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Change of Infection Properties of Subcultured Marine Birnavirus in Several Fish Cell Lines

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Marine birnavirus (MABV) has wide host range in marine organisms. To clarify various infection properties of MABV in different host species, *in vitro* study was performed by subculture for 10 passages in several fish cell lines. In CHSE-214, RTG-2 and RSBK-2 cells, the virus produced high yield of virus. Typical CPE with high protein expression was observed in these cells. On the contrary, the virus grown in EPC, FHM and BF-2 cells exhibited no CPE appearance although virus protein was detected. In EPC and FHM cells, the virus titer increased in later passages. The plaque size was distinctly bigger in CHSE-214, RTG-2 and RSBK-2 cells than in other cell lines. The nucleotide sequence of VP2/NS junction region on genome segment A exhibited one specific nucleotide change at 195. The different infection properties in several cell types performed in the present work might reflect *in vivo* MABV infection in various host species occurring in natural conditions.

Key words: Marine birnavirus, Subculture, Host cells, Adaptation, Mutation

Members of the family Birnaviridae have a bisegmented double-stranded RNA genome consisting of two segments (A and B) within an unenveloped, icosahedral capsid of 60 nm (Dobos *et al.*, 1979; Brown, 1986). The birnaviruses isolated from fish include infectious pancreatic necrosis virus (IPNV) of young salmonid fishes and marine birnavirus (MABV) including yellowtail ascites virus (YAV) and related strains isolated from marine fishes (Sorimachi and Hara, 1985; Novoa and Figueras, 1996).

After the discovery of YAV in yellowtail *Seriola quinqueradiata* (Sorimachi and Hara, 1985), the similar viruses have been isolated from the japanese flounder *Paralichthys olivaceus*, red sea bream *Pagrus major*, sea bass *Lateolabrax japonicus*, amberjack *Seriola dumerili* and other marine fishes (Kusuda *et al.*, 1989; Hosono, 1996).

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Recently, it has been known that MABV infects not only fish but shellfish such as jack knife clam *Sinonovacura constricta* and japanese pearl oyster *Pinctada fucata* although the pathogenicity was not strong in experimental infection (Chou *et al.*, 1994; Suzuki *et al.*, 1997; Suzuki *et al.*, 1998). Rivas *et al.* (1993) reported that birnaviruses distribute widely in wild fish, molluscs and sediment around fish farm. The investigations above indicate that the birnaviruses show wide host range and present in natural reservoirs.

In general, when a virus infects a host, a clone which can appropriately produce progeny virus becomes major population in the host. Such adaptation is accompanied by genetic mutations as reported in several viruses (Symington and Schlesinger, 1975; Miyamura *et al.*, 1980; Tantawi and Al-Sheikhly, 1980; Weaver *et al.*, 1993). Since MABV shows wide host range as mentioned above, it seems that there is genetic variation throughout the infection process in various hosts.

In this study, in order to clarify the mutation/variation of MABV occurring in the various hosts and in the process of passage, I characterized the virus growth, antigen production, cytopathogenicity, plaque morphology and nucleotide sequences in the variable region of segment A when the MABV strain Y-6 was continuously subcultured in various fish cell lines.

Materials and Methods

Cells and virus

Chinook salmon embryo (CHSE-214), rainbow trout gonad (RTG-2), red seabream kidney (RSBK-2), epithelial papilloma of carp (EPC), fathead minnow epithelium (FHM) and bluegill fin (BF-2) cell lines were propagated in Eagle's minimal essential medium (Nissui) supplemented with 10% fetal bovine serum.

The cloned MABV Y-6 strain, originally isolated from yellowtail was employed in this study. The titer of the starting virus was 6.8×10^8 p.f.u. (plaque forming unit)/ml. The virus was infected to each cell line at low multiplicity (0.01 p.f.u./cell). Then harvested 40 h after inoculation followed by titration by plaque method with RTG-2 cells according to the suspension assay of Russell (1962) using an overlay medium containing low melting agarose (BRL). The subcultures were performed 10 passages in each cells. In case the virus titer is not high enough to adjust 0.01 p.f.u./cell, 1 ml of virus suspension was inoculated into the confluent cell sheet in 25 cm^2 flask (Sumilon).

