Establishment, Characterization and Application of Monoclonal Antibodies against Eel Virus European (EVE)

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A panel of six monoclonal antibodies (MAbs) against eel virus European (EVE) isolated from eel (*Anguilla japonica*) with branchiononephritis was established in the present study. These systems have been applied for a rapid identification and presumptive serotyping of aquatic birnavirus isolates using western immunoblot assay. Amongst these six MAbs, four were demonstrated to be able to react with viral 7-polypeptide, whereas the other two were specific to viral β -polypeptide. Three MAbs identified epitopes that were highly conserved among members of AB serotype. One MAb recognized an epitope present on AB and SP serotype strains. Two MAbs exhibit the common epitopes observed on AB, SP and VR299 serotypes of infectious pancreatic necrosis virus (IPNV). One of these two MAbs could react with all aquatic birnavirus isolates from various areas including Asia, North America and Europe. Six isolates from Asia exhibiting five varying reaction patterns were demonstrated to be distinct from AB, SP and VR299 serotypes.

The Birnaviridae are characterized by bisegmented double stranded RNA, medium size, an unenveloped and single layer icosahedral capsid (Dobos and Roberts, 1982). These viruses however, consist of infectious bursal disease virus (IBDV), Drosophila X virus (DXV) and aquatic birnaviruses.

Some aquatic birnaviruses such as infectious pancreatic necrosis virus (IPNV) are demonstrated to be etiological agents of economical species of fish and shellfish which may initiate infectious pancreatic necrosis of salmonid, branchiononephritis and nephroblastoma of eels, and gill necrosis of clams (Wolf *et al.*, 1960; Sano, 1976; Sano *et al.*, 1981; Ueno *et al.*, 1984; Lo *et al.*, 1988). In addition, spleen and kidney of loach (*Misgurnus anguillicaudatus*) and sand goby (*Oxyeleotris marmoratus*) may also be seriously affected by some aquatic birnaviruses (Chen *et al.*, 1984; Hedrick *et al.*, 1986).

Amongst aquatic birnaviruses, IPNV was found to be the most important. A serological study showed that most aquatic birnaviruses are antigenically related. However, using reciprocal cross neutralization and polyclonal antiserum, three serogroups of IPNV designated as AB, SP and VR299

can be distinguished (MacDonald and Gower, 1981; Okamoto *et al.*, 1983). Except the described serotypes, Hill and Way (1983) further identified six additional serotypes designated Hect (He), Tellina (Te), Jasper (Ja), Canada 1 (C1), Canada 2 (C2) and Canada 3 (C3). These nine serotypes constitute a major group (serogroup A), and four other isolates represent a minor unrelated serogroup (serogroup B). Viruses isolated from the United States are restricted to VR299 serotype, whereas four serotypes (Ja, C1, C2, and C3) are only detected in Canada and four serotypes (SP, AB, He and Te) are found in Europe.

To investigate the antigenic relationship among these viruses in detail, several monoclonal antibody systems against different serotypes of various aquatic birnaviruses have been established within recent years (Wolski *et al.*, 1986; Caswell-Reno *et al.*, 1986; Caswell-Reno *et al.*, 1989). Lipipun *et al.* (1989) reported that ten viruses isolated from Taiwan were distinct from the nine known serotypes. It was also found that an isolate from Thailand was SP serotype (Lipipun *et al.*, 1989).

The present paper attempts to describe an establishment of the monoclonal antibodies (MAbs) against eel virus European (EVE). The charac-

terization and application of these MABs were also described.

