Lymphocystis Virus: Isolation and Propagation in Centrarchid Fish Cell Lines

Abstract. *A virus from fish with lymphocystis disease was isolated in fish cell cultures. Eleven serial transfers were made and the pathognomonic lymphocystis cells were produced in vitro in each transfer. Fish inoculated with 6th- and 9th-passage material developed the disease, and virus was reisolated from them.*

Lymphocystis disease is a common, chronic, benign infection of fishes. Certain cells of the connective tissue group, which serve as specific targets, are excited to monstrous, albeit more or less normally proportioned, enlargement. After infection they are termed lymphocystis cells (Fig. 1). Weissenberg, who first proposed that lymphocystis disease was a viral infection, has just published a review of the research on this disease (*1*). Until the present time, however, the causal agent has not been isolated. Grützner (*2*) found that an inoculum of homogenized lymphocystis lesions from plaice (*Pleuronectes platessa*) evoked increased degenerative changes in cultures of tissues from guppy (*Lebistes reticulatus*) and paradise fish (*Macropodus opercularis*). The cytoplasm of some cells condensed into multiple discrete droplets; small vacuoles developed in other cells, or their nuclei became granular; and among some cultures there were cells that had prominent granular cytoplasmic inclusions, which were similar to the inclusions of lymphocystis cells but their specificity was uncertain. Using primary monolayer cultures of predominantly epithelioid cells from ovaries of bluegills (*Lepomis macrochirus*) and virus from the same species, Wolf (*3*) could not find development of lymphocystis cells in vitro. Similarly, material which was infectious for centrachid fish failed to effect changes in cultures of the fibroblastic RTG-2 cell line from rainbow trout (*Salmo gairdneri*). Wolf (*4*) also noted that 3-day-old lymphocystis cells failed to develop further when explanted, with surrounding tissue, to a culture system. On the other hand, the disease is clearly infectious and some biological properties of the virus are known (*3*, *4*). Electron microscopy has shown unequivocal evidence of virus—often in crystalline array—associated with the intracytoplasmic Feulgen-positive inclusions (*5*, *6*). We now report successful isolation of lymphocystis virus in two separate laboratories and describe some of its characteristics.

During June and July 1964, we began development of permanent cell lines from juvenile centrachid fishes: bluegills at Lehigh University (LU), and bluegills and largemouth bass (*Micropterus salmoides*) at the Eastern Fish Disease Laboratory (EFDL).

At both laboratories the cells were grown in Eagle's minimum essential medium containing 10 or 15 percent fetal bovine serum. The complete medium also contained 100 units of penicillin, 100 μg of streptomycin, and 25 units of nystatin per milliliter. The growing cells were incubated at 23° ± 2°C.

Primary monolayer cultures and early passages of cells from largemouth bass were inoculated with filtrates of homogenized lymphocystis lesions (*7*), and within several days the cells showed increased microscopic refractility and contraction. During the next 2 to 3 weeks the size of such cells increased markedly, and the nucleus became more distinct, the nucleolus especially so. A capsule formed, and stained preparations showed that the cells had the essential features of lymphocystis cells. Bluegills infected with the same strain of virus at about the same animal passage were taken to LU . There the virus was transferred to healthy young bluegills, and, when disease was evident, lesions were homogenized and inoculated into cultures of bluegill cells. Confirmatory development of pathognomonic lymphocystis cells occurred.

Since isolation, the material has been passaged 11 times at the EFDL for a total dilution of 10^{-27} of the original material. At LU the virus has been passaged nine times; the final passage attained a dilution of 10^{-20} and showed no diminution of infectivity. Thus far, only largemouth bass and bluegill cell cultures have been susceptible. Permanent cell lines from rainbow trout, fathead minnow (*Pimephales promelas*), and brown bullhead (*Ictalurus nebulosis*) were refractory.