Immunofluorescence test

The immunofluorescence technique was employed to detect viral antigen in EPC, FHM and BF-2 cells which did not show typical cytopathic effect (CPE) in infected cells. The virus was inoculated to cells grown on 13.5 mm diameter cell disk (Sumilon) in 24 wells plate (Sumilon). Forty hours after infection, cells on cell disk were fixed with 100% ethanol for 10 min. After blocking with 5% skim milk, predetermined dilutions (1/200) of MABV Y-6 rabbit antiserum produced in our laboratory were applied, and incubated at 37°C for 1 h. Then cells were reacted with 1/200 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Dako) as a secondary antibody. For the negative control, normal rabbit serum and uninfected cells were prepared by the same procedure.

Nucleic acid extraction

The harvested virus from several cell lines was centrifuged at $2,000\times g$ for 20 min to remove cellular debris. Proteinase K (TaKaRa) was added to virus fluid as a final concentration of 100 μg/ml and digested at 42°C for 1 h. The solution was extracted with TE (50 mM Tris, 1 mM EDTA) buffer-saturated phenol, mixture of phenol-chloroform (1 : 1, v/v) and chloroform. Then the nucleic acids in the aqueous phase were purified by ethanol precipitation.

cDNA synthesis

The cDNA synthesis was performed as described by Suzuki *et al.* (1997). Briefly, purified nucleic acid fraction was mixed with 100 pmol of the oligonucleotide primers, P1 : 5'-AGAGATCACTGACTTC-ACAAGTGAC-3' and P2 : 5'-TGTGCACCACAG-GAAAGATGACTC-3' then heated at 100°C for 5 min followed by cooling on ice. Five μl of the reaction mixture was taken and mixed with 15 μl of a cocktail containing 250 mM Tris, 375 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTP mixture, 20 U of RNase inhibitor (HRPI) and 200 U of moloney murine leukemia virus reverse transcriptase (BRL). The reverse transcription reaction was performed at 37°C for 1 h.

RT- and nested PCR

The resulting cDNA was heated at 100°C for 5 min and cooled on ice. PCR was carried out in a total volume of 100 μl containing 5 μl of cDNA fraction, 100 mM Tris, 500 mM KCl, 15 mM $MgCl_2$, 0.2 mM dNTP mixture and 2.5 U of Taq DNA poly-

merase (TaKaRa). The mixture was subjected to 30 cycles (denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min) in a thermal-cycler (TaKaRa TP2000).

Because of low virus titers in EPC, FHM and BF-2 cells, RT-PCR products was not detected on agarose gel electrophoresis. To detect virus from these cells, two step PCR amplifying inside RT-PCR region was performed. 3 μl of the RT-PCR product was employed for nested PCR with a primer set with P3 : 5'-CAACACTCTTCCCCATG-3' and P4 : 5'-AGAACCTCCCAGTGTCT-3' in the same condition as RT-PCR with exception of annealing at 48°C.

PCR products were analyzed on 1.5% agarose (BRL) gels in TBE buffer (0.45 M Tris, 0.45 M Boric acid, 0.01 M EDTA) at 100 V for 40 min. The gels were stained with ethidium bromide.

Sequence analysis

Sequence analysis was conducted with a procedure described by Suzuki *et al.* (1998). Direct sequencing of the PCR products electroeluted from the agarose gel was conducted using a DNA sequencer 373A (Applied Biosystems) and an ABI PRISMTM Dye Terminator Cycle Sequencing Kit (Perkin Elmer). The sequence was analyzed using the Genetyx-Mac version 8.0.

Results

Virus replication and antigen detection

The virus was harvested at 40 h after infection from each culture. The virus titers in each passage in different cell lines are shown in Fig. 1. In CHSE-214, RTG-2 and RSBK-2 cell lines, typical CPE was observed and most of the cells were detached from the flask bottom after 40 h. These cases gave high virus yields. On the contrary, there was no CPE appearance in EPC, FHM and BF-2 cells. The virus titer was below detection limit (5.0×100 p.f.u./ml) in FHM cells through 2nd to 5th passage and in certain passages in EPC and BF-2 cells (Fig. 1). In later

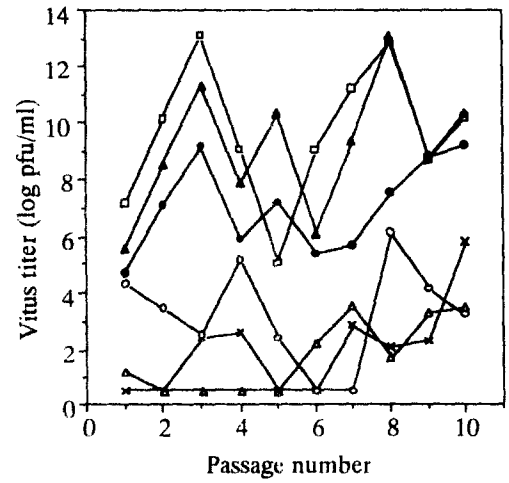

Fig. 1. Virus titers of each passage in various cell lines. -□-, CHSE-214; -▲-, RTG-2; -●-, RSBK-2; -○-, BF-2; -×-, EPC and -△-, FHM cells.

