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# A Retrovirus in Chinook Salmon (*Oncorhynchus tshawytscha*) with Plasmacytoid Leukemia and Evidence for the Etiology of the Disease<sup>1</sup>

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## ABSTRACT

A plasmacytoid leukemia (PL) has caused mortalities in chinook salmon (*Oncorhynchus tshawytscha*) reared in seawater netpens in western British Columbia, Canada, since 1988. Kidney or eye tissues from 11 of 13 fish from netpens with clinical PL had reverse transcriptase (RT) activity. This RT activity was associated with virus particles of retrovirus morphology and buoyant density. In a transmission experiment, PL-positive donor fish tissues also had RT activity and virus particles of retrovirus morphology and buoyant density, as did recipient fish tissues following development of the disease 6 weeks postinjection with a tissue homogenate from the donor fish. Kidney and spleen tissues from fish that developed PL following injection with an inoculum that was passed through a 0.22- $\mu$ m filter, in a separate experiment (M. L. Kent and S. C. Dawe. Further evidence for a viral etiology in the plasmacytoid leukemia of chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org., in press, 1992), also exhibited RT activity.

The virus particles observed by electron-microscopic examination of tissues or sucrose fractions from PL-positive fish were enveloped and were about 110-nm diameter with a central electron-dense core. Polypeptides of about *M*<sub>r</sub> 120,000, 80,000, 42,000, 27,000, 25,000, 22,000, and 19,000 were observed when purified virus particles were examined by polyacrylamide gel electrophoresis analysis.

Many infectious neoplasms of animals, including fishes, are caused by retroviruses. The evidence in this study shows the presence of a retrovirus in chinook salmon with PL and further suggests a retroviral etiology of the disease. We are tentatively calling this virus salmon leukemia virus.

## INTRODUCTION

A PL<sup>3</sup> of chinook salmon (*Oncorhynchus tshawytscha*), referred to as marine anemia by fish farmers, has caused extensive mortality at numerous seawater netpen facilities in western British Columbia, Canada, since 1988 (1, 2). The disease has been characterized as a proliferation and infiltration of plasmablasts into the visceral organs and retrobulbar tissue of the fish (1). Although no etiological agent of PL was identified, the disease has been shown to be caused by an infectious agent, inasmuch as intraspecific transmission was repeatedly achieved by injection of thoroughly homogenized tissue from affected chinook salmon (2, 3).

The disease can be transmitted from chinook salmon to sockeye (*Oncorhynchus nerka*) and Atlantic salmon (*Salmo salar*) (2, 3). Transmission between different species (chinook and sockeye salmon) and different genera (chinook and Atlantic salmon) support the assumption that the disease is caused by an infectious agent, as opposed to transplantation of neoplastic

cells. Furthermore, PL has been recently transmitted with an inoculum passed through a 0.22- $\mu$ m filter.<sup>4</sup>

Infections by the intranuclear microsporidium, *Enterocytozoon salmonis*, and bacterial kidney disease, caused by *Renibacterium salmoninarum*, are frequently observed in fish with PL. However, transmission studies demonstrated that these 2 conditions were not the primary cause of PL, as the disease could be transmitted in the absence of either of these pathogens (2, 3). Essentially all infectious neoplasms are caused by oncogenic viruses, and these transmission studies led us to hypothesize that PL is caused by an oncogenic virus, perhaps a retrovirus.

Retroviruses are known etiological agents of many leukemias and leukemia-like diseases in higher vertebrates (4), and have also been associated with hemic (5-9) and nonhemic neoplasms (10-17) in several fish species. In this paper, we describe the presence of a retrovirus in chinook salmon with PL and the evidence suggesting a retroviral etiology of the disease.

