	−	−	−	−	−	+	−	+	+

[a]Original stock virus population.
[b]var = MAb variant (e.g., AS-1 var = escape variant selected with MAb AS-1).

Table 5. Neutralization of parental virus populations and MAb neutralization-resistant virus populations with a panel of ten MAbs

	MAbs									
Virus	AS1	W1	W2	W3	W4	W5	E1	E3	E5	E6
ASV[a]	+	−	−	−	−	−	−	−	−	−
ASV/AS-1 var[b]	−	−	−	+	+	−	−	−	−	−
EEV[a]	+	−	−	−	−	−	−	+	−	−
EEV/E3 var	+	−	−	+	+	−	−	−	−	−
EEV/AS-1 var	−	−	−	−	−	−	+	−	−	−
WBV[a]	+	−	−	+	+	−	−	−	−	−
WBV/W3 var	+	−	−	−	−	−	−	−	−	−
WBV/W4 var	+	−	−	−	−	−	−	−	−	−
FV[a]	+	−	−	+	+	−	−	−	−	−
FV/W3 var	+	−	−	−	+	−	−	−	−	−
FV/W4 var	+	−	−	−	−	−	−	−	−	−

[a]Original stock virus population.
[b]var = MAb variant (e.g., AS-1 var = escape variant selected with MAb AS-1).

MAb variants was different in comparison to the respective parental virus strain. The plaque titer of EEV/E3 var was higher than that of the parental strain of EEV, whereas, the titers of WBV/W3 var, WBV/W4 var, and FV/W4 var were lower than the respective parental populations. These differences in virus yields between variant and parental virus types corresponded with larger or smaller plaque sizes compared to these viruses. Plaque sizes of EEV/E3 var and EEV/AS-1 var were larger than EEV. On the other hand, the plaque size of the original WBV population was larger than variants WBV/W3 var and WBV/W4 var. There were no significant differences in plaque size between the original ASV population and the ASV/AS-1 variant. The same result also was observed between the original FV population and the FV/W3 variant.

Infectivity of original virus populations and MAb selected variant viruses in different cell cultures

Since several MAb neutralization-resistant viruses exhibited apparent differences in infectivity for CHSE-214 cells, the cell culture used for standard propagation of the original populations of viruses and selection and propagation of the variants, the infectivity titers of approximately equal concentrations (as determined by infectivity in CHSE-214 cell cultures) of each virus were compared in four other fish cell cultures by 50% end-point dilution assays. Several MAb neutralization-resistant viruses exhibited differences in infectivity in certain cell lines compared to the parental virus in the population from which they were selected (Table 7). Most notable were the EEV/E3 var which replicated to high titers in FHM cells, cells in which the parental EEV virus is incapable of replication, and WBV/W4 var and FV/W4 var which did not replicate in FHM cells, a cell culture in which both parent virus populations replicate to high titers. Also, the ASV/AS-1 var exhibited higher titers in all four other cell cultures (particularly FHM and RTG-2) compared to the original ASV population, whereas the EEV/AS-1 var showed a lower titer in RTG-2 cells and the WBV/W3 var showed a lower titer in BB cells.

Molecular weights of virion proteins (VP) of original predominating virus types and variant viruses

In an attempt to investigate the structural differences associated with the observed antigenic and host range differences among the viruses selected for MAb neutralization resistance, the molecular weights of the virion proteins (VP) of each virus were compared by radioimmune precipitation of ^{35}S-methionine-labeled individual virion proteins and their precursors from infected cells

Table 6. Plaque titer and plaque size in CHSE-214 cell cultures of parental virus populations and MAb neutralization-resistant virus populations

Virus	Titer ± SD (log_{10} of PFU[a]/ml)	Size ± SD (mm)
ASV[b]	7.08 ± 0.02	0.76 ± 0.24
ASV/AS-1 var[c]	7.00 ± 0.05	0.99 ± 0.26
EEV[b]	7.58 ± 0.03	0.81 ± 0.21
EEV/E3 var	8.26 ± 0.06	2.08 ± 0.20
EEV/AS-1 var	7.30 ± 0.01	1.18 ± 0.42
WBV[b]	8.17 ± 0.06	2.28 ± 0.36
WBV/W3 var	7.00 ± 0.01	1.22 ± 0.38
WBV/W4 var	7.40 ± 0.03	1.15 ± 0.33
FV[b]	8.28 ± 0.08	1.84 ± 0.33
FV/W3 var	8.12 ± 0.05	1.89 ± 0.46
FV/W4 var	7.53 ± 0.03	1.00 ± 0.18

[a]PFU = plaque forming unit.
[b]Original stock virus population.
[c]var = MAb variant (e.g., AS-1 var = escape variant selected with MAb AS-1).

