NOTE

An infectious pancreatic necrosis virus isolated from common dab *Limanda limanda* previously affected with X-cell disease, a disease apparently unrelated to the presence of the virus

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ABSTRACT: Infectious pancreatic necrosis (IPN) virus has been isolated for the first time from common dab *Limanda limanda* L. during a search for a possible viral etiology of the X-cell condition. The virus was identified by electron microscopy as a 60 nm diameter spherical particle. It produced IPN-typical cytopathic effects in chinook salmon embryo (CHSE-214) cells and was neutralized by rabbit IPN-antisera. Experimental inoculations of dab with the virus failed to generate X-cell lesions, further supporting the currently held view that X-cells are not virally transformed fish cells.

The nature and origin of X-cell disease, a condition documented in over 20 species of marine teleosts, is unknown despite the considerable research efforts invested in it over the past decade (for review see Mix 1986). The viral etiology of X-cells has been argued in the past (Peters et al. 1978, 1981), but no virus could be demonstrated to be associated with the condition in the Pacific Ocean (Ito et al. 1976, Wellings et al. 1976). In the present study, common dab *Limanda limanda* L. from the North Sea, some with gross X-cell lesions, others that had recovered from the condition, were surveyed for presence of virus.

Methods and Results. Common dab were sampled during May 1986 off the coast of northeast Scotland. Approximately 100 fish with X-cell lesions were transported alive to the Marine Laboratory, Aberdeen, where they were transferred to tanks supplied with flowing filtered (but otherwise untreated) seawater at ambient temperature. The X-cell diseased fish were severely stressed from capture and transport, and 70 %

died within 24 h of capture. After 7 d, only 4 individuals remained (see Diamant & McVicar 1987). Tissues pooled from two of these yielded a viral agent (see later).

To determine whether antigens characteristic of the agent occurred in gill X-cell tissue, antiserum was raised in a rabbit against an X-cell tissue extract (see later). X-cell material for this study was obtained from affected dab collected in the southern North Sea (Dogger Bank) and had been stored in liquid nitrogen.

To determine whether the agent reproduced the X-cell condition in healthy dab, the latter were exposed to the agent by bathing and injection (described later). Healthy dab for this purpose were taken from commercial catches off the Aberdeenshire (NE Scotland) coast. No cases of X-cell lesions had ever been recorded from dab populations in this area and thus the dab were considered to be naive with respect to the X-cell agent. To obtain a regular supply of X-cells, an attempt was made to culture them. Gill tissue affected with X-cell lesions was trypsinized. After overnight decontamination at 15°C in Hank's balanced salt solution (HBSS) containing antibiotics (penicillin 500 IU ml⁻¹, streptomycin 500 µg ml⁻¹, kanamycin 500 µg ml⁻¹, and fungizone 12.5 µg ml⁻¹), cells released by 3 trypsin treatments were collected (Wolf & Quimby 1969) and cultured in marine-adjusted (0.206 M NaCl) Eagles minimum essential medium containing 20 % foetal calf serum (mMEM-20) in sealed flasks. Viable cells attached to the plastic and were maintained for several weeks. However, the cells did not grow or multiply, and eventually they became necrotic and disintegrated.

In an initial attempt to determine whether a virus was associated with the X-cell condition, X-cell gill tissue from 3 fish of 15 to 21 cm total length (TL) was pooled

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and homogenized in 20 vols. of HBSS containing antibiotics to decontaminate them as before. Cultures of CHSE-214 and BF-2 cells were inoculated with samples of the homogenates and incubated at 18°C. No cytopathic effect (CPE) was observed during 14 d of incubation. In addition, explants of gill X-cell lesions were prepared, overlaid with mMEM-20, and incubated at 18°C. However, no growth was detected within 21 d, during which the cells slowly deteriorated, became necrotic, and disintegrated.

