A retrovirus isolated from cell lines derived from neurofibromas in bicolor damselfish (Pomacentrus partitus)

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Damselfish neurofibromatosis (DNF) is a naturally occurring, neoplastic disease affecting bicolor damselfish (Pomacentrus partitus) living on coral reefs in southern Florida, USA. The disease consists of multiple neurofibromas, neurofibrosarcomas and chromatophoromas and has been proposed as an animal model for neurofibromatosis type 1 in humans. DNF is transmissible by injection of crude tumour homogenates, cell-free filtrates of homogenates or cells from tumour cell lines. An analysis of tumorigenic cell lines derived from fish with spontaneous or experimentally induced DNF revealed virus particles budding from cells and present in conditioned media. The 90–110 nm particles resembled type C retroviruses. This virus exhibited a buoyant density of 1.14–1.17 g/cm² in sucrose, at least six virus proteins of 15 to 80 kDa and reverse transcriptase (RT) activity. RT activity was maximized with a poly(rC)-oligo(dG) template primer combination and Mn²⁺ at a concentration of 0.5–1.0 mM. The optimum temperature for RT was determined to be 20 °C, a finding consistent with the ambient temperatures encountered by this species. This retrovirus, tentatively named damselfish neurofibromatosis virus (DNFV) may be the aetiologic agent of DNF. Whether DNFV or another, as yet unidentified, virus is the cause of DNF, this agent may be unique in virus oncogenesis; neoplastic transformation of the cell types involved in DNF, Schwann cells and chromatophores, has not been documented in any other transmissible tumour.

Introduction

Damselfish neurofibromatosis (DNF) is a neoplastic disease affecting the peripheral nervous system and chromatophores of bicolor damselfish (Pomacentrus partitus) living on coral reefs in southern Florida, USA. (Schmale, 1991). The disease consists of multiple neurofibromas, neurofibrosarcomas and chromatophoromas (Schmale et al., 1983, 1986). DNF has been proposed as an animal model for neurofibromatosis type 1 (NF1) in humans, based on the similarity of the plexiform neurofibromas observed in these diseases. The gene responsible for NF1, an autosomal dominant inherited disorder, has been identified (Gutmann & Collins, 1993; Viskochil et al., 1993). However, the mechanisms by which alterations in the protein product of this gene, neurofibromin, lead to the development of neurofibromas and other features of NF1 are unknown. Experimental studies of NF1 have been limited by the lack of good animal models of this disease.

DNF can be transmitted by intramuscular injection of tumour homogenates or cell-free filtrates of these homogenates into healthy fish (Schmale & Hensley, 1988; Schmale, 1995). These findings indicate that DNF is caused by a subcellular agent. In addition, several tumour cell lines established from fish with spontaneous or induced DNF are tumorigenic when injected, whereas cells cultured from healthy fish are not (Schmale, 1995). These data demonstrate that the aetiologic agent of DNF is present in the tumour cell lines. These cell lines consist entirely of neoplastic Schwann cells, the predominant cell type involved in formation of neurofibromas (Schmale et al., 1994a).

Many neoplasms in fish have been suspected of being associated with viruses and/or of being transmitted horizontally. However, conclusive transmission experiments have been conducted with only a few of these tumours from which viruses have been isolated. Retroviruses have been shown to be the aetiologic agents of lymphosarcomas in pike and other esocids (Papas et al., 1976), walleye dermal sarcoma (WDS; Martineau et al., 1991) and plasmacytoid leukaemia in coho salmon (Eaton & Kent, 1992). Epidermal papillomas in masu salmon and carp have been shown to be caused by herpesviruses (Anders & Yoshimizu, 1994). As yet, none of these oncogenic viruses have been isolated in cell culture.

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The majority of infectious neoplasms in animals are leukaemias, lymphomas and sarcomas. These tumours arise from haematopoietic, lymphoid and connective tissue cells, respectively. DNF appears to be the only naturally occurring, transmissible cancer arising from cells of the central or peripheral nervous system or from tissue cells, respectively. DNF appears to be the only leukaemias, lymphomas and sarcomas. These tumours arise from haematopoietic, lymphoid and connective tissue cells, respectively. DNF appears to be the only aetiological agent that affects two neuroectodermal cell types, Schwann cells and chromatophores. Here, we describe a retrovirus isolated from several DNF tumour cell lines. Cells from these lines have previously been shown to induce tumours typical of DNF when injected into healthy fish (Schmale, 1995).

