UNIVERSITY OF MIAMI

A research paper submitted in partial fulfillment of the requirements for the degree of Master's of Arts

# A Recent Review of Retroviral Diseases of Fish and Suggestions for Future Research

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[Restored and transferred to electronic form by Damon J. Gomez (NOAA/RSMAS) in 2002 as part of the Coastal and Estuarine Data/Document Archeology and Rescue (CEDAR) for South Florida. Sponsored by the South Florida Ecosystem Restoration Prediction and Modeling Program. Original stored at the Library, Rosenstiel School Marine and Atmospheric Science, University of Miami. Minor editorial changes were made.]

March 16, 1995

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## Acknowledgements

First and foremost I would like to thank Mike Schmale for the guidance, insight and understanding he has shown me, on a professional and personal level, since my arrival in his lab in April 1991. He gave me the opportunity to further my education and grow as a researcher and as a person. I hope to carry with me many of the positive qualities I have learned from him. Jerry Ault, who impressed me with his interest in the students and the academics of MBF to such a degree, that I asked him to be my member from the Academic Committee. Even though molecular biology is not his area of research, I knew his attention would be sincere. My thanks also go to the past and present members of the Schmale lab for helping create an enjoyable atmosphere and a tight-knit group of friends. Maia McGuire, who saw me through some of the roughest times with her kindness. EGSV. Chris, who has been there, when I needed him the most, with love and patience. The encouragement, understanding and love my family has given me, even though most of it was long distance, has been invaluable. Finally, to my ever faithful companions, Tinsel and Oso.

## I. Introduction

During the past two decades no other group of viruses has been studied in such depth as the retroviruses. They have been found in a wide range of vertebrate hosts, including a few of the lower vertebrates; species of fishes and snakes have been investigated most thoroughly (Bowser and Casey 1993). Retroviruses have the ability to mutate and recombine quickly. This is probably best evidenced by the battle researchers are fighting with the human immunodeficiency virus (HIV) that causes AIDS. Many diseases are associated with retroviruses, including: wasting diseases, malignancies, immunodeficiencies, neurological disorders and lifetime viremia.

The family *Retroviridae* is divided into three subfamilies: (1) *Oncovirinae* contains the oncogenic viruses that are able to acquire and mutate cellular genes into cancer causing genes, referred to as '*onc* genes' or oncogenes; and many of the closely related nononcogenic members; (2) *Spumavirinae* contains the "foamy" viruses that cause persistent infection with no clinical disease; and (3) *Lentivirinae* contains the "slow" viruses that are associated with neurological and immunological disorders; HIV is part of this subfamily (Fields *et al.* 1990).

## II. Retroviral Characteristics

Despite an extensive range of hosts and diseases, all retroviruses have a common virion structure and unique characteristics (Fig. 1). Retroviruses are split into four morphological types, A to D, based on differences in virion structure. Subtleties in characteristics; protein weights, enzyme preferences, virion diameter, buoyant density, also aid in classification.

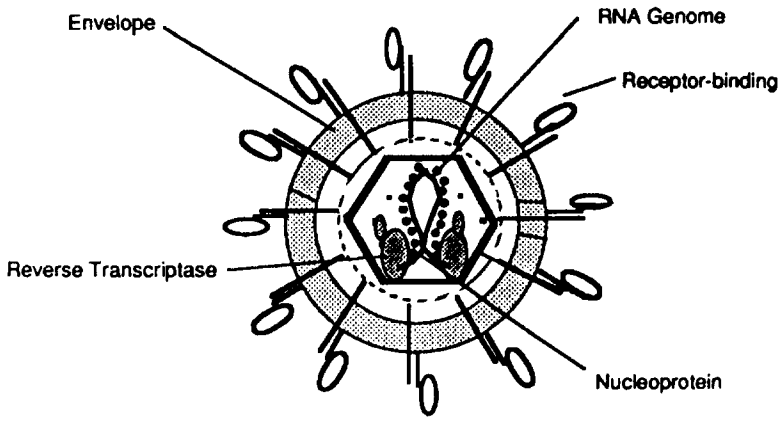


**Fig. 1.** Simplified schematic of the retrovirus virion. Modified from Fields *et al.* (1990).

Retroviruses have diameters ranging from 80-120 nm and lipid envelopes with glycoprotein spikes that recognize receptors on host cells enabling infection. They have a buoyant density of 1.16-1.18 g/ml in sucrose. The nucleocapsid, or core, is conical to spherical and contains several proteins that are enzymatically active during viral replication.

The retroviral genome is comprised of two identical positive-sense stranded RNA molecules, 7-13 kilobases (kb) in length. Consistently, the sequence for the genes encoding the structural proteins is *gag-pol-env*. The RNA molecules are modified in ways that are similar to cellular mRNA modifications; a 3' polyadenylation and a 5' capping. One of the major features of retroviruses is that their genome replication goes through a double-stranded DNA intermediate, the provirus. This is accomplished by the enzyme reverse transcriptase (RT). *Retroviridae* is the only family of viruses in which every member utilizes RT. This makes detection of the enzyme an important research finding. However, there are a couple unrelated exceptions that exhibit RT activity. For example, Hepatitis B virus (HBV) and cauliflower mosaic viruses (Varmus 1988). Two properties of RT further help classification: template preference and ion preference. Certain RTs have a greater affinity for a heteropolymeric template, such as poly (rC):oligo (dG); others for a homopolymeric template such as poly (rA). All RTs, *in vitro*, rely on a divalent cation for polymerase activity. So far, the majority of retroviruses studied prefer $Mg^{2+}$, however, the C-type viruses prefer $Mn^{2+}$ (Fields *et al.* 1990).

## III. Replication

Retroviral replication can be separated into two stages: (a) the use of viral or host proteins; and, (b) the presence or absence of viral gene expression. In the first stage, only proteins that are contained within the virion are used and no viral gene expression takes place. The first stage includes: (1) binding of the virion to the surface receptor of the cell; (2) infection of cellular cytoplasm by a viral particle; (3) reverse transcription of viral single-stranded RNA into double-stranded DNA, the provirus; (4) transport of the DNA to the nucleus; and, (5) insertion of the provirus into the host genome. The second stage proceeds solely with host proteins and the subsequent expression of viral genes. The steps in the second stage are: (1) generation; and, (2) organization of viral genomes, mRNAs, and proteins. The final steps for the production of viral particles are: genome encapsidation, nucleocapsid association with the host cell membrane, and virion budding. The lipid envelop is acquired during budding and is made from the host cell membrane (Fields *et al.* 1990).

## IV. Provirus

The provirus is the double stranded DNA intermediate that results from reverse transcription of the viral RNA genome; it is inserted into the host genome. Insertion could have three possible effects on the host cellular genes: (1) no effect; (2) activation; or, (3) inactivation. So far no pathogenic effects have been found when proviral sequences interrupt the normal cellular gene function. Two reasons for this are: (1) the majority of cells infected are diploid; and, (2) the probability of a second insertion in the remaining gene is low (Fields *et al.* 1990).

As mentioned, other viruses use reverse transcription in their replication cycles. However, only retroviruses have the unique feature of being able to stably insert the product into the host DNA. Weiss *et al.* (1982) suggested that proviral integration has a tendency to occur in areas of the host genome that are more transcriptionally active to ensure that the provirus will be expressed. Not all viral DNA synthesized each cycle is integrated; this may be greatly influenced by cell cycle stage and retrovirus subfamily. Lentiviruses do not integrate the majority of their provirus. Since some of the DNA is unintegrated, it can be detected as a separate species by Southern blotting allowing the researcher to size the viral genome.

## V. Lifestyle

Retroviruses may be either endogenous or exogenous. Endogenous viruses are proviruses integrated into the host genome. They are transmitted vertically, as stable Mendelian genes, because they reside in the host germline. Expression of endogenous retroviruses has been reported in several tumor cell lines. Only a few tumors and immunological disorders in mice can be attributed to nondefective endogenous retroviruses (Weiss *et al.* 1982). Löwer *et al.* (1993) consider human endogenous retroviruses (HERVs) as mobile genetic elements with the potential to trigger disease development through reinsertion of proviruses at new genomic sites. It has not been shown if endogenous proviruses benefit their host (Weiss *et al.* 1982).

Exogenous viruses, on the other hand, are infectious virions that become integrated after reverse transcription has taken place. The fact that they are infectious does not necessarily mean they are pathogenic. If insertion of an exogenous virus occurs in the germline then it may become an endogenous virus.

## VI. Host Range

An important element of retroviral biology is of cell-tropism. Cell-tropism is the ability of a virus to use particular host cell receptors. There are two types of cell-tropism, ecotropism and xenotropism. Ecotropic viruses can use the receptors of the species from which it was isolated. Accordingly, it can replicate well in that species, but does not grow well in other species. Ecotropic viruses, both endogenous and exogenous, may be pathogenic. Xenotropic viruses are endogenous to a species, but cannot use the receptors of that species, therefore, it can not complete replication; e.g., it will not grow However, if introduced into another organism, it can grow well and replicate. Xenotropic viruses have not shown pathogenicity in any organism. The majority of endogenous viruses studied to date, have shown a xenotropic host range after activation. This suggests that the host genome has evolved several mechanisms that prevent reinfection (Weiss *et al.* 1982; Fields *et al.* 1990).

## VII. Transmission

Retroviral transmission can occur either horizontally or vertically. Horizontal transmission can occur by direct contact, or possibly by air or water-borne routes. Vertical transmission has two modes; (1) genetic transmission; or, (2) congenital infection. The first mode takes place as an integrated provirus in the germline; endogenous viruses. Transmission by this route allows the virus to bypass all host-range restrictions, e.g., receptors, cell specificity, and immunity. Congenital infection occurs by infectious particles released by the mother and transported via the egg (fish or bird), or the placenta in mammals (Weiss *et al.* 1982). It is unknown what effect vertical transmission has in fish retroviral diseases.

## VIII. Defective Viruses

Defective viruses can not undergo complete replication because part of the *gag-pol-env* sequence has been lost. For a complete replication cycle to be achieved, the defective virus must coinfect the cell with a helper virus. This helper virus is replication competent and provides the missing proteins for replication. All viruses that contain an oncogene, with the exception of a few strains of the Rous sarcoma virus (RSV), are replication defective. In these viruses the oncogene has replaced some of the essential genes.

