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NOTE

A virus isolated from juvenile Japanese black abalone *Nordotis discus discus* affected with amyotrophia

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ABSTRACT: The disease amyotrophia causes serious morbidity and mortality in juvenile abalones at several hatcheries in Japan. The etiology of the disease has not been established; however, a filtrable agent has been associated with the disease. In the present study, a virus was isolated in a primary culture of abalone hemocytes from Japanese black abalones *Nordotis discus discus* histopathologically diagnosed to be affected with amyotrophia in 6 different prefectures. However, pathogenicity of the virus was not confirmed in a transmission experiment with juvenile black abalones, while a filtered homogenate prepared from the same batch of affected abalones reproduced the disease.

KEY WORDS: Abalone · Amyotrophia · Abalone virus · Hemocyte culture

To augment coastal fisheries stocks, seed production and release of various marine fish and shellfish have been conducted in Japan for the past 30 yr. Abalones (major species: Japanese black abalone *Nordotis discus discus* and ezo abalone *N. discus hannai*) have also been involved in such a program and more than 40 million juvenile abalones were produced and released in 1995. Epizootic mortalities have been observed in juvenile black abalone during seed production and subsequent nursery stages at several hatcheries since the early 1980s. The major species for seed production was changed from black abalone to ezo abalone in some hatcheries because the latter species seemed to be resistant to the disease. However, later, ezo abalone was also found to be vulnerable to the disease. Affected abalones develop muscle atrophy in the mantle and epipodium followed by impaired growth of the shell, which impedes adhesion to the substrate and feeding of juveniles (Nakatsugawa et al. 1988). Based on these pathological features, the disease was named amyotrophia (Nakatsugawa 1991). A transmission experiment with a filtrate (0.22 µm membrane filter) prepared from a homogenate of affected abalones revealed that the disease is caused by a filtrable agent (Nakatsugawa 1990). However, virus particles were not found in histological preparations (K. Momoyama pers. comm.). The causative agent of the disease is still unknown. Recently we isolated a virus from diseased black abalones, although its pathogenicity was not observed.

Materials and methods. Samples of diseased black abalones, 10 to 25 mm in shell length, fixed in 10% formalin solution in 1992, 1993 or 1997 were obtained from 9 different Prefectural Fisheries Research Stations and submitted to a histological examination through ordinary processes of paraffin embedding, sectioning, and staining (hematoxylin and eosin, H & E).

Virus isolation was conducted by inoculating filtered (0.45 µm membrane filter) homogenate of affected black abalones obtained from 6 different prefectures into primary hemocyte cultures from healthy black abalones which were maintained in modified Leibovitz L-15 medium (Nagai et al. 1998). One hundred microliters of a filtrate of affected abalones diluted 1:5 with Hank's balanced salt solution (HBSS) was inoculated on a 24 h primary culture, and incubated at 20°C for about 1 mo. A control primary culture was inoculated with the same volume of HBSS. In one trial, idoxuridine (IUdR: antiviral agent) was added at 50 µg ml^{-1} following inoculation of the abalone filtrate. Hemocytes collected from cultures showing a cytopathic effect (CPE) were fixed in a 2% glutaraldehyde-2.25% paraformaldehyde mixture in 0.07 M cacodylate buffer (pH 7.2) for transmission electron microscopy (TEM).

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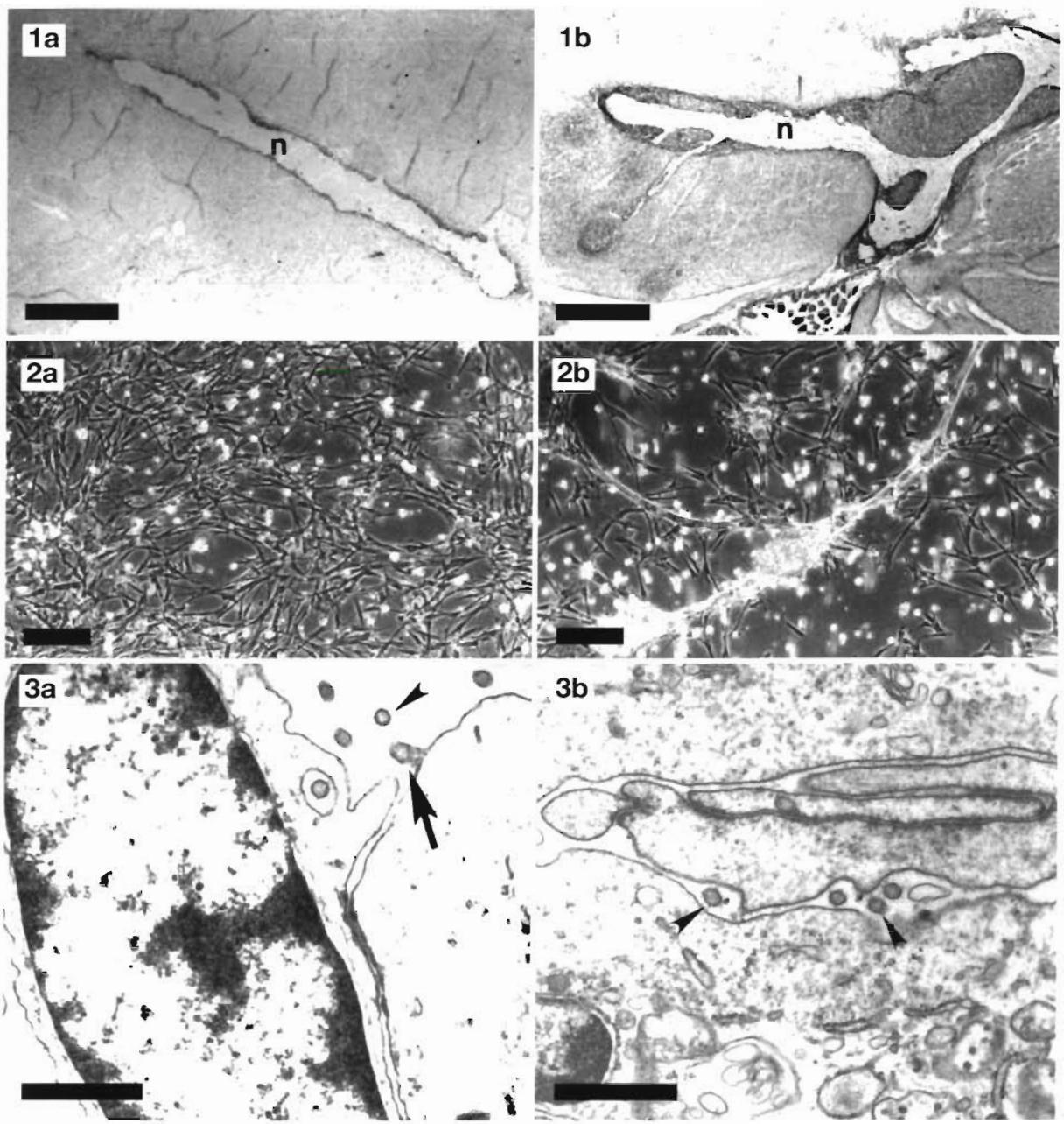

