

Hematopoietic intranuclear microsporidian infections with features of leukemia in chinook salmon *Oncorhynchus tshawytscha*

R. P. Hedrick¹, J. M. Groff¹, T. S. McDowell¹, M. Willis², W. T. Cox²

¹ Department of Medicine, School of Veterinary Medicine, University of California, Davis, California 95616, USA

² California Department of Fish and Game, Fish Disease Laboratory, Rancho Cordova, California 95670, USA

ABSTRACT: Intranuclear infections of hematopoietic cells with characteristics of lymphoblasts were detected in juvenile chinook salmon *Oncorhynchus tshawytscha* with a leukemic condition. The microsporidian infection was associated with an anemia secondary to the proliferation of hematopoietic cells in the kidney and spleen. Many of the nuclei of these lymphoid cells contained plasmodia and sporogonic stages of the microsporidian. Infected cells occurred in the kidney and spleen but were also found in the blood, eye, brain, muscle, liver, pancreas, intestine, peritoneum and gill. Spores develop from multinucleated sporogonial plasmodia which contain polar tube precursors. Spores are ovoid ($1.0 \times 2.0 \mu\text{m}$), have a thin exospore and poorly developed endospore surrounding a complex of membranes (polaroplast), a posterior vacuole, nucleus and cytoplasm containing a polar tube with 4 to 5 turns. The characteristic sporogony and spore morphology of the salmonid microsporidian is found only in the genus *Enterocytozoon*. The microsporidian stimulates an abnormal proliferation of host lymphoblasts and the subsequent migration and invasion of these infected host cells into various tissues resulted in a leukemic condition. A similar disease has recently been described among adult chinook salmon reared in seawater net-pens in British Columbia, Canada. The microsporidian was transmitted to previously uninfected kokanee salmon *O. nerka* by intraperitoneal injections of cells obtained from kidney homogenates of naturally-infected chinook salmon. These kokanee salmon also developed a similar leukemic condition to that observed in chinook salmon.

INTRODUCTION

Microsporidians are intracellular parasites of many animals and several genera are found in fish (Canning & Lom 1986). Several species have been detected in salmonids but there are only 3 reports of microsporidia that are found within the nuclei of parasitized cells. Modin (1981) detected a microsporidian, *Microsporidium rhabdophilia*, in the nuclei of the rodlet cells of several salmonid species in California, USA. Although *M. rhabdophilia* was found to be widely distributed among salmonids, no pathological changes were reported among infected fish (Modin 1981). Elston et al. (1987) detected infections associated with an anemia in 3-yr-old chinook salmon *Oncorhynchus tshawytscha* reared in seawater net-pens in Washington state, USA. An identical infection to that described by Elston et al. (1987) was later detected in juvenile chinook salmon reared in freshwater also from

Washington state (Morrison et al. 1990). In the latter 2 reports, the cell type infected by the microsporidian was clearly not a rodlet cell. Elston et al. (1987) thought the principal cell type involved was a blood-cell precursor but in a more detailed examination of the staining and structural characteristics, Morrison et al. (1990) determined the affected cell population most closely resembled lymphoblasts. In both of their reports the paucity of mature spores prevented further ultrastructural descriptions of the parasite found in affected lymphoblasts.

The purposes of the following report are to (1) describe further characteristics of the disease induced by the microsporidian and its similarities to a recent report of plasmacytoid leukemia in chinook salmon (Kent et al. 1990), (2) to provide further details on spore morphology of the parasite, and (3) to describe transmission of the microsporidian and disease to kokanee salmon *Oncorhynchus nerka*.

MATERIALS AND METHODS

Fish. Juvenile chinook salmon (30 to 50 g) were obtained directly from the Darrah Springs Hatchery, State of California, Department of Fish and Game (CDFG) USA. The fish were examined several times for the presence of pathogenic agents, from October through December of 1989, by standard procedures (Amos 1985). Blood from 39 fish with gross signs of the disease (anemia) was collected in microhematocrit tubes and was used to determine the packed cell-volume of erythrocytes. Hematocrits were also taken from uninfected chinook salmon (57 g) from the same origin but held at a site free of the disease. In December 1989 a 100-fish sample was taken to determine the incidence of gross signs of infection. Healthy kokanee salmon *Oncorhynchus nerka* (2.5 g) obtained from the Yountville Isolation Facility (CDFG) were used in transmission trials.

Light microscopy. Portions of the kidney, spleen, liver, intestine, eye, body musculature, gill, heart and brain were placed into Davidson's fixative (Humason 1979). After 16 h fixation, samples were transferred to 70% ethanol and processed for standard paraffin embedding and sectioning. Tissue sections (5 μ m) were stained with hematoxylin and eosin, Giemsa or Brown and Brenn Gram reagents. Imprints made directly from infected tissues were air dried for 30 min, fixed in 100% methanol for 5 min and stained with Leishman-Giemsa (Yasutake & Wales 1983).