In a second attempt to isolate a virus from X-cell lesion material, 2 fish of 14 and 20 cm (TL) with moderate to heavy gill lesions were held in a tank supplied with flowing seawater and examined on Day 57 following capture. They showed dramatic regression of the X-cell lesions. The 2 fish were sacrificed and the anterior kidneys, spleens, and gills were pooled (by tissue) for virus isolation by either co-cultivation or homogenate overlay. Tissues were homogenized and decontaminated with antibiotics overnight (as above) at 15°C and at room temperature. Cells were prepared for co-cultivation by a modification of the procedure of Agius et al. (1982) (Smail unpubl.). The homogenate was clarified by a brief, low speed centrifugation ($10 \times g$ for 2 min), and cells were collected from the supernatant by $1500 \times g$ centrifugation for 15 min. Pellet dilutions at $100 \times$ and $500 \times$ were made in maintenance medium, and 0.5 ml of each was added directly to the medium overlying CHSE-214 25 cm² flask cultures 24 h after seeding. For homogenate overlay, the cell-free super-

natant from the homogenate was passed through a HA (0.45 μ m) filter, and the filtrate was diluted 5-fold in HBSS. A volume of 0.5 ml of diluted filtrate was adsorbed for 30 min to drained CHSE-214 cells and then maintenance medium was added to the culture. Cultures were incubated at 15°C and examined daily for signs of cytopathic effect.

Cytopathic effect was observed 8 d after inoculation in co-cultivation cultures that received gill, kidney, or spleen cells. No CPE was observed in those receiving filtered (cell-free) inocula. On first passage, the CPE from cultures inoculated with gill and spleen cells was evident in 3 d and the CPE was characterized by cell rounding, with foci of rounded necrotic CHSE cells. Subsequent passage of filtered medium from the cultures showing CPE to fresh monolayers of CHSE cells resulted in identical CPE. At 24 h post-inoculation, CPE was already evident, and by 48 h the cell monolayer was completely destroyed (Fig. 1).

The medium overlying infected cells was titrated by agarose plaque assay (Wolf & Quimby 1973). After 3 d, the cells were fixed with buffered formol saline, the agarose removed, and the cells stained with 1 % crystal violet. A plaque count indicated a titer of 6.3×10^4 plaque forming units (PFU) per ml. This was a relatively low titer for released virus and may indicate that much virus remained cell-associated at the stage of harvesting.

To determine whether X-cells contain IPN virus antigen, antiserum was prepared against an X-cell extract

Fig. 1. (a) Normal CHSE-214 culture (phase contrast, $\times 175$). (b) Dab virus infected CHSE-214 culture 48 h post-inoculation, showing gross CPE (phase contrast, $\times 175$)

Table 1 Plaque neutralization of dab virus (DV) by various antisera and the homologous end points of the rabbit anti-IPN virus antisera from previous tests. Data are ND₅₀ values: reciprocal of antiserum dilution reducing plaque count by half (log₁₀ values in brackets). x: ratio of homologous to heterologous ND₅₀ value. Homology of strains is suggested when x = < 10

Antisera	DV	Ab	Sp	Virus SS	VR-299	TV-2	x
Rabbit anti IPN							
Ab	955 (2.98)	32 360 (4.51)					34
Sp	7 943 (3.90)		3 548 000 (6.55)				466
SS	95 499 (4.98)			707 945 (5.85)			7.4
VR-299	372 (2.57)				177 800 (5.25)		478
TV-2	4 786 301 (6.68)					794 328 (5.90)	< 1
Anti X-cell	85 (1.93)						
Dab^a							
X-cell	4 898 (3.69)						
^a Serum was obtained from a specimen severely affected with X-cell disease (IPN virus presence in this individual was not tested)							

by homogenizing cryopreserved pieces of gill from X-cell diseased dab with Freund's complete adjuvant and injecting the mixture into a rabbit. The rabbit received an intramuscular injection at Weeks 0 and 4 and was bled at 6 and 8 wk after the first injection. Antisera against IPN serotypes VR-229, Ab, Sp, and SS (=salmon strain, isolate SS/Sh80, a member of the Te serotype of Hill [Hill pers. comm.]) were raised in rabbits using the optimum regime described by Hill et al. (1981), i.e. intramuscular injection at Week 0 followed by ear vein injection of purified virus at 4 and 6 wk and final bleeding at 7 or 8 wk. Antiserum to TV2 was kindly supplied by K. Way (Ministry of Agriculture, Fisheries and Food, Weymouth, UK). Plaque neutralization of the dab virus (DV) was carried out on CHSE-214 cells using rabbit antisera. The plaque neutralizing activity of serum from a dab (IPN virus infection status unknown) with severe X-cell gill lesions was also tested. Normal rabbit serum was used as the control. The results are presented in Table 1 and suggest an antigenic relationship between DV, SS, and the TV2 strain of IPN virus, the last virus also being a member of the Te serotype, according to the nomenclature proposed by Dr B. J. Hill (Ahne 1985). The poor neutralization of DV by the X-cell antiserum suggests that there was no virus in the X-cell material. The significant neutralization of DV by dab serum suggests that the fish may have been infected with the virus for some