The *onc* gene in the defective virus confers transforming abilities to the virus. The *onc* sequences show homology to the normal cellular gene from the host. It has been suggested that these oncogenic viruses are

recombination and mutation products due to events that enabled the virus to gain control of the cellular gene transcription and translation and increase the rate of insertion. A wide variety of neoplastic diseases have been linked to these defective transforming viruses; these include carcinomas, leukemias and sarcomas (Weiss *et al.* 1982).

## IX. Retroviral Infections of Fishes

The diseases discussed in this paper will be presented based on the data for retroviral etiology. Diseases that have an established retroviral etiology and those in which C-type viral particles have been found and transmission experiments have been successful, will be presented as: Confirmed or Strong Evidence for Retroviral Etiology. The remaining diseases will be presented as: Suspected Retroviral Etiology.

### A. Confirmed or Strong Evidence for Retroviral Etiology

#### 1. Damselfish Neurofibromatosis (DNF)

The information presented on Damselfish neurofibromatosis (DNF) is unpublished data from Dr. Michael C. Schmale's laboratory at RSMAS, University of Miami, Miami, FL unless otherwise cited.

Neurofibromatosis type-1 (NF-1) is a genetic disorder affecting 1 in 3,000 people (Crowe *et al.* 1956). It is characterized by benign tumors; occasional malignant lesions are present. The pattern of inheritance is autosomal dominant and the disease has a very high spontaneous mutation rate, roughly one-half of all new cases (Riccardi 1981). The high number of spontaneous mutations can partially be attributed to the large locus of the NF-1 gene (Fountain *et al.* 1989).

Damselfish neurofibromatosis (DNF) is a disorder affecting the bicolor damselfish (*Pomacentrus partitus*) in the Florida Keys with a prevalence of 0.4-23.8% (Schmale 1991). *P. partitus* resides in the Caribbean and South Florida. Histopathological characteristics of DNF are similar to several features of NF-1; these include: malignant schwannomas; hyperpigmented epidermal lesions; and multiple, disseminated neurofibromas, including plexiform growths which are the hallmark of human NF-1. Main differences between piscine and human NF are that the piscine tumors show a greater degree of malignancy; neoplastic cells constitute the majority in hyperpigmented growths instead of benign cells; and DNF is transmissible (Schmale and Hensley 1988).

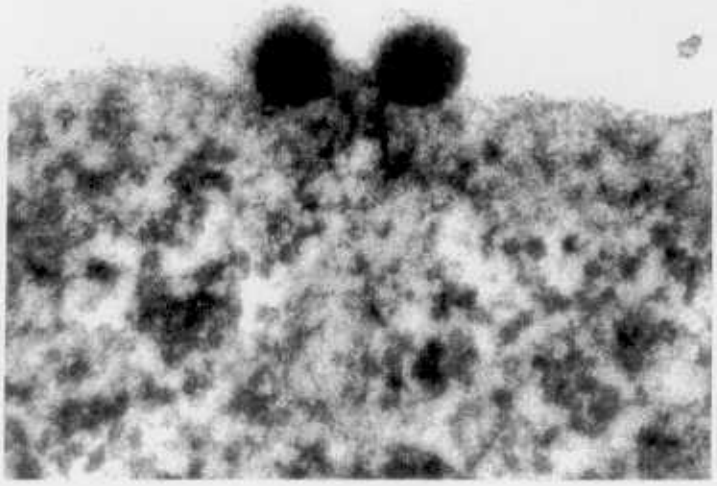

**Fig. 2.** EM of C-type viral particles budding from a DNF tumor cell line. 100 nm = ——. Modified from Schmale *et al.* (1993).

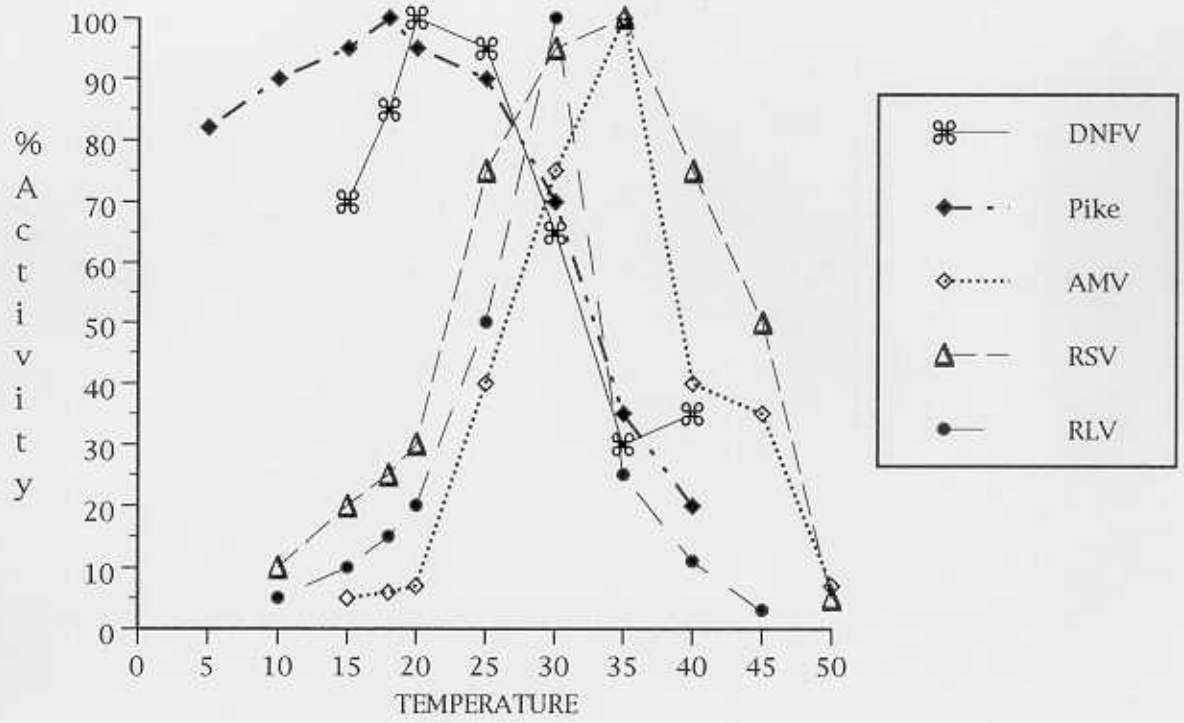


Fig. 3. Optimum temperature curves for several C-type viral RTs. AMV: avian myeloblastosis virus; DNFV: Damselfish neurofibromatosis virus; RLV: Rauscher leukemia virus; RSV: Rous sarcoma virus. Modified from Schmale et al., 1993.

C-type viral particles have been observed budding from the cytoplasmic membrane of tumor cell lines (TF) in EM studies (Fig. 2). These virion have also been visualized in viral isolates (VI) from fractionated TF culture media with a buoyant density of 1.157 g/ml. The viral particles are 80-100 nm in diameter and posses an electron-dense core surrounded by an electron-lucent region. Reverse transcriptase (RT) activity was detected in the 1.157 g/ml fraction. No activity was seen in healthy fish (HF) cell lines at any buoyant density. DNF virus (DNFV) RT shows a cation preference for 1 mM $Mn^{2+}$ and a template primer preference for poly(rC):oligo(dG).

Retroviruses from both mammalian and piscine species have exhibited a peak activity at certain temperatures (Papas *et al.* 1976) (Fig. 3). A temperature curve assay showed the optimal temperature for DNFV RT is 20°C; 90% of the peak activity is still present at 25°C. However, activity drops off at temperatures of 15°C (70%) and 30°C (65%). These findings are consistent with habitat temperatures of *P. partitus* (20-28°C).

Several TF cell lines have been established that actively produce viral particles. These cell lines are the virion sources for nucleic acid and protein work. Serial passages of tumor homogenates have reduced the latency of experimentally induced tumors (Schmale *et al.* 1993). Tumor transmission has been accomplished by injection of TF cell lines, tumor tissue homogenate, and cell-free filtrates of homogenate. To date, transmission experiments to other Pomacentrid species, *P. variablis* (cocoa damselfish) and *P. planifrons*, (threespot damselfish) have been unsuccessful, unlike plasmacytoid leukemia in which transmission to different salmonid genera and species has been accomplished [see below (Newbound and Kent 1991)].

Attempts at isolating viral RNA for Northern blot analysis have been unsuccessful. Nevertheless, several molecular techniques have been employed to characterize the virus associated with DNF. Complementary DNAs (cDNAs) have been made from small amounts of viral RNA and probes have been synthesized using degenerate primers for highly conserved regions of the *pol* (RT) gene. The Polymerase Chain Reaction (PCR) is the technique employed (Donehower *et al.* 1990). A 1.1 kb transcript has been seen in one clone from the viral cDNAs. This transcript demonstrates homology to the polymerase gene (RT). A cDNA library, constructed from pooled tumor tissue, is being analyzed. TF cell line fractions with RT activity and C-type particles were analyzed on SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). HF fractions with the same buoyant density, 1.157 g/ml, were analyzed and no RT activity and C-type particles were observed. Proteins characteristic of retroviruses were seen in the TF samples, but not in the HF samples.

Several immunological studies have been performed on fish affected with DNF. These fish show non-specific suppressed immune responses, especially individuals in the advanced stages of the disease (McKinney and Schmale 1994). Degenerative histological changes in the kidney and spleen are also apparent (Schmale and McKinney 1987). Some fish in transmission experiments never (after several years) develop tumors. It is possible that the immune response, natural killer cells and cytotoxic T cells, of these fish was great enough to overcome the disease process.

## 2. Esocid Lymphosarcoma

Bowser and Casey (1993) report three diseases affecting *Esox* sp.; Esocid lymphosarcoma (North America, Ireland and Finland) Esox sarcoma (Baltic coasts of Finland and Sweden) and Pike epidermal proliferation (North America). After reviewing the literature, I feel Esox sarcoma and Esocid lymphosarcoma are the same disease for the following reasons.