Table 7. Infectivity titer of parental virus populations and MAb neutralization-resistant virus populations in different fish cell cultures

	Cell Cultures				
Virus	BB	BF-2	CHSE-214	FHM	RTG-2
ASV[a]	7.25[b]	7.00	8.50	4.75	6.25
ASV/AS-1 var[c]	9.25	9.00	9.25	8.00	9.00
EEV[a]	9.75	9.50	10.00	< 1.00	9.00
EEV/E3 var	9.25	8.75	9.25	8.75	7.50
EEV/AS-1 var	8.50	8.50	8.75	< 1.00	6.50
WBV[a]	9.75	9.00	9.75	6.50	8.00
WBV/W3 var	7.50	7.75	8.25	7.75	6.50
WBV/W4 var	8.50	8.00	9.50	< 1.00	7.00
FV[a]	9.75	9.50	9.25	8.25	7.75
FV/W3 var	8.50	9.00	9.25	8.00	7.50
FV/W4 var	8.50	9.50	8.75	< 1.00	7.25

[a]Original stock virus population.
[b]Log_{10} of $TCID_{50}$/ml.
[c]var = MAb variant (e.g., AS-1 var = escape variant selected with MAb AS-1).

followed by SDS-PAGE and autoradiography as described in the Materials and Methods section. The results of a typical autoradiogram are shown in Fig. 1. Faint bands of VP1 were observed on autoradiograms but were not visible on photographs. From the autoradiogram, the molecular weights of the parental virus type in the original population and variant virion proteins were determined (Table 8). No differences in the molecular weights of VP1 were detected between the original parental virus type and variant viruses. Also, in only two cases were alterations in the size of VP3 detected; VP3 of FV/W3 var and FV/W4 var were 31.0 and 31.4 kDa, respectively, compared to 32.8 kDa for the original parental FV virus.

The molecular weights of VP2 of several MAb-selected viruses were significantly higher than those of the parental virus type in the original population. The molecular weight of VP2 of ASV/AS-1 var was 56.6 kDa compared to 52.7 kDa for standard ASV (Fig. 1, lanes 1 and 2; Table 8). In some cases, VP2 precursor proteins (pVP2) were also precipitated. As with VP2 itself, the molecular weight of pVP2 of ASV/AS-1 var (60.4 kDa) was higher than that of pVP2 of standard ASV (59.4 kDa). Similarly, the molecular weights of both VP2 (57 kDa) and pVP2 (60.4 kDa) of EEV/E3 var were higher than the molecular weights of VP2 (50.8 kDa) and pVP2 (55.1 kDa) of the parental virus type in the EEV population (Fig. 1, lanes 3 and 4; Table 8). VP2 of EEV/AS-1 was not precipitated in detectable amounts by the MAbs used (Fig. 1, lane 5; Table 8).

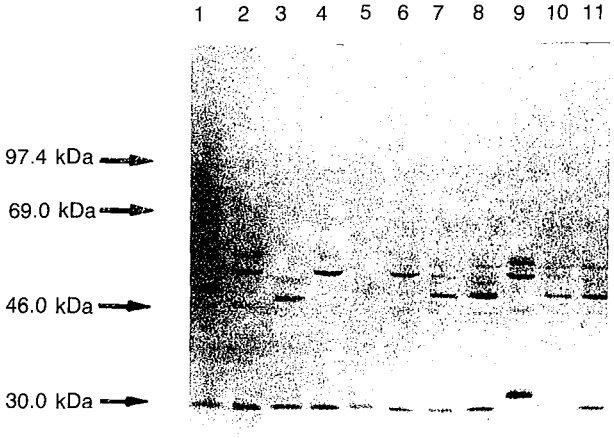

Fig. 1. Autoradiogram of virion proteins immunoprecipitated from infected cells.
Lane 1 = ASV; Lane 2 = ASV/AS-1 var; Lane 3 = EEV; Lane 4 = EEV/E3 var; Lane 5 = EEV/AS-1 var; Lane 6 = WBV; Lane 7 = WBV/W3 var; Lane 8 = WBV/W4 var; Lane 9 = FV; Lane 10 = FV/W3 var; Lane 11 = FV/W4 var