Electron microscopy. Samples from the kidney of infected fishes were placed into 2.5% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.4) and fixed for 24 h at 4°C. Tissues were rinsed twice in buffer and then post-fixed in 1% aqueous OsO₄, dehydrated through a graded ethanol series, infiltrated and embedded in epoxy resin. Thin sections (10 to 20 nm) were stained with 4% uranyl acetate and lead citrate prior to examination with a Philips EM 400 electron microscope at 80 kV.

Transmission trial. Experimental transmission of the parasite was attempted by inoculation of juvenile kokanee salmon with kidney tissue homogenates from infected chinook salmon. A kidney from a heavily infected chinook salmon (based on gross signs) was aseptically removed and placed into a sterile petri dish. A small fragment was used to make imprints that were later stained with Leishman-Giemsa reagents for detection of the parasite. The remainder of the kidney was homogenized in 10 ml (1:10 w/vol.) of minimal essential medium (MEM), without antibiotics or serum, by forcing through a screen with a glass rod. Two groups of 30 kokanee salmon *Oncorhynchus nerka* received an intraperitoneal injection with 0.1 ml of kidney homogenate. A third group of 30 kokanee

received 0.1 ml of MEM only. All 3 groups were maintained in 20 l aquaria receiving 12°C well water.

Kidney imprints from moribund and dead fish were stained with Leishman-Giemsa and visceral organs, kidney and gill fixed for later microscopic examinations of hematoxylin and eosin stained tissue sections. All fish (including 20 controls) remaining 45-d after infection were euthanized and examined in the same manner as dead and moribund fish.