Methods

Cell culture. Tumour cell lines were derived from neurofibromas and neurofibrosarcomas of bicolor damselfish. The isolation, propagation and characteristics of these cell lines have been described previously (Schmale et al., 1994 a). Cell lines used in the present study included 89-05, which was derived from a spontaneous tumour, and 88-503, derived from a experimentally induced tumour. Both cell lines were composed entirely of neoplastic Schwann cells. Cultures of healthy cells used as controls were primary cultures, several weeks in age, derived from fins of bicolor damselfish showing no signs of DNF. These cultures contained mixtures of fibroblasts, chromatophores and Schwann cells.

Purification of virus. Conditioned medium was harvested from cultures once each week. Virus material was concentrated by centrifugation of conditioned medium as follows. Cellular debris was removed from the media by centrifugation at 300 g for 1 h. The supernatant was then centrifuged at 16000 g for 1 h with both the pellet (termed the P-16 fraction) and the supernatant retained. The supernatant was centrifuged at 100000 g for 1 h and the pellet retained (termed the P-100 fraction). Both pellets were resuspended in 10 mM-Tris–HCl, 100 mM-NaCl, 1 mM-EDTA pH 7-5 (TNE) and layered on a 20/50% sucrose step gradient and resuspended in a final volume of TNE equal to approximately 1/2500 of the initial starting volume of conditioned medium.

Electron microscopy. Cultured cells were fixed and embedded on the culture dish for examination of virus particles associated with cells. Cells were fixed with 2% gluteraldehyde in 0.1 M-cacodylate pH 7.2, post-fixed in 1% OsO4, dehydrated in a graded series of ethanol and embedded in a mixture of Polybed 812 and Araldite 502 (Polysciences) (Schmale et al., 1994 b). Blocks were sectioned at 80-100 nm thickness, mounted on uncoated grids, stained with lead citrate and uranyl acetate and viewed with a Phillips 300 electron microscope at 60 kV. Virus particles were prepared for electron microscopy by embedding pelleted virus particles (the sucrose-purified P-100 fraction before final resuspension in TNE) in 1% agarose. Pellets were then fixed, dehydrated, embedded in Araldite 502, sectioned and stained as described above and examined at 80 kV.

Protein analysis. After concentration by sucrose gradient ultracentrifugation, virus polypeptides were analysed by SDS-PAGE on vertical slab gels with 13% polyacrylamide. Proteins were visualized by staining with Coomassie Blue. Molecular masses were calculated by comparison with known size standards.

Reverse transcriptase (RT) assay. Assays of RT activity were based on measurement of rates of incorporation of 3H[GTP] or 3H[TTP] in the presence of an appropriate template: primer molecule and reaction buffer. Reactions were initiated by combining 35 µl of a virus isolate, distilled water (for blanks) or an RT standard with 75 µl of RT reaction mixture. Half of each reaction was incubated for 60 min (T60) and half was not incubated (To). Unless otherwise indicated, incubations were conducted at 37 °C. Avian myeloblastosis virus (AMV; Promega) RT was used as a standard at 1–5 U per reaction. Reaction mixtures contained 2.5 mM-CaCl2 3H[GTP] or 3H[TTP] (Amersham or NEN) and additional unlabelled GTP or TTP to yield a final concentration of 5 µM, 20 µg/ml poly(rC)-oligo(dG) (for GTP), or poly(rA)-oligo(dT) or poly(dA)-oligo(dT) (for TTP) (Pharmacia), 100 mM-Tris–HCl pH 8.3, 4 mM-DTT, 50 mM-KCl, 0.2% NP-40 and either MgCl2 or MnCl2 at 0.1–10 mM. Reactions were stopped by transfer to Whatman GF/C filters containing 100 mM-sodium pyrophosphate, followed by washing for 5-10 min each in 10% TCA, 5% TCA, 5% TCA with 1% SDS and 95% ethanol. 3H levels were then determined by liquid scintillation counting. Nucleotide incorporation levels were expressed as the mean difference between the counts obtained at 0 and 60 min on triplicate samples.