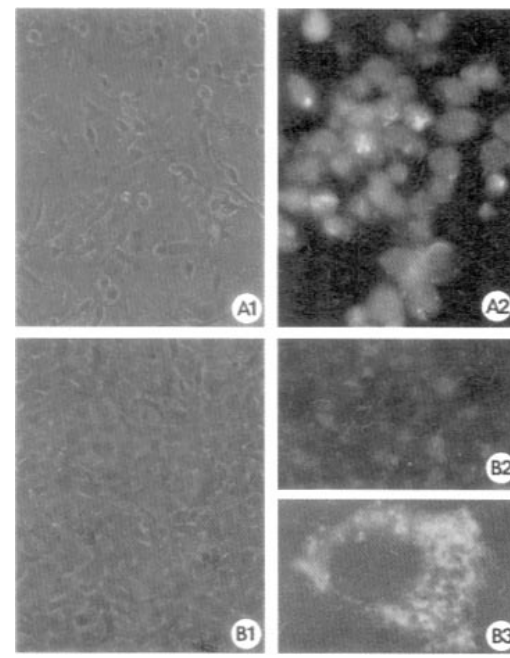

Fig. 2. Representative cell morphology and immunofluorescence in the permissive and nonpermissive cell lines in 5th passage of MABV. A1; CHSE-214 cells showing round and lytic cytopathic effect. A2; Strong fluorescence of CHSE-214 cells with anti-MABV serum. B1; Virus infected FHM cells showing indistinguishable morphology with normal cells. B2; Granular fluorescence in the cytoplasm of FHM cells and its high magnification (B3).

passages, the virus titer was increased in EPC and FHM cell suggesting that MABV adapted to grow in these cells. The CPE was not detected for 7 d of observation in EPC, FHM and BF-2 cells although virus titer was increased in later passages.

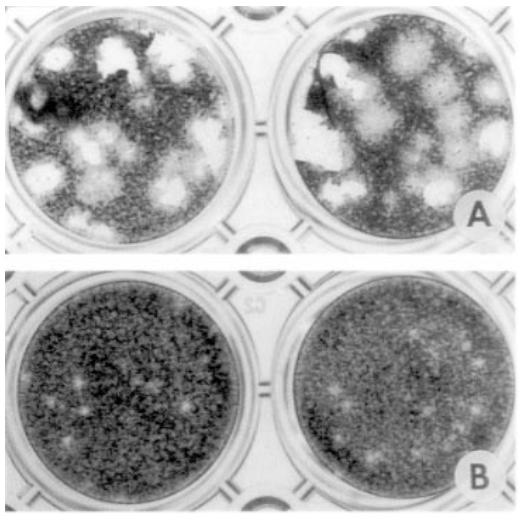

Fig. 3. Representative photograph of plaque on RTG-2 cells. Tenth passage of MABV Y-6 cultured in CHSE-214, RTG-2 and RSBK-2 cells produced bigger plaque than the virus from EPC, FHM and BF-2 cells.

To determine whether viral proteins were expressed or not in the cells employed, immunofluorescent staining with MABV Y-6 antiserum was conducted. As shown in Fig. 2, the cells producing high virus yields such as CHSE-214, RTG-2 and RSBK-2 cells show strong fluorescence in the cytoplasm. In EPC, FHM and BF-2 cells, less amount of virus specific antigen was detected as granular fluorescence in cytoplasm indicating the expression of the virus proteins occurred even in the nonpermissive cells. No fluorescence was observed in the control groups.

The subcultured virus had different plaque sizes in RTG-2 cells. The virus harvested from EPC, FHM and BF-2 cells exhibited distinguishably smaller plaque size (0.58 mm in average) than the virus grown in CHSE-214, RTG-2 and RSBK-2 cells (1.53 mm) (Fig. 3).