Several pieces of information conflict in Bowser and Casey's paper (1993) regarding the two diseases. Firstly, they cite Ljungberg and Lange's work (1968) on a Baltic pike population for both Esox sarcoma and Esocid lymphosarcoma; but the paper discusses a single disease. Second, Bowser and Casey (1993) state the lesions only involve connective tissue and skin, not muscle, citing Ljungberg and Lange (1968). However, Ljungberg and Lange (1968) clearly report, "Downward infiltration in the muscles was frequently observed." Third, Bowser and Casey explain the tumor cells from Esox sarcoma "can be differentiated from Esocid lymphosarcoma in that cells of Esox sarcoma contain many lipid droplets...." (pp. 220). On the preceding page their description of tumor cells from Esocid lymphosarcoma includes the "presence of large numbers of lipid droplets."

Esocid lymphosarcoma affects northern pike (*Esox lucius*) and muskellunge (*E. masquinongy*). The disease has been reported in North America, Ireland, and along the Baltic coasts of Finland and Sweden (Sonstegard 1976; Mulcahy 1976; Thompson 1982; Ljungberg and Lange 1968; Ljungberg 1976). Esocid lymphosarcoma shows one of the highest frequencies of malignant neoplasms in a noncaptive vertebrate; DNF (Schmale 1991) and Walleye dermal sarcoma [see below (Martineau 1991)] show similar frequencies. Prevalence for Esocid lymphosarcoma is as high as 20.9% (Mulcahy and O'Rourke 1964; Sonstegard 1976).

External lesions are always present (Sonstegard 1976), but target areas vary with geographic location. Mulcahy (1976) reported the head, buccal cavity and mouth as primary sites for tumor development in Ireland. Ljungberg and Lange (1968) in Sweden, Sonstegard (1976) in North America and Thompson (1982) in Finland all reported the flanks as the

principal regions for lymphosarcoma growth. External lesions appear nodular and pink in the early stages, as the disease progresses they become white and ulcerated (Sonstegard 1976) (Fig. 4). Tumor diameter can be several centimeters. Growths are highly aggressive and infiltrate underlying muscle and connective tissue (Mulcahy 1963; Ljungberg and Lange 1968; Sonstegard 1976; Thompson 1982). Metastases are common in the spleen and kidneys (Mulcahy 1963; Sonstegard 1976). Affected tissues increase in size, up to four times larger than normal, due to infiltration of lymphocytes (Mulcahy 1963).

Tumor cells are transformed lymphocytes, 1.5-2 x larger than normal fish lymphocytes (Mulcahy 1970). The nucleus is rounded or oval and sometimes exhibits a distinct indentation in one side giving it a characteristic "kidney" shape. A small number of cells are multinucleated and a few mitotic figures are always seen (Sonstegard 1976). EM studies revealed the infiltrating cells contained numerous lipid droplets, lacked tight junctions and had collections of granules that are consistent with primary lysosomes (Thompson and Miettinen 1988).

Immunological studies done by Thompson and Kostiala (1988) revealed that mononuclear cells from lymphoid organs of healthy and diseased *Esox lucius* presented cytoplasmic and surface immunoglobulin (Ig) while tumor cells did not. This finding strongly suggests the neoplasm is not a B cell lymphoma or a plasmacytoma since both present Ig. The tumor has been diagnosed as a histiocytic lymphoma (Bowser and Casey 1993).

C-type viral particles and RT activity were seen in tumor homogenates with a buoyant density of 1.16 g/ml. Peak enzymatic activity was at 20°C, 82% of the optimum activity is seen at 5°C and only 35% at 35°C (Papas *et al.* 1976). *Esox* RT prefers the template primer poly(Cm):oligo(dG). Figure 5. summarizes the response of several RTs to various template primers (Papas *et al.* 1977).

Information from the RT temperature curve is very important due to the seasonality of esocid lymphosarcoma in North America. Nigrelli (1954) was the first to recognize a seasonal cycle for tumor formation and progression. Sonstegard (1976) showed the disease is highly seasonal in muskellunge. June-July lesion number is extremely low; during the late summer/early fall (August-September) there is a significant increase (up to 10%) in lesion number. March-April mirror the lesion number of the fall months, however, the disease has progressed to more advanced stages. May-June there is an increase in advanced tumors and a subsequent die-off of affected fish. It is hypothesized that the disease progresses during the colder fall (12°C) and winter (4°C) months when a high degree of RT activity is still present due to permissive temperatures. Tumor regression

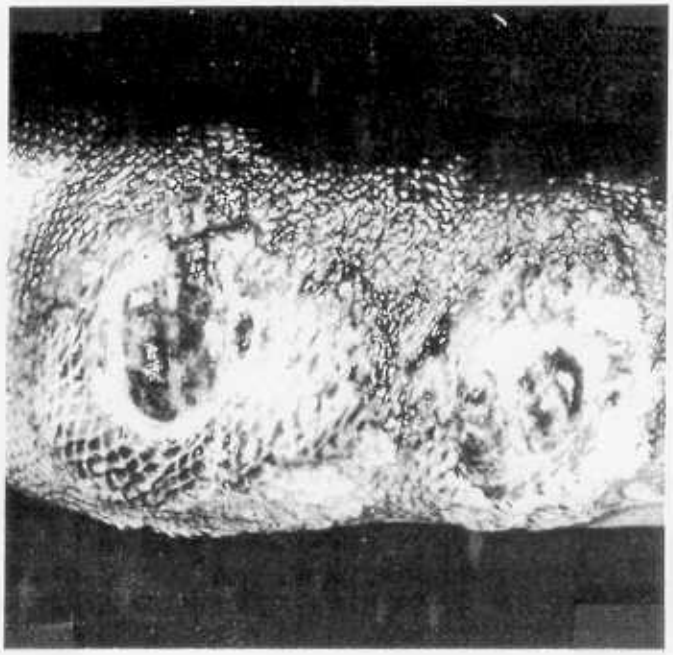

**Fig. 4.** Two ulcerated lesions from *E. masquinongy*. Note the severe ulceration and necrosis indicating an advanced stage of the disease. From Sonstegard (1976).

| | cpm | | | |
|---|---|---|---|---|
| DNA polymerase | Poly(rA): oligo(dT) | Poly(dT): oligo(rA) | Poly(rC): oligo(dG) | Poly(Cm): oligo(dG) |
| AMV | 26,051 | 257 | 126,967 | 6,365 |
| MLV | 36,746 | 311 | 47,957 | 36,883 |
| RLV | 63,727 | 394 | 52,743 | 254,232 |
| RSV | 3,175 | 59 | 8,174 | 1,972 |
| Pike lymphoma | 4,125 | 260 | 8,710 | 16,135 |

AMV: avian myeloblastosis virus; MLV: murine leukemia virus; RLV: Rauscher leukemia virus; RSV: Rous sarcoma virus.

**Fig. 5.** RT template primer preference. Modified from Papas *et al.* (1977).

occurs in summer due to warmer non-permissive temperatures; 25-34°C (Papas *et al.* 1977). It has been suggested, to explain the relationship between host temperature and optimum temperature for enzymatic activity, that there has been a long association between host and virus or that viral RT might be derived from the host (Papas *et al.* 1977).

A correlation between age and sex and onset of the disease has been observed. Tumors are found only in pike three years or older; males 5-6 years are affected most often. The sex ratio (male/female) of diseased fish is 2.5/1 and in non-diseased fish 1.1/1. The contrast between sexes may be due to hormonal differences and/or rates of contact during spawning. Males are more aggressive than females during the mating season allowing for more contact and greater transmission of the disease (Thompson and Kostiala 1990).

Transmission experiments have been successful with tumor explants; cell-free filtrates passed through a 0.45 $\mu$m filter; and tumor homogenate injections (Mulcahy and O'Leary 1970; Brown *et al.* 1975; Ljungberg 1976; Sonstegard 1976). A drop in latency was observed in cell-free filtrate experiments, due to serial passages increasing the infectivity of the virus (Ljungberg 1976); this is also seen with DNF (Schmale *et al.* 1993). Sonstegard (1976) showed successful transmission occurred with cell-free filtrates from tumor material collected in the spring, but not from summer material. This finding corroborates the data of permissive/non-permissive temperatures for viral activity and tumor development. To date attempts at viral propagation in cell culture have failed.

### 3. Plasmacytoid Leukemia (PL)

In British Columbia, Canada, net-reared, chinook salmon (*Onccorhynchus tshawytscha*) have been affected by a plasmacytoid leukemia (PL) since 1988. Fish farmers named the disease "marine anemia". Affected fish exhibit anemia, enlargement of the spleen, kidneys and ascites and bilateral exophthalmia ("pop-eye") resulting from neoplasms in the retrobulbar area. Histologically, tumors are proliferations of plasmacytoid cells in various organs; kidneys, spleen, pancreas, liver, heart, eye orbit and intestinal lamina proporia (Eaton and Kent 1992).

EM studies of cell lines displaying cytopathic effects (CPEs) revealed C-type retrovirus-like particles budding from intercellular membranes (Fig. 6). Virions were 110 nm in diameter with an electron-dense core. Reverse transcriptase activity was present in fractions of affected tissue with a buoyant density of 1.16-1.18 g/ml. Temperature curves for optimal RT activity have not been attempted, but plasmacytoid leukemia virus (PLV

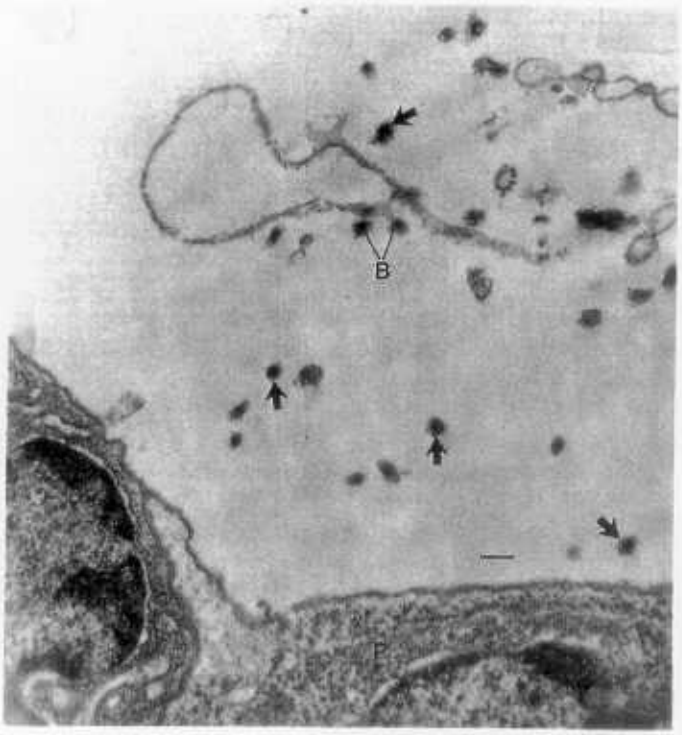


Fig. 6. Virus-like particles budding (B) from membrane sections. Particles (arrows) in intercellular spaces between neoplastic plasmablasts. Bar, 100 nm. From Eaton and Kent (1992).