RESULTS

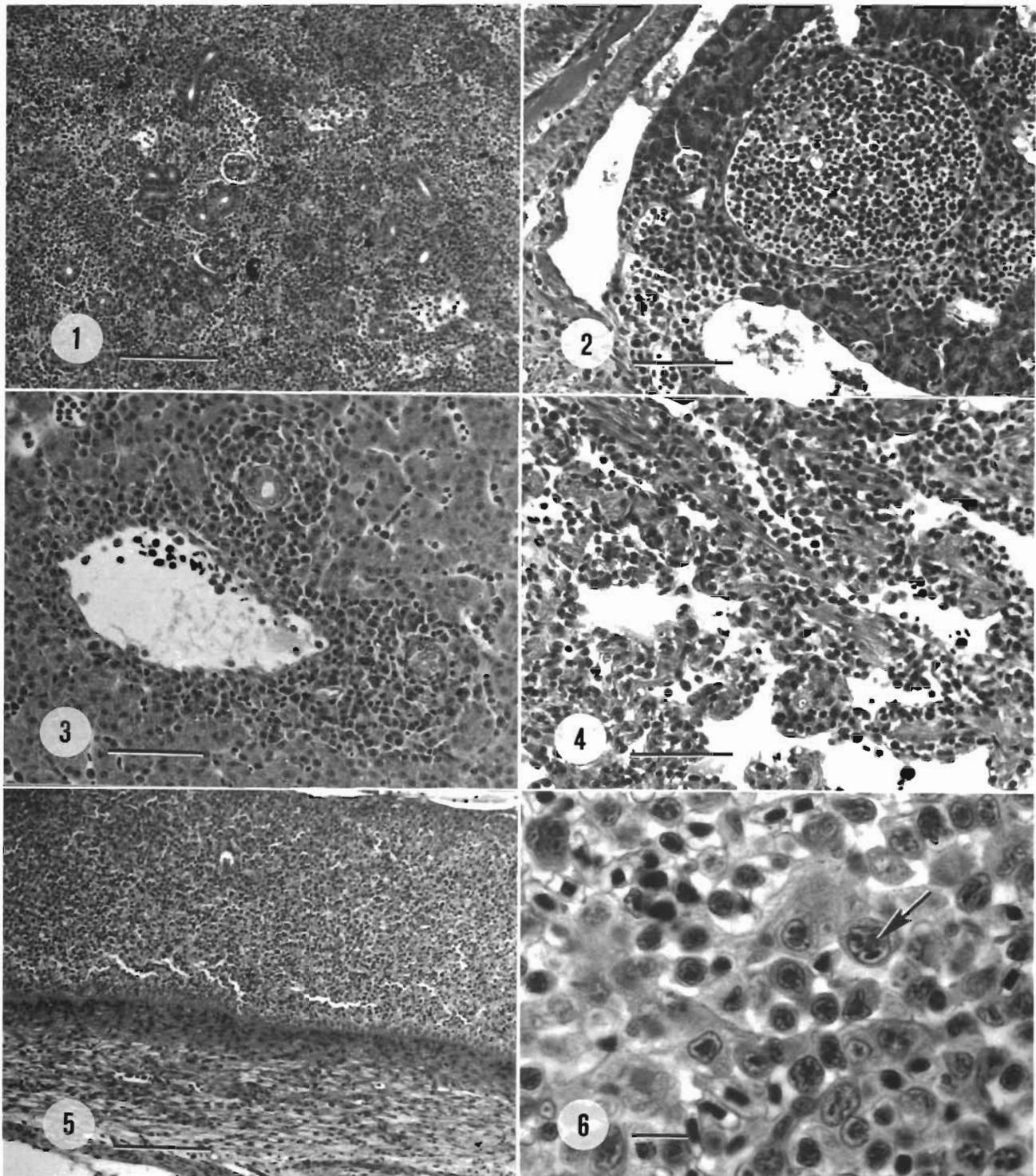
Gross signs

There were few remarkable external signs associated with naturally-infected juvenile chinook salmon *Oncorhynchus tshawytscha*. The first indication of the disease was above normal mortality in the hatchery population which occurred principally after feeding or handling. Exophthalmos was evident in a small number of fish but the most prominent external sign in moribund fish was moderate to severe gill pallor. Hematocrits averaged 17.5% (SD = 8.5, n = 39) in affected fish compared to 38.4% (SD = 1.7, n = 20) in normal fish. Internally, the kidney and spleen were enlarged but otherwise normal in color. The pyloric ceca and intestines were hyperemic and swollen in some fish. Moderate amounts of ascites, sometimes containing blood, were occasionally observed. A random sample of 100 fish from the affected population in November showed a 12% prevalence of kidney and spleen swelling.

Light microscopy

Microscopic changes observed in hematoxylin and eosin stained tissue sections were characterized by a moderate to severe hyperplasia of hematopoietic cells in the kidney (Fig. 1) and spleen. There was vascular migration of affected cells often with disruption of the vascular endothelium particularly in the renal and pancreatic (Fig. 2) sinuses. Affected cells occurred throughout the hepatic sinusoids although there was a characteristic perivascular orientation (Fig. 3). Similar cells were also found within the sinuses of the heart principally associated with reticuloendothelial cells lining the atrium (Fig. 4). Affected cells were found in the body musculature, lamina propria of the small and large intestine, pyloric ceca, the mesenteric membranes, choroid gland of the eye, the meninges of the spinal cord and myelencephalon and metencephalon, dermis and epidermis (Fig. 5).

Infected cells were pleomorphic but generally characterized by a large irregular and lobate nucleus



Figs. 1 to 6. *Oncorhynchus tshawytscha*. Hematoxylin stained tissue sections from fish infected with an intranuclear microsporidian. Fig. 1. Kidney with hyperplasia of hematopoietic cells; bar = 2 mm. Fig. 2. Large occluded vein in the pancreas containing infected lymphoblasts; bar = 1 mm. Fig. 3. Vein, artery and bile duct of the liver with populations of affected cells; bar = 1 mm. Fig. 4. Trabeculae of atrium with internalized affected cells; bar = 1 mm. Fig. 5. Accumulation of affected cells in the epidermis; bar = 2 mm. Fig. 6. Infected cells in the interstitium of the kidney. Arrow shows intranuclear stage of the microsporidian; bar = 10 μ m

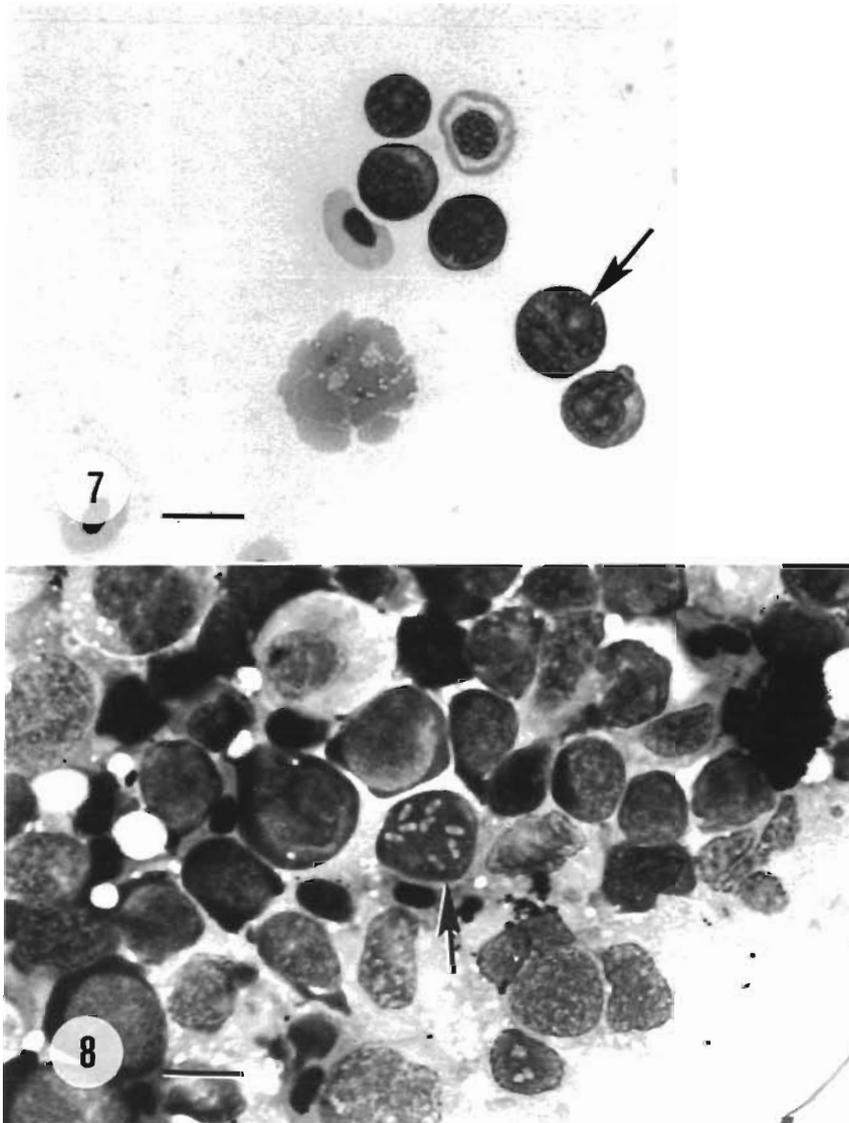
and an increased nuclear to cytoplasmic ratio. The nucleus of affected cells contained eosinophilic bodies which ranged from circular to rod-shaped (Fig. 6). In certain nuclei, 2 to 3 of these intranuclear inclusions were observed. Apparent binucleate and mitotically active cells were common even in peripheral blood (Fig. 7). These infected cells had amphophilic to basophilic cytoplasm with irregular plasmalemma. Intranuclear stages were also found in many dead or smudged cell nuclei (Fig. 7). The infection was most easily detected by observation of prespore stages and spores within the nucleus of cells following Leishman-Giemsa staining of kidney imprints (Fig. 8). Although well developed spores were infrequent, some nuclei contained up to 8 or 16 spores (Fig. 8). The spores stained poorly and were ovoid, approximately $1.0 \times 2.0 \mu\text{m}$ (width by length) and contained a small centrally