## MATERIALS AND METHODS

**Source of Material: Field Fish.** Thirteen different chinook salmon with clinical PL from several private aquaculture facilities were brought to the Department of Fisheries and Oceans Pacific Biological Station in Nanaimo, British Columbia, Canada. All had been reared in seawater netpens that contained fish experiencing a PL-associated epizootic. The fish showed typical signs of PL, such as swollen kidney and spleen, pale gills, bloody ascites, and bilateral exophthalmia. Samples were collected and processed for histological and electron-microscopic examination. Approximately 10 g of kidney tissue from 11 of these fish and 10 g of tumor tissue from the orbit of the eye from 2 of these fish were removed and processed for RT analysis. Ten g of a dermal sarcoma from walleye pike (*Stizostedion vitreum*) were used as a positive control, as RT activity and retrovirus particles have been observed in this neoplasm (15, 17). In addition, 20 ml of supernatant from a BF-2 cell culture 5 days postinoculation with the snakehead retrovirus (18) were centrifuged at 100,000  $\times$  g for 1 h and resuspended in 0.5 ml of TNE and then used as an additional positive control for the RT assay. Ten g of kidney tissue from normal chinook salmon from an unaffected population were used as a negative control.

**Source of Material: Transmission Study Fish.** A transmission study was conducted using a tissue homogenate prepared from kidney and spleen of PL-affected fish. Kidney tissues from 3 PL-positive chinook salmon from one of the netpen facilities previously mentioned were diluted 1:4 (v:v) in minimum essential medium and thoroughly homogenized with a Polytron (Brinkmann Instruments) at 4°C. The homogenate was examined by wet mount preparation (5 fields of view) to ensure that no intact cells remained. This homogenate was used as the donor inoculum for the transmission study. The donor inoculum was examined for RT activity and by electron microscopy.

Thirty apparently healthy chinook salmon (average weight, 60 g) were used as recipient fish and were given i.p. injections of the donor inoculum (0.5 ml/fish). The fish were held in tanks with flow-through fresh water at about 13°C. Twenty control fish were given injections of 0.5 ml TNE and similarly maintained. At 6 weeks PI, kidney and spleen from 3 recipient fish with clinical and histological signs of PL and 3 control fish were collected for RT analysis. The tissues from these

<sup>4</sup> M. L. Kent and S. C. Dawe. Further evidence for a viral etiology in the plasmacytoid leukemia of chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org., in press, 1992.

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<sup>3</sup> The abbreviations used are: PL, plasmacytoid leukemia; RT, reverse transcriptase; TNE, 0.01 M Tris-HCl, pH 7.5, 1.0 mM EDTA, 0.1 M NaCl; PI, postinjection; TCA, trichloroacetic acid; DNAP, DNA polymerase; SLV, salmon leukemia virus.

2 groups were separately pooled to supply 10 g of either PL-positive or PL-negative tissue, then processed for RT analysis and electron-microscopic examination.

PL-affected donor and recipient tissues from a separate cell-free filtrate transmission study reported by Kent and Dawe<sup>4</sup> were acquired and also analyzed for RT activity. The samples analyzed were from PL-positive donor kidney and spleen tissues, and kidney and spleen tissues from recipient fish after they developed PL 100 days following injection with the donor inoculum that had passed through a 0.22- $\mu$ m filter. Ten g of donor, 10 g of recipient, and 10 g of negative control fish tissues (pooled from 4 fish) were analyzed for RT activity.

**Tissue Processing for Reverse Transcriptase Analysis.** All tissues to be analyzed (approximately 10 g/sample) were frozen and thawed, then diluted 1:4 (w:v) in 0.01 M Tris-HCl, pH 7.5, 1.0 mM EDTA, 0.1 M NaCl, 5% sucrose and homogenized with a Polytron at 4°C. Homogenates were centrifuged for 30 min at 10,000  $\times$  g, and the supernatants collected and centrifuged at 100,000  $\times$  g for 90 min. The pellets were resuspended in 1 ml 0.01 M Tris-HCl, pH 7.5, 1.0 mM EDTA, 0.1 M NaCl, 5% sucrose; layered over a 15, 35, and 50% sucrose step gradient; and centrifuged for 90 min at 100,000  $\times$  g. The region at the 35–50% sucrose interface was removed, centrifuged at 100,000  $\times$  g for 90 min, and resuspended in 1 ml of TNE and 0.01 M dithiothreitol. These samples and the snakehead retrovirus pellet described above were used as crude samples for RT analysis.

