Antigenic Analysis of Asian Aquatic Birnavirus Isolates Using Monoclonal Antibodies

V. LIPUN^{*1,5}, P. CASWELL-RENO^{*1}, Y.-L. HSU^{*2}, J.-L. WU^{*2}, M.-C. TUNG^{*3},
P. W. RENO^{*1}, W. WATTANAVIJARN^{*4} and B. L. NICHOLSON^{*1}

^{*1}*Department of Microbiology and Center for Marine Studies, University of Maine,
Orono, Maine, 04469, USA*

^{*2}*Institute of Zoology, Academia Sinica, Taipei, Taiwan, Republic of China*

^{*3}*National Pingtung Institute of Agriculture, Pingtung, Taiwan, Republic of China*

^{*4}*Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand*

(Received March 23, 1989)

A panel of monoclonal antibodies (MAb) was used in an immunodot assay to investigate the antigenic relationships of a variety of aquatic birnaviruses from Asia. All of the Asian birnaviruses tested shared a serogroup-specific epitope with related viruses from North America and Europe. Six viruses from Taiwan were identical in MAb reaction patterns to European eel virus (EEV) and differed from the European Ab virus only by the presence of a single epitope. A single isolate from Thailand was identical to the European Sp virus. Ten viruses from Taiwan exhibited a variety of MAb reaction patterns that were distinct from those previously reported for the nine known serotypes of North America and Europe.

The aquatic birnaviruses are the largest and most diverse group of viruses in the family Birnaviridae and include a variety of viruses from numerous species of fish and marine molluscs worldwide (BOVO *et al.*, 1984; DOBOS *et al.*, 1979; HEDRICK *et al.*, 1986; HILL, 1976; SCHUTZ *et al.*, 1984; SONSTEGAARD *et al.*, 1972; STEPHENS *et al.*, 1980; UNDERWOOD *et al.*, 1977; WOLF and MANN, 1980). Some of these viruses are the etiological agents of diseases in a number of important fish species used in fish farming and aquaculture such as infectious pancreatic necrosis (IPN) in salmonids and branchionephritis in eels (SANO, 1976; SANO *et al.*, 1981; WOLF *et al.*, 1960). Serological studies have demonstrated that most aquatic birnaviruses are antigenically related but significant antigenic differences exist among the numerous isolates. On the basis of differences in reciprocal cross neutralization titers of polyclonal rabbit antisera with selected virus isolates three serotypes designated VR299, Sp, and Ab have been identified (MACDONALD

and GOWER, 1981; OKAMOTO *et al.*, 1983). In similar studies of a large number of birnavirus isolates worldwide, HILL and WAY (1983) confirmed these three serotypes although changing the designation of serotype VR299 to West Buxton (WB). In addition, these investigators demonstrated six additional serotypes which together with the original three represent a single major serogroup (Serogroup A) and four individual isolates that represent a second serogroup (Serogroup B).

Although there are no apparent correlations between the aquatic birnavirus serotypes and specific diseases or host species, individual serotypes of Serogroup A are generally restricted to one or more broad geographical areas. West Buxton (WB) is the predominant, if not sole, serotype detected in the United States whereas there are four serotypes found only in Canada (Canada 1, Canada 2, Canada 3, and Jasper) and four serotypes in Europe (Sp, Ab, Hecht, and Tellina). Aquatic birnaviruses from Asia have not been as well studied, although most isolates to date have been reported to belong to either the WB or Ab serotypes with the majority of isolates included in the latter

^{*5} Present address: Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

(HEDRICK *et al.*, 1985; LIPIUN *et al.*, 1988). Whereas Ab serotype viruses from Asia have been isolated from a variety of fish and shellfish species, most WB serotype viruses from Asia have been limited to salmonids.

Monoclonal antibodies (MAb) have been developed and used in several investigations for antigenic characterization and improved diagnosis of the aquatic birnaviruses (CASWELL-RENO *et al.*, 1986; WOLSKI *et al.*, 1986). However, most of these serological studies using MAbs have concentrated on viruses from North America and Europe. Little information is available about the detailed antigenic characteristics of individual birnavirus isolates from Asia with the exception of one report demonstrating that EEV virus (LIPIUN *et al.*, 1988), an Asian birnavirus isolated from eels and belonging to the Ab serotype, could be distinguished from the Ab type strain by the presence of at least one epitope recognized by one MAb.

Recently, a panel of MAbs has been developed and used in a simple immunodot assay for rapid screening as well as serotyping viruses of Serogroup A (CASWELL-RENO *et al.*, 1989). In this report we describe the use of this panel of MAbs to investigate the antigenic relationships of a variety of aquatic birnaviruses from Asia.

