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Etiology of Clam Leukemia 30 pp

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Narragansett Bay Estuary Program

TITLE: Etiology of Clam Leukemia

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EXECUTIVE SUMMARY

The Etiology of Clam Leukemia

The viral etology of hematopoietic neoplasia in soft-shell clams has not been resolved in this study. Although a virus was isolated from neoplastic clams, no evidence was found to associate the virus as the causative agent of hematopoietic neoplasia of clams.

The virus was isolated only in 50% of the time from neoplastic clams (4 out of 8 attempts), and transmission study failed to induce neoplasia in clams following the inoculation of the purified virus. Electron microscopy study of hemocytes and tissues of neoplastic clams did not reveal any intracellular virus in the neoplastic cells.

The isolated virus is small (25-30 nm) with isometric morphology, and has a buoyant density of 1.33 - 1.35 g/cm^3 in CsCl. They resemble some picronavirus (Poliovirus,Echovirus, and Coxackie virus) and bacteriophage MS2, which are normally present in sewage and estuaries where sewage outfall is deposited. Because of similar size and density, it is speculated that the isolated virus probably is a picornavirus or a bacteriophage, and an incidental passenger in the clams. In this study, it was found that the prevalence of hematopoietic neoplasia in soft-shell clams in the southernpart of Narragansett Bay varied from 0% to 15% with an average of 6.3%. Such prevalence is considered low compared to those reported in the Falmouth, Massachusetts area (11-18% in 1981-82); New Bedford, Massachusetts area (18-90% in 1983); Westport, Connecticut area (13% in 1984-85); and Chesapeake Bay area (42-65% in 1983-84). The prevalence reported currently in Allen Harbor (0.6-15% average of 6.3%) was much less than those reported in Allen Harbor (1977-79)by Cooper, et al, which ranged from

19% - 43% and averaged 30%. It appears that hematopoietic neoplasia in Allen Harbor and most of the southern part of Narragansett Bay area, though present, is less severe than many other areas of New England and Chesapeake Bay, and at Allen Harbor the number of neoplastic clams has decreased dramatically from those reported 10-12 years ago.

Recommendations to the Narragansett Bay Governing Board regarding the management of the disease leukemia in soft-shell clams, are premature at this point of the project. The virus isolated in the neoplastic clams in the early part of this study could no longer be isolated in the later part of this study. Also, the transmission study showed no induction of neoplasia following the inoculation of the purified virus. Therefore, no evidence can be presented to associate the 25-30 nm virus as the causative agent of hematopoietic neoplasia of clams.

Work is continuing in this laboratory toward resolving the role of a transmissible agent in causing hematopoietic neoplasia of clams.

INTRODUCTION

The harvesting of soft-shell clams, _Mya arenaria_ in the Northeastern states is a multi-million dollar industry. In 1987, the estimated gross income from the East Coast area exceeded 19.8 million dollars[1].

In certain areas of New England[3,4,6,19,20] and Chesapeake Bay[9,10], an epizootic disease involving 50 to 65% of the feral clam population occurs in which the clam's hematopoietic tissue becomes neoplastic. The neoplasm is invasive, replacing the clam's normal tissue with neoplastic cells. The resulting conditions lead to the death of the clams[7,8,10,12,13]. Although no data is available, mass mortality of clams probably occurs in the field, costing the shellfish industry millions of dollars in potential lost harvest[12].

The causes of hematopoietic neoplasia (HN) have not been clearly delineated. HN has been associated with pollutants such as petroleum-derived hydrocarbons[2,21,22]. However, Brown, _et al_[5] reported that clams with HN were found in non-polluted pristine areas. They later demonstrated that a transmissible agent was involved in the causation of the neoplasm. Oprandy, _et al_[15] isolated a virus from neoplastic clams which had a morphology similar to that of a B-type retrovirus. Reinisch reported that polychlorinated biphenyl found in New Bedford Harbor may be a causative agent[20]. Sunila and Farley reported the environmental limits for the survival of neoplastic cells from clams[18]. The conflicting findings suggest that the causation of HN in clams may be multifactoiral, and that chemical pollutants, transmissible agents, and environmental factors could all play a role in the induction of HN.

To arrive at some rational measure to control the disease, it is necessary to first establish its etiology. The interplay of the several reported causes need to be delinieated. The objective of this first study is to determine the

viral etiology of HN in clams.

