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CLEVELAND Lakeside Hospital	October 17, 1966
DISTRICT OF COLUMBIA Naval Medical Research Institute	October 27, 1966
NORTHWEST Battelle Memorial Inst., Richland, Wash.	November 11-12, 1966
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Inhibition of Effects of Leukemogenic Viruses in Mice by Extracts of *Mercenaria mercenaria.** (31471)

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For several years as a result of the reports of Szent-Gyorgyi *et al*(1,2), there has been increased interest in the oncostatic effect of certain normal cell constituents. Their work described the inhibition of gross tumors in animals and malignant cell cultures by a variety of tissue extracts derived from a number of different species. The biological property of oncostasis is thought by Szent-Gyorgyi to be part of the mechanism by which mitosis is regulated in multicellular organisms. This property has been particularly investigated in extracts of shellfish in which both antiviral and antitumor activities have been described. Schmeer(3,4) demonstrated that extracts of *Mercenaria mercenaria,* the common edible quahog or clam, prevented growth of transplanted Sarcoma 180 and Krebs 2 tumors in Swiss mice. Li *et al*(5) subsequently demonstrated inhibition of tumor growth induced by human adenovirus in hamsters treated with clam extracts.

These studies suggested the possibility that clam extracts might prevent or inhibit the development of leukemia in susceptible mice inoculated with murine leukemogenic viruses. Experiments were then conducted to determine whether clam extracts have any effect on the development of virus-induced leukemia in mice.

Materials and methods. Live clams obtained during the summer months were used

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immediately or were quick-frozen at —70°C after being shucked, because of reports(6) that clam extracts possess greater antitumor activity during this season. Clams were processed according to the following modification of Schmeer's method.

Shucked clams were homogenized in a sterilized stainless steel Waring blendor and an equal volume of 20% ammonium sulfate added to the homogenate. The mixture was centrifuged at 4000 RPM for 30 minutes. The supernate was dialyzed against distilled water at 4°C for 48 hours and then lyophilized at —30°C. The powder product was yellow-tan and completely water soluble. The yield was approximately 1% of wet weight of the shucked clams.

The powder was rehydrated in sterile 0.1 M NaCl and eluted from a Sephedex® G-25 column using 0.1 M NaCl as the elution fluid. All elutions were performed at room temperature. Five grams of lyophilized powder were dissolved in 50 ml eluent