RT) performs best with poly(rA):oligo(dT) template primer and $Mn^{2+}$ as the cation (Eaton *et al.* 1993). Fractions demonstrating positive RT activity and C-type particles exhibit proteins characteristic of retroviruses when analyzed by gel electrophoresis (Eaton and Kent 1992).

Tumor cell lines were established, characteristic proliferative plasmablast-like cells were numerous. CPEs, such as vacuolation and syncytium formation, were seen that are similar to retroviral infections by bovine leukemia virus (Burny *et al.* 1978), feline leukemia virus and caprine arthritis-encephalitis virus (Dahlberg 1988). Tumor cells present immunoglobulin on their surfaces (Bowser and Casey 1993). Transmission of the cell culture associated virus, into salmonid and non-salmonid cell lines, was unsuccessful (Eaton *et al.* 1993). However, experimental transmission via tumor homogenate and cell-free filtrate injections (0.2 $\mu$m) have been successful (Newbound and Kent 1991; Eaton and Kent 1992; Kent and Dawe 1993).

Newbound and Kent (1991) demonstrated interspecies transmission of plasmacytoid leukemia in salmonid fishes. Chinook salmon, coho salmon (*O. kisutch*), sockeye salmon (*O. nerka*), rainbow trout (*O. mykiss*) and Atlantic salmon (*Salmo salar*) are important commercial and sport fishes. Since they are raised in netpens, their susceptibility to PL was examined. Kidney homogenate from diseased chinook salmon was used for intraperitoneal injections. All 5 species exhibited pathological changes from 6 to 16 weeks post injection. Sockeye and chinook salmon displayed gross and histological changes characteristic of PL. Atlantic salmon showed only histological changes. Rainbow trout and coho salmon developed minor growths: renal interstitial hyperplasia and foci in the mesenteries that resembled leukemic plasmablasts; neither developed signs of PL.

Even though PL has not been reported to show seasonal variation, a temperature curve assay, for PLV RT, would prove insightful. If there are permissive and non-permissive temperatures for viral RT, then this information could be a useful tool in controlling the spread of disease in hatchery situations. Rearing fish in temperatures that are sub-optimal for tumor growth, but within the natural temperature range of the species, transmission of the disease, and the subsequent mortality, could be decreased.

### 4. Walleye Dermal Sarcoma (WDS)

Dermal lesions on walleye perch (*Stizostedion vitreum*) from the Great Lakes and Lake Oneida, New York were reported as early as 1947 by Walker. The prevalence in adults is 27% in Lake Oneida (Martineau *et al.* 1991). Lesions, originate from the scales, are firm, smooth and nodular and sometimes occur as multiples. The diameter can be greater than 1 cm. Histologically, tumors vary in collagen content and cellularity. Tumors are not locally invasive and no metastases occur, which is consistent with benign lesions. Tumor cells appear oval to fusiform in shape. They contain well-developed granular endoplasmic reticulum, lipid droplets and large mitochondria. Few mitotic figures are visualized. Many cells in the late stages of tumor development have an anaplastic appearance (Martineau *et al.* 1990).

C-type viral particles have been observed in tumor tissue and budding into intercellular spaces in EM studies (Walker 1969; Bowser *et al.* 1988 and Martineau *et al.* 1990). Visualized were characteristic spikes protruding from the virion envelope and a central, electron-dense core surrounded by an electron-lucent area (Martineau *et al.* 1991). Walker (1969) and Martineau *et al.* (1990) sized the particles at 100 nm, but Bowser *et al.* (1988) reported virions sized at 135 nm. The size difference may be due

to different measurement techniques employed (Martineau *et al.* 1991). RT activity was present at a buoyant density of 1.18 g/ml in tumor tissue. Healthy tissue was negative for RT activity (Martineau *et al.* 1991). No experiments have been attempted to look at template primer or cation preference of WDS virus (WSDV) RT.

Bowser *et al.* (1988) reported a seasonal occurrence of dermal sarcomas in Lake Oneida (Fig. 7). A high number of lesions were seen in early spring, low numbers in summer and high numbers again in late fall (Bowser *et al.* 1990). This seasonality is also seen in Esocid lymphosarcoma. Temperature dependence for lesion development could be linked to several factors; especially the permissive temperature range for WDSV RT. It has been shown for DNFV RT and Esocid lymphosarcoma RT that temperature plays a major role in enzymatic activity. A temperature curve assay for WDSV RT would be helpful in proving this relationship.

Experimental evidence for permissive and non-permissive temperatures for tumor development was shown by Bowser *et al.* (1990). Cell-free filtrates were injected into healthy walleye fingerlings. Fish were held at three different temperatures during the study: 10°C (fall temperature), 15°C (late spring, mid-fall) and 20°C (late spring/early summer). The most successful transmission of lesions occurred at 15°C, which is the temperature for mid-late fall when tumor development is thought to occur. This study ruled out developmental maturity as an influential factor for tumor growth since transmission to immature fingerlings was successful (Bowser *et al.* 1990).

| Season | Temperature (°C) | Dermal sarcoma* |
|---|---|---|
| Early spring | 0-5 | 14 |
| Late spring | 15-20 | 14 |
| Summer | 20-25 | 4 |
| Early fall | 10-15 | 25 |
| Late fall | 5-10 | 27 |

* Percent of fish caught.

**Fig. 7.** Relationship between temperature and seasonal growths on walleye in Lake Oneida, NY, 1986. Adapted from Bowser *et al.* (1988).

Tumor growth and regression in *S. vitreum*, appear to be associated with permissive and non-permissive temperatures and the species immune response (Bowser *et al.* 1988; Bowser and Casey 1993). When temperatures drop during the fall, tumor growth occurs; during the winter, when temperatures decline even further, tumor growth slows. The

immune response is opposite the seasonal cycle. In the spring and early summer an infiltration of lymphocytes into the overlying dermis and epithelium is seen. Inflammation is not seen in the fall (Bowser *et al.* 1988). Several studies have shown initial immunosupression in several species of fish at colder temperatures (Avtalion *et al.* 1973; Clem *et al.* 1984; Schneider and Ambrosius 1987). Once acclimation has occurred, the immune system will function, but at a much slower rate (Avtalion *et al.* 1973; Avtalion 1981). Avtalion (1981) demonstrated antibody production and release occurred at low temperatures.

Bowser and Casey (1993) suggest there may be a multifactorial etiology. Other elements may include physiology, sunlight, genetics, environmental carcinogens. The last factor could be of special interest regarding immunosupression and occurrence of lesions on the white sucker in more polluted watersheds than in more pristine ones [see below (Sonstegard 1977)]. Later in this paper three piscine cell lines that produce retroviral particles when exposed to a carcinogenic substance will be discussed. Could mutagens and/or carcinogens in the environment be the underlying cause for some retroviral diseases found in nature?

Viral polyadenylated RNA from RT positive fractions of tumor homogenate was isolated. cDNAs were synthesized from this RNA and used as probes for Southern blot analysis of tumor and healthy tissue DNA. A 13.2 kb unintegrated viral DNA was visualized on Southern blots in WDS-affected individuals only. DNAs from non-tumor and non-piscine organisms were negative. Northern blot analysis of viral RNA revealed a 12 kb band representing the viral genome. Viral RNA was always present in tumor tissue and when analyzed on a Northern blot several message lengths were seen (Martineau *et al.* 1991).

Several findings suggest that WDSV may be a lentivirus or spumavirus, not an oncovirus. First, copy number/tumor cell of viral DNA. WDSV has a copy number of 7-50/cell. Lentiviruses and spumaviruses have copy numbers in this range because of the large proportion of unintegrated provirus (Bowser and Casey 1993). Second, WDSV contains a unique single-stranded region that is seen in spumaviruses and some lentiviruses in their unintegrated viral DNA. This sequence is thought to be an additional template primer for second strand DNA synthesis (Martineau *et al.* 1992). Third, the length of the WDSV DNA corresponds more closely with spumaviruses. This is the strongest piece of evidence for classification in the subfamily *Spumavirinae* and not in *Oncovirinae*. To date spumaviruses are not known as disease causing pathogens (Weiss *et al.* 1982; Bowser and Casey 1993).

Several pieces of evidence support a causal relationship between WDS and WDSV: (1) Southern blots of tumor and non-tumor DNA are positive with tumor samples only; (2) no sequence homology to WDSV by non-tumor DNA: and (3) WDSV hybridized to a 13.2 kb fragment demonstrating sequence homology to uncut tumor DNA (Martineau *et al.* 1992).

Many advances have been made in comprehending the etiology, histology and nuances of WDS since it was first reported by Walker (1947). However, several aspects of research need to be fulfilled to further understand the disease. Attempts, by several researchers, at culturing the virus in tissue culture have failed (Bowser and Casey 1993). Cell culture is a powerful tool when observing cytopathic effects (CPEs) of pathogens on cells. Knowledge of the cellular progression of WDS could be obtained when stable virus producing cell lines are established. As mentioned above, a few molecular assays will aid in viral characterization of WDSV: (1) template primer and cation preferences for WDSV RT would pinpoint details about the virus; (2) a temperature curve for WDSV RT would strengthen the relationship between tumor growth/regression and water temperature; and (3) the identification of viral proteins, by gel electrophoresis, would enhance the description of WDSV.

Bowser and Casey (1993) mention WDS may have a multifactorial etiology. Experiments designed to use the elements together and interchangeably could prove to be very interesting. Some retroviral diseases may be the result of interacting factors in a species' habitat and physiology.