MATERIALS AND METHODS

Sources and maintenance of neoplastic and non-neoplastic clams

Soft-shell clams were obtained from Allen Harbor, Prudence Island (Coggshall Cove), Marsh Point (East Greenwich), Duck Cove (North Kingstown), Narragansett Pier and Warwick in Rhode Island; New Bedford, Massachusetts; Machiasport, Maine; and New Brunswick, Canada. The clams were bled and their hemocytes were diagnosed as neoplastic or non-neoplastic by an *in vivo* diagnostic system. The neoplastic clams were maintained at 6°C in a stationary 20 gallon glass aquaria without sediment. The aquaria were held in Woodward Hall, University of Rhode Island. The sea water in the aquaria was obtained from the Narragansett Bay (at the US-EPA Laboratory, Narragansett, RI) with salinity which varied from 29°/oo to 30°/oo. The non-neoplastic clams were maintained at ambient temperature in a flow through seawater system at the Environmental Protection Laboratory, US-EPA Laboratory, Narragansett, RI.

Diagnosis of neoplastic clams in vivo

A system to diagnose neoplasia in the live clams was developed. The system was a modification of that described by Farley, *et al*[11,14]. The method involved bleeding the clam from the adductor muscle with a 25 gauge needle, and allowing the hemocytes to attach for 2 hours to microslides which has been coated with poly-L-lysine. The cells were then fixed in 1% glutaraldehyde/4% formaldehyde in seawater and stained with Papanicolaou stain. The monolayer of cells was examined for neoplastic and non-neoplastic cells (Figure 1 and 2).

Isolation, concentration, purification and identification of virus

For each attempt at virus isolation, a 20% homogenate of 3-6 whole clams,

exclusive of siphons, was made by mixing the clam tissues with TNE buffer (10mM Tris-HCL, 400mM CaCl, and 1mM EDTA, pH 7.4) and 1% Triton X-100 in a Waring blender for 2 min. at room temperature. After processing, the homogenate was filtered through glass wool to remove any large fragments of tissues. The homogenate was clarified by centrifugation at 5000 Xg for 30 min. at $4^{o}C$ in a Sorvall RC2-B centrifuge, and again at 10,000 Xg for 30 min. at $4^{o}c$. The supernatant was collected and layered onto one ml of 50% CsCl, which served as a cushion in a 30 ml centrifuge tube. The tube was centrifuged at 83,000 xg for 3 hrs. using a Sorvall AH627 rotor. The visible band above the cushion was harvested. The density of the harvested fluid was adjusted to $1.34 g/cm^3$ with the addition of CsCl salt, and centrifuged to produce a gradient at 140,000 xg for 16 hrs. in a Sorvall AH650 rotor. Following centrifugation, 10-12 fractions were collected from the centrifuge tube. The density of each fraction was determined by its refractive index. A similar number of non-neoplastic clams were processed. A total of 8 isolation attempts were made between February of 1989 through February 1990.

Electron microscopy

Samples of each of the 12 fractions of normal and neoplastic clams were stained with 2% phosphotungstic acid, pH 7.4, by the pseudoreplication technique[17], and examined with a Hitachi HS-9 electron microscope operating at an acceleration voltage of 75 KV.

Transmission study

Virus that had been purified and concentrated through isopycnic centrifugation, was dialyzed against phosphate buffered saline (PBS) solution overnight at $6^{o}C$. The virus was diluted 1:2 with sterile seawater and inoculated into the adductor muscle of 14 non-neoplastic clams. Three clams were bled and

examined for neoplasia prior to the inoculation and were found to be negative. Each clam received 0.2 ml of the virus and was maintained at $6^{0}C$ in a 20 gal. glass aquarium without sediment. The seawater had a salinity of approximately 30‰. The seawater was changed weekly. Similar inoculants were prepared for control clams using the same fraction of non-neoplastic clam tissue.

All clams were bled after 4, 8, and 12 weeks post-inoculation, and the hemocytes were examined for neoplasia as previous described.

Detection of viral specific antigen in neoplatic and non-neoplastic cells by the indirect immunoperoxidase procedure

Virus that had been purified and concentrated through isopycnic centrifugation was dialyzed against PBS at $6^{0}C$ overnight. It was then mixed with an equal volume of Freund's complete adjuvant (0.5 ml), and inoculated intramuscularly into a rabbit at biweekly intervals for three times to produce hyperimmune serum. The rabbit was bled at 14 days after the last injection, and serum was collected. The indirect immunoperoxidase procedure was used. It is described by Smolowitz and Reinisch[16].