Tissues from pen-reared PL-positive chinook salmon (nos. 2, 8, and 10) and PL-negative chinook salmon, the pooled donor and pooled recipient fish from the crude tissue homogenate transmission study, and the negative control fish from the transmission study were further processed through a 10–60% continuous sucrose gradient at 100,000  $\times$  g for 20 h. After centrifugation, the gradients were fractionated into 0.3-ml samples. The refractive index of each fraction was determined with a refractometer, the buoyant density was then calculated, and the RT activity of each fraction was determined.

**Reverse Transcriptase Assay.** The RT assays were performed as described by Tomley *et al.* (19) with the following exceptions: poly r(A):oligo d(T) was used as the primer for RT activity, [<sup>3</sup>H]TTP was used as the radiolabel, 0.2% Nonidet P-40 (final concentration) was added to in the cocktail, and the Whatman GF/C filter disks containing the samples were washed for 5 min in each of the following: 10% TCA, 5% TCA, 5% TCA in 1% sodium dodecyl sulfate, and 95% ethanol prior to determining the acid-precipitable cpm.

The RT activity was determined by subtracting the acid-precipitable cpm of a sample at the start of the incubation period ( $T_0$ ) from the cpm of the sample after 60 min incubation ( $T_{60}$ ). An identical procedure was conducted using the cellular DNAP primer poly d(A):oligo d(T) to provide a measure of DNA-dependent DNAP activity. The ratio of the activity at  $T_{60}$ - $T_0$  using the poly r(A) primer and the poly d(A) primer was calculated  $\{T_{60}$ - $T_0$  [poly r(A)]: $T_{60}$ - $T_0$  [poly d(A)] $\}$ . A ratio  $\geq 4$  was considered positive for RT activity and not due to DNA-dependent DNA polymerase activity. The RT assays performed on the gradient fractions were only reported as the  $T_{60}$ - $T_0$  [poly r(A)] values as these samples originated from crude samples shown to be RT-positive.

**Electron Microscopy and Histology.** Tissues for electron-microscopic examination were fixed in 4% glutaraldehyde in Millonig's phosphate buffer, postfixed in 1% OsO<sub>4</sub>, embedded in Epon plastic, sectioned, and stained with lead citrate and uranyl acetate. Gradient fractions were centrifuged for 60 min at 100,000  $\times$  g, resuspended in 100  $\mu$ l TNE. Carbon-nitrocellulose grids were floated onto a drop of the suspension for 10 min, the excess fluid removed, then placed onto a drop of 2% glutaraldehyde (prepared in 0.1 M cacodylate buffer) for 10 min, the excess fluid again removed and the grid transferred to 2% uranyl acetate at pH 4 for 2 min. The excess stain was removed, the grid dried, and then examined.

Tissues for histological examination were preserved in Davidson's solution (20), processed for histology using standard methods, and examined by light microscopy to confirm the presence or absence of PL.

**Virus Purification from PL+ Tissues and Analysis.** One hundred g of kidney and spleen tissues from fish with PL from one of the previously mentioned aquaculture facilities were processed through fractionation on sucrose gradients. Fractions with a buoyant density of 1.16–

1.18 g/ml were pooled, and the RT activity was determined, then processed, and examined by electron microscopy. The sample was analyzed by polyacrylamide gel electrophoresis using standard methods (21). Tissues from normal chinook were similarly processed and analyzed as negative controls.

## RESULTS

**Gross and Histopathological Changes.** All fish collected from the field exhibited gross and histopathologic changes consistent with PL, as described previously (1). The fish were anemic and exhibited serosanguinous ascites and reno- and splenomegaly. Two fish exhibited severe bilateral exophthalmos due to accumulation of neoplastic tissue in the retrobulbar area. Histological examination revealed infiltration of plasmablasts in the visceral organs and in the retrobulbar tissue of the affected eyes.