At 23° to 25°C the sequence of changes which occur in vitro parallels development of lymphocystis cells in fish at 25°C (*8*). Newly infected cells increase in size and become basophilic. Six days after inoculation, Feulgen-positive, basophilic inclusions appear in the cytoplasm, and the hyaline capsule begins to form at about 10 days. The

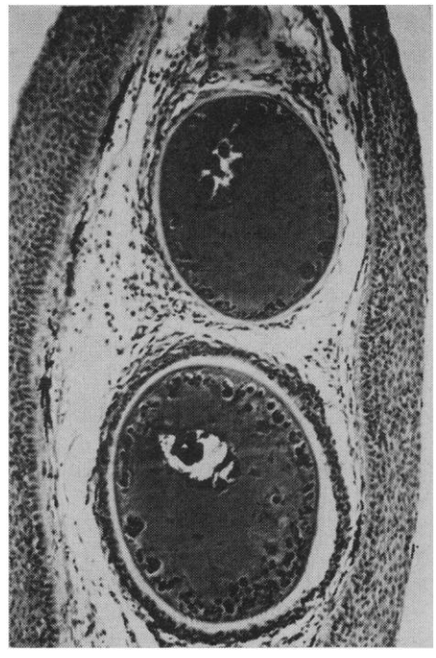

Fig. 1. Section through two mature lymphocystis cells from a natural infection in an orange-spotted sunfish (*Lepomis humilis*). The larger cell measures 280 by 213 μ. Cells of normal size comprise the surrounding tissue.

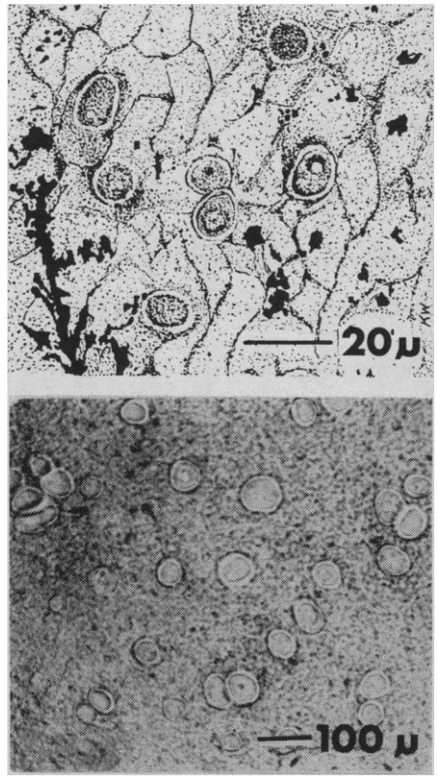

Fig. 2. (Top) Fresh, whole mount of caudal fin membrane of *Acerina cernua*, showing 2-week-old lymphocystis cells already encapsulated. [After Weissenberg, 1920] (Bottom) Centrarchid cell culture with a low population density of lymphocystis cells. Incubation had been at 23°C for 23 days.

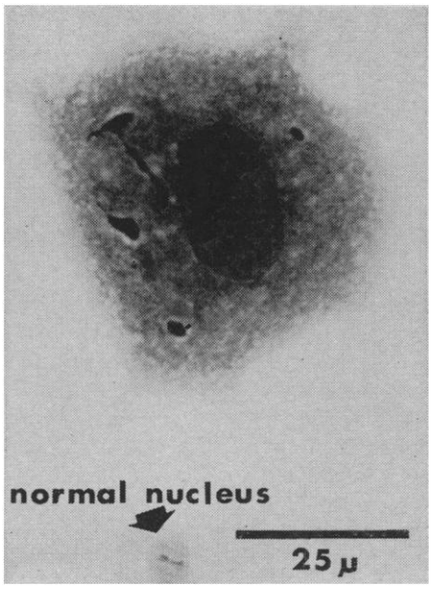

Fig. 3. Mature, 24-day-old, unencapsulated lymphocystis cell produced in vitro and stained with May-Grünwald-Giemsa. Encapsulation normally obscures internal detail: therefore, a nonencapsulated cell was selected for illustration.

cells are mature at 3 to 4 weeks and have the identical appearance of natural lymphocystis cells in fin webs of perch (*Acerina cernua*) (*9*) (Fig. 2). Lymphocystis cells that develop from centrarchid cells in vitro may reach a length of several hundred microns before becoming encapsulated but afterward they are smaller than those which occur in the fish. Encapsulated mature cells in the bluegill are usually over 100 μ in greatest length and commonly measure several hundred microns (*10*). In contrast, encapsulated cells in culture seldom exceed 100 μ in length and the largest are usually 40 to 90 μ. Inclusion bodies, the apparent sites of viral replication (*4*, *5*), form extensive fenestrated networks in infected centrarchids. Similar differentiation occurs in some cells in culture (Fig. 3), but in others the inclusions remain dense and homogeneous although there usually are several inclusions in each mature cell.

