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NOTE

Generation of monoclonal antibodies specific to hepatopancreatic parvovirus (HPV) from *Penaeus monodon*

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ABSTRACT: Hepatopancreatic parvovirus (HPV) was isolated from the hepatopancreas (HP) of slow growth *Penaeus monodon* by urografin gradient centrifugation. The presence of HPV in the fraction was monitored by PCR and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Only 1 major 54 kDa protein band was observed in the strong PCR-positive fractions used to immunize mice for monoclonal antibody production. After cell fusion, the first step in selecting specific antibodies was performed by dot-blot assay with purified HPV viral particles. The second screening step was carried out using Western blots of purified HPV proteins and immunohistochemistry of HPV-infected HP tissue. Four monoclonal antibodies were isolated; these bound to the 54 kDa protein in Western blots and to intranuclear inclusion bodies in tubule epithelial cells of HPV-infected prawn tissue by immunohistochemistry. None of the antibodies showed cross-reactivity either to uninfected shrimp tissue or to other shrimp viruses tested. These reagents have potential for use in developing a highly sensitive immunoassay such as sandwich ELISA or a convenient kit for detection of HPV infection.

KEY WORDS: Dot-blot · Hepatopancreatic parvovirus · HPV · Immunohistochemistry · Monoclonal antibody · *Penaeus monodon*

INTRODUCTION

Hepatopancreatic parvovirus (HPV) has been known to infect several penaeid shrimp in Asian, Australian and African regions since 1985 (Lightner et al. 1993). HPV was first reported from Thailand infecting *Penaeus monodon* in 1993 (Flegel & Sriurairatana 1993). Shrimp infected with HPV usually show non-specific gross signs of disease. However, infection is statistically related to retarded growth in *P. monodon* (Flegel et al. 1999). HPV virions are icosahedral, approximately 22 nm in diameter and contain ssDNA of 6 kb in size. This probably comprises overlapping genes for at least 2 polypeptides. The virus was classified in the family *Parvoviridae* based on these characteristics (Bonami et al. 1995, Spann et al. 1997, Sukhumsirichart et al. 1999). Detection was traditionally dependent upon the histological demonstration of basophilic intranuclear inclusions in tubular epithelial cells of the hepatopancreas (HP) by hematoxylin and eosin (H&E) or Giemsa staining (Lightner 1996). DNA probes and PCR primers have been developed and

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proven to be powerful tools for diagnosis of shrimp HPV infection (Sukhumsirichart et al. 1999, 2002, Pantoja & Lightner 2000, Phromjai et al. 2001, 2002). Under optimized conditions, DNA approximately equivalent to that in 300 viral particles could be detected (Pantoja & Lighner 2000). However, there are practical limitations to using PCR in widespread applications. These include the need for special equipment and highly trained personnel and the costs incurred for small sample numbers. Moreover, HPV isolates from various shrimp species and geographic regions seem to be genetically different. For example, HPV in *P. chinensis* from Korea (Bonami et al. 1995), *P. monodon* from Thailand (Sukhumsirichart et al. 1999) and *Macrobrachium rosenbergii* from Malaysia (Lightner et al. 1994) are different. PCR primers designed for HPV from *P. chinensis* were not particularly efficient for PCR amplification of HPV DNA from *P. monodon* (Phromjai et al. 2001) and probes designed from the same source did not hybridize with DNA of HPV from *M. rosenbergii* (Lightner et al. 1994).

Immunologically based diagnosis, especially using monoclonal antibodies (MAbs), has proven to be an effective, alternative method for diagnosis of various viral diseases including those of shrimp, e.g. yellow head virus (YHV) (Sithigorngul et al. 2002). A series of MAbs specific to YHV was produced, most of which could recognize various isolates of YHV complex such as gill associated virus from Australia and nonvirulent YHV-like virus from Thailand. Few recognized only the virulent YHV isolate from Thailand (Soowannayan et al. 2003). MAbs can be adapted to various formats for use in convenient diagnostic kits. Here we describe preliminary trials to produce a MAb that could be used to develop a specific, sensitive and convenient test kit for HPV detection.