Results

Ultrastructural examination of the 88-503 cell line revealed virus particles budding from cell membranes into the extracellular spaces (Fig. 1). No intracellular particles were observed. Analysis of sucrose gradient fractions from conditioned medium of 88-503 and 89-05 cell lines revealed similar viruses that could be visualized by negative staining (data not shown) or sectioning of pelleted virus isolates (Fig. 1). Particles were 90–110 nm, round and multilayered with an outer, relatively thin, electron-lucent layer probably representing an envelope and a dense inner nucleocapsid ring 45–70 nm in diameter. The core within the capsid was round, centrally located and either very electron dense or somewhat less dense. No conspicuous projections were seen on the outer surface of viruses budding from the cells in culture or on particles prepared by ultracentrifugation.

Analysis of conditioned medium from 88-503 and 89-05 cell lines using SDS–PAGE indicated the presence of proteins considered to be of virus origin, due to the absence of similarly-sized proteins in conditioned medium from healthy primary cultures (Fig. 2). Comparisons with media of primary cultures from healthy, unaffected fish (HF media) were based on the P-16 (16000 g pellet) rather than the P-100 (100000 g pellet) fraction, due to the lack of detectable protein in the P-100 fraction from HF media. Six virus proteins were observed in the media from both the 88-503 and 89-05 cell lines. Estimated molecular masses were 80, 69, 27, 25, 18 and 15 kDa from both cell lines. These proteins were recovered from sucrose gradient fractions with buoyant
Fig. 1. Virus particles associated with cells and conditioned media of the 88-503 cell line. (a) Particles resembling retroviruses can be seen outside of or attached to cell membranes. (b) Same culture as in (a) showing budding of particles (arrows) from cell membrane. (c) Virus particle isolated from conditioned medium of an 88-503 culture using sucrose gradient ultracentrifugation. The capsid layer (arrow) is visible surrounding the less electron dense core of the particle. Bars represent 100 nm.

Densities of 1.12 to 1.2 g/cm³, with the majority of protein being localized in the 1.14 to 1.17 g/cm³ fractions (Fig. 2b).

RT activity was detected in both tumour cell lines but not in healthy primary cultures (Table 1). RT activity was four- to fivefold greater in conditioned media from
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Sucrose density (g/ml)

Fig. 2. Polypeptide analysis of virus purified from conditioned media using SDS–PAGE. (a) Media from the 88-503 cell line and HF primary culture purified using a 20/50% sucrose step gradient. Lane 1, 88-503 media concentrated by centrifugation of 16000 g supernatant at 100000 g. Lane 2, 88-503 media concentrated by centrifugation at 16000 g. Lane 3, media from HF primary cultures concentrated by centrifugation at 16000 g. Identical concentrations of conditioned media were used in all sample lanes (the equivalent protein isolated from 40 ml of media, i.e. a 2500-fold concentration). (b) Media from the 89-05 cell line (100000 g fraction) separated on a 20–60% linear sucrose gradient. Lanes are labelled with the density of the sucrose fraction. Equal volumes were loaded from each fraction. The molecular masses of the virus proteins are shown, estimated using molecular mass standards run on the same gel.

Table 1. RT activity of selected virus isolates with selected template–primer combinations*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Poly(rC)·oligo(dG)</th>
<th>Poly(rA)·oligo(dT)</th>
<th>Poly(dA)·oligo(dT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>88-503</td>
<td>25/70 ± 1697</td>
<td>10742 ± 3174</td>
<td>136 ± 58</td>
</tr>
<tr>
<td>89-05</td>
<td>5592 ± 789</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HF1†</td>
<td>158 ± 155</td>
<td>324 ± 506</td>
<td>−14 ± 4</td>
</tr>
<tr>
<td>AMV§</td>
<td>55000 ± 19308</td>
<td>200413 ± 15186</td>
<td>937 ± 521</td>
</tr>
<tr>
<td>No sample</td>
<td>−603 ± 870</td>
<td>−41 ± 58</td>
<td>−85 ± 110</td>
</tr>
</tbody>
</table>

* Assays were performed at 1 mM-MnCl₂ using the P-100 fraction. Results are mean change in c.p.m. ± 1 SD; negative values occur when T₀ > T₉₀. ND, Not done.
† Primary culture derived from a healthy fish.
‡ Only a single sample run rather than a triplicate.
§ 5 U sample.

the 88-503 cell line than that from the 89-05 cell line. The activity from 88-503 media was approximately 2-5-fold greater in the presence of a poly(rC)·oligo(dG) template–primer than the more commonly used poly(rA)·oligo(dT) (Table 1). This was in contrast to the activity of AMV RT, which was almost fourfold higher using poly(rA)·oligo(dT). No significant activity was detected using a poly(dA)·oligo(dT) template–primer combination on either the 88-503 or AMV RT. The RT activity in the 88-503 culture medium was primarily associated with fractions of linear sucrose gradients with a density of approximately 1.16 g/cm³ (Fig. 3).