Materials and Methods

Viruses and Production of Monoclonal Antibodies

The viruses listed in Table 1 were used in the

Table 1. Aquatic birnavirus isolates

Virus	Country	Host
VR-299	USA	Rainbow Trout
WF	USA	Rainbow Truout
Buhl	USA	Rainbow Trout
CL	USA	Coho Salmon
Coho	USA	Coho Salmon
AB	Denmark	Rainbow Trout
SP	Denmark	Rainbow Trout
SPC30	Denmark	Rainbow Trout
FR21	France	Rainbow Trout
Fog	France	Oyster
SO ₇ T ₁	Japan	Rainbow Trout
Kagoshima	Japan	Rainbow Trout
Nichiro	Japan	Rianbow Trout
Oippe	Japan	Rainbow Trout
Matsuhisa	Japan	Rainbow Trout
EVE	Japan	Eel
Jov	Japan	Oyster
Thai-X (SGV)	Thailand	Sand Goby
DS 1-5	Taiwan, R.O.C.	Eel
DS 6-10	Taiwan, R.O.C.	Eel
LK 46-50	Taiwan, R.O.C.	Eel
LK 51-55	Taiwan, R.O.C.	Eel
LK 61-65	Taiwan, R.O.C.	Eel
LK 76-80	Taiwan, R.O.C.	Eel
IPN 009	Taiwan, R.O.C.	Eel
IPN 010	Taiwan, R.O.C.	Eel
PTE-83	Taiwan, R.O.C.	Eel
DSE	Taiwan, R.O.C.	Eel
DS 16-20	Taiwan, R.O.C.	Eel
Tilapia 1-5	Taiwan, R.O.C.	Tilapia
Tilapia 6-10	Taiwan, R.O.C.	Tilapia
Tilapia F10-12	Taiwan, R.O.C.	Tilapia
LKT	Taiwan, R.O.C.	Tilapia
KRBT	Taiwan, R.O.C.	Rainbow Trout
IPN 002	Taiwan, R.O.C.	Milk Fish
SWB-83	Taiwan, R.O.C.	Sea Bass (Barramundi)
SWB 2/83	Taiwan, R.O.C.	Sea Bass (Barramundi)
IPN elver	Taiwan, R.O.C.	Elver
TS-1 CV	Taiwan, R.O.C.	Clam
TS-8 CV	Taiwan, R.O.C.	Clam
HB-1	Taiwan, R.O.C.	Clam
Lin-2	Taiwan, R.O.C.	Clam
IPN 004	Taiwan, R.O.C.	Clam
TV	Taiwan, R.O.C.	Tellina
OV-2	Taiwan, R.O.C.	Oyster
J4	Mainland	Oyster

present study. VR299 and Sp were kindly provided by Professor T. Sano of Tokyo University of Fisheries, Japan. Jov, Lin-2, J4 and Fog were isolated from shellfish by our laboratory in the Department of Zoology, National Taiwan University. The other viral isolates of viruses were kindly provided by Dr. R. P. Hedrick, University of California, Davis, U.S.A.

Each virus was cloned by terminal end dilution and propagated in CHSE-214 or TO-2 cells at 18°C in Leibovitz's L-15 medium supplemented with 2% foetal bovine serum (FBS) as described by Hedrick *et al.* (1983). EVE used for Balb/C intra-venous immunization and ELISA screening was cloned by plaque assay twice, propagated in TO-2 cells purified by polyethylene glycol (PEG; mol. wt. 10000) concentration, Freon 113 extraction, and 40% cesium chloride continuous gradient centrifugation as described by Caswell-Reno *et al.* (1989) with slight modification.

Eight-week-old Balb/C mice were injected with purified EVE (25 µg) mixed with complete Freund's adjuvant and 4 weeks later boosted intravenously with a dose of 50 µg without adjuvant. Splenic cells were harvested 3 days later and fused with mice myeloma cells (FO cell line) at a ratio 10:1 by using PEG (mol. wt. 1500). Fused cells were resuspended at a concentration of 1×10^5 cells/ml in growth medium (GM) consisting of RPMI 1640 (Flow Laboratories) with 15% FBS, 2 mM glutamine and 1 mM sodium pyruvate, dispensed 96-well microculture plates in 0.1 ml/well and incubated in 5% CO₂ at 37°C. On the first day of test, 0.1 ml GM supplemented with hypoxanthine (0.1 mM), aminopterin (4×10^{-4} mM) and thymidine (1.6×10^{-2} mM) (GM-HAT) was added into each well. HAT selection continued for 2 weeks by changing half volume of supernatant of 96-well microculture plates with GM-HAT at each 3 consecutive days. Positive hybridomas were cloned by limiting dilution. The cells after cloning were supplemented with conditioned medium of splenic cells or FO cells. Monoclonal antibody isotypes were determined using an ELISA isotyping kit (SBA Clonotyping System III CAT. NO. 16-808-49). Ascites of selected monoclones were made by I.P. injection of 1×10^6 hybridoma cells into Balb/C mice 10 days after inoculation of Prestain (2, 6, 10, 14-tetramethylpentadecane, Sigma, T-7640).