PCR and nucleotide sequence

The starting virus, 5th and 10th passage viruses were employed for PCR. The nucleotide sequencing of the RT-PCR product (359 bp) of the virus from CHSE-214, RTG-2 and RSBK-2 cells and nest-

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                    CTCCC AACCT CAAAA GCCTG GGGGT GGAGA GACAT TGTGA GAGGC ATCCG  50
                    GAAGG TGGCA GCACC AGTGC TGTCA ACACT CTTCC CCATG GCGGC ACCAC 100
                    TCATC GGAGC CGCCG ACCAA TTCAT CGGAG ACCTG ACCAA GACCA ACGCA 150

Starting  Y-6     GCCGG AGGCC GCTAC CTAAC ACATG CAGCA GGAGG ACGCT ACACT GACGT 200
CHSE-214  (5th)   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 200
CHSE-214 (10th)   ..... ..... ..... ..... ..... ..... ..... ..... ....C ..... 200
RTG-2  (5th)      ..... ..... ..... ..... ..... ..... ..... ..... ....C ..... 200
RTG-2  (10th)     ..... ..... ..... ..... ..... ..... ..... ..... ....C ..... 200
EPC  (5th)        ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 200
EPC  (10th)       ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 200
FHM  (5th)        ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 200
FHM  (10th)       ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 200
BF-2  (5th)       ..... ..... ..... ..... ..... ..... ..... ..... ....C ..... 200
BF-2  (10th)      ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... 200

                    AATGG ACTCC TGGGC CAGCG GCACA GACAC TGGGA GGTTC TCACG CAACC 250
                    TCAAA GACCG GCTGG AGTCA AACAA CTATG AGGAG ATGGA ACTTC CTCCA 300
                    CCAAC GAAAG                                               310
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Fig. 4. The nucleotide sequence of MABV Y-6 strain of starting, 5th and 10th passage virus on VP2/NS junction region in several cell lines. The 310 bp of RT-PCR product from CHSE-214, RTG-2 and RSBK-2 cells, and 134 bp of nested PCR product fromEPC, FHM and BF-2 cells are shown. Underlined region is the sequences of nested PCR region. Sequence between #151 to #200 is compared among the virus obtained from various cell lines.

ed-PCR product (168 bp) from EPC, FHM and BF-2 cells were performed, then compared with the sequence of starting virus. Fig. 4 shows the nucleotide sequences of the PCR products of starting virus, 5th and 10th passage viruses without primer sequences. There were no mutations of the viruses grown in EPC and FHM cells for 10 passages. Only one nucleotide, T at 195 was changed to C in CHSE-214 cells at 10th passage, in RTG-2 cells at 5th and 10th passage and in BF-2 cells at 5th passage. These base substitutions were silent indicating that the primary amino acid sequences in VP2/NS region was not changed in all samples.

Discussion

The objective of this study was to determine the infection properties of MABV in several cell lines, and to know whether genomic mutations in the process occur or not. The high yields of virus with abundance of virus antigen were obtained in CHSE-214, RTG-2 and RSBK-2 cells. In EPC, FHM and BF-2 cells, virus proteins were expressed but the infectious virion was not detected in early passages, suggesting that MABV occurs abortive infection in these stages. However, virus yield increased in later passages although CPE was not appeared. This indicates that the virus causes nonlytic but productive infection in EPC, FHM and BF-2 cells. The *in vitro* models of acute and subclinical persistent infections were reported in IPNV (Hedrick and Fryer, 1981; 1982). They established persistent infection state of IPNV in three salmonid cell lines, CHSE-214, steelhead trout (STE-137) and RTG-2 cells, by inoculation with high multiplicity of the virus. Similar persistence was observed in their *in vitro* system and IPNV infected carrier brook trout *Salvelinus fontinalis*.

IPNV causes acute disease with high mortality in young salmonid fish but usually subclinical or persistent infection is caused in a various species of nonsalmonid fish and older salmonid fish (Wolf, 1988). The persistently infected fish were asymptomatic, but they contain virus in many visceral organs and can vertically transmit the virus to their progeny (Billi and Wolf, 1969; Yamamoto, 1975; Reno *et al.*, 1978; Ahne and Negele, 1985; Dorson and Torchy, 1985). In case of MABV, acute and persistent infection in various species of marine fishes, shellfish and rotifer has been reported (Sorimachi and Hara, 1985; Lo *et al.*, 1988; Comps *et al.*, 1991; Rivas *et al.*, 1993; Novoa and Figuers, 1996). But the viral replication, protein expression and genomic changes of MABV from one strain in different hosts are not understood. The results of this study can be a model of MABV infection in permissive and nonpermissive host.