Table 8. Molecular weights of virion proteins of parental virus populations and MAb neutralization-resistant virus populations

	Molecular weight (kDa)				
Virus	VP1	pVP2a	pVP2b	VP2	VP3
ASV[a]	93.0	59.4	—[b]	52.7	30.4
ASV/AS-1 var[c]	93.0	60.4	—	56.6	30.0
EEV[a]	93.0	55.1	—	50.8	30.0
EEV/E3 var	93.0	60.4	—	57.0	30.0
EEV/AS-1 var	93.0	—	—	—	30.4
WBV[a]	93.0	60.4	—	56.5	30.0
WBV/W3 var	93.0	59.4	56.5	52.2	30.0
WBV/W4 var	93.0	59.4	55.6	52.2	30.4
FV[a]	96.3	960.4	—	57.0	32.8
FV/W3 var	95.2	59.4	57.5	52.7	31.0
FV/W4 var	95.2	59.4	55.9	52.7	31.4

[a]Original stock virus population.
[b]pVP2b was not detected.
[c]var = MAb variant (e.g., AS-1 var = escape variant selected with MAb AS-1).

In several cases, the molecular weights of VP2 of MAb-selected viruses were lower than those of the parental virus in the parental population (Fig. 1, lanes 6, 7, and 8; Table 8). The molecular weights of VP2 of both WBV/W3 var and WBV/W4 var were 52.2 kDa compared to 56.5 kDa for VP2 of standard WBV. Interestingly, two VP2 precursor proteins (pVP2) were precipitated with both of these variants whereas no pVP2 was detected in cells infected with the original WBV preparation. The molecular weights of these two pVP2 proteins were 59.4 kDa and 56.5 kDa and 59.4 kDa and 55.6 kDa for WBV/W3 var and WBV/W4 var, respectively. As with WBV, the molecular weights of VP2 for variants selected from stock FV virus with MAbs W3 and W4 (52.7 kDa) were lower than those of original FV predominating virus (57.0 kDa) (Fig. 1, lanes 9, 10, and 11). One pVP2 of molecular weight 60.4 kDa was detected in cells infected with the original population of FV virus whereas two smaller pVP2 proteins were precipitated from cells infected with each variant virus selected from this population (59.4 and 57.5 kDa for FV/W3 var; 59.4 and 55.9 kDa for FV/W4 var).

DISCUSSION

Four neutralizing MAbs (AS-1, W3, W4, and E3) from a panel of aquatic birnavirus-specific MAbs previously developed in our laboratory

(Anderson 1986, Caswell-Reno et al. 1986, Lipipun 1988, Caswell-Reno et al. 1989) were used independently to inhibit the replication of the parental antigenic type in stock populations of several different strains of aquatic birnaviruses and to select variants representing one or more normally undetectable subpopulations of virus. MAb AS-1 is a group reactive MAb recognizing an epitope common to all Serogroup A aquatic birnaviruses (the predominant serogroup worldwide) (Caswell-Reno et al. 1986, Caswell-Reno et al. 1989, Lipipun et al. 1992b). The reactivities of MAbs W3 and W4 are more restrictive, reacting only with viruses of three (WB, Sp, and Ja) and two (WB and Ja) serotypes, respectively, of the nine well recognized serotypes of Serogroup A (Caswell-Reno et al. 1989). MAb E3 is a strain-specific antibody, reacting only with EEV and certain other members of the Ab serotype (Caswell-Reno et al. 1989). As shown in Table 3, viruses completely resistant to neutralization were selected for each of the four MAbs.