MATERIALS AND METHODS

Antigen preparation. Growth retarded shrimp *Penaeus monodon* (1 to 5 g) were collected from ponds in Chonburi province, Thailand. Hepatopancreata were dissected out and frozen immediately in liquid nitrogen and stored at –70°C until use. The HP was homogenated in NaCl-Tris-EDTA buffer (NTE; 0.2 M NaCl, 0.02 M Tris-HCl, 0.02 M EDTA pH 7.4). Cell debris was removed by centrifugation at both 7000 and 13 000 × *g* for 15 min. The supernatant was centrifuged at 140 000 × *g* for 1 h and the pellet was resuspended and further separated by gradient ultracentrifugation in (20 to 40%) urografin for 3 h. Fractions were collected, diluted with NTE and centrifuged at 140 000 × *g* for 3 h. Pellets were resuspended in Tris-EDTA buffer (TE; 10 mM Tris-HCl, 1 mM EDTA pH 7.4) at 4°C and checked for the presence of HPV by PCR and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Positive fractions were divided into small aliquots and stored at –70°C. The viral suspension was used for immunization and for screening of MAbs by dot blot and Western blot analysis.

Part of the purified virus preparation was denatured by mixing with treatment buffer (SDS and β-mercaptoethanol) dialyzed against phosphate buffered saline (PBS; 0.15 M, pH 7.2), then divided into small aliquots and used for immunization. Part of an HPV-infected HP was fixed in Davidson's fixative and processed for paraffin sectioning (Lightner 1996) and used for immunohistochemical screening of antibodies against HPV.

PCR detection of HPV. HPV-specific primers 121F (5′GCA CTT ATC ACT GTC TCT AC 3″) and 276R (5′GTG AAC TTT GTA AAT ACC TTG 3″) were used in PCR amplification (Sukhumsirichart et al. 1999) to yield a 156 bp amplicon specific for HPV. A stored viral fraction was thawed and treated with 50 µg ml^{-1} of Proteinase K for 30 min at 37°C followed by 0.5% sarkosyl treatment for 1 h at 65°C. The nucleic acid was then extracted using phenol and chloroform-isoamyl alcohol (24:1:1 v/v) and precipitated with 70% ethanol. The DNA pellet was washed with 70% ethanol, air dried and resuspended in TE buffer. The purified DNA was amplified by PCR in a 50 µl reaction mixture (Sukhumsirichart et al. 1999).

Immunization. Three Swiss albino ICR mice were first immunized intraperitoneally with 100 µl of denatured HPV (0.2 mg ml^{-1}) mixed with complete Freund's adjuvant (1:1). The mice were boosted 3 times at 2 wk intervals with the same preparation mixed with incomplete Freund's adjuvant. After the fourth injection, sera were collected and tested for HPV immunoreactivity by immunohistochemistry and Western blot analysis.

Monoclonal antibody production. The best performing mouse was boosted 3 d before hybridoma production. A cell fusion procedure was adapted from the method developed by Köhler & Milstein (1976), with modifications described by Mosmann et al. (1979). A P3X myeloma cell line was used as the fusion partner. Fusion products from 1 mouse were plated on 30 microculture plates (96 wells per plate). After identification of positive cultures by the screening methods described below, cells were recloned by the limiting dilution method.

Immuno dot-blots. Purified virus (0.1 mg ml^{-1}) was spotted onto nitrocellulose membranes (1 µl $spot^{-1}$) that were subsequently baked at 60°C for 10 min and then incubated with hybridoma conditioned media from each culture well (1:20 dilution in 5% Blotto: 5% nonfat dry milk, 0.1% Triton-X-100 in PBS) for 4 h. After extensive washing in 0.5% Blotto, the membrane

was incubated in horseradish peroxidase conjugated goat anti-mouse immunoglobulin G (IgG) heavy and light chain specific antibody (GAM-HRP; BioRad) at 1:1000 dilution for 3 h. The membrane was then washed as extensively as before and incubated in a substrate mixture containing 0.006% hydrogen peroxide, 0.03% diaminobenzidine (DAB), 0.05% cobalt chloride in PBS. Hybridoma clones producing antibodies that displayed immunoreactivity were confirmed for viral specificity by Western blot and immunohistochemistry before cloning and cryo-preservation for further investigation.

Western blots. Proteins from partially purified HPV were separated by 10% SDS-PAGE according to the method described by Laemmli (1970). Samples were electrophoresed for 1 h at 100 V and part of the gel was stained using Coomassie brilliant blue R-250. For Western blot analysis, the samples resolved by SDS-PAGE were electroblotted onto nitrocellulose membranes using a Transblot apparatus (BioRad). The nitrocellulose membrane was incubated in 5% Blotto for 10 min, treated with 1:200 of each MAb for 4 h and then processed as described in the dot-blotting section above.