The DNF RT was also shown to have a preference for Mn²⁺ at 0.5 to 1.0 mM concentrations as a required cation in these reactions, with very little activity at lower or higher concentrations (Fig. 4). RT activity was essentially zero in the presence of 5 mM-Mg²⁺ (data not shown).

An analysis of the relationship between RT activity and temperature for this virus indicated that RT activity in the 88-503 cell line was optimum at 20 °C, with
Although the viruses observed in the 88-503 and 89-05 cell lines exhibited similar morphologies and protein compositions, we have not determined the similarity of these viruses at the nucleic acid level. We have tentatively named this virus damselfish neurofibromatosis virus (DNFV) due to its association with this disease. At this time we have not determined if this virus is the aetiological agent of DNF.

Several features of DNFV identify it as a type C retrovirus. These include the symmetrical location of the core, the lack of any intracellular particles or precursors and the lack of conspicuous glycoprotein spikes. The observed variations in core density are typical of C particles, with the immature stage having a relatively open appearing core which matures into a condensed, more electron-dense form (Coffin, 1992). The type C morphology suggests that DNFV may be related to either of the currently recognized genera that exhibit such structure, the murine leukaemia-related viruses and the avian leukosis-related viruses. However, taxonomic classification of DNFV will require comparison of sequence information with viruses from these groups.

All retroviruses contain at least eight virus-derived proteins. In the majority of type C viruses these proteins range in size from 10–85 kDa (Weiss et al., 1982). The six proteins observed in isolates of DNFV are consistent with this pattern. The failure to observe more than six proteins may have been due to an inability to distinguish proteins with very similar molecular masses or to identify some of the less conspicuous proteins present on gels as virus-derived because of their similarity in size to cellular proteins. Identification of the complete range of proteins contained in DNFV will require sequence analysis of the virus genome or development of antibodies to the virus proteins.

The pattern of RT activity observed was typical of that reported for other retroviruses. Virus RT enzymes can act on homopolymers composed of DNA primers on RNA templates but not those on DNA templates. The lack of activity on a DNA template demonstrated that the assay was measuring RNA-based DNA polymerase activity and not DNA polymerase or terminal transferase (Baltimore & Smoler, 1971). The higher RT activity observed for DNFV in the presence of poly(rC)-oligo(dG) relative to the poly(rA)-oligo(dT) template-primer is not common among retroviruses, although many viruses have not been tested with poly(rC)-oligo(dG). The esocid haematopoietic neoplasm (EHN)-associated retrovirus exhibited higher activity in the presence of poly(rC)-oligo(dG) (Papas et al., 1977) while a retrovirus isolated from an embryo-derived cell line of the platyfish (Xiphophorus maculatus) showed several-fold higher activity with poly(rA)-oligo(dT) (Petry et al., 1992). The preference for poly(rC)-oligo(dG) in DNFV may indicate a broader range of host cells or apecies-specific preference.

Discussion

The morphology, buoyant density, protein composition and RT activity of the virus isolated from the DNF tumour cell lines indicate that it is a retrovirus (the co-occurrence of the virus particles, proteins and RT activity at the same buoyant density in sucrose gradients indicate that all of these features are attributable to the virus).

Fig. 3. RT activity of the 88-503 cell line in different buoyant density fractions from conditioned media separated on a linear sucrose gradient. RT activity was measured as [3H]GTP incorporation using a poly(rC)-oligo(dG) template-primer in the presence of 1 mM-MnCl₂. Incorporation is given as c.p.m. T₆₀−T₀±1 s.d.

Fig. 4. RT activity as a function of concentration of MnCl₂. RT activity measured as [3H]GTP incorporation using a poly(rC)-oligo(dG) template-primer. Incorporation is given as c.p.m. T₆₀−T₀±1 s.d.

activity dropping to less than 50% of maximum levels above 30 °C or below 15 °C (Fig. 5). This was similar to the activity range reported for a retrovirus isolated from lymphosarcomas in another fish, the northern pike, and much lower than the ranges reported for AMV or Rous sarcoma virus (RSV) of birds and the murine Rauscher leukaemia virus (RLV; Papas et al., 1977).
for the Mn\(^{2+}\) cation over Mg\(^{2+}\) is not uncommon among retroviruses. Several fish retroviruses have been shown to prefer Mn\(^{2+}\), including viral erythrocytic infection (VEI) virus and isolates from four freshwater fish cell lines (Frerichs et al., 1991; Pinto et al., 1995). Several other fish-derived viruses have been shown to use Mn\(^{2+}\), including WDS virus and salmon leukaemia virus (SLV) (Martineau et al., 1991; Eaton & Kent, 1992).