Western Immunoblot Test

Western immunoblot technique as described by Lo *et al.* (1988) was used for detection of specificity of MABs to viral polypeptides and to the known IPNV serotypes. The similar technique was also employed to identify the antigenicity in various isolates of aquatic birnaviruses.

The polypeptides of virus obtained from the lysates of infected cells were separated by SDS-PAGE (mini-gel, 4.5% stacking gel, 10% separating gel, 120 volt, 1 h), and blotted to nitrocellulose (NC) membrane (1A, 20 min). NC membranes were then soaked in 3% skim milk tris buffered saline (TBS) for 1 hour, incubated with each MAB for 2 hours at room temperature, washed with TBS, reacted with peroxidase conjugate goat system for 1 hour, and stained with substrate containing 60 mg 4-chloronaphthol in 20 ml methanol plus 60 µl H₂O₂ in 100 ml TBS. Rabbit anti-IPNV sera were used as positive control, and virus-free cell pellets were used as negative control in each test.

Results

Production of Hybridomas

Using ELISA technique, antibody titers of the Balb/C mice immunized with purified EVE were approximately 1:51200. The fusion efficiency

Fig. 1. Western immunoblot of reactions of 6 MABs with viral polypeptides of EVE. a, viral polypeptides reacted with rabbit anti-IPNV serum; b to g, viral polypeptides reacted with MAB Er1, Er2, Er3, Er4, Eb1 and Eb2.

was 87%, and the frequency of positive hybridomas was 60%. Six monoclones designated as Er1, Er2, Er3, Er4, Eb1 and Eb2 were obtained for further characterization. The isotype for each MAb was determined as follows: Er1=IgG1; Er2=IgG2a; Er3=IgG1; Er4=IgG1; Eb1=IgG2a; Eb2=IgG2a. All MAbs were demonstrated to possess K light chains.

Polypeptide Specificity for MAbs

Western immunoblot assay was employed to determine the specificity of viral polypeptides

against the above described six MAbs and the results were presented in Fig. 1. Er1, Er2, Er3 and Er4 specifically reacted with γ polypeptide. However, Eb1 and Eb2 were β polypeptide specific.

Serotype Specificity for MAbs

The results on the serotype specificity test for six MAbs were shown in Fig. 2 and Table 2. MAb Er1, Er2 and Er3 were specific to AB serotype of IPNV. MAb Eb2 reacted with an epitope on both AB and SP serotypes. No precipitation line was observed when MAb Eb2

Fig. 2. Western immunoblot of reactions of 6 MAbs with AB, SP and VR299 serotype strains. (A), serotype specificity of MAb Eb1; (B), serotype specificity of MAb Eb2 and Er1; (C), serotype specificity of MAb Er2, Er3 and Er4. The first lane of (A) indicated the result of viral polypeptides reacting with polyclonal antiserum against IPNV.

Table 2. Reaction of MAbs with representative aquatic birnaviruses

Virus	Reaction of following MAbs					
	Er1	Er2	Er3	Er4	Eb1	Eb2
AB serotype						
AB	+	+	+	+	+	+
EVE	+	+	+	+	+	+
DSE	+	+	+	+	+	+
LKT	+	+	+	+	+	+
TS-1	+	+	+	+	+	+
TS-8	+	+	+	+	+	+
HB-1	+	+	+	+	+	+
SP serotype						
SP	—	—	—	+	+	+
SPC 30	—	—	—	+	+	+
VR299 serotype						
VR299	—	—	—	+	+	—
Buhl	—	—	—	+	+	—

reacted with VR299 strain. MAb Er4 and Eb1 reacted with common epitopes on AB, SP and VR299 serotypes.