In the crude tissue homogenate transmission study, all the recipient fish developed PL and began to die with clinical and histological signs of the disease at 4 weeks postinjection. Three fish with the most severe clinical signs of PL were killed at 6 weeks postinjection for RT analysis.

**RT Activity.** Eleven of the 13 crude samples from the PL-positive field fish, the walleye pike dermal sarcoma, and the snakehead virus crude samples showed RT activity with  $T_{60}$ - $T_0$ [poly r(A)]: $T_{60}$ - $T_0$ [poly d(A)] ratios as high as 14.6 (Table 1). The PL-negative and the remaining 2 PL-positive fish crude samples did not show RT activity, nor was there any DNA-dependent DNAP activity associated with any of the field fish samples. The RT activity from fish nos. 2, 8, and 10 was associated with a buoyant density of about 1.16 to 1.18 g/ml in sucrose, and no RT activity was associated with any fraction from the normal chinook salmon tissues (Fig. 1).

Results of the crude tissue homogenate transmission study showed that by 6 weeks PI, all the recipient fish exhibited gross and histological evidence of PL, while the negative control fish did not. The 3-fish pooled tissue samples from the recipient and donor fish showed RT activity, with  $T_{60}$ - $T_0$  [poly r(A)]: $T_{60}$ - $T_0$  [poly d(A)] ratios up to 14.1, whereas the negative control fish did not (Table 1). In addition, the RT activity in both the recipient and donor samples was associated with a buoyant density of 1.16 to 1.18 g/ml in sucrose (Fig. 2). No RT activity was associated with any fraction from the negative control fish tissues, and there was no DNA-dependent DNAP activity associated with any samples from the transmission study.

The PL-positive donor and recipient tissues acquired from the cell-free filtrate transmission study reported by Kent and Dawe<sup>4</sup> also had RT activity,  $T_{60}$ - $T_0$ [poly r(A)]: $T_{60}$ - $T_0$ [poly d(A)] ratios as high as 27.6, while the negative controls did not (Table 1).

**Purified Virus Polypeptide Analysis.** The pooled fractions with a buoyant density in sucrose of 1.16–1.18 g/ml derived from PL-positive tissues showed positive RT activity (Table 1). When the sample was analyzed by polyacrylamide gel electrophoresis, 7 polypeptides  $\sim M_r$  120,000, 80,000, 42,000, 27,000, 25,000, 22,000, and 19,000 were observed (Fig. 3). There were no bands of similar molecular weight in samples from normal chinook salmon.

**Electron Microscopy.** Transmission electron-microscopic examination of tissues from the field fish revealed the presence of enveloped virus particles about 110 nm diameter, with a well defined electron dense core. Virus particles were seen in extracellular spaces near plasmablasts and apparently budding from intercellular membrane debris (Figs. 4 and 5). Examination of negative stains of RT-positive fractions with a 1.16 to 1.18 g/ml

Table 1 RT and DNAP directed activity in crude samples from PL-positive and PL-negative fish tissues

Sample	Synthetic template (cpm) <sup>a</sup>		
	poly r(A) (T <sub>60</sub> -T <sub>0</sub> )	poly d(A) (T <sub>60</sub> -T <sub>0</sub> )	r(A):d(A)
Pike dermal sarcoma	6,438	1,254	5.1
Snakehead retrovirus	8,766	1,192	7.3
Normal chinook kidney	2,540	1,101	2.3
<b>PL-positive chinook salmon</b>			
Fish no. 1-kidney	12,204	2,340	5.2
Fish no. 2-kidney	16,427	1,376	11.9
Fish no. 3-eye tumor	4,507	1,119	4.0
Fish no. 4-kidney	1,561	945	1.7
Fish no. 5-kidney	17,347	1,187	14.6
Fish no. 6-kidney	21,670	2,259	9.6
Fish no. 7-kidney	20,090	2,137	9.4
Fish no. 8-eye tumor	15,081	1,935	7.8
Fish no. 9-kidney	2,206	596	3.7
Fish no. 10-kidney	10,595	1,847	5.7
Fish no. 11-kidney	6,333	1,255	5.0
Fish no. 12-kidney	4,806	907	5.3
Fish no. 13-kidney	3,530	563	6.2
<b>Crude tissue homogenate transmission study<sup>b</sup></b>			
Negative controls	1,089	678	1.6
PL+ donor tissue	20,935	1,481	14.1
Recipient fish (6 wk PI)	12,580	1,695	7.4
<b>Cell-free filtrate transmission study<sup>c</sup></b>			
Negative controls	1,344	685	2.0
PL+ donor tissue	27,033	979	27.6
Recipient fish (6 wk PI)	16,580	1,624	10.2
<b>Purified virus<sup>d</sup></b>			
	10,595	1,622	6.5