Analyses of these viruses revealed a number of interesting variations and relationships in antigenic as well as biological properties in comparison to the parental antigenic type in the population. In some cases, as would be expected, selected variants not only were resistant to neutralization by the MAb used for selection (Table 3) but also failed to react with this MAb in immunobinding assays (Table 4), indicating a complete absence of this epitope. Interestingly, in some cases, the variant, although resistant to MAb neutralization, was capable of binding to the MAb. Similar results were reported by Lipipun et al. (1992b) for other aquatic birnavirus MAb neutralization-resistant variants as well as by Caswell-Reno et al. (1986) for naturally occurring variants. One possible explanation for such a phenomenon is that the bulk of the variant virus population consisted of viruses completely resistant to MAb neutralization, but that a small residual population of the parental antigenic type may have persisted that was sufficient to yield positive results in the immunodot assay. However, such a situation is generally unlikely, considering that the viruses were plaque-cloned, the relative insensitivity of the immunodot assay, and the results, of SDS-PAGE analyses of virion proteins discussed below. Another possibility is that these epitopes were not altered sufficiently to prevent MAb recognition and binding, but they were altered in such a way that binding no longer resulted in neutralization or else the antibodies bound with less avidity. Alternatively, neutralization by these MAbs may not have resulted as a direct reaction with the target epitope but, rather, as an indirect conformational alteration of one or more other functionally related epitopes and it was in one of these other epitopes in which the MAb neutralization-resistant virus differed from the parental antigenic type.

Analyses of the reactivities of the 10 MAbs with each MAb neutralization-resistant virus (Table 4) and the ability of the four neutralizing MAbs to neutralize each virus (Table 3) demonstrated a number of epitope differences in comparison with the parental virus types in the original populations. For example, the EEV/AS-1 var, in addition to the AS-1 epitope, also lacked epitopes W1 and W2 which are present on standard EEV. Similarly, WBV/W3 var did not react with MAbs W1, W2, W4 or W5 in immunodot assays and it also was resistant to neutralization by MAb W4. In contrast to the original population of FV virus, FV/W3 var did not react with MAbs W1, W2, or W4 and it was highly resistant to neutralization by MAb W4. WBV/W4 var and FV/W4 var, in contrast to their parental virus populations, did not react in immunodot assays with MAbs W1, W2, W3, or W5 and were resistant to neutralization by MAb W3.

A surprising result was discovery that some viruses selected for resistance to neutralization by a specific MAb reacted with one or more MAbs that do not react with the original stock virus population (Table 4). The ASV/AS-1 var reacted in immunodot assays with MAbs W1, W2, W3, W4, and W5; preparations of the original ASV virus population do not bind to any of these five MAbs. Similarly, EEV/E3 var, in contrast to the parental EEV population, reacted with MAbs W3, W4, and W5. Further, both variants were highly sensitive to neutralization by MAbs W3 and W4 (Table 3).

From these data, it is clear that normally undetectable antigenic variants that represent one or more subpopulations of viruses can exist in stock populations of aquatic birnaviruses. Moreover, these variants can exhibit considerable differences in antigenic epitopes in comparison to the parental virus type. This is not surprising, however, considering the "quasi-species" concept of RNA virus populations. Natural populations of RNA viruses (e.g., foot and mouth disease virus, poliovirus, and influenza A virus) have been demonstrated to be highly heterogeneous in terms of genomic sequences (Domingo et al. 1980, Kew et al. 1981, Sobrino et al. 1983, Parvin et al. 1986, Hsu et al. 1995). Even in plaque-cloned populations of RNA viruses, the predominant viruses represent only

15%-20% of the total population. This heterogeneity results from the fact that RNA polymerases lack proofreading capabilities; thus, the replication of RNA viruses is characterized by high mutation rates (10^{-3} to 10^{-4} as compared to $\leq 10^{7}$ changes per nucleotide per replication cycle). Therefore, in the heterogeneous populations of aquatic birnaviruses, selective pressures exerted by neutralizing MAbs will result in shifts in the predominant population based on relative resistance to MAb neutralization.

Aquatic birnaviruses exhibit a great deal of variation in several biological properties including virulence for a given species, host range, and optimum temperature of replication. Therefore, it was of interest to determine whether any of the antigenic variants obtained by selection for resistance to MAb neutralization also differed from the parental antigenic type virus in biological properties. The in vitro replication characteristics of the MAb-selected viruses and parental virus populations were compared. In several cases, the yields of MAb neutralization-resistant viruses in CHSE-214 cell cultures differed in comparison to the respective parental virus population (Table 6). Whether these differences in in vitro replication ability correlate with differences in virulence for fish is unknown. Nevertheless, the data suggest that immunoselection pressures may result in selection of antigenically different virus subpopulations with either increased or decreased virulence.