Reactivity of MAbs with Heterologous Aquatic Birnaviruses

Thirty three isolates of aquatic birnaviruses were tested for their specific reaction against the above described six MAbs, and the results were listed in Table 3. The three viral isolates from USA belonged to VR299 serotype, and the two

isolates from Europe were demonstrated to be SP serotype. However, the reaction patterns of the isolates from Asia against those MAbs showed a great diversity. Most of them were AB serotype. The second large group of Asian isolates belonged to SP serotype, and 5 isolates of this group originated from Japan. Only two isolates from eel (*Anguilla japonica*) and sea bass (*Lates calcarifer*) cultured in Asia were VR299 serotype. It is noted that 6 isolates, LK51-55, OV-2, LK46-50, Jov, Lin-2 and Thai-X (SGV)

Table 3. Reaction of MAbs with unserotyping aquatic birnavirus isolates

Virus	Reaction of following MAbs						Suggested serotype
	Er1	Er2	Er3	Er4	Eb1	Eb2	
USA isolates							
WF	—	—	—	+	+	—	VR
CL	—	—	—	+	+	—	VR
Coho	—	—	—	+	+	—	VR
Europe isolates							
FR 21	—	—	—	+	+	+	SP
Fog	—	—	—	+	+	+	SP
Asia isolates							
LK61-65	—	—	—	+	+	—	VR
SWB-83	—	—	—	+	+	—	VR
Kagoshima	—	—	—	+	+	+	SP
Nichiro	—	—	—	+	+	+	SP
SO ₇ T ₁	—	—	—	+	+	+	SP
Oippe	—	—	—	+	+	+	SP
Matsuhisa	—	—	—	+	+	+	SP
LK76-80	—	—	—	+	+	+	SP
DS16-20	—	—	—	+	+	+	SP
TV	—	—	—	+	+	+	SP
DS1-5	+	+	+	+	+	+	AB
IPN002	+	+	+	+	+	+	AB
IPN008	+	+	+	+	+	+	AB
IPN009	+	+	+	+	+	+	AB
IPN010	+	+	+	+	+	+	AB
PTE-83	+	+	+	+	+	+	AB
Tilapia 1-5	+	+	+	+	+	+	AB
Tilapia 6-10	+	+	+	+	+	+	AB
Tilapia F10-12	+	+	+	+	+	+	AB
SWB 2/83	+	+	+	+	+	+	AB
IPN004	+	+	+	+	+	+	AB
IPN elver	+	+	+	+	+	+	AB
J4	+	+	+	+	+	+	AB
LK51-55	+	—	+	+	+	+	
OV-2	—	—	+	+	+	+	
LK46-50	—	—	+	+	+	—	
Jov	—	—	—	—	+	—	
Lin-2	—	—	—	—	+	—	
Thai-X (SGV)	—	—	+	—	+	—	

exhibited distinct reaction patterns between viral polypeptides and established MABs that were significantly different from those obtained from known AB, SP and VR299 serotypes of IPNV (Tables 2 and 3).

MAB Eb1 was demonstrated to be able to react with AB, SP and VR299 serotypes which could recognize an epitope shared by all aquatic birnaviruses tested in the present study. MAB Er4 was also able to react with 3 known serotypes but unable to react with Jov, Lin-2 and SGV. Meanwhile, Jov, Lin-2 and viral isolate from Thailand Thai-X (SGV) did not react with Er1, Er2 and Eb2 MABs. When MAB Er3 was used, Thai-X (SGV) revealed a positive reaction (Table 3).