Comparison of the virus proteins observed from cell lines 88-503 and 89-05 suggests that these virus populations are similar. Differences in the RT activity levels may have been due either to qualitative differences in the function of the enzymes from the virus in these two cell lines (i.e. sequence differences) or to the quantity of virus particles present. Although identical volumes of spent media were concentrated for each assay from the two cell lines, no attempts were made to standardize the number of cells in these cultures. Thus, higher levels of RT activity observed in 88-503 may have been due to more virus particles being produced per cell and/or a higher average number of cells per culture in 88-503 cell lines.

The temperature optimum of approximately 20 °C observed for RT activity of DNFV is consistent with the typical range of ambient temperatures encountered by bicolor damselfish in southern Florida, 21 to 31 °C (Ogden et al., 1994). These observations suggest that RT activity in vivo may be highest during winter months if the virus derived from the 88-503 cell line is representative of virus present in wild fish. However, no seasonal differences have been observed in rates of progression of DNF (Schmale et al., 1986). Thus, the relationship of the temperature function in RT activity to the pathogenesis of DNF is not clear. Seasonal fluctuations have been observed in at least six neoplastic diseases of fish associated with retroviruses. One of these diseases, WDS, is characterized by dramatic regression of tumours during periods of warm water temperatures (Poulet et al., 1994). The relationship between seasonal changes in tumour development and RT activity at different temperatures has not been investigated in these diseases. The lack of obvious seasonal fluctuations observed in DNF may be due to the relatively minor seasonal changes encountered in the tropical environments inhabited by the bicolor damselfish. In contrast, all of the diseases exhibiting seasonal changes occur in temperate environments characterized by relatively large seasonal temperature ranges.

In addition to DNFV, there are currently at least 11 retroviruses suspected of causing proliferative diseases and one of causing anaemia (VEI) in fish (Poulet et al., 1994; Pinto et al., 1995). DNF and three other neoplastic diseases, EHN, WDS and salmon leukaemia, as well as VEI, have been propagated by cell-free filtrates of homogenates from affected tissues (Martineau et al., 1991; Eaton & Kent, 1992; Pinto et al., 1992; Schmale, 1995). Several additional retroviruses have been isolated from fish cell lines that are not associated with any specific pathology (Frerichs et al., 1991). All of these viruses, except those observed in VEI and fibromas of hooknose, display morphology typical of type C retroviruses.

None of the pathogenic fish retroviruses described previously has been propagated in cell culture. The virus isolated in culture by Eaton et al. (1993) from fish with leukaemia appears to be similar to SLV. However, the cultures were incapable of inducing the disease when injected into healthy fish. In contrast, cell lines 88-503 and 89-05, as well as several other DNF-derived cell lines, are capable of inducing DNF when injected into healthy fish (Schmale, 1995). These data indicate that the aetiological agent(s) of DNF is present in the cell cultures. However, we have not been able to isolate DNFV or any other virus from either spontaneous or experimentally induced cases of DNF. Infection of cells with retroviruses often does not result in the production of detectable numbers of virus particles if the infected cells are non-permissive for virus replication, regardless of the effects that the integrated retrovirus may have on these cells (Weiss et al., 1982). In these cases, virus particles may not be detectable until the cells are maintained in culture for some time. Alternatively, retrovirus particles derived from endogenous sequences with no pathogenic significance may be produced after cells have been propagated in culture systems.

DNF is unique in virus oncogenesis because neoplastic transformation of the cell types involved, Schwann cells and chromatophores, has not been documented in any other transmissible tumour. Animal models of Schwann cell tumours are rare, regardless of aetiology. This
situation has complicated efforts to understand the pathogenesis of NF1 in humans. We are presently attempting to determine if DNFV is the aetiological agent of DNF using molecular probes based on sequences obtained from the virus isolates described here. Regardless of the identity of the agent(s), the uniqueness of the cell types transformed in this disease suggests that unusual mechanisms may be involved in the pathogenic process. Investigation of these mechanisms may contribute to an understanding of neoplastic transformation in NF1.

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