<sup>a</sup> Samples were processed and analyzed for RT and DNAP activity as described in text using 20 µg of poly r(A):oligo d(T) or poly d(A):oligo d(T) primers, 50 µl sample, and 50 µl cocktail (0.1 M Tris-HCl, pH 8.3, 0.5 M KCl, 1.0 mM MnCl<sub>2</sub>, 4.0 mM dithiothreitol, 0.2 % Nonidet P-40, and 20 µCi/ml [<sup>3</sup>H]TTP). The acid-precipitable cpm were determined from each sample at time 0 (T<sub>0</sub>) and after 60-min incubation at 22°C (T<sub>60</sub>). A T<sub>60</sub>-T<sub>0</sub> [poly r(A)]:T<sub>60</sub>-T<sub>0</sub> [poly d(T)] ratio ≥4 is considered positive for RT.

<sup>b</sup> A crude tissue homogenate from PL+ donor fish was injected into apparently healthy fish as described in the text.

<sup>c</sup> A crude tissue homogenate from PL+ donor fish was passed through a 0.22-µm filter, then injected into apparently healthy fish as described in the text.

<sup>d</sup> Virus was purified from PL+ fish received from a netpen facility.

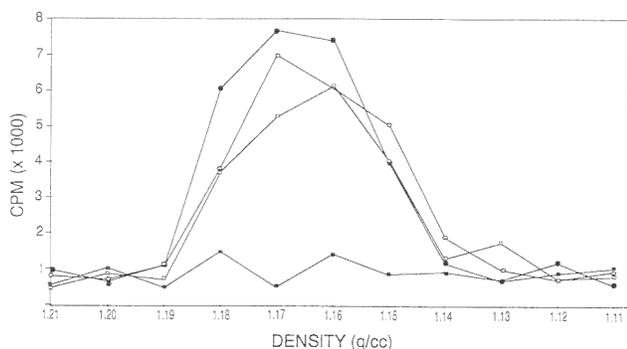


Fig. 1. Buoyant density and RT activity associated with sucrose gradient fractions from 3 PL-positive and 1 PL-negative field fish tissue extracts. The RT activity was the ratio of the acid-precipitable cpm at T<sub>60</sub> and T<sub>0</sub> following an RT assay (●, PL-positive fish no. 2; □, PL-positive fish no. 8; ○, PL-positive fish no. 10; ■, PL-negative fish).

buoyant density from field fish nos. 2, 8, and 10, the donor and recipient fish from the transmission study, and the purified virus samples from pooled PL-positive tissues used for polypeptide analysis revealed similar virus particles (Fig. 6).

**DISCUSSION**

Retroviruses have been associated with several neoplasms of fishes. C-Type retrovirus particles have been found in northern pike (*Esox lucius*) with epidermal hyperplasia (10, 14), walleye pike (*S. vitreum*) with dermal sarcoma (15, 17), Atlantic salmon (*S. salar*) fibrosarcomas of the swim bladder (13), white sucker (*Catostomus commersoni*) papillomas (12), and lentivirus-like particles in hooknose (*Agonus cataphractus*) fibromas (16). A

C-type retrovirus has also been described in 4 cell lines derived from 3 different species of fish from southeast Asia (18).