## B. Suspected Retroviral Etiology

### 1. Angel Fish Lip Fibroma

Benign lip fibromas were observed in a small group (n=20) of angel fish from commercial operations and a hobbyist. They were investigated at the University of Florida fish disease laboratory (Francis-Floyd *et al.* 1993).

Tumors were firm, conical, multinodular and raised. They occurred singularly or in multiples. Lesion diameter ranged from 3 to 9 mm. Infiltration into surrounding tissue was not observed. Histologically, tumors were comprised of thick fibrovascular connective tissue covered by an enlarged, stratified squamous epithelium. Mitotic figures were not present. Slight mononuclear infiltrate was seen near the dermal/epidermal boundary (Francis-Floyd *et al.* 1993).

EM studies were performed on tumor material. Two types of retrovirus-like particles were observed: A-type and C-type. A-type particles had diameters of 90 nm with electron-dense cores, while C-type particles were enveloped and had 150 nm diameters (Francis-Floyd *et al.* 1993).

Transmission experiments with cell-free filtrates were unsuccessful. Virus isolation in tissue culture has not been attempted (Francis-Floyd *et al.* 1993).

## 2. Atlantic Salmon Papilloma

Benign epidermal papillomas have been observed throughout the United States, Sweden, Norway and England, in both free-living and cage-reared Atlantic salmon (*Salmo salar*). Lesions are commonly found during the summer on second-year fresh water salmon (parr) and occasionally on young adult fish which have adapted to salt water (smolts and grilse) (Carlisle 1977).

Lesions are wart-like, occur singularly or in multiples and are found anywhere on the body except the head. Tumors have distinct boundaries, up to 4 cm in diameter, and elevated areas 2-5 mm in height. The affected tissue is squamous epithelium exhibiting diminished numbers of mucous cells. The tumor is not locally invasive even though there is an increase in the number of mitotic figures (Carlisle 1977).

The observed immune response is highly variable, ranging from a few leukocytes to massive infiltration. In more advanced tumors , necrosis and elevated numbers of macrophages and neutrophils are present. The final stage is tissue ulceration. In cases without complications, the ulcer is covered with a new epithelial layer resulting in recovery. The most common complication is a fatal secondary infection by bacteria or fungi (Carlisle and Roberts 1977).

Tissue samples from affected fish were prepared for electron microscopic (EM) studies. Retrovirus-like particles were seen associated with the plasma membrane. They were 125-150 nm in diameter and possessed an electron-dense core and an electron-lucent area surrounding the core. Attempts to establish virus-producing cell lines have been unsuccessful (Carlisle 1977).

Carlisle (1977) proposes a multifactorial etiology for Atlantic salmon papilloma development. Growths are found mainly on parr during the summer and do not remain through the winter (Carlisle 1977). Lesion growth could be effected by factors such as developmental maturity (hormones, osmotic regulation) of the fish and environmental

temperature. A few diseases with retroviruses or retrovirus-like particles have demonstrated permissive and non-permissive temperatures for tumor growth; Esocid lymphosarcoma (Sonstegard 1976) and Walleye dermal sarcoma (Bowser *et al.* 1990). Atlantic salmon papilloma is the only retrovirally associated disease reported in fish, that affects juveniles more frequently than adults. There is usually an increase in lesion number/size with age, exhibited by Damselfish neurofibromatosis (Schmale *et al.* 1986); Esocid lymphosarcoma (Sonstegard 1976) and White sucker papilloma (Sonstegard 1973).

### 3. Atlantic Salmon Swim Bladder Sarcoma

Reports of tumors on the swim bladders of caged-reared Atlantic salmon (*Salmo salar*) in Scotland have been investigated (Duncan 1978; McKnight 1978). Growths are found primarily on second year smolts and occasionally on one-year-old smolts with a prevalence of 4.6% (McKnight 1978).

Lesions are firm and well differentiated protruding 5-10 mm from the organ with diameters of 15-30 mm. No invasion into surrounding tissues or organs is present, but growth is locally aggressive. There are no metastases.

Duncan (1978) reports the tumor as a fibrosarcoma, citing his EM studies and McKnight's histological work (1978). EM reveals aggregates of virions associated with "semi-ordered" fibrils believed, by Duncan, to be collagen fibers. However, McKnight (1978) reports little collagen content in the tumor and describes it as a leiomyosarcoma. McKnight also cites the following characteristics for his identification: blunt tapering nuclei; mitotic figures; local invasion and interwoven cell bundles.

Enzinger and Weiss (1983) describe leiomyosarcomas with the following characteristics: blunt-ended nuclei ("cigar shaped"); highly aggressive, intertwining fasicular growth and high numbers of mitotic figures. The majority of tumors have little collagen content (Bennington 1984). These properties are consistent with McKnight's findings. Enzinger and Weiss (1983) report the majority of fibrosarcomas contain interwoven collagen fibers between the individual cells.

Based on the sparse evidence reported, I am inclined to agree with McKnight's conclusion: the tumors are leiomyosarcomas. Immunohistochemistry would be an important tool in differentiating between a leiomyosarcoma and a fibrosarcoma. A distinct feature of leiomyosarcomas is cytoplasmic desmin filaments. Desmin filaments are

found principally in muscle tissue; they would not be detected in fibrosarcomas. Reticular fibers, however, are detected in fibrosarcomas. They are narrow bundles of collagen fibrils that form a meshwork between individual cellular components (Enzinger and Weiss 1983; Bennington 1984; Mies personal communication).

A viral etiology is suggested because C-type viral particles were visualized in tumor material by EM. Virions were seen budding from cells into extracellular spaces and often appeared in extensive aggregates. Particles were electron-dense and 110 nm in diameter (Duncan 1978).

There have been no attempts at viral isolation in tissue culture or experimental transmission experiments.

### 4. European Smelt Fin Papilloma

Spawning papillomatosis in European smelt (*Osmerus eperlanus*) has been reported from the River Thames, England (Lee and Whitfield 1992); the Elbe estuary, Germany (Anders and Möller 1985); the Bothnian Sea, Finland (Anders 1989); and the southern Baltic Sea (Breslauer 1916).

Two tumor types are present, fin and trunk tumors. Fin tumors are found primarily on the fins, but occasionally on the head. Lesions are white, ovoid and have diameters less than 5 mm. Trunk tumors are flat and can cover a large surface area of the fish (Anders and Möller 1985).

A herpesvirus is believed to be the etiological agent of the papillomas and lesion outbreak is most-likely linked to the spawning cycle of European smelts. However, retrovirus-like particles are found in 10% of the tumors. Two different virion sizes are observed, 88-101 nm and 55-76 nm (Anders 1989). The role of the retrovirus-like particles in papilloma development is unknown. These particles may be endogenous retroviruses that are packaged as the result of stress on the organism. Stress factors could include pollution, papillomas, hormonal changes during spawning. Aaronson *et al.* (1974) describe induction of endogenous viral packaging in cell lines exposed to the base analog 5-bromo-2'-deoxyuridine (BrdU). Propagation of retrovirus-like particles in cell culture and transmission of the disease experimentally have been unsuccessful (Bowser and Casey 1993).

## 5. Hooknose Fibroma

In 1988-89 a survey in the German Wadden Sea uncovered a previously unreported disease of the hooknose poacher (*Agonus cataphractus*) with a prevalence of 0.7%. Little is known about the population biology of *A. cataphractus* except it is a bottom-dwelling species that feeds primarily on brown shrimp (Anders *et al.* 1991).

Tumors are flat or nodular and have diameters of 3-9 mm. Histologically, most lesions arise from connective tissue and are benign, however, one growth observed invaded surrounding muscle tissue. Tumors are classified by color: yellow or skin-colored (reddish to black). Yellow lesions contain multi-nucleated giant cells and large numbers of lymphocytes and granulocytes. Skin-colored lesions are characterized by intact basal lamina, extensive proliferation of connective tissue with interweaving collagen, proliferation into epidermal layers and highly vascularized tumor tissue. Tumor cells are characterized by crystalline structures, numerous cytoplasmic vacuoles, dense endoplasmic reticulum and enlarged mitochondria. Tumor cells are morphologically similar to lymphocytes (Anders *et al.* 1991).

Lentivirus-like particles were seen in EM studies with a diameter of 86-132 nm and were primarily found in yellow tumors. Virions were classified as lentivirus particles because of size, occasional double cores, lipid envelopes, lateral bodies and occurrence in cytoplasmic vacuoles (Anders *et al.* 1991).

Virus isolation in cell culture and experimental transmission experiments have not been attempted. Identification of lentivirus-like particles in hooknose poacher is significant since it is the first report in a lower vertebrate (Anders *et al.* 1991).

## 6. Pike Epidermal Proliferation

A second disease in northern pike (*Esox lucius*), pike epidermal proliferation, has been reported in North America (Yamamoto *et al.* 1983) and Europe (Winqvist *et al.* 1968).

Epidermal lesions are smooth, translucent and plaque-like; in some instances hemorrhaging occurs. Growths are 5-20 mm in diameter and 2 mm in relief. Tumor cells are undifferentiated and randomly arranged. This results in uneven stratification in epidermal layers. Mitotic figures are present and internal organs are not involved (Yamamoto *et al.* 1983).

EM studies revealed C-type particles budding from cytoplasmic membranes into intercellular spaces. Particles have a diameter of 150 nm; the virion capsid is 120 nm in diameter and the core is 80 nm (Yamamoto *et al.* 1983). Isolation of viral particles in cell culture has been unsuccessful. Experimental transmission studies have not been attempted.

### 7. Walleye Discrete Epidermal Hyperplasia

Walleye (*Stizostedion vitreum*) from Lake Oneida, New York and central Canada display another dermal lesion associated with a retrovirus, discrete epidermal hyperplasia (Walker 1969; Yamamoto *et al.* 1985). Lesions occur anywhere on the body, but Walker (1969) observed them most often on the caudal fin. Tumors are mucoid, often occur in multiples and exhibit a distinct boundary. Diameters are up to 1 cm and relief is 0.25-1.5 mm. Although locally aggressive, no infiltration of surrounding tissue occurs and there are no metastases. Tumor cells are cuboidal and contain high numbers of mitotic figures (Yamamoto *et al.* 1985).

EM studies by both Walker (1969) and Yamamoto *et al.* (1985) revealed C-type retroviral particles budding from the cells. They do report, however, a marked size difference in particle diameter, 80 nm by Walker (1969) and 120 nm by Yamamoto *et al.* (1985). The difference in size may be the result of different measurement techniques employed.