Owing to the reaction patterns of LK51-55 and OV-2 against MAB Er1 and Er2 indicated in Table 3, these two isolates were distinguished from the other AB serotype isolates. Although OV-2 reacted weakly with MAB Er3, but no reaction was observed between this virus and MAB Er1, or with Er2. It is therefore suggested that this isolate is more closely related to SP than to AB serologically.

Discussion

In the present study, a panel of six MABs against EVE has been developed and these MABs have been applied for the detection of the antigenic relationship among a variety of aquatic birnavirus isolates from different areas including North America, Europe and Asia.

FO cells were used for the present study. These cells provided sufficient results on fusion efficiency and high frequency of positive hybridomas compared with SP2-Ag-14 myeloma cells used by Caswell-Reno *et al.* (1986). Following a serological study on IPNV, Ishiguro *et al.* (1984) speculated that common antigens occur on the inner part of the virion and strain specific determinant sites located on the surface. However, the results revealed that both common and specific epitopes might exist on the inner protein (γ polypeptide) of aquatic birnaviruses.

Because MAB Eb1 reacted with all the viruses in the present study, it possesses a great potential to be a good tool for screening aquatic birnaviruses from a batch of unknown viruses. Although MAB Er4 reacted with all 3 serotype strains, it

did not react with SGV, Jov and Lin-2, and this MAB can be useful for screening the isolates from Asia.

AB serotype specific MABs Er1, Er2 and Er3 were demonstrated to react with different epitopes. Therefore these three MABs can be applied on the strain identification for AB serotype. Several viruses isolated from different hosts can be distinguished by the established monoclonal antibodies due to the presence or absence of one or two epitopes. For example, the only difference between LK51-55 from eel and OV-2 from oyster (*Ostrea edulis*) relies on their binding with MAB Er1. Therefore the utility of this MAB as a probe for detecting IPNV derived from various hosts is becoming increasingly apparent.

According to the reaction patterns of these MABs against AB, SP and VR299 serotypes, presumptive serotyping of members of the three serotypes is readily accomplished. Previous studies using polyclonal antisera indicated that in Asia the majority of aquatic birnavirus isolates belonged to AB serotype, and only very few isolates were VR299 serotype (Ueno *et al.*, 1984; Lo *et al.*, 1988; Lipipun *et al.*, 1989; Hedrick *et al.*, 1983; Hedrick *et al.*, 1985). It was also noted that most VR299 isolates from Asia have been limited to salmonids. However, in the present study the host species of VR299 serotype were not only restricted to salmonid fish, but also found in eel and sea bass. Besides, the frequency of SP serotype isolates increased very significantly, and the majority of these isolates originated from Japan.

The serologic reaction patterns of Jov and Lin-2 with MABs were distinct to those of AB, SP and VR299. SGV was isolated from sand goby with ulcer disease in Thailand by Hedrick *et al.* (1986). Although the virus was an aquatic birnavirus, it was also different from the AB, SP and VR299 serologically using cross neutralization assay. SGV was demonstrated to be able to react with MAB Eb1, but did not interfere with MAB Er1, Er2, Er4 and Eb2. This result therefore confirms the previous conclusion obtained by Hedrick *et al.* (1986) that SGV is unidentical to these three known serotypes.

Western immunoblot assay using a battery of those MABs provides an useful tool for resolving the serotyping controversy. The use of MABs is a better technique than traditional usage of poly-

clonal antisera since it provides stable and specific conditions for the detail identification of viruses. The time required to complete the whole process using MAbs for detection is approximately 8 hours. The minimum virus titer for detection is approximately 10^4 TCID₅₀/0.1 ml. Crude cell associated virus (CAV) of infected cells is sufficiently for the assay. Therefore MAb system is strongly recommended for the investigation of serotyping of IPNV. In recent years, several monoclonal antibodies systems against birnaviruses have been developed (Wolski *et al.*, 1986; Caswell-Reno *et al.*, 1986; Caswell-Reno *et al.*, 1989). These results indeed are beneficial to a more detailed study on the serological study of the fish viruses.

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