located vacuole or polar body as measured from Leishman-Giemsa stained preparations (Fig. 8).

Electron microscopy

Prespore and spore stages were observed by electron microscopy in many cells in the kidney interstitium (Figs. 9 and 10). The host cell-type infected with the microsporidian was characterized by a large nucleus with dense chromatin, and a cytoplasm with abundant endoplasmic reticulum often with a concentric array (Fig. 11).

Plasmodia within affected nuclei had a single simple plasmalemma and contained abundant endoplasmic reticulum and ribosomes but no mitochondria. Sporogonic plasmodia with lamellar precursors of the polar tube, vacuoles and pronounced endoplasmic



Figs. 7 and 8. *Oncorhynchus tshawytscha*. Leishman-Giemsa stained cells from the blood and kidney, respectively, of microsporidian infected fish. Fig. 7. Blood smear with infected lymphoblasts, one undergoing division with intranuclear microsporidian stages (arrow) and a smudge cell with similar intranuclear forms; bar = $10 \mu\text{m}$. Fig. 8. Kidney imprint showing an infected cell with 8 spores within the nucleus (arrow); bar = $10 \mu\text{m}$

reticulum were the stages most often detected (Fig. 10). Several nuclei were found dispersed throughout the cytoplasm of these developing stages prior to fission into sporoblasts (Fig. 10). Mature spores were rare and contained a polar tube with 4 to 5 turns, a dense matrix surrounded by a thin exospore and a poorly differentiated endospore (Fig. 11). A central vacuole was evident but lamellar membranes or the polaroplast were not prominent. The few nuclei observed in spores did not appear to be diplokaryotic. Many degenerative spores were found within phagolysosomes of macrophages (Fig. 12). Although the spore size, shape and wall were intact, few internal structures were recognizable.

Transmission trial

The transmission trial showed that kokanee salmon *Oncorhynchus nerka* were susceptible to intraperitoneal injections of the parasite as contained within kidney homogenates from infected chinook salmon. All but 4 (56/60) kokanee salmon died over a period of 45 d. Dead and moribund fish from the experimental groups had slightly swollen kidneys and spleens, and ascites were evident. Examinations for systemic viral or bacterial pathogens were negative. Kidney imprints of these fish revealed the presence of the intranuclear microsporidian. Microscopic examinations of stained tissue sections from several of these fish showed massive numbers of lymphoblast-like cells in the mesenteries, spread throughout the kidney, spleen, pancreas and on and below the endothelium of the major veins in the liver. Although intranuclear microsporidian stages were found, they were in lesser abundance compared to naturally-infected chinook salmon. The systemic nature of the disease was most severe in the four kokanee salmon remaining at the end of the study (45 d post-injection). These fish had moderate gill pallor and the kidney and spleen were enlarged and pale. Microsporidian-infected lymphoblasts were found in stained tissue sections, kidney imprints and blood films from all four fish. No microsporidians were observed in the 20 control fish examined at the end of the study.

DISCUSSION

Parasitism of the nucleus of hematopoietic cells with characteristics of lymphoblasts was found to be associated with a moderate to acute anemia secondary to a leukemic-like condition of juvenile chinook salmon *Oncorhynchus tshawytscha*. The presence of well developed spores in these fish and the first experimental transmission of the parasite build on prior observations of the parasite and disease.