Hemolymph from neoplastic and non-plastic clams were withdrawn from the adductor muscle blood sinus of the clam (0.5 ml) into a syringe containing 0.5 ml of seawater. The cell suspension was placed on a 0.1% poly-L-lysine coated microscope slide, and allowed to attach to the slide for 2 hours. The attached cells were then fixed in glutaraldehyde 1%/formaldehyde 4% for 2 hours. The fixed cells were stained by the indirect immunoperoxidase (IP) procedure, which is described in Appendix 1. The stained cells were overlaid with coverslip using Permount mounting fluid and examined at 400X magnification. The positive cells were stained with a brown coloration. For positive controls, the rabbit anti-virus serum, diluted 1:50 in PBS, was applied to neoplastic hemocytes collected from a neoplastic clam. The negative controls consisted

of hemocytes collected both from neoplastic and non-neoplastic clams and incubated with normal rabbit serum (1:50) instead of the rabbit anit-virus serum. Another negative control consisted of normal hemocytes collected from non-neoplastic clams and incubated with rabbit anti-virus serum (1:50).

Electron microscopy of hemocytes and tissues of neoplastic and non-neoplastic clams

Two mm pieces of clam tissues and aliquots of hemocytes freshly bled from clams were fixed in cold 2% gtularaldehyde (in 0.1M cacodylate buffer) and 12% sucrose for 60 min. The tissues and cell specimens were rinsed in a gradient of sucrose of 12%, 9%, 6%, 3%, and 0% in cacodylate buffer for 15 min. each. They were then post-fixed in 2% OsO_4 (pH 7.4) for 2 hrs. followed by dehydration in acetone. The cells and tissues were then embedded in Spurr resin, stained with uranyl acetate and lead citrate, and examined in a Hitachi HS-9 electron microscope.

Prevalence of hematopoietic neoplasia in clams in the southern part of Narragansett Bay

Soft-shell clams were collected from Allens Harbor, Marsh Point, Prudence Island, Narragansett Pier, Warwick, and Duck Cove. The clams were diagnosed for hematopoietic neoplasia, and their prevalences of neoplasia calculated.

RESULTS

Virus isolation

From February 1989 to February of 1990, eight trials were made to isolate virus from clams. In each trial, separate attempts were carried out to isolate virus from neoplastic and non-neoplastic clams. Depending on the availability of neoplastic clams, isolations were made from clams from various areas. Table 1 presents the dates of isolation, the extent of each clam's neoplasia, the source of clams used, and the results of virus isolation. The data showed that from February 1989 to May of 1989, 4 trials were attempted and in all 4 trials, a virus measuring 25-30 nm in size with a buoyant density of 1.34 g/ml was isolated (Figure 3). Because of its small size, whether the virus has 2-3-5 symmetry with icosahedral shape is difficult to determine.

The later 4 trials of virus isolation, carried out from July 1989 to February of 1990, yielded no virus (Table 1).

Transmission study

Of the 14 non-neoplastic clams which were inoculated with the purified virus, none come down with neoplasia 4 and 8 weeks post-inoculation. By 12 weeks post-inoculation, only 6 out of the 14 clams were surviving and none of them demonstrated neoplasia (Table 2) All control clams which received only normal clam tissue fluid remained negative throughout the experiment (Table 2).

Detection of viral antigen in neoplastic cells using the indirect immunoperoxidase (IP) procedure

Neoplastic hemocytes when reacted with rabbit anti-virus serum followed by anti-rabbit peroxidase-linked serum, showed positive IP reaction of dark brown color on the cells membrane (Figure 4). When normal non-neoplastic cells were stained with rabbit anti-virus serum, they also exhibited an IP reaction of

dark brown color on the cell membrane (Figure 5). However, when normal rabbit serum was used, no IP reaction was seen in the non-neoplastic and neoplastic cells (Figures 6 and 7).

Electron microscopy of hemocytes and tissues of neoplastic and non-neoplastic clams

No 25-30 nm intracellular virus particles were found in the electron micrograph of hemocytes and tissues of neoplastic and non-neoplastic clams (Figure 8). Particles of 30 nm were observed in the cytoplasm of one neoplastic cell, but its identification could not be differentiated from glycogen granules (Figures 9 and 10).

Prevalence of HN of clams at Narragansett Bay

The prevalence of HN in soft-shell clams at 6 locations in Narragansett Bay is presented in Table 3. The peak prevalence of 15% and 13% was found in Marsh Point and Allen Harbor, respectively, in January 1989. On the average, the prevalence of HN at these 6 locations was 6.3%. No neoplastic clams were found at Prudence Island.