Immunohistochemistry. Cephalothoraces from HPV-infected prawns were fixed in Davidson's fixative solution for 24 h before processing for paraffin sectioning. Serial sections (8 µm thickness) were prepared and processed for indirect immunoperoxidase staining using various MAbs and GAM-HRP diluted 1:1000 with 10% calf serum in PBS. Peroxidase activity was revealed by incubation with 0.03% DAB and 0.006% hydrogen peroxide in PBS. Preparations were counterstained with H&E, dehydrated in graded ethanol series, cleared in xylene and mounted in permount (Sithigorngul et al. 1999). Positive reactions were visualized as brown coloration against pink cytoplasm and purple nuclei. Tissues from *Penaeus monodon* infected with YHV, nonvirulent YHV, monodon baculo virus (MBV) and white spot syndrome virus (WSSV) were also used to determine cross-reactivity of the MAbs by immunohistochemistry.

Monoclonal antibody class and subclass. Class and subclass of mouse IgG produced by the hybridomas were determined by sandwich ELISA using Zymed's Mouse MonoAb ID Kit (HRP).

RESULTS AND DISCUSSION

SDS-PAGE of proteins from partially purified HPV revealed a major protein band of 54 kDa (Fig. 1A) similar to that reported for HPV from *Penaeus chinensis* (Bonami et al. 1995). Therefore, the preparation was considered of sufficiently high purity to use for immunization. After the fourth immunization, antisera from all 3 mice bound specifically to basophilic intra-nuclear inclusion bodies of HPV-infected HP tubule epithelial cells in immunohistochemistry assays and strongly to 2 specific protein bands of 54 and 97 kDa in Western blot assays. One mouse was used as a spleen donor and the yield of hybridoma clones was approximately 1000 (30% of wells from 30 plates). Only 10 clones showed specific binding to viral protein by dot-blot assay. After Western blot and immunohistochemistry analyses, only 4 clones showed specific binding to the 54 kDa protein (Fig. 1B) and to HPV intra-nuclear inclusion bodies but not to uninfected tissues (Fig. 2). Antibodies from these clones also recognized the 97 kDa protein. It may be a precursor of the 54 kDa protein, since it appeared as a minor protein but cross-reacted with the MAb for the major 54 kDa protein. Specificity of the selected MAbs was verified when negative immunohistochemical reactions were obtained in shrimp tissues infected with virulent YHV, nonvirulent YHV, WSSV, and MBV (Table 1). Immunohistochemical studies using MAb HPV16-9C, revealed that some inclusion bodies were not well recognized (Fig. 2). A similar result was observed in *in situ* hybridization tests using *P. monodon* HPV probe (Sukhumsirichart et al.

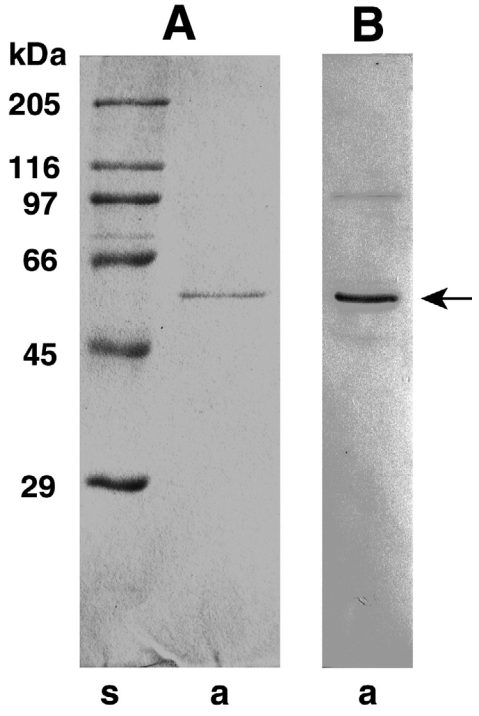

Fig. 1. SDS-PAGE and Western blot analysis. (A) SDS-PAGE of hepatopancreatic parvovirus (HPV) preparation stained with Coomassie blue. (B) Western blot of HPV preparation treated with MAb HPV16-9C that binds to the main HPV protein band at 54 kDa (arrow) and a minor band at 97 kDa. s: standard proteins; a: HPV preparation