Retroviruses have also been implicated as etiological agents of at least 2 fish hemic neoplasias. A plasma cell leukemia in brown bullhead (*Ictalurus nebulosus*) was found to be transmissible and associated with high RT activity (9), and a lymphosarcoma of northern pike was transmissible, and associated with high RT values and C-type virus particles (5-8). Our study showed the presence of a retrovirus in chinook salmon with PL, which we are tentatively calling SLV. In addition, our results suggest that SLV is the etiological agent of the disease.

Tissues from 11 of 13 clinically PL-positive field fish had RT activity. In addition, this RT activity was associated with virus

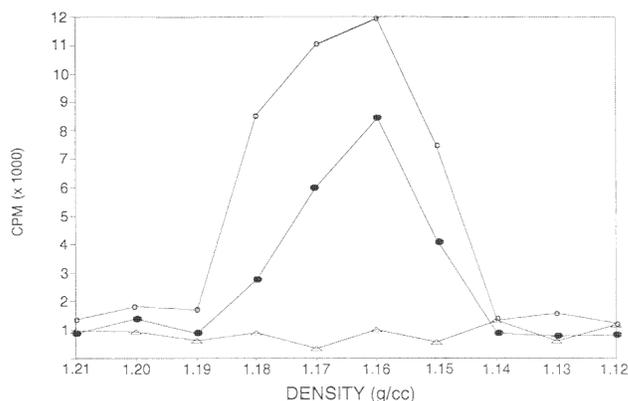


Fig. 2. Buoyant density and RT activity associated with sucrose gradient fractions from PL-positive donor and recipient fish, and negative control fish following a transmission study. The RT was the ratio of the acid-precipitable cpm at T<sub>60</sub> and T<sub>0</sub> following an RT assay (○, PL-positive donor fish; ●, PL-positive recipient fish 6 weeks postinjection; △, negative control fish).

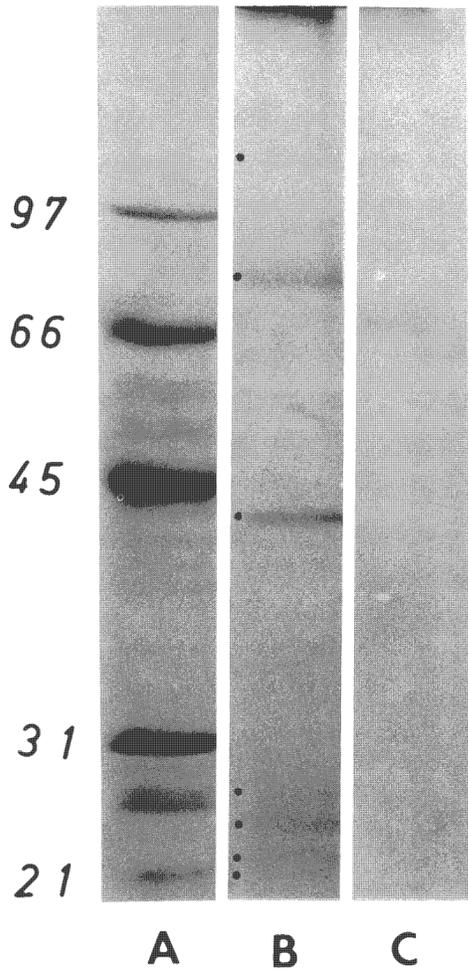


Fig. 3. Polyacrylamide gel electrophoresis of polypeptides associated with RT-positive virus particles, purified from PL-positive fish tissues (*Lane A*, molecular weight markers; *Lane B*, purified virus from PL-positive tissues; *Lane C*, PL-negative tissue extract treated in a manner identical to that of the virus samples).

particles of retroviral buoyant density and morphology. The crude tissue homogenate transmission study showed that PL-positive donor fish tissues had RT activity associated with virus particles of retroviral density and morphology, and that these characteristics could be transmitted to normal recipient fish along with the disease. In addition, donor fish tissues used for the cell-free filtrate transmission study of Kent and Dawe<sup>4</sup> had RT activity, as did the recipient fish tissues following development of PL 100 days after injection with the cell-free filtrate.