DISCUSSION

The viral etiology of hematopoietic neoplasia in soft-shell clams has not been resolved in this study.

A 25-30 nm virus with a buoyant density of 1.33-1.35 g/cm^3 was isolated several times from whole neoplastic clams. Between February and May 1989, the virus was isolated from neoplastic clams which were collected in Allen Harbor and at Marsh Point, but not from non-neoplastic clams collected in Prudence Island. However, during a latter period between July 1989 and January 1990, no virus was isolated from either neoplastic or non-neoplastic Rhode Island clams. The reason for the discrepency in the isolation of the virus from neoplastic clams at two periods of time is unknown. It maybe associated with the time and site where the clams were collected.

The isolated virus was small (25-30 nm) with isometric morphology (Figure 3), and had a buoyant density of 1.33-1.35 g/cm^3 in CsCl. Since some of the picornavirus (Poliovirus, Echovirus, and Coxsackie viruses) and bacteriophage (MS2) have similar size (25-30 nm) and density (1.33-1.35 g/cm^3) and share similar isometric morphology, it is possible that the isolated virus could have been a picornavirus or bacteriophage. Picornaviruses and bacteriophage MS2 are commonly found in sewage and estuaries where sewage outfall is deposited. During feeding, clams concentrate water particles including phage-carrying bacteria and viruses. These types of viruses have never been shown to induce neoplasia in animals, and were possible incidental passengers in the clams.

A transmission study with the 25-30 nm virus in 14 clams has not shown any neoplasia at 4, 8, and 12 weeks post-inoculation. Brown, et al induced hematopoietic neoplasia in clams via effluent from affected clams between 2 - 4 months[5]. Oprandy, et al induced tumors in clams in 2 months[15]. Farley also reported the induction of neoplasia via transplantation of neoplastic cells in

one and one-half months[13]. It appears that three months used in this transmission study should be sufficient time to induce neoplasia. Clams which were inoculated suffered unusually high mortality at three months in this study, and could have affected the results. A larger number of samples should be considered in future studies.

Efforts to detect viral antigens on neoplastic cells using the indirect immunoperoxidase procedure were inconclusive. The antigen which was used to immunize the rabbit to produce specific anti-virus serum could possibly have contained some clam cellular protein, thus producing the non-specific reaction with normal hemocytes as well as neoplastic ones (Figures 4 and 5). It was interesting to note that normal rabbit serum did not react with either neoplastic or non-neoplastic cells (Figures 6 and 7), indicating that the color produced was not due to the non-specific reaction with the peroxidase labeled secondary antiserum.

Electron microscopy of hemocytes and tissues of neoplastic and non-neoplastic clams gave no evidence of intracellular particles similar to the 25-30nm virus which was isolated by the virus purification procedure. Particles of 30 nm were observed in the cytoplasm of neoplastic cells, but its identification could not be diffenentiated from glycogen granules.

Oprandy, <u>et al</u>[15] showed that a B-type retrovirus with a buoyant density of 1.17 g/cm^3 could be isolated from neoplastic soft-shell clams. The present study found only membranous-like material at this density and no virus could be identified.

This study showed that in 1989, the prevalence of HN in the southern part of Narragansett Bay varied from 0% to 15% with an average of 6.3%. Such prevalence is low when compared to those reported in Falmouth, Massachusetts

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(11-18% in 1981-82)[20], New Bedford, Massachusetts (18-90% in 1983)[20], Westport, Connecticut (13% in 1984-85)[6], and Chesapeake Bay (42-65% in 1983-84)[10]. Also, the current prevalence of HN at Allen Harbor (0.6 - 15%, average of 6.3%) was much less than that reported by Cooper, *et al*[8] some 10-12 years ago. Cooper, *et al* showed that in 1977-79, the prevalence of HN at Allen Harbor ranged from 19-43% and averaged 30%. It appears that HN in the southern part of Narragansett Bay, though present, is less serious than many other areas of New England and Chesapeake Bay. At Allen Harbor, the number of neoplastic clams has decreased dramatically from those reported 10-12 years ago.