In view of previous work (Nicholson et al. 1979) which showed that the ability of some aquatic birnaviruses to replicate in different fish cell cultures is the result of selection of naturally occurring host range variants present in small numbers in the population, the ability of each variant and parental virus population to infect several other fish cell cultures was investigated (Table 7). Several MAb-resistant viruses exhibited significant differences in infectivity (either higher or lower) in certain cell cultures compared to the respective parental population. Of particular interest were three variants which exhibited major differences in in vitro host range. EEV/E3 replicated to high titers in FHM cells, cells in which the parental EEV population appeared incapable of replication. Conversely, WBV/W4 var and FV/W4 var did not replicate in FHM cells, a cell culture in which the parental antigenic type viruses in both parental populations replicate to high titers. Thus several variant strains selected for resistance to neutralization by a specific MAb also were in vitro host range variants as well.

In order to determine whether these differences were associated with any major differences in virion proteins (VP), the molecular weights of the VPs of both parental type viruses and the respective MAb neutralization-resistant viruses were compared by radioimmune precipitation (RIP) of individual VPs and, in some cases, their precursors (pVP) from infected cells followed by SDS-PAGE and autoradiography (Fig. 1). No alteration in the size of VP1 was detected between any parental and variant viruses. In only two cases were relatively small differences detected in the size of VP3. However, in every case, VP2 of the MAb neutralization-resistant viruses differed significantly in molecular weight compared to VP2 of the parental antigenic type in the respective parental virus population. In some cases, the VP2 protein of the variant virus was larger than that of the parental virus type, and in other cases it was smaller. These data indicate that there can be relatively large differences in the size of VP2 of selected MAb neutralization-resistant viruses compared to the parental virus in the parental population and, therefore, they suggest that differences in the size and/or structure of VP2 may be involved in the observed variations in antigenic characteristics, virulence, and/or host range.

The results of this investigation have important implications for understanding the adaptation and evolution of aquatic birnaviruses. First, these results indicate that populations of aquatic birnaviruses can exhibit a great deal of variation in antigenic and biological properties and that differences in specific properties such as host range and resistance to antibody neutralization are possibly related to each other and to differences in specific virion proteins and antigenic epitopes. Particularly interesting are the implications that antigenic variants representing one or more normally undetected subpopulations within a parental population that survive immunological selective pressures can also differ in levels of infectivity or virulence as well as host range compared to the parental virus type. Such variations have potential significance in regard to the development of effective diagnostic assays and vaccines. Also, the selection of such variant strains of aquatic birnaviruses in the natural environment may be the basis by which these viruses have become so ubiquitous and are able to infect different host species and apparently cause different diseases. Additional studies of this type together with nucleotide sequence analyses should be performed with cell cultures infected with plaque-purified virus

preparations followed by progressive in vitro propagation to follow the appearance and emergence of individual variant populations.

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經由中和單株抗體篩選之病毒株證明水生兩段雙股核醣核酸病毒族群中抗原及寄主範圍之變異性

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爲了研究水生兩段雙股核醣核酸病毒(birnaviruses)的族群差異性，我們以不同的病毒株培養在含有中和病毒感染力之單株抗體之培養基中以抑制原血清型病毒的複製，而分離出抗中和單株抗體的病毒族群。篩選出的抗中和單株抗體病毒經病毒斑純化，並對其抗原及各種生物特性加以鑑定。這些篩選出來的變異病毒株中，有些除了對於用以篩選的單株抗體不能反應之外，也無法與其它原可反應之單株抗體反應；另外一些變異病毒株則雖不會被用以篩選的單株抗體中和，但可與之反應。某些不能被特定單株抗體中和的變異病毒株，卻與能其它一種或多種不能與親株病毒反應之單株抗體結合，並能被其中和。此外，某些篩選出來的變異病毒株在感染細胞後，複製出比親株病毒高的力價，並產生較大的病毒斑。有三個變異病毒株的寄主範圍亦產生變化，其中兩株已無法感染 fat head minnow (FHM)細胞，而一株則於感染FHM細胞後複製出較親株病毒猶高之力價。當病毒顆粒蛋白比較分子量時，發現變異株的VP1 和VP3 與親株幾乎沒有差異，而不同變異株的VP2 大小皆不相同，有些變異株的VP2 先趨物較親株爲大，有些則較小。

關鍵詞：感染性胰臟壞死病毒, 抗原變異株, 抗中和變異株。

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