Microsporidians are commonly encountered parasites of fish but only rarely are they associated with severe diseases (Dyková & Lom 1980). In Pacific salmon, *Loma salmonae* can be associated with serious gill infections and accompanying mortality (Morrison & Sprague 1981, Hauck 1984, Kent et al. 1989). Vascular lesions associated with the rupture and release of this microsporidian from parasitized host cells and a subsequent intense inflammatory response have been cited as causes of the gill pathology (Hauck 1984, Kent et al. 1989).

A quite different response is associated with recent reports of an intranuclear microsporidian of chinook salmon in Washington state. Elston et al. (1987) and Morrison et al. (1990) have reported anemia and mortality among adult and juvenile chinook salmon parasitized by an intranuclear microsporidian.

In both reports, infections were limited to the nucleus of a specific hematopoietic stem cell. Elston et al. (1987) believed this to be an erythroblast, although Morrison et al. (1990) and the results of our study suggest an affinity for cells with morphological properties of lymphocyte precursors. The large nucleus (and nuclear to cytoplasmic ratio), compact chromatin, a thin basophilic cytoplasm with occasional pseudopodia are consistent morphological characteristics of lymphocytes and the cells infected with the microsporidian in our study (Etlinger et al. 1976, Yasutake & Wales 1983).

Although observations of further developmental stages and ultrastructural details of the spore are needed, sufficient characteristics are present for comparison to other known genera of the phylum Microspora. The microsporidian observed in our study, and those of Elston et al. (1987) and Morrison et al. (1990), is most similar to descriptions of *Enterocytozoon bieneusi* as observed in the enterocytes (cells of the epithelium of the intestine) of human patients with acquired immune deficiency syndrome (Desportes et al. 1985). The human microsporidian's development is unique among the microsporidia. A cytoplasmic plasmodial stage develops into multinucleated presporoblastic forms with a concurrent development of the extrusion apparatus (which includes the polar tube) prior to fission of this presporoblastic plasmodia into sporoblasts (Desportes et al. 1985). The precursors of the polar tube appear prominently in these presporoblastic cells and these were observed by both Elston et al. (1987) and Morrison et al. (1990) and in sporogonic plasmodia in our study (Fig. 10). At least 8 spores are formed from a single presporogonic plasmodia with both *E. bieneusi* and the intranuclear salmonid microsporidian. Both spore types have a polar tube with 4 to 5 turns, contain a central vacuole and lack a well developed endospore. These shared characteristics with *E. bieneusi* are sufficient to identify the salmonid microsporidian as a new