Materials and Methods

Viruses: The viruses used and their source of origin are listed in Table 1. The isolation and propagation of EEV and EIS viruses have been described previously (SANO *et al.*, 1981; WU *et al.*, 1987). The other viruses used in this study were isolated by workers in several laboratories over a period of several years as part of fish disease research and/or diagnostic programs. MFK, TF, CV-1, and PV viruses were isolated by Y.-L.H. and J.-L.W. in northern and central Taiwan; CV-HB-1 and CV-TS-1 were isolated in northern Taiwan by Dr. C.-F. Lo, Fu-Jen University, Taipei, R.O.C.; DPL virus was isolated by W. W. in Thailand; and the remaining viruses were isolated by M.-C.T. in southern Taiwan. For this study, all viruses were propagated in CHSE-214 cell cultures at 20°C and cloned by end-point dilution as pre-

viously described (CASWELL-RENO *et al.*, 1986).

Monoclonal antibodies (MAb): Hybridoma development and characterization of the MAbs (AS-1, W1, W2, W3, W4, W5, E1, E2, E3, E5 and E6) used in this study have been described previously (CASWELL-RENO *et al.*, 1986; LIPIUN *et al.*, 1989; CASWELL-RENO *et al.*, 1989). The source of MAbs for this investigation were supernatant fluids from hybridoma cultures propagated at 37°C in Dublecco's modified Eagle's Minimum Essential Medium (MEM) with D-glucose (4.5 mg/ml), NCTC 109 medium (10%), bovine pancreatic insulin (0.2 units/ml), oxaloacetic acid (1 mM), pyruvic acid (0.45 mM), and fetal bovine serum (15%).

Immunodot test: The enzyme immunodot test was performed as outlined previously (LIPIUN *et al.*, 1988; CASWELL-RENO *et al.*, 1989). Briefly, two μ l aliquots of infected cell culture supernatants containing virus at a minimum titer of 10^4 TCID₅₀/ μ l were "dotted" onto nitrocellulose strips (Bio-Rad Laboratories, Richmond, CA) and air dried at room temperature. After washing four times with Tris-buffered saline (TBS), pH 7.4, the remaining non-specific protein binding sites were blocked with 3% (w/v) bovine serum albumin (BSA) and 10% (v/v) calf serum (CS). Individual strips were then incubated for 3 h at room temperature in each MAb at concentrations at least two times the minimum previously determined to give positive results with homologous virus. After washing and blocking as above, the strips were incubated for 2 h with goat anti-mouse immunoglobulin conjugated with horseradish peroxidase, washed, and incubated for 10 min in substrate (0.5 mg of 4-chloronaphthol and 10 μ l of 3% H₂O₂ in 1 ml TBS). A positive reaction was indicated by a visible purple color.

Results

A panel of 11 monoclonal antibodies (MAbs), including a group reactive as well as serotype or strain specific MAbs, has been developed for the aquatic birnaviruses and used in a variety of serological assays for studying antigenic characteristics, general virus screening, and serotyping (CASWELL-RENO *et al.*, 1986; LIPIUN *et al.*, 1988; CASWELL-RENO *et al.*, 1989).

Table 1. Asian aquatic birnavirus isolates

Virus	Country	Host
European eel virus (EEV)	Japan	Eel (<i>Anguilla anguilla</i> L.)
E1S	Taiwan, R.O.C.	" (<i>Anguilla japonica</i>)
288	Taiwan, R.O.C.	" " "
321	Taiwan, R.O.C.	" " "
339	Taiwan, R.O.C.	" " "
409	Taiwan, R.O.C.	" " "
553	Taiwan, R.O.C.	" " "
3371	Taiwan, R.O.C.	" " "
3372	Taiwan, R.O.C.	" " "
3748	Taiwan, R.O.C.	" " "
6194	Taiwan, R.O.C.	" " "
MFK	Taiwan, R.O.C.	Milkfish (<i>Chanos chanos</i>)
TF	Taiwan, R.O.C.	Trout (<i>Oncorhynchus mykiss</i>)
CV-1	Taiwan, R.O.C.	Clam (<i>Meretrix lusoria</i>)
CV-HB-1	Taiwan, R.O.C.	Clam " "
CV-TS-1	Taiwan, R.O.C.	Clam " "
PV	Taiwan, R.O.C.	Perch <i>Lateolabrax japonicus</i>)
DPL	Thailand	Snakehead (<i>Ophicephalus striatus</i>)

In this report these MAbs were used in an immunodot assay to compare the antigenic relationships of a variety of aquatic birnavirus isolates from Asia. These viruses and the species from which they were isolated are listed in Table 1. The cross-reactivity of each MAb with these viruses is shown in Fig. 1. MAb AS1, which previously has been shown to react with all Serogroup A birnaviruses tested, and MAb E1 reacted with all 18 viruses in this study. Three MAbs (W1, W2, and E2), which recognize epitopes occurring on all Ab serotype viruses tested in previous studies, reacted with EEV and all virus isolates from Taiwan but did not react with isolate DPL from Thailand. Similarly, MAb E5, which in other studies reacted with all isolates tested from North America and Europe, reacted with most of these isolates from Asia but, interestingly, did not react with two isolates from eels in Taiwan. Also, MAb E6, which reacts with all Ab serotype viruses, only reacted with eight viruses here. The epitope defined by MAb E3, which distinguishes EEV from the Ab strain of IPNV, was detected on nine of the viruses in this group. MAb W3 reacted with only five of these viruses and MAbs W4 and W5 failed to react with any of the Asian isolates.