Viral propagation in cell culture has not yet been successful, but Yamamoto *et al.* (1985) mention RT assays to be tried on viral isolations from tumor tissue. No transmission experiments have been attempted.

### 8. White Sucker Papilloma

Epidermal papillomas of white suckers (*Catostomus commersoni*) have been reported throughout eastern and central Canada and the United States. Pollution may be an influencing factor since tumor prevalence is higher in polluted waters, lesion growths are larger in polluted areas and multiple lesions occur more often in polluted watersheds (Sonstegard 1973; Sonstegard 1977; Smith *et al.* 1989a; Smith *et al.* 1989b; Harshbarger and Clark 1990; Hayes *et al.* 1990).

Lesions are white to pinkish, benign with no metastases and are not locally invasive. Growths form anywhere on the body including eyes, operculum, flank, fin, but on the lips in particular (Sonstegard 1977). Papillomas are similar in structure to virally induced papillomas in other fish species; salmon (*O. masou*) brown bullhead (*Ictalurus nebulosus*) (Pilcher and Fryer 1980). Lip lesions are round or oval and raised 0.5 cm. Body

tumors have two distinct morphs; papillomas and plaques. Papillomas are circular (up to 0.75 cm in diameter), focal, firm and have a discrete boundary. Plaques appear as low, mucoidal growths with varied shapes They can become quite large, covering a large surface area (Smith *et al.* 1989b).

Several biochemical assays have been performed on all three lesion types. The data are inconclusive as to the role xenobiotics play in lesion development and severity (Hayes *et al.* 1990).

Tumor regression studies have been performed by removing affected specimens to laboratories with clean water (Smith and Zajdlik 1987; Hayes *et al.* 1990). Smith and Zajdlik (1987) found regression rates of 22% for lip papillomas, 64% for body papillomas and 79% for body plaques. Neutrophil infiltrate is present in all tumor types. This demonstrates a relationship between an immune response and regression of a tumor (Smith *et al.* 1989a). New tumor development was observed in a large number of individuals suggesting a factor other than environmental pollution as the cause for lesion growth (Smith and Zajdlik 1987). Smith and Zajdlik (1987) also report that temperature does not have a significant role in tumor development, as it does in other species (Sonstegard 1976; Papas *et al.* 1977; Bowser *et al.* 1990).

Sonstegard, (1973), reported C-type viral particles in tumor tissue seen in EM studies (Fig. 8). They were 100 nm in diameter, found budding from cytoplasmic membranes and in intracellular spaces. However, in 1976, he published the same electron micrograph of viral particles in an Esocid lymphosarcoma paper citing northern pike tissue as the source of the virions (Sonstegard 1976). To further complicate the issue, Sonstegard (1977), reports RT activity in epidermal papilloma tissue, from a white sucker, at a buoyant density of 1.15-1.16 g/ml. Unfortunately, no other EM studies have revealed viral particles in tumor tissue from white suckers (Harshbarger 1990).

Etiology of epidermal lesions on white suckers is still largely unknown; a multifactorial etiology seems most plausible. *C. commersoni* is a bottom-dwelling species resulting in constant exposure to accumulated carcinogens and pollutants in sediments. Possible activation of latent viruses or increased virulence of existing ones may be the result (Sonstegard 1977; Smith *et al.* 1989a and 1989b). Lip papillomas might arise from mechanical abrasion allowing infectious and/or carcinogenic agents into the epidermis (Sonstegard 1977). Age might be another influencing factor. Tumor development does not occur in individuals less than 4 years.; white suckers become sexually mature at this age. This observation can be interpreted at least three different ways. One, there is

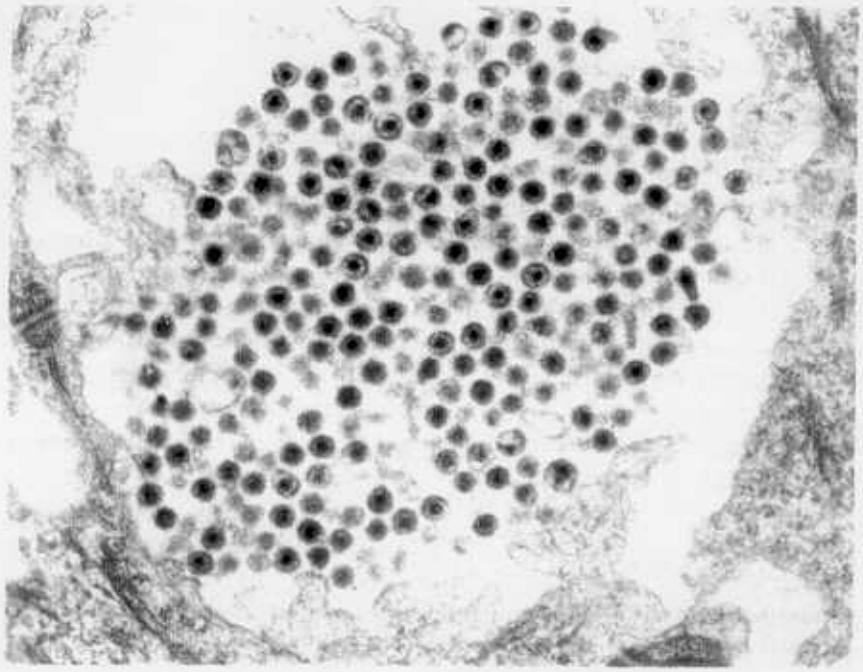

Fig. 8. EM of C-type viral particles reported from both *C. commersoni* and *E. lucius*. From Sonstegard (1973 & 1976).

horizontal transmission of an infectious agent during spawning, as seen with Esocid lymphosarcoma (Thompson & Kostiala 1990); two, manifestation of the disease is the result of hormonal changes from sexual maturation; or three, it is a time dependent event, such as Huntington's chorea in humans.

Attempts at culturing a virus in tissue culture have been unsuccessful. Experimental transmission via tumor explants, cell-free filtrate injections and healthy individuals exposed to tumor-bearing ones have also failed (Sonstegard 1977). Retroviral etiology for the epidermal lesions cannot be ruled out even though the data are still inconclusive. Latent viruses could be activated by carcinogens in the environment (Black 1982), or chemicals and viruses could work in tandem as co-carcinogens (Sonstegard 1973; Smith *et al.* 1989a). Pollution assays need to be done to clarify the role of environmental pollutants in the development and progression of papillomas. The presence or absence of viral particles associated with the lesions also requires resolving.

### 9. *Xiphophorus* sp. Hybrid Neuroblastoma

The fish genus *Xiphophorus*, especially the hybrid platyfish (*X. maculatus*) x swordtail (*X. helleri*), has been studied as a model for cancer for over 60 years. The majority of tumors are caused by genomic oncogenes present in several copies (Petry *et al.* 1992). However, retroviruses have been implicated in two instances (Kollinger *et al.* 1979; Perlmutter and Potter 1987).

Aaronson *et al.* (1974) describe induction of endogenous viral packaging when cell lines are exposed to the base analog 5-bromo-2'-deoxyuridine (BrdU). Neuroblastomas injected with BrdU produce two particle types; B-type virions with an eccentric core and C-type particles with a central core (Kollinger *et al.* 1979; Perlmutter and Potter 1987).

Retrovirus-like particles are present in an embryonic cell line. The virion diameter is 100 nm and their buoyant density is 1.16 g/ml. An electron-lucent area surrounding the core is not seen. RT assays performed on the 1.16 g/ml fraction showed positive results in the presence of 0.4 mM $Mn^{2+}$, a template primer preference for poly(rA):oligo(dT) and an optimal temperature between 28°C and 32°C. (Petry *et al.* 1992)

cDNAs were made from viral RNA and were used as probes for Northern and Southern blots. Northern blots of viral RNA revealed three transcripts, the largest is thought to be the viral genome. Southern blot analysis revealed homologous sequences between both *Xiphophorus* sp. and the cell line genomic DNA. Homology also exists between the

*Xiphophorus* sp. genome and the LTR-gag region of the feline leukemia virus (FeLV) (Petry *et al.* 1992). SDS-PAGE was performed on virion containing fractions and proteins characteristic of retroviruses were seen. Western blotting was also done on these fractions. The 70, 65 and 28 kiloDalton (kDa) proteins showed strong reactivity to FeLV anti-p27 serum (Petry *et al.* 1992). The 70 kDa protein is the surface protein made from the *env* gene; p65 is the RT synthesized from the *pol* region and p28 is the capsid protein made from the *gag* gene. The significance of this cross-reactivity is two-fold. One, it confirms these proteins, from *Xiphophorus*, are derived from a retrovirus because the FeLV serum contains antibodies to the corresponding feline proteins. Second, it helps demonstrate the long evolutionary relationship between retroviruses in vertebrates.

Infectivity studies in cell culture were attempted and failed. Petry *et al.* (1992) suggest several explanations for the lack of infectivity. Particles visualized in EM studies displayed immature retroviral particle morphology. If this is the case, then transformation of a cell line would not occur since immature virions are not infectious. A second explanation is that the virus is xenotropic, a characteristic of most endogenous retroviruses. A third interpretation for lack of infectivity, not suggested by the investigators, is the viral isolation technique. As discussed in the introduction, retroviruses are infectious when their glycoprotein spikes are present on the envelope surface. EM photographs from Petry *et al.* (1992) show no spikes on the viral surface; he even states this observation in the text.

## 10. Piscine Cell Culture Retroviruses

Frerichs *et al.* (1991) report spontaneous production of C-type retroviral particles from three Southeast Asian piscine cell lines; climbing perch (*Anabas testudineus*), snakehead fish (*Ophicephalus striatus*) and snakeskin gourami (*Trichogaster pectoralis*). Fiebiger (1909) proposed a viral etiology for papillomas in climbing perch in aquariums based on epidemiology and cytology. However, experimental evidence was never obtained to support viral involvement for transmission and development of lesions. Nigrelli (1952) also observed papillomas in climbing perch residing at the New York Aquarium. Lesions were seen on the lips, operculum and fins, but attempts to transmit the disease were unsuccessful and no viral particles were detected.