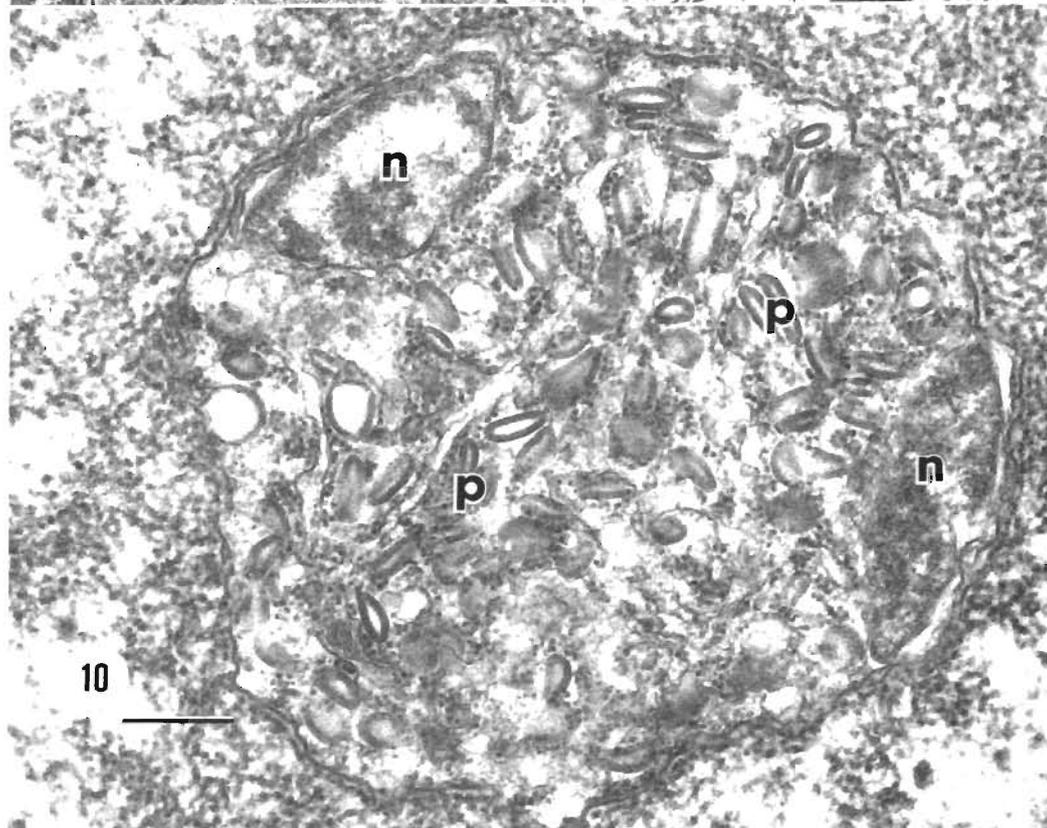
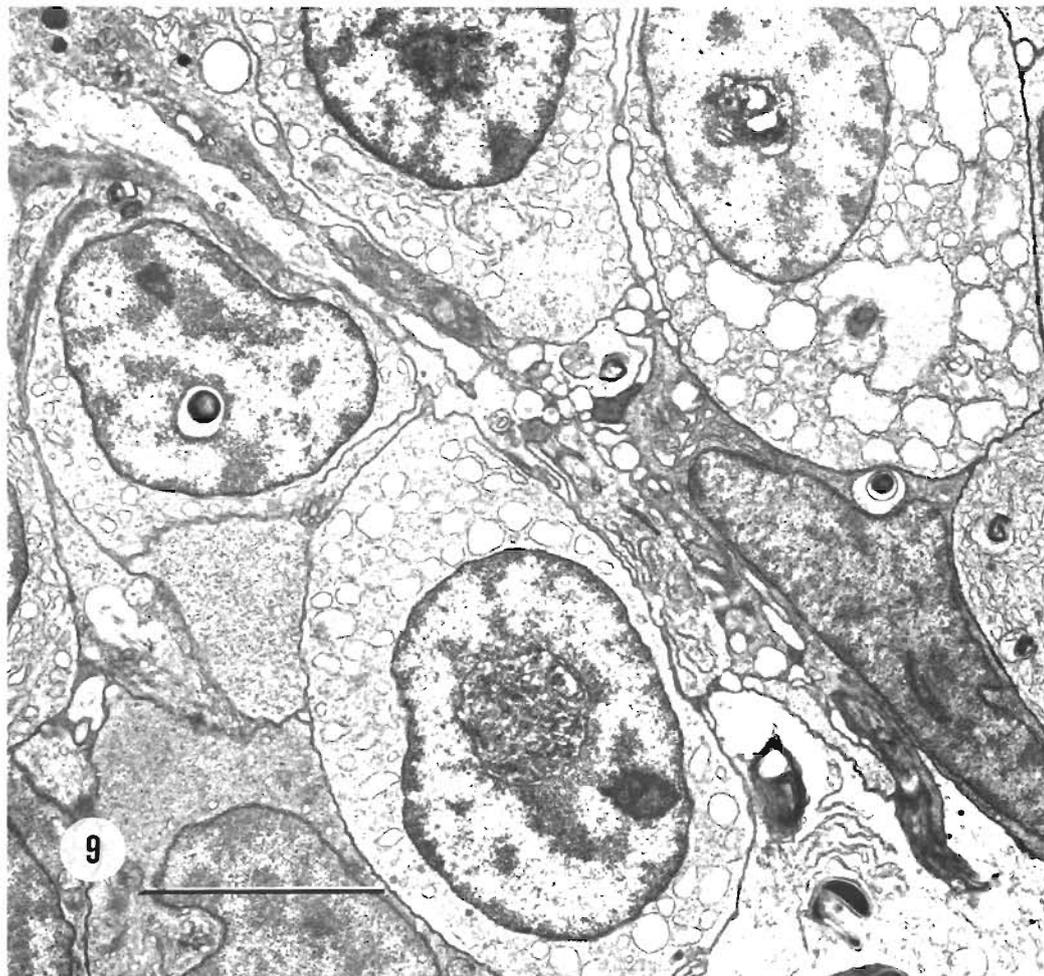




Fig. 11. *Oncorhynchus tshawytscha*. Electron-micrograph of mature (with at least 4 turns on polar tube) spores within the nuclei of infected cells from the kidney of chinook salmon; bar = 1 μ m

Figs. 9 and 10. *Oncorhynchus tshawytscha*. Electron micrographs of microsporidian prespore and spore stages within the nuclei of infected cells from the kidney of chinook salmon. Fig. 9 Four cells with intranuclear stages; bar = 5 μ m Fig. 10 Sporogonic stage prior to division into sporoblasts showing polar tube precursors (p), nuclei (n) and endoplasmic reticulum; bar = 0.5 μ m