Virus grown in EPC, FHM and BF-2 cells exhibited distinguishable smaller plaque than in CHSE-214, RTG-2 and RSBK-2. Chen *et al.* (1990) described that the clones of infectious hematopoietic necrosis virus producing small and large plaque are genetically distinct. The differences of plaque size may reflect mutation of viral genomes. There are many reports about the relationship between plaque size or shape and pathogenicity (Takemoto, 1966; Douglas *et al.*, 1974; Cosby *et al.*, 1981). Cosby *et al.* (1981) demonstrated large plaque strain of canine distemper virus produces an acute neurological illness in weanling hamsters, whereas small plaque virus fails to produce any clinical signs until 3 months. The different plaque size variant of rift valley fever virus shows less pathogenicity to several kinds of host animals (Takemoto, 1966; Moussa *et al.* 1982). Historically, the plaque mutants were used as materials in the development of live poliovirus vaccine (Sabin, 1965). It is useful to determine the pathogenicity of MABV forming small plaque for the development of attenuated vaccine.

Heppell *et al.* (1992) reported that VP2/NS junction region is suitable for the analysis of genomic variation between IPNV strains. Havarstein *et al.* (1990) also demonstrated that the part of the NS and the central region of VP2 are the most variable section in genome segment A by comparing nucleotide sequences of the Jasper and N1 strains. These geno-

mic parts of the MABV was analyzed by Hosono *et al.* (1996), and the complete nucleotide sequence of the segment A was recently reported (Suzuki *et al.*, 1998). The nucleotide sequence analysis in this study appeared that one nucleotide at 195 was changed in four cases (Fig. 4). The T of the starting virus at 195 changed to C in these cases, suggesting that the nucleotide change occurs in specific place. But a unity of the mutation with passage number or type of cell lines was not occurred in this region. The nucleotide sequence of MABV segment A (Suzuki *et al.*, 1998) shows that the variable region was observed in the central region of VP2 as similar to IPNV and avian infectious bursal disease virus. The central variable region in VP2 should be analyzed in order to find the mutations caused by passage number and/or host differences in further study.

In this study, it was found that the one cloned parental virus evoluted different ways in different hosts. The virus was cytolytic in some cell types but caused persistent infections in other cell types. The virus adaption and plaque size differences might be caused by mutation of viral genome. The corresponding genomic regions of these changes may be on other part(s) of VP2/NS region. These kinds of changes might occur in natural conditions in various kinds of host species of MABV.

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어류 주화세포에서의 계대배양에 의한 해양버나바이러스의 감염특성의 변화

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해양버나바이러스(MABV)는 여러 종의 해양생물에 감염되며 숙주역이 넓다. 다양한 종의 숙주에 감염되었을 때의 MABV의 감염 특성을 규명하기 위하여, 주화세포 내에서 바이러스를 10대 계대 배양하여 *in vitro*로 연구했다. CHSE-214, RTG-2와 RSBK-2 세포에서는 전형적인 CPE를 보이며 많은 양의 바이러스가 생산되었고, 높은 바이러스 단백질의 발현도 관찰되었다. 이에 반하여, EPC, FHM과 BF-2세포에서는 형광항체법에 의하여 바이러스단백질은 검출되었으나 CPE는 나타나지 않았다. EPC와 FHM 세포에서는 계대를 할수록 바이러스의 역가가 높아져, 바이러스의 숙주세포에의 적응이 일어난 것으로 보인다. 플라크의 크기는 CHSE-214, RTG-2와 RSBK-2 세포에서 계대한 것이 다른 세포에서 계대한 것보다 커, 숙주세포의 종류에 따른 변이가 바이러스에 일어난 것으로 추측되었다. 게놈분절 A에 존재하는 VP2/NS 경계영역의 염기배열에서는 195번째의 염기에 특이적인 변이가 보였다. 숙주세포의 종류에 따라 다른 MABV의 감염특성은 자연계에서 다양한 숙주종에서 일어나는 *in vivo*에서의 감염특성을 반영하는 것으로 생각된다.

Key words: Marine birnavirus, Subculture, Host cells, Adaptation, Mutation