months before bleeding. It is also possible that neutralization of the IPN virus by dab serum may have been due to presence of a non-induced virus-neutralizing factor such as the 6S protein found in rainbow trout (Dorson & de Kinkelin 1974). This possibility was not investigated but it seems unlikely in view of the high titer noted (Table 1).

To observe the morphology of the virus, CHSE cells were harvested at 24, 48, and 72 h after infection. The cells were prepared for electron microscopy by fixation in 2.5 % glutaraldehyde in Millonig's buffer and by concentration by centrifugation onto a pellet of 2 % Noble agar (Difco) in the same buffer. The material was post-fixed in 2.5 % osmium tetroxide (OsO₄), dehydrated in an ascending acetone series and embedded in Agar-100 resin blocks. Sections of 0.1 µm were stained with uranyl acetate and lead citrate and observed in a Hitachi H-300 electron microscope at 60 kV. Infected CHSE cells contained numerous spherical particles of 60 nm average diameter, usually in cytoplasmic vacuoles but also free in the cytoplasm and in the nucleus of disintegrating cells (Fig. 2). Clumps of virus, morphologically indistinguishable from IPN, were also seen among disintegrating cells but no virus was observed in control uninfected CHSE cells.

To study the infectivity of DV for dab, one fish was bathed for 1 h in 5 l of seawater to which a lysate of CHSE virus-infected cells was added. This gave 6×10^2

Fig. 2. Section through a CHSE-214 cell 3 d following infection with dab IPN virus. Spherical particles with distinct capsids and cores are dispersed in the degenerate cytoplasm and disintegrating nucleus ($\times 32\,000$). Inset: high power magnification of viral particles ($\times 100\,000$)

PFU ml⁻¹ in the water. A second fish was infected by intraperitoneal (i.p.) injection (6×10^3 PFU in MEM) as well as by bathing. A control dab (TL 21 cm) was bathed for 1 h in seawater to which was added an uninfected CHSE cell lysate. The 2 virus-exposed dab did not develop signs of X-cell disease during 31 d of holding at 18°C. Virus was recovered from the pooled kidney and spleen of each of the virus-exposed dab at 31 d post-challenge (tissue titer of >40 PFU g⁻¹). Virus was also recovered from the gill tissue of these fish by co-cultivation. By contrast, the control dab was virus-negative. These results show that the virus was not only capable of persisting in dab tissues but that it was also invasive for dab when administered by the bath method.

Discussion. The virus described here is clearly an IPN strain, and, to our knowledge, represents the first IPN virus isolated from dab. Though possible, it is unlikely that the dab were accidentally exposed to IPN virus in the laboratory because all dab were kept in separate tanks that had previously been dry for many months and because no other species were being held in the aquarium facilities at the time. Moreover, a

similar, possibly identical, virus has subsequently been found in dab off the Danish coast (S. Møllergaard pers. comm.), but serological comparisons have yet to be carried out. A possible adenovirus was also recently reported from epidermal hyperplasias of dab, but not isolated or characterized (Bloch et al. 1986). The only other case of an IPN strain isolated from a pleuronectid was that reported by McAllister et al. (1984) in southern flounder *Paralichthys lethostigma* from the Atlantic coast of North America.

The failure of dab challenged with the present virus to develop X-cell lesions strongly suggests that no relationship exists between this virus and X-cell disease. This is consistent with the growing body of evidence that the X-cell condition is due to other than viral factors (Dawe 1981, Morrison et al. 1982, McVicar et al. 1987, Diamant & McVicar unpubl.).

Studies are continuing to further characterize the IPN isolate, particularly with respect to its serological relationship to other European IPN serotypes.

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