Cell cultures and young susceptible fish have comparable sensitivity to lymphocystis virus. Homogenates of mature lesions contained about 10^7 ID_{50}/ml (infective dose, 50 percent effective) when assayed in young bluegills (*4*, *8*). Infectivity of similar material in bluegill cell cultures was determined at both laboratories and found to be about $10^{6.5}$ ID_{50}/ml. Though not intended as a comparison, infected cell cultures (tubes, 16 by 125 mm, seeded with 1.0 or 1.5 ml cell suspension) at maturity generally had a titer of $10^{4.5}$ to 10^5 ID_{50}, most but not all of which was present in the cell sheet.

During the long incubation necessary to develop mature lymphocystis cells, *p*H of the culture medium dropped from an initial 7.6 or 7.8 to 7.0 or lower. Changes of medium were not required, but when the medium was renewed, young lymphocystis cells appeared, generally in "colonies," and this was interpreted as infection by first-generation virus.

Several methods were used successfully to prepare virus suspensions for transfer. Cell sheets were covered with water, exposed to three cycles of freezing and thawing, or simply scraped from culture vessels, and homogenized in tissue grinders.

At LU, 6th-passage virus in cell culture (10^{-15} dilution from original) was homogenized and inoculated into bluegills and into bluegill cell cultures. The titer in cell culture was $10^{6.5}$ ID_{50}/ml, and lymphocystis lesions were grossly evident in fish inoculated with the lower dilutions of virus. Lymphocystis in fish was confirmed by histological examination, and virus was reisolated in cell culture. Cell homogenates from uninfected control cultures produced no lesions in fish.

At EFDL, 9th passage of the virus in cell culture (10^{-20} dilution from original) was similarly used to infect young bluegills. Lymphocystis cell culture medium (0.05 ml) was injected into young bluegills and all developed lesions; histologic confirmation was obtained and virus was reisolated. Fish inoculated with control-culture homogenates did not develop lesions.

Cell-culture homogenates were tested for hemagglutination at *p*H 7.2 to 7.3, 4° and 20°C. Washed cells from the following animals were tested: bluegill, largemouth bass, bullfrog (*Rana catesbeiana*), painted turtle (*Chrysemys picta*), chicken (*Gallus domesticus*), rabbit (*Oryctolagus cuniculus*), guinea pig (*Cavia porcellus*), sheep (*Ovis musimon*), and man (*Homo sapiens*) (O+). With the possible exception of material diluted no more than 1:2 with frog and turtle cells at 4°C, lymphocystis virus did not hemagglutinate, neither was there hemadsorption of any erythrocytes by infected cultures at 20°C. Newcastle disease virus, used as a positive control, agglutinated fish, frog, turtle, chicken, and human O+ cells.

When the method of Marmur was used (*11*) spectacular yields of DNA were extracted from lymphocystis lesions in fish. DNA was also extracted by the cold phenol method of Ito (*12*). Neither extract showed infectivity in cell cultures that were susceptible to intact virus, and fish were similarly unaffected.

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Lobster Hemocyanin: Properties of the Minimum Functional Subunit and of Aggregates

Abstract. *Lobster hemocyanin dissociates into a functional subunit of 68,000 to 70,000 molecular weight when Ca^{2+} ions are removed from an alkaline solution of low ionic strength. Succinylation results in a further dissociation into two nonfunctional subunits of approximately 34,000 to 35,000 molecular weight. Amino acid analysis and tryptic peptide patterns indicate that the functional subunit is composed of at least two polypeptide chains which are similar.*

Both the dissociation of hemocyanins into subunits and their equilibrium with oxygen are strongly influenced by divalent cations, *p*H, and ionic strength (*1*). This relation suggests that the degree of dissociation into subunits may be