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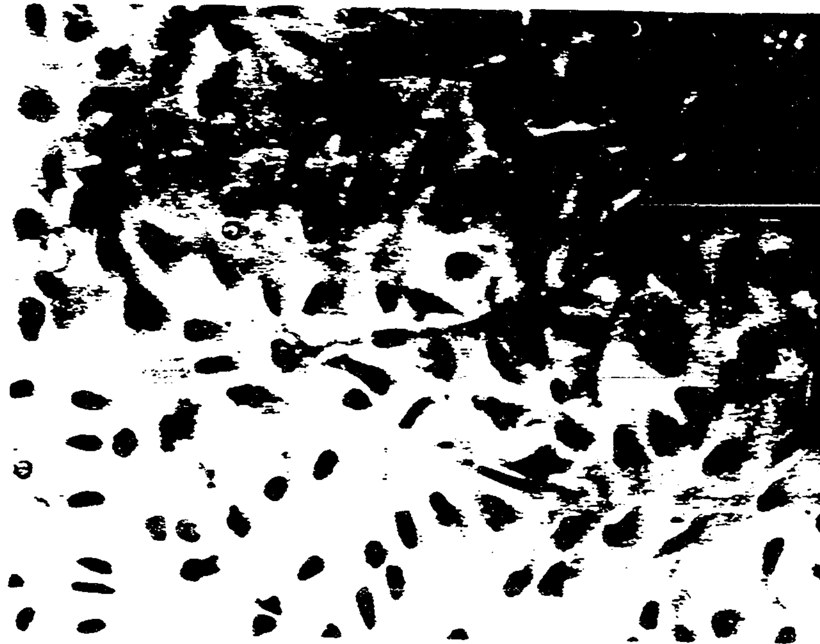

Figure 1. Monolayer of hemocytes from a non-neoplastic soft-shell clam stained with Papanicolaou stain (400X).

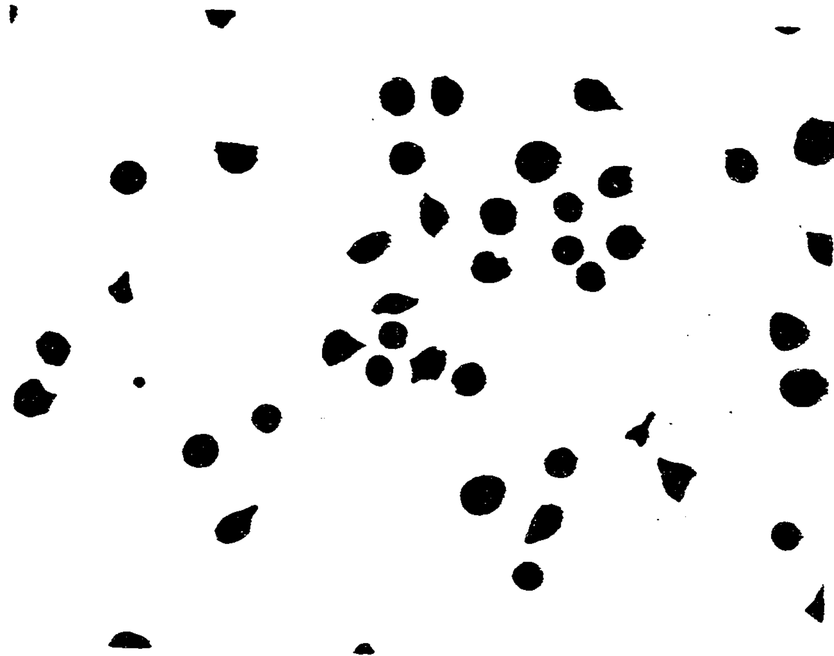

Figure 2. Monolayer of hematopoietic neoplastic cells from soft-shell clam, stained with Papanicolaou stain (400X).

Figure 3. 25-30 nm virus isolated from neoplastic soft-shell clams (Bar = 50 nm).

Figure 4. Neoplastic hemocytes stained with the indirect peroxidase stain using rabbit anti-virus serum as the primary antibody. Cells are stained positive for antibody reaction. (400X)

Figure 5. Normal non-neoplastic hemocytes stained with the indirect peroxidase stain using rabbit anti-virus serum as the primary antibody. Cells are stained positive for antibody reaction. (400X)