Frerichs *et al.* (1991) isolated C-type viral particles from cell culture supernatant of the three species, with a buoyant density of 1.15-1.16 g/ml. Virions had a diameter of 85-90 nm, an electron dense core and an electron-lucent ring surrounding the core. RT activity was found at the same density; it was $Mn^{2+}$ and poly(rA):oligo(dT) dependent.

Transmission experiments in cell culture were performed; a blue gill fry cell line was inoculated with the above supernatant. CPEs were apparent after 6-10 days with a drop in cell number. Between 10-14 days complete destruction of the cell monolayer had occurred. No experimental transmission studies to members of these piscine species have been undertaken (Frerichs *et al.* 1991).

Frerichs *et al.* (1991) discuss further research to determine whether the retroviruses are horizontally transmitted as infectious exogenous particles, or vertically transmitted as endogenous particles in the germline. The presence or absence of proviral sequences in the genomic DNA of these species may clarify the mode of acquisition.

### 11. Miscellaneous References

Two other piscine diseases, brown bullhead (*Ictalurus nebulosus*) plasma cell leukemia and splake (lake trout *Salvelinus namaycush* x brook trout *S. fontinalis*) lymphosarcoma were reported to have hematopoietic neoplasms associated with retroviruses, RT activity and transmissibility (Sonstegard 1979). Unfortunately, the sole reference found was an abstract.

## X. Discussion

Interpretation, impact, screening, monitoring and prevention of retroviral diseases in fish are complex undertakings. Demonstrated throughout this paper, the exact cause of disease might range from cytopathic effects produced by a virus to a multifactorial etiology. The impact of retrovirally transmitted diseases is largely unknown because there is no system to easily assay populations and the majority of species affected are not economically important. Screening and monitoring assays and vaccines need to be developed for commercially important species to prevent epidemics in hatchery situations.

### A. Interpretation of Retroviral Diseases

Interpretation of disease etiology presented in this paper points to three possible categories: (1) naturally occurring retroviruses, (2) chemical enhancement of retroviruses and (3) induction of endogenous retroviruses.

Infection via naturally occurring retroviruses is represented by Walleye dermal sarcoma, Plasmacytoid leukemia, Esocid lymphosarcoma and Damselfish neurofibromatosis. In each disease the causal factor for development is presence of retroviral particles. Even though more work is needed to conclusively establish a viral etiology, several factors have been ruled out. Positive transmission experiments, including juveniles, have eliminated sexual maturity, and environmental variables such as pollutants and carcinogens.

Chemical enhancement of retroviral diseases, is an important area for research. Pollutants in rivers, lakes and oceans continue to increase giving pre-existing diseases an advantage by creating more virulent strains of viruses, rendering organisms more susceptible to disease, and causing genetic damage to organisms. Casto (1974) demonstrated an increase in viral transformation of cells when exposed to chemical carcinogens. The increase is believed to take place due to gaps created in host DNA by the carcinogens. The virus has been provided additional sites for integration.

White sucker papilloma exhibits increased lesion development in more heavily polluted waters (Sonstegard 1973; Sonstegard 1977; Smith *et al.* 1989a; Smith *et al.* 1989b; Harshbarger and Clark 1990; Hayes 1990). Tumor regression experiments linked tumors to chemically laden water. A significant percentage of lesions regressed when affected animals were removed to a clean environment (Smith and Zajdlik 1987; Hayes *et al.* 1990). Another possibility is that the viruses are acting as co-carcinogens with the pollutants (Sonstegard 1973; Smith *et al.* 1989a).

Induction of endogenous retroviruses is exhibited by *Xiphophorus* sp. hybrid neuroblastoma and the three piscine cell lines discussed (Kollinger *et al.* 1979; Perlmutter and Potter 1987; Frerichs *et al.* 1991). *Xiphophorus* sp. produced viral particles after exposure to BrdU (Kollinger *et al.* 1979; Perlmutter and Potter 1987). Spontaneous production of retrovirus particles occurred in three Southeast Asian fish cell lines (Frerichs *et al.* 1991).

There is no clear relation between *Xiphophorus* sp. and the piscine cell lines. Induction of endogenous viral packaging by injection of a chemical known to have this effect is straight-forward. On the other hand, production of retroviral particles by the cell lines could be attributed to adaptations by cells to grow in culture since normal *in vivo* regulation parameters have been removed. Adaptations include: lower growth factor requirements; absence of differentiation; reduced cohesiveness; and immortality.

## B. Impact of Retroviral Diseases

The impact of retroviral diseases on fish populations is largely unknown, except for a few commercially important, hatchery-raised species. Assessment of wild populations would be difficult, costly and time consuming.

Carlisle and Roberts (1977) reported the most common complication of Atlantic salmon papilloma was a fatal secondary infection by fungi or bacteria. Ulceration of tissue created an entry point for a second pathogen. Weakened by the viral infection, the immune system might not be able to fight the secondary disease. Deaths attributed to bacteria, fungi and other pathogenic microorganisms may have an underlying retroviral cause.

One impact, not addressed by the researchers, is the possible reservoir of retroviruses in the environment. Asymptomatic fish can not only infect healthy individuals of their own species, but interspecies transmission has been demonstrated with Plasmacytoid leukemia (Newbound and Kent 1991). Exotic species could prove to be a source of viruses pathogenic to native species. These reservoirs for disease would have the strongest impact on commercially important species, such as the salmonids.

## C. Detection and Monitoring of Fish Pathogens

Management of major fish diseases in hatchery situations depends on detection/screening and monitoring procedures that are reliable, fast and cost effective. Detection of a pathogen (virus, bacteria, fungi) involves discovery of foreign antigens, usually proteins, from the invading microorganism. Monitoring populations through antibody titers to a pathogen or through antigen titers, enables identification of new infections, monitoring of current infections and determination of exposure to specific diseases.

Advances in biotechnology have made available several extremely sensitive and specific assays. Unfortunately, specificity or sensitivity of the assay is sacrificed when trying to develop tests that are easy to perform (Hardy 1991). The most sensitive and specific techniques are: (1) immunofluorescent antibody test (IFA); (2) radioimmunoassay (RIA); (3) immunoblot (Western blot); (4) the Polymerase Chain Reaction (PCR); and (5) nucleic acid probes. Main drawbacks of these assays are the need for specialized equipment and personnel, and they are time consuming. The Enzyme-Linked Immunosorbent Assay (ELISA) is sensitive, does not require highly specialized equipment or personnel, but is less specific (Hardy 1991).

IFA can be used for screening and monitoring populations since it can be designed to detect antigens or antibodies. Swanson (1981) employed IFA to monitor the presence of infectious pancreatic necrosis virus (IPNV) antigens in brook trout. Vestergaard *et al.* (1991) detected rainbow trout (*Oncorhynchus mykiss*) antibodies to infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) by IFA. Arnzen *et al.* (1991) coupled monoclonal antibodies to the glycoprotein and nucleoprotein of IHNV with IFA. Detection of infected fish or eggs was accomplished in 10-12 hours. Arnzen *et al.* (1991) feel this is a rapid test, especially when faced with mandatory destruction of samples proven or suspected of contamination.

RIA can be used for screening populations because it detects host antibodies. Western blots can be useful in both screening and monitoring because they identify foreign proteins. Until a reliable PCR diagnostic assay was developed for HIV detection, a Western blot was used to confirm viral presence.

PCR is another assay that can be employed as both a screening and monitoring tool. PCR detects unique sequences in the pathogen's nucleic acid. Ideally, a conserved sequence should be targeted to detect more than one strain. PCR is a valuable assay not only due to its high specificity and sensitivity, but also its ability to detect integrated and latent viruses (Arakawa *et al.* 1990). Amplification of a nucleoprotein gene sequence of IHNV was reported by Arakawa *et al.* (1990).

Nucleic acid probe assays are similar to PCR assays because they detect unique pathogen sequences; different strains can be identified if conserved regions are probed; and they can be used for screening and monitoring. Batts *et al.* (1993) synthesized DNA probes to specific sequences of the nucleoprotein gene of VHSV. Three probes were developed, with slightly altered sequences; they recognized: (1) the European strain; (2) the North American strain; and (3) both the European and North American strains.

ELISA, like IFA, can be designed to detect antigens or antibodies and therefore serve to screen and monitor populations. Due to its low cost in time and materials, as well as its relatively high sensitivity and reproducibility, ELISA is a valuable assay for screening large numbers of organisms (Vestergaard *et al.* 1991). Detection of rainbow trout antibodies to IHNV and VHSV by ELISA was reported by Vestergaard *et al.* (1991). Domínguez *et al.* (1990) also report ELISA detection of monoclonal antibodies to IPNV. Several commercial ELISA kits are available for feline leukemia virus (FeLV) diagnosis (Tonelli 1991).

## D. Vaccine Development

Ellis (1988a) outlines three main stages for vaccine development: (1) identification of protective antigens, antigens capable of eliciting a protective immune response; they are involved in virulence or pathogenicity of the invading organism; (2) antigen production; and (3) antigen delivery in an immunogenic form, a form that will invoke a strong immune response and long-lasting protection; antigen modification may be necessary.

An effective vaccine must be safe, immunogenic and protective. Stimulation of the immune system must occur without producing clinical illness. Inactivated viruses are safer to use than live attenuated viruses, but both may create unwanted side effects. Not all antigens associated with virulence are strongly immunogenic, when this is the case the antigen may be altered to induce a better response. Some modifications include attachment of adjuvants, immunostimulators, or cleavage of the antigen molecule to remove epitopes that stimulate T suppressor cells. The main goal of vaccination is to provide immunity. As mentioned, not all protective antigens induce a strong response, however, they must be included in the vaccine to stimulate antibodies against virulence factors of the pathogen (Ellis 1988a).

Advances in biotechnology are also seen in vaccine development, unfortunately these techniques have similar drawbacks to the detection/monitoring assays. They are not cost effective, due to the limited market for fish vaccines, and require specialized equipment and personnel. Some of the advances include: (1) genetic recombination, which is used to mass produce antigens by inserting the antigen encoding gene into a microorganism that is easily cultured; (2) genetic attenuation, that involves insertion or deletion mutations in genes that code for some of the virulent antigens; wild type reversion should be impossible; (3) protein engineering, which improves immunogenicity of an antigen by cleavage of epitopes that stimulate T suppressor cells; it is also used to mass produce protective antigens that are short amino acid sequences, "peptide vaccines"; and (4) anti-idiotype vaccines, which are antibodies. They are produced by making antibodies (idiotype) to the antigen and then raising antibodies (anti-idiotypic antibodies) to the first antibodies. The anti-idiotype can be used as a vaccine because the antigen-binding site resembles stoichiometrically the original antigen. This route is favorable when the protective antigen is difficult to obtain or produces severe side effects (Ellis 1988a).