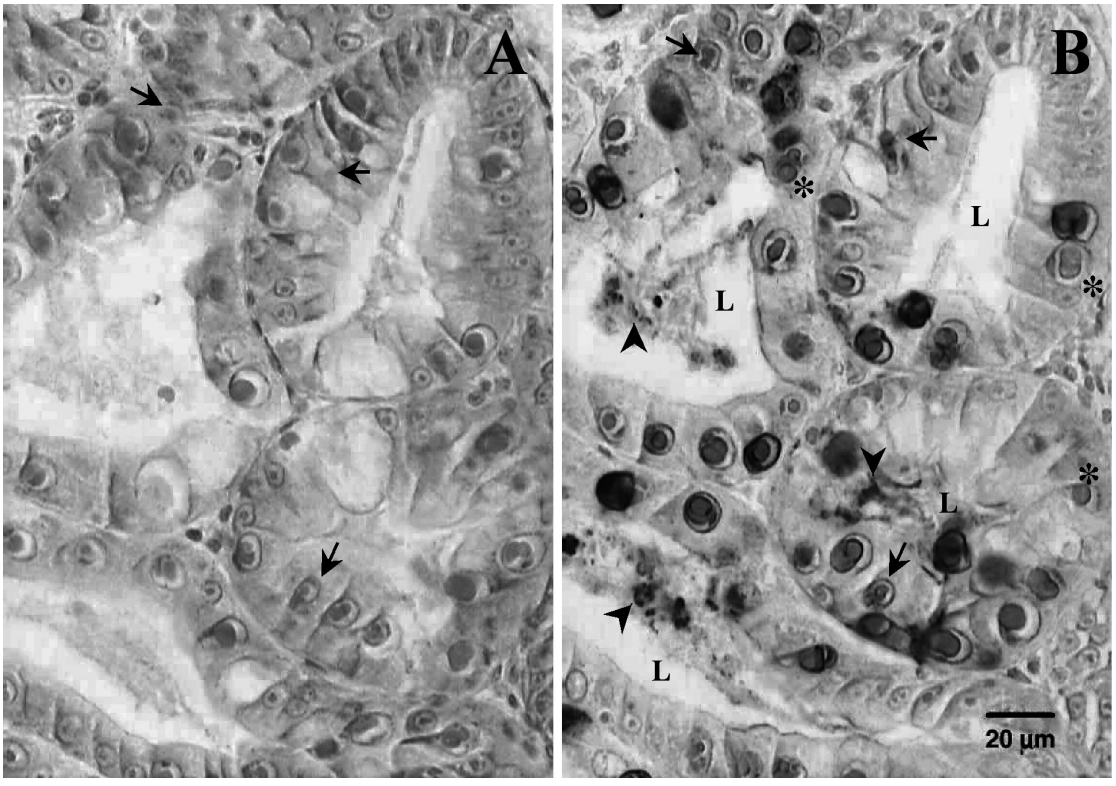

Fig. 2. *Penaeus monodon* infected by immunohistochemistry of hepatopancreas from HPV-infected *P. monodon*. (A) Tissue section stained with H&E without antibody treatment. (B) Adjacent section treated with MAb HPV16-9C and counter stained with H&E. Arrows indicate immunoreactivity in nuclei at the early stage of HPV infection. HPV immunoreactivity is also observed in the lumen (L) of the HP tubule (arrowhead). With some inclusion bodies, the immunoreactivity is demonstrated only at the peripheral surface (*)

1999). The lack of reaction with some inclusion bodies may result from high density preventing MAb access, or from mixed infections with different types of HPV-like viruses.

All MAbs were of the IgG class. Two were IgG_1 and 2 were IgG_{2b} (Table 1). The latter 2 appeared to bind to the HPV protein stronger than the former. These MAbs may be useful for further development of a highly sensitive immunoassay such as a sandwich ELISA for HPV detection in feces, similar to the PCR method (Pantoja & Lightner 2000). A convenient strip test would be another choice for rapid assays in the field. Generation of more MAbs against various epitopes would increase the sensitivity of the assay. Molecular cloning and expression of a high quantity of HPV protein might also be a useful approach for the production of polyclonal antibodies in rabbits.

Table 1. Characterization of monoclonal antibodies specific to hepatopancreatic parvovirus (HPV). –: no immunoreactivity; +: light immunoreactivity; ++: strong immunoreactivity; MAb: monoclonal antibody; IHC: immunohistochemistry; WSSV: white spot syndrome virus; MBV: monodon baculo virus

MAb (subclass)	Dot blot	Western blot	IHC	YHV	YHV (non-virulent)	WSSV	MBV
HPV6-12B (IgG_{2b})	+	++	++	–	–	–	–
HPV12-10C (IgG_1)	+	+	+	–	–	–	–
HPV16-9C[a] (IgG_{2b})	+	++	++	–	–	–	–
HPV 17-1G (IgG_1)	+	+	+	–	–	–	–

[a]Antibody used for characterization

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