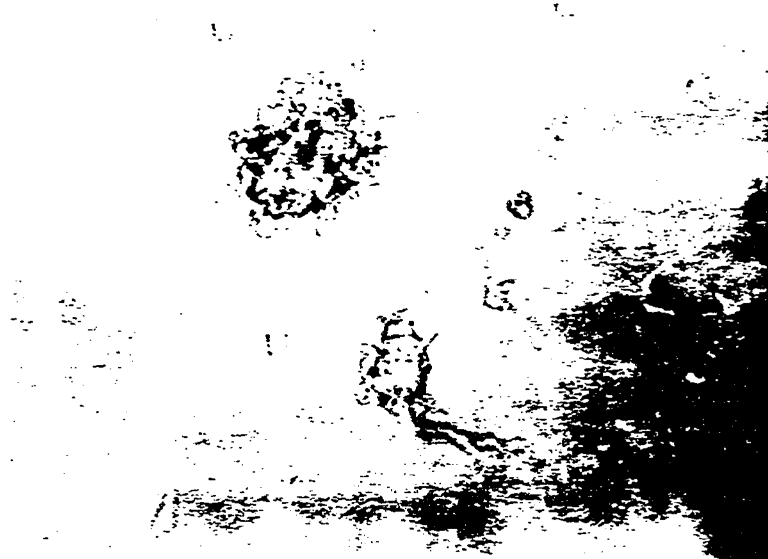

Figure 6. Neoplastic hemocytes stained with the indirect peroxidase stain using normal rabbit serum as the primary antibody. Cells are stained negative for antibody reaction. (400X)

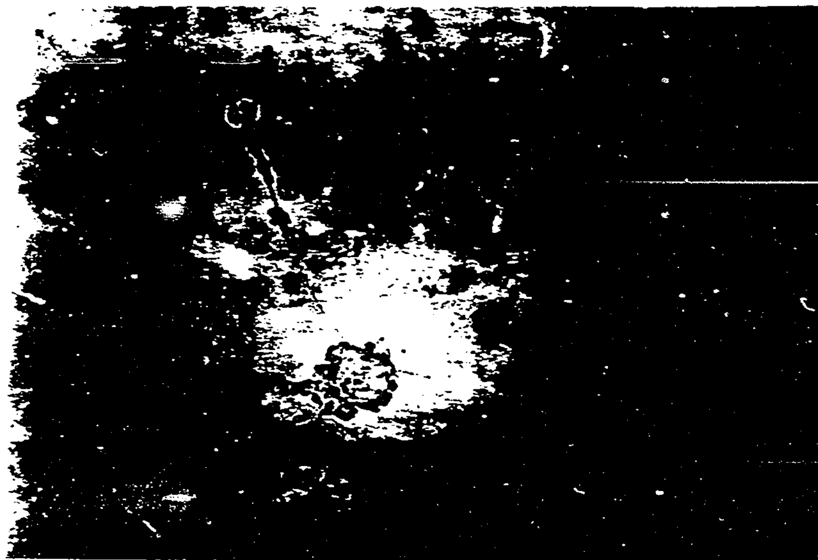

Figure 7. Normal non-neoplastic hemocytes stained with indirect peroxidase stain using normal rabbit serum as the primary antibody. Cells are stained negative for antibody reaction. (400X)

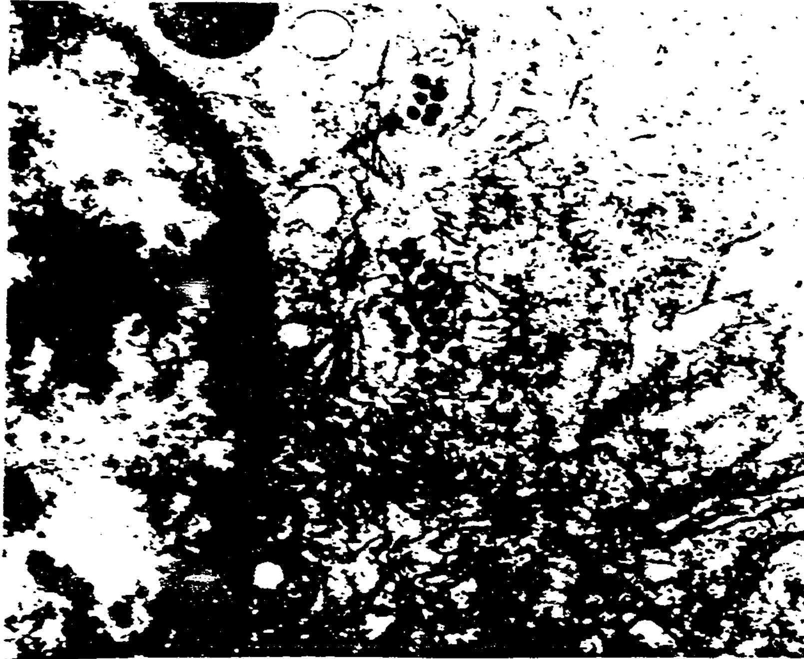

Figure 8. Electron micrograph of a neoplastic cell. Large granules in the cytoplasm (arrow) measures 90 nm and are probably glycogen granules. Bar = 300 nm.