There will be an increased demand and market for effective and economical protection against fish diseases in the future. Natural fisheries are being depleted, fish farming is becoming established in developing countries and the world population is expected to double in 40 years (Lantigua 1995). New techniques may provide manufacturers with more efficient ways to mass produce vaccines.

Several viral pathogens of fish have been studied and vaccine development investigated. To date only one of the following diseases has a commercially available vaccine.

Channel catfish virus (CCV) is a herpesvirus that is transmitted horizontally; outbreaks are restricted to the United States. Inactivated virus elicits a poor immune response, but live attenuated virus has shown more promising results. No commercial vaccine is available (Plumb 1988).

IHNV is a rhabdovirus enzootic to western North America that affects salmonids. It has traveled to Europe, Japan and Taiwan via transportation of infected stocks (Vestergaard *et al.* 1991). IHNV is highly destructive, directly through mortality and indirectly through mandatory destruction of infected fish and eggs; transmission is vertically and horizontally (Batts *et al.* 1991). Three vaccine types have been studied; inactivated, attenuated and peptide, all three produce varying degrees of protective immunity (Leong *et al.* 1988).

IPNV is a birnavirus that affects salmonids world-wide (Domínguez *et al.* 1990); it is highly destructive, vertically and horizontally transmitted and survivors of outbreaks can become carriers and shed virus for life (Swanson 1981; Dorson 1988). Formalin-killed vaccines showed positive results with no risk of infecting healthy fish. Immunization of broodstocks 3-4 months prior to spawning allows enough time for production of neutralizing antibody titers sufficient to produce IPNV-free sex products (Sano *et al.* 1981). Live attenuated vaccines have also been studied; no vaccine has been marketed (Dorson 1988).

Spring viraemia of carp (SVC) is caused by a rhabdovirus. It has a European distribution, seasonal occurrence and horizontal transmission; survivors can become carriers. Bioveta, CSSR, manufactures the only commercially available vaccine; it contains two inactivated strains of the virus (Fijan 1988).

VHSV is a rhabdovirus that affects salmonids in Europe and North America. It is highly destructive, directly through mortality and indirectly through mandatory destruction of infected fish (Batts *et al.* 1993).

Transmission is horizontal and survivors can become carriers. Inactivated and live attenuated vaccines were studied; no vaccine has been marketed (de Kinkelin 1988).

When dealing with an unknown or highly variant pathogen, e.g. *Vibrio sp.*, a non-specific defense mechanism may be advantageous to vaccination (Anderson 1988). Immunostimulants can produce a non-specific response, especially in the phagocytic system (Ellis 1988b). Substances that provide immunostimulation, in fish and related species, include: Ete, Freund's Complete Adjuvant, FK-156, (Kitao and Yoshida, 1986), and levamisole (Siwicki 1987).

The viral pathogens and vaccination research discussed above do not include retroviruses. Since retroviruses have the ability to mutate and adapt to their environment rapidly, development of vaccines against them is even more challenging. I will discuss research of vaccine development and the problems encountered with two retrovirally transmitted diseases, FeLV and HIV.

### E. Feline Leukemia Virus

FeLV vaccinations are routinely administered to domestic cats, even though their value has been controversial. Efficacy, the desired immune response to protect against the disease, varies from study to study (Pollock and Haffer 1991; Sebring *et al.* 1991). Several problems researchers faced during development of the FeLV vaccine include: type specific vs. group specific antigens, immunosupression and possible oncogenesis from live attenuated vaccines (Hardy and Zuckerman 1991; Pollock and Haffer 1991).

FeLV has several strains and genetic variation is expressed as type specific antigens; the envelope antigens (Hardy and Zuckerman 1991). Basing a vaccine on group specific antigens, antigens identical for all subgroups, would bypass variations from strain to strain. Unfortunately, low immune responses to internal, group specific antigens of FeLV are seen (Hardy and Zuckerman 1991).

Immunosupression, by early versions of FeLV vaccine, was associated with the envelope protein, p15e, which inhibits lymphocyte development. Live attenuated vaccines of FeLV were not considered practical due to possible genetic recombination or oncogenesis (Pollock and Haffer 1991).

In recent efficacy testing, Sebring *et al.* (1991), concluded a live virus recombinant vaccine provided protection against FeLV, but was not considered a good candidate because of environmental safety issues in obtaining a USDA license. Several adjuvant systems were tested; type and concentration of adjuvant were critical for vaccine performance. Whole killed virus preparations conferred immunity to >90% of cats vaccinated.

## F. Human Immunodeficiency Virus

Limited AIDS vaccinations began in 1987, however, no full scale testing is in place due to controversy, scientific and ethical, surrounding HIV vaccines. Several candidate vaccines did not pass critical laboratory testing last year, but clinical trials to determine their effectiveness are still sought. Ethically, scientists are wrestling with their moral obligation to educate volunteers in a vaccination program to better protect themselves against HIV. This would unfortunately bias the results (Cohen 1994).

Researchers have encountered unique problems in developing a vaccine against HIV: immunosupression; enhancement of viral entry, escape mutants (Merigan and Kundu 1994); and use of native vs. denatured protein in vaccines (Scandella *et al.* 1993).

Merigan and Kundu (1994) report three areas of difficulty in HIV vaccine development. First, immunosupression by inhibition of lymphocyte development. As seen with FeLV, immunosupression is associated with an envelope protein, gp120. Second, enhancement of viral entry into cells. This phenomenon may result from immunization antibodies or production of cytotoxic cells that kill host cells. Third, production of escape mutants. HIV has the ability to rapidly mutate to its environment and escape mutants are the result. They are not affected by vaccines or drug therapy.

Using a conserved discontinuous epitope on gp120, Scandella *et al.* (1993) demonstrated that the native structure of gp120 is important for immune recognition. A subset of antibodies isolated from HIV-positive patients binds to native gp120, but not denatured gp120. Primates injected with native gp120 neutralize geographically diverse HIV-1 isolates; injection with denatured gp120 does not elicit this response.

The World Health Organization Working Group (WHOWG) (1994) summarized experimental vaccines that are in development, they include: (1) whole inactivated virus; (2) subunit antigens produced by recombinant DNA techniques; (3) synthetic peptides; and (4) live attenuated virus. Live attenuated vaccines are considered too dangerous due to possible recombination, oncogenesis or transmission through sexual contact, blood, or gametes (WHOWG 1994; Merigan and Kundu 1994).

Recombinant technology may prove to be the best research avenue. Anderson (1988), Ellis (1988a), and Merigan and Kundu (1994) discuss the appeal of bulk antigen production, greater purity and reproducibility through genetic engineering. Merigan and Kundu (1994) also mention the flexibility of recombinant vaccines; modifications to create appropriate second and third generation vaccines in culture, might provide a way for researchers to keep pace with HIV mutations.

## G. Commercial Production and Licensing of Fish Vaccines

Commercial production and licensing of fish vaccines are costly and extensive processes. Even though aquaculture is a rapidly growing and commercially important industry, demand is not yet strong enough to support the financial investment needed to develop, manufacture and license commercial vaccines.

Development of commercial vaccines has three basic phases: (1) identification of protective antigens; (2) establishment of a protective antigen source; and (3) production and stability of protective antigens (Horne and Ellis 1988).

Identification of protective antigens may yield one that elicits a stronger immune response than the others. This may be important when selecting the vaccination route: oral, immersion or injected because there is the potential for variation of efficacy (Horne and Ellis 1988).

Strain variation within pathogens will play a role in antigen source for a vaccine. If different serotypes produce a protective response, then the various strain antigens must be incorporated into the vaccine for complete protection. Vaccines of this type are multivalent (Horne and Ellis 1988).

Viability and antigen expression after long-term storage is an important factor in vaccine development. Strain variation may lead to identification of an optimal type for vaccine use. During initial stages of development, strains are stored by routine methods such as liquid nitrogen, lyophilization and ultra-low freezing. Samples are periodically reconstituted to determine viability and antigen expression. Strains are also tested for ease of mass culturing. Special culturing techniques and media may be necessary for optimal growth, expression and harvesting of the desired antigens (Horne and Ellis 1988).

Licensing a new vaccine may take as long as the development. Licensing authorities, such as the Food and Drug Administration (FDA), require commercial vaccines to perform consistently in specific areas; potency, safety and stability (Horne and Ellis 1988).

A minimum standard of potency is set for each vaccine and every batch must meet or exceed this value. Potency testing involves vaccination and challenge, exposure to the pathogen after vaccination. Mortality is then compared to mortality in unvaccinated control fish exposed to the pathogen (Horne and Ellis 1988).

Fish safety is the main concern and reason for vaccine development. Licensing authorities usually require a toxicity testing of the vaccine; this involves a "double-safe" procedure. Fish are exposed to twice the recommended concentration for commercial use. Care must be taken when introducing a vaccine into a species not previously tested. Sensitivity varies from species to species, fresh water to salt water, and temperature to temperature (Horne and Ellis 1988).

As with any drug, shelf-life must be determined prior to commercial sale of the vaccine. An important element affecting long-term stability of an antigen is the container type in which it is stored, glass or plastic. The manufacturer must then establish the shelf-life by storing several batches following the recommended storage instructions. Periodically, aliquots are tested for efficacy to determine expiration dates (Horne and Ellis 1988).

The unusual mix of aquaculture growth and advancements in protein and genetic engineering may provide a unique stimulus for a serious investment in the mass production of fish vaccines. The potential market will continue to grow as fish farming is established in third world countries, while new techniques in biotechnology fields will provide more efficient and cost effective ways to manufacture vaccines to fish diseases Short-term goals should include development of reliable, rapid and affordable screening and monitoring assays. Until vaccines are widely available, management of fish disease will depend on identification of pathogens to control epidemics, therefore controlling losses.

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