Although the activities of our RT assays were of low to moderate magnitude, the lack of any significant activity using poly d(A):oligo d(T) primer shows that the activity was not due to DNA-dependent DNAP, but was due to RT. Reverse transcriptase values as low or lower than those observed associated with SLV have been associated with retroviruses isolated from fish, dogs, rabbits, baboons, and humans with lymphosarcomas or leukemias (7, 19, 22–25). We are currently characterizing the RT activity of SLV with respect to requirements of MnCl<sub>2</sub> concentrations, temperature, and incubation period in an attempt to optimize the assay.

Many retroviruses produce polypeptides with molecular weights similar to those associated with SLV in the current study (26–28). The *M<sub>r</sub>* 42,000, 80,000, and 120,000 SLV proteins may represent structural proteins for the virus. Similar sized proteins are known to be structural components of bovine or murine leukemia viruses. They may also represent capsid and

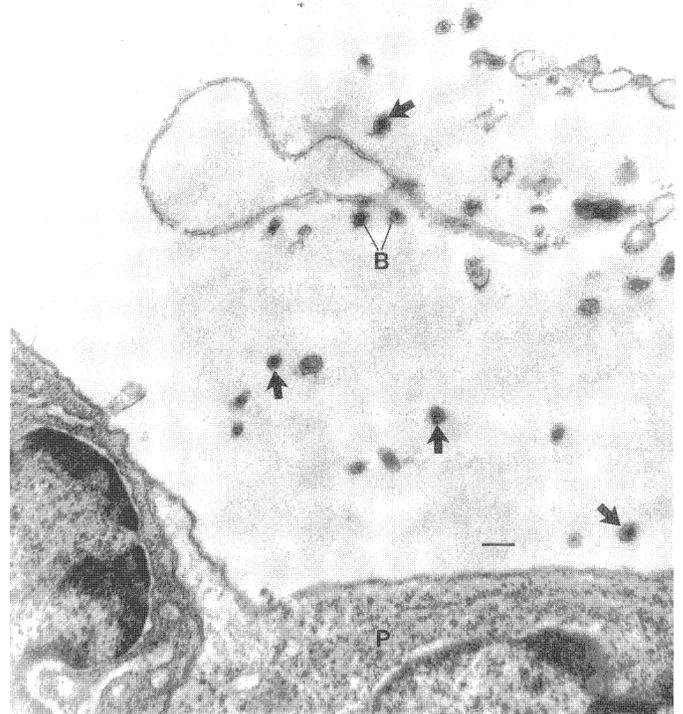


Fig. 4. Virus-like particles (*arrows*) in intercellular spaces between neoplastic plasmablasts (P) in reticuloblastic tissue. B, budding particles from membrane debris. Bar, 100 nm.