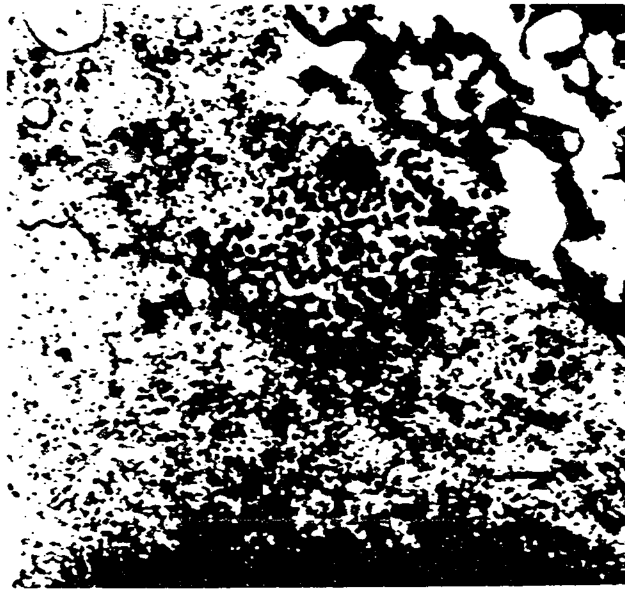

Figure 9. Aggregates of 30 nm particles in the cytoplasm of a neoplastic hemocyte and probably consist of glycogen. Bar = 200 nm.

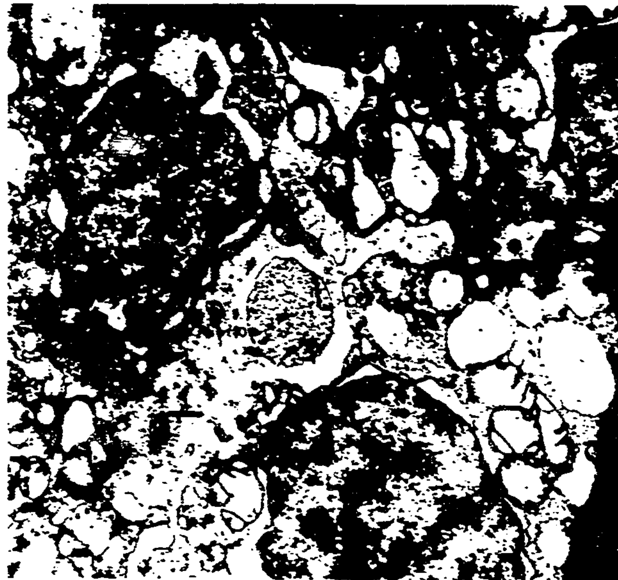

Figure 10. Small particles measuring 30 nm and enclosed in an envelope in the cytoplasm of a neoplastic cell. The particles resemble those described in Figure 9. Bar = 5 u.

TABLE 1 Isolation of 25-30 nm virus from neoplastic and non-neoplastic clams

Date of isolation	Sources of Clams: neoplastic	Sources of Clams: non-neoplastic	Virus isolation
1989, 2/21	M8(4+), M35(3+), M61(2+),A53(3+)	P4(-), P23(-), P35(-), P123(-)	yes 25-30 nm virus
3/15	M18(2+),M55(4+) M66(4+),A7 (2+) A92(4+)	P43(-),P46(-), P55(-) P58(-),P75(-)	yes 25-30 nm virus
4/27	M42(4+),A8 (4+) A223(1+)	P146(-),P148(-), P150(-)	yes 25-30 nm virus
5/23	M45(2+) M30(2+) F9 (2+)	P140(-) P151(-) P152(-)	yes 25-30 nm virus
7/18	A412(3+),A454(4+) A463(4+),A470(4+) A492(3+),M78 (3+)	P147(-) P139(-) P156(-) P140(-) P137(-) P142(-)	no 25-30 nm virus
12/27	X6(4+) X41(3+),X50(2+)	X35(-) X61(-) X83(-)	no 25-30 nm virus
1990, 1/17	W7(4+) W36(4+)	W121(-) W1-9(-)W105(-)	no 25-30 nm virus
1/22	D18(4+),D79(4+) D81(4+),D84(4+)	X57(-),X86(-) X240(-),X243(-) X246(-)	no 25-30 nm virus

Stages of Neoplasia: (-) = negative, (1+)<10% cells neoplastic, (2+) 10-30% cell neoplastic, (3+)30-70% cells neoplastic, (4+)70-100% cells neoplastic.