Figs. 1 to 3. *Haliotis discus discus*. Fig. 1. Section of a juvenile Japanese black abalone *H. discus discus*. (a) Healthy abalone; (b) abalone affected with amyotrophia showing tumor-like masses in the nerve trunk. n: nerve trunk of pedal ganglia. Scale bars = 500 µm. Fig. 2. *H. discus discus*. (a) Uninoculated primary culture of hemocytes; (b) primary cell culture inoculated with a filtered homogenate from affected abalone with amyotrophia (14 d after inoculation). Scale bars = 100 µm. Fig. 3. (a,b). Virus-like particles in a primary hemocyte culture showing CPE induced by inoculation with the affected abalone homogenate. Small arrowheads indicate virus-like particles. Large arrow indicates virus-like particle in the budding state. Scale bars = 500 nm

Apparently healthy juveniles of black abalone, 12 mm in average shell length, were challenged with concentrated supernatant of a CPE-forming primary culture and the filtered homogenate of affected abalones. The supernatant of a primary culture with CPE was concentrated by 2 centrifugations (4000 × *g* 20 min and 80 000 × *g* 2 h, both at 4°C), and the resulting pellet suspended in phosphate-buffered saline (PBS) was layered onto 20, 35 and 50% (w/v) sucrose incontinuous gradients and centrifuged at 80 000 × *g* for 100 min at 4°C. The resulting unclear band formed between the 35 and 50% sucrose layers was submitted to the above

challenge test and TEM examination. Three groups of 12 abalones each were injected intramuscularly with 10 µl of the diseased abalone filtrate, the concentrated culture supernatant, or modified L-15 medium (control) and reared separately for 90 d, receiving a commercial abalone feed, at 18 to 22°C.

Results and discussion. Tumor-like cell masses were observed in the nerve trunks of pedal ganglia and their transverse commissures of naturally affected juvenile abalones (Figs. 1 to 3). The nuclei of these cells were contracted and the center of the tumor became necrotic. These changes are in accordance with the pathognomonic features of amyotrophia described by Nakatsugawa et al. (1988). Lesions were observed in 26 of the 34 juveniles examined including at least 1 specimen from every prefecture, indicating that the disease has spread throughout western Japan.

A CPE-like change characterized by aggregated cell masses (Fig. 2) developed in primary hemocyte cultures 1 wk after the inoculation with the filtrate, and cells were detached from the bottom of the culture flask after 3 wk. No changes were observed in the control culture. The second passage of the supernatant harvested from CPE-forming cultures resulted in formation of a similar CPE in another primary culture, although only 3 of the 10 trials were successful. The development of CPE was not inhibited by IUdR, which is known as an antiviral substance against herpesvirus and some other DNA viruses. Virus-like particles, icosahedral and 120 nm in diameter, were observed outside the cells or just in the budding state (Fig. 3). These observations seem to suggest a resemblance of the present virus to a retro-like virus isolated from soft-shell clam *Mya arenaria* (Oprandy et al. 1981). The presence of the similar virus particles were confirmed in all CPE-forming primary cultures inoculated with affected abalone filtrates from 6 different prefectures, indicating that the isolated virus would be the causative agent of the disease.

However, the isolated virus in the concentrated supernatant of the CPE-forming hemocyte culture did not introduce disease in juvenile abalones. In the transmission experiment, amyotrophia was produced in the group of abalones injected with the filtrate of affected abalones with a mortality of 55% within 90 d, but no histological changes were produced in the group injected with the infected cell culture supernatant. This failure might have occurred due to the small quantity or low infectivity of cultured virus. Also, the *in vitro* passages were only partially successful and no decisive figures of purified virus particles were demonstrated in the 35 to 50% sucrose band by TEM.

Recently Otsu & Sasaki (1997) reported the presence of virus particles in the cytoplasm of cells near the nerve trunk of black abalone affected with the same disease in Japan, but the pathogenicity of that virus has not been tested yet. In the USA, the disease withering syndrome causes lesions in the digestive system (this was not observed in our case) and atrophy of foot muscle. Recently, a rickettsia was reported as the causative agent (Gardner et al. 1995). Overall, further investigations are required to determine the etiology of amyotrophia of Japanese black abalone.

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