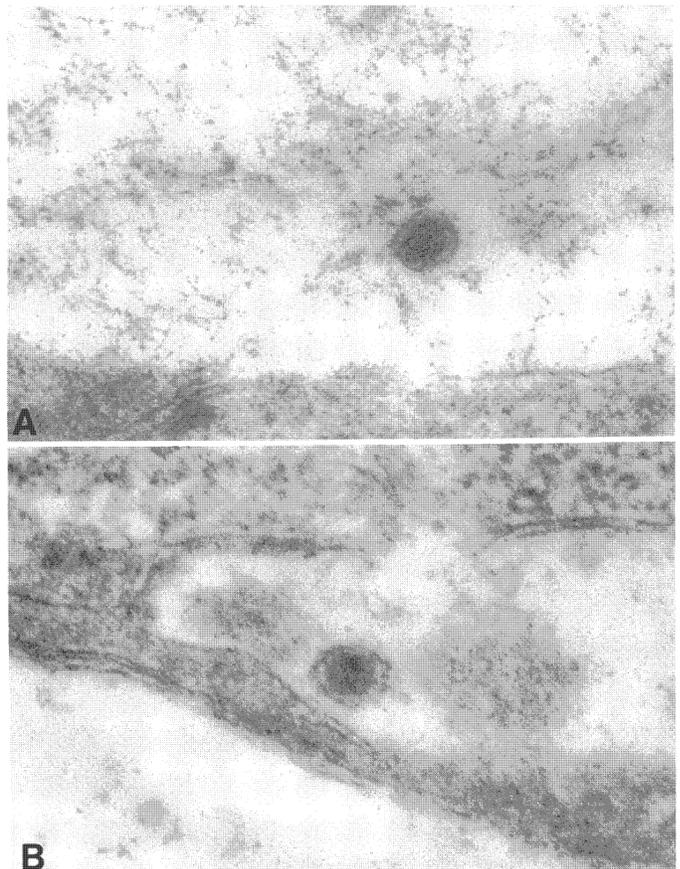


Fig. 5. Transmission electron micrographs of tissues from field fish with plasmacytoid leukemia. Enveloped virus particles of 110–120-nm diameter with a dense core are seen in extracellular spaces near plasmablasts (*A* and *B*). Bars, 100 nm.

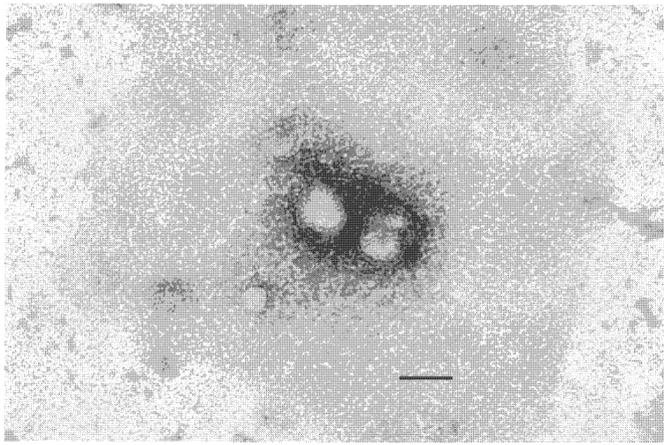


Fig. 6. Negative stain electron micrograph of virus particles from pooled sucrose gradient fractions with reverse transcriptase activity and buoyant densities of 1.16–1.18 g/ml derived from PL-positive tissues. The particles are enveloped, have a capsid, a double envelope, and are about 110-nm diameter. Bar, 100 nm.

capsid-associated precursor polypeptides, as retroviruses often have such proteins in these molecular weight ranges. The  $M_r$  19,000–27,000 proteins may represent the capsid and capsid-associated proteins of SLV, as numerous retroviruses have such structural proteins in the  $M_r$  24,000–30,000 and 11,000–20,000 ranges, respectively.

To determine whether the polypeptides associated with SLV are final structural components or precursor proteins, and whether other proteins are present that may not have been observed, we are conducting glycosylation staining and inhibition and pulse-chase experiments using purified SLV from PL-positive fish and virus grown in a cell culture derived from PL-positive tissues that appears to be similar to SLV.<sup>5</sup>

In summary, data presented here and previous observations indicate that: (a) PL is caused by an infectious agent that is transmissible through a 0.22- $\mu$ m filter (*i.e.*, a virus); (b) most infectious neoplasms, particularly hemic neoplasms, are caused by retroviruses; (c) PL-affected tissues consistently showed RT activity associated with retrovirus buoyant densities; (d) retrovirus-like particles were associated with this RT activity; (e) polypeptides consistent with those of retroviruses were associated with the retrovirus-like particles and RT activity; (f) the disease has been transmitted in the absence of other infectious agents; and (g) recipient fish showed RT activity and retrovirus-like particles. Consequently, a retrovirus, SLV, is associated with chinook salmon with PL, and we believe it to be the etiological agent of the disease. Work is currently under way in our laboratories to confirm the etiology of PL and to develop more rapid diagnostic procedures using cDNA probes and monoclonal antibodies.

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<sup>5</sup> W. D. Eaton, unpublished data.

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