M = Marsh Point; A = Allen Harbor; P = Prudence Island; F = FDA in Quonsett.

X = Narragansett (location unknown); W = Warwick; D = Duck Cove, Hamilton

TABLE 2 Transmission Study

Clam no.	Treatment	Results on Neoplasia			
		Date of Bleeding			
		5/1/89	5/25/89	6/23/89	7/25/89
41	I[b]	(-)[a]	(-)	(-)[a]	
75		(-)	(-)	(-)	(-)[a]
76		(-)	(-)	(-)	(-)
A212		(-)	(-)	(-)	(-)
A251		(-)	(-)		
A260		(-)	(-)		
A275		(-)	(-)	(-)	
E1		(-)	(-)		
F21		(-)	(-)		
F40		(-)	(-)	(-)	(-)
P59		(-)	(-)	(-)	(-)
P105		(-)	(-)	(-)	(-)
P144		(-)	(-)		
R17		(-)	(-)	(-)	
243	C[c]	(-)	(-)	(-)	
A52		(-)	(-)	(-)	(-)
A60		(-)	(-)	(-)	
A68		(-)	(-)	(-)	(-)
A81		(-)	(-)	(-)	
M33		(-)	(-)	(-)	
M37		(-)	(-)	(-)	
M52		(-)	(-)	(-)	
M74		(-)	(-)	(-)	
M85		(-)	(-)	(-)	(-)
P7		(-)	(-)	(-)	(-)
P23		(-)	(-)	(-)	
P31		(-)	(-)	(-)	
X36		(-)	(-)	(-)	(-)

[a](-): clam was diagnosed negative for neoplasia by examination of stained hemocytes.

[b]I : clams were inoculated with 0.2 ml of purified virus (25-30 nm).

[c]C : control clams were inoculated with 0.2 ml of clam tissue fluid.

TABLE 3 Prevalency of Hematopoietic Neoplasm (Clam Leukemia) of *Mya arenaria* in Rhode Island

Date	Site	Total No. Tested	No. of Clams With Neoplasia	% Neoplasia
1/2/89	Prudence Island	137	0	0
1/9/89	Allens Harbor	109	14	13.0
1/14/89	Marsh Point	76	12	15.0
4/7/89	Allens Harbor	160	1	0.6
4/20/89	Marsh Point	24	2	8.3
4/20/89	Allens Harbor	46	1	0.6
4/20/89	Prudence Island	15	0	0
6/15/89	Allens Harbor	55	2	3.6
6/30/89	Allens Harbor	61	6	9.8
7/6/89	Allens Harbor	92	7	7.1
7/6/89	Marsh Point	30	3	10.0
9/22/89	Allens Harbor	10	1	10.0
12/13/89	Warwick area	73	2	2.7
12/13/89	Narragansett Pier area	132	6	4.5
1/16/90	Duck Cove (Hamilton)	125	11	8.9

APPENDIX 1

Procedure for Indirect Immunoperoxidase (IP) Procedure

Treatments and Solutions	Incubation time in min.
Fixation	
Cells fixed in glutaraldehye 1%/formaldehyde 4% in sea water	120
Washing	
PBS, 3 changes, 5 min each change	15
Blocking	
3% hydrogen peroxidase in methanol	30
Rehydrate	
50% ethanol	1
Washing	
PBS, 4 changes, 5 min each change	20
Blocking	
Normal goat serum 1:10 in PBS	30
Dry by blotting	
Primary serum	
Rabbit anti-virus serum (1:50 in PBS) or normal rabbit serum (1:50 in PBS)	60
Washing	
PBS, 3 changes, 5 min each change	15
Labeling reagent	
Peroxidase-labeled goat anti-rabbit immuno-globulin G (1:1000)	30
Washing	
PBS, 2 changes, 5 min each	10
Chromogen (mix before using)	
10ug DAB in 9 ml .1M Tris buffer plus 20 ul H_2O_2 (30%) plus 10 ml PBS	
Washing	
Running water	5
Dehydrate	
50% ethanol	2
100% ethanol	2
Xylene	2
Mount with cover-slip	