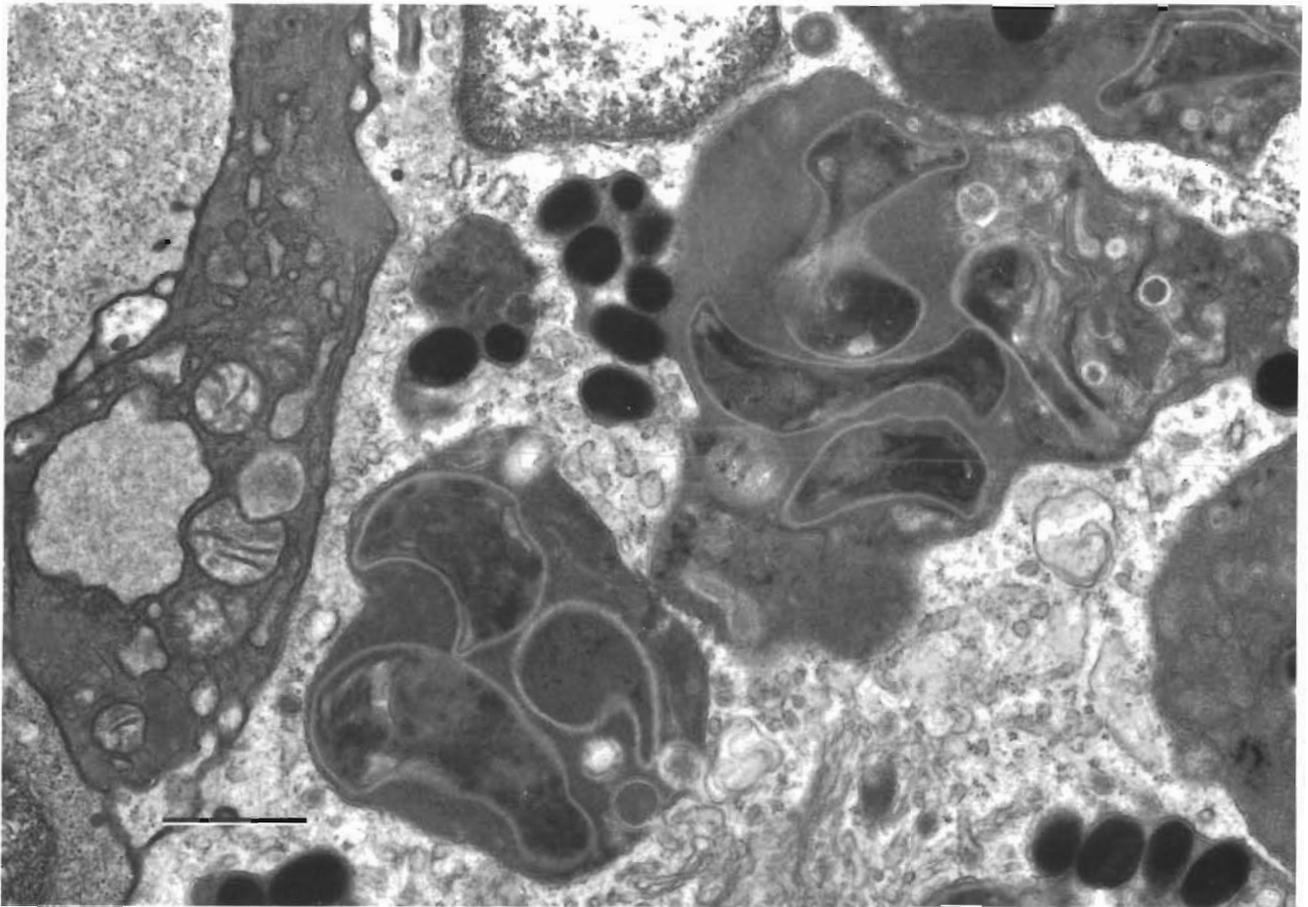


Fig. 12. *Oncorhynchus tshawytscha*. Electron-micrograph of degenerative spores within a phagolysosome of a macrophage of infected kidney; bar = 1 μ m

Enterocytozoon sp. The intranuclear development, larger size and different host for the salmonid microsporidian however, clearly separates it from *E. bieneusi*.

The possible relationship of the microsporidian we observed to *Microsporidium rhabdophilia* needs to be further examined. Although no ultrastructural studies were conducted, Modin (1981) described a spore of similar size and intranuclear location in several salmonid species, including chinook salmon. He did not, however, observe the parasite other than in rodlet cells. The gross and microscopic pathology associated with *M. rhabdophilia* however, is clearly different to that observed in chinook salmon in our study. An ultrastructural examination of *M. rhabdophilia*, as present in rodlet cells, is needed to determine whether possibly this microsporidian is related to the parasite we have found in lymphoblasts of chinook salmon.

The migration via the vasculature, adherence to endothelium of vessels and migration and establishment of microsporidian-infected cells in surrounding tissues are features of a neoplastic rather than pro-

liferative response (Cotran et al. 1989). Populations of these cells were found in nearly every tissue examined, although they were most abundant in the hematopoietic organs of chinook salmon (e.g. spleen and kidney). There was no evidence of tumor like growths in naturally or experimentally-infected fish.