Fig. 1. Reaction of monoclonal antibodies (MAB) with Asian birnaviruses in immunodot assay. ■=positive reaction, □=negative reaction

Overall, this panel of MABs displayed eight distinct patterns of cross reactions with the 18 viruses.

Discussion

The availability of a number of new birnavirus isolates from a variety of fish and shellfish species in Asia provided an opportunity to use a panel of recently developed monoclonal antibodies (MAB) (CASWELL-RENO *et al.*, 1986; LIPIPUN *et al.*, 1988; CASWELL-RENO *et al.*, 1989) to investigate the antigenic relationships of a group of relatively uncharacterized birnaviruses from Asia, a geographical area where little is known about the detailed antigenic characteristics of such indigenous viruses. Previous studies using polyclonal antisera have been shown that most aquatic birnaviruses from Asia, particularly those from non-salmonid species, belong to the Ab serotype of Serogroup A, one of four serotypes occurring in Europe. However, it was not known whether all of these isolates were identical to each other or to the European Ab virus in antigenic characteristics except for a recent report showing that EEV, an Asian Ab serotype virus, differs from the European Ab type strain virus in at least one epitope (LIPIPUN *et al.*, 1988).

All of the viruses included in this study reacted with MAB AS1 which also has been shown to react with all Serogroup A aquatic birnaviruses tested from North America and Europe (CASWELL-RENO *et al.*, 1989). These results support the view that this MAB recognizes a Serogroup A reactive epitope. Furthermore, the fact that this epitope apparently has been conserved among all the aquatic birnaviruses worldwide suggests that it serves a relatively important structural or functional role and is, therefore, a prospective target for general diagnostic assays and vaccine development.

The data presented here show that five of these new isolates from several species including eel, perch, trout, and clams were identical to EEV in MAB reaction patterns. We have previously shown (CASWELL-RENO, *et al.*, 1989) that EEV and several of these viruses differ from the Ab serotype strain only in one epitope

(E3) described by this panel of MABs. Therefore, it appears that many of the aquatic birnaviruses in Asia are closely related serologically to members of one of the major serotypes found in Europe. All but one of the other isolates examined in this study also reacted with MABs E2, W1 and W2 which is characteristic of Ab serotype viruses; however, the reaction patterns of 10 of these isolates with the entire panel of MABs varied and were not consistent with known Ab serotype viruses nor with those of any of the other eight serotypes (CASWELL-RENO *et al.*, 1989). Particularly interesting was the fact that two viruses did not react with MAB E5 which reacts with all aquatic birnaviruses tested from North America and Europe indicating that some Asian viruses are antigenically distinct in that they lack this particular epitope which has been highly conserved among similar viruses occurring on other continents. Whether these viruses actually are members of the Ab serotype and the current criteria using MABs for classifying isolates as Ab type must be revised or whether they represent one or more new serotypes remains to be determined.

The DPL virus isolate from snakehead fish in Thailand appeared significantly different from the other isolates from Taiwan and Japan. In fact, by MAB reaction patterns described by CASWELL-RENO *et al.* (1989), this virus appeared from the data in this study to belong to the Sp serotype. Recently, another birnavirus isolate belonging to the Sp serotype was reported from snakehead fish in Thailand (WATTANAVIJARN *et al.*, 1988). Previously, the only report of an Sp serotype virus in Asia was from imported rainbow trout (*O. mykiss*) in the Peoples Republic of China (YULIN and ZHENGJIU, 1987). Also, HEDRICK *et al.* (1986) have described a birnavirus isolated from sand goby in Thailand that appears significantly different in serology and biochemical characteristics from other aquatic birnaviruses, including Sp. Most aquatic birnavirus isolates from Asia have come from Japan, Korea, and Taiwan. Because the three recent isolates from species indigenous to Thailand exhibit significant antigenic differences from these other viruses, it would be interesting to investigate

the antigenic relationships of additional birnaviruses from Thailand as well as viruses from other countries in Southeast Asia.

Overall, based on the different patterns of cross-reactivity with this panel of MABs, at least eight different strains of aquatic birnaviruses were identified in this group of new isolates. These isolates were obtained from several species from a variety of geographical sites over a period of several years. No apparent correlations were detected between individual antigenic strains and host species or geographic origin.

In summary, this investigation has demonstrated that all of the Asian aquatic birnaviruses examined share a serogroup specific epitope with related viruses from North America and Europe and that six of these new Asian viruses were identical to EEV and could be distinguished from the European Ab virus only by the presence of a single epitope recognized by MAB E3. One isolate from Thailand appeared similar to the European Sp strain and 10 viruses gave varying reaction patterns that were distinct from those previously reported for the nine known serotypes of North America and Europe.

Acknowledgments

This research was supported by grants from the National Oceanic and Atmospheric Administration (NOAA) University of Maine Sea Grant College Program (No. R/FMD-177), the National Science Foundation (No. INT-810447), the Maine Agriculture Experiment Station, and the University of Maine Center for Marine Studies. Publication No. 1394 of the Maine Agriculture Experiment Station.

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