The microscopic signs associated with microsporidian infection in our study are nearly identical to a recently described plasmacytoid leukemia in chinook salmon reared in seawater net-pens in British Columbia (Kent et al. 1990). Kent & Dawe (1990) were able to transmit the disease by intraperitoneal injections of homogenized kidney from infected chinook, sockeye (*Oncorhynchus nerka*) and Atlantic salmon (*Salmo salar*). Development of the disease and mortality began 1 to 2 mo post-injection at water temperatures of 12°C. Although the microscopic signs of the plasmacytoid leukemia and the intranuclear microsporidian infections are similar, microsporidian stages have not been observed in experimentally-induced plasmacytoid leukemia in chinook salmon in British Columbia (M. L.

Kent pers. comm.). Studies on the possible relatedness of the 2 conditions is in progress. The microsporidian may be acting as a factor stimulating a leukemic condition perhaps in a fashion similar to the haemogregarina-induced lymphoma in cultured turbot (*Scophthalmus maximus*) as reported by Ferguson & Roberts (1976). Resolution of the microsporidian infection in affected chinook with persistence of the proliferative/neoplastic condition may then result in a condition identical to the plasmacytoid leukemia described by Kent et al. (1990).

Acknowledgements. This work was supported in part by Dingell-Johnson/Wallop-Breaux Fish Restoration Act funds administered through the California Department of Fish and Game. Thanks to the technicians of the Veterinary Medicine Teaching Hospital for their preparations of stained tissue sections for light microscopy and Mr R. Munn for his electron microscopy expertise. We thank Mr J. Morrison for his valuable insight in reviewing the microscopic materials.

LITERATURE CITED

- Amos, K. (1985). Procedures for the detection of certain fish pathogens. Fish Health Section, blue book. American Fisheries Society, Bethesda, Maryland
- Canning, E. M., Lom, J. (1986). The microsporidia of vertebrates. Academic Press, London
- Cotran, R. S., Kumar, V., Robbins, S. L. (1989). Pathologic basis of disease, 4th edn. W. B. Saunders, Philadelphia
- Desportes, I., Le Charpentier, Y., Galian, A., Bernard, F., Cochand-Priollet, B., Lavergne, A., Ravisse, P., Modigliani, R. (1985). Occurrence of a new microsporidian: *Enterocytozoon bieneusi* n.g.n.sp., in the enterocytes of a human patient with AIDS. J. Protozool. 32: 250–253
- Dyková, I., Lom, J. (1980). Tissue reactions to microsporidian infections in fish. J. Fish Dis. 3: 265–283
- Elston, R. A., Kent, M. L., Harrell, L. H. (1987). An intranuclear microsporidium associated with acute anemia in the chinook salmon. J. Protozool. 34: 274–277
- Etlinger, H. M., Hodgins, H. O., Chiller, J. M. (1976). Characterization of lymphocytes in a primitive teleost, *Salmo gairdneri*. In: Wright, R. K., Cooper, E. L. (eds.) Phylogeny of thymus and bone marrow-bursa cells. North-Holland Publishing Co., New York, p. 83–91
- Ferguson, H. W., Roberts, R. J. (1976). A condition simulating lymphoma, associated with a sporozoan infection in cultured turbot (*Scophthalmus maximus* L.). Prog. exp. Tumor Res. 20: 212–216
- Hauck, A. K. (1984). A mortality and associated tissue reactions of chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), caused by a microsporidian *Loma* sp. J. Fish Dis. 7: 217–229
- Humason, G. L. (1979). Animal tissue techniques. W. H. Freeman, San Francisco
- Kent, M. L., Dawe, S. C. (1990). Experimental transmission of a plasmacytoid leukemia of chinook salmon (*Oncorhynchus tshawytscha*). Cancer Res. (in press)
- Kent, M. L., Elliot, D. G., Groff, J. M., Hedrick, R. P. (1989). *Loma salmonae* (Protozoa: Microspora) infections in seawater reared coho salmon (*Oncorhynchus kisutch*). Aquaculture 80: 211–222
- Kent, M. L., Groff, J., Traxler, G. F., Zinkl, J. G., Bagshaw, J. W. (1990). Plasmacytoid leukemia of chinook salmon (*Oncorhynchus tshawytscha*). Dis. aquat. Org. 8: 199–209
- Modin, J. C. (1981). *Microsporidium rhabdophilia* n. sp. from the rodlet cells of salmonid fishes. J. Fish Dis. 4: 203–211
- Morrison, C. M., Sprague, V. (1981). Microsporidian parasites in the gills of salmonid fishes. J. Fish Dis. 4: 371–386
- Morrison, J. K., MacConnell, E., Chapman, P. F., Westgard, R. L. (1990). A microsporidium-induced lymphoblastosis in chinook salmon (*Oncorhynchus tshawytscha*) in fresh water. Dis. aquat. Org. 8: 99–104
- Yasutake, W. T., Wales, J. H. (1983). Microscopic anatomy of salmonids: an atlas. United States Department of the Interior, Fish and Wildlife Service, Resource Publication 150, Washington D.C.

Responsible Subject Editor: Professor W. Körting, Hannover, F. R. Germany

*Manuscript first received: February 8, 1990
Revised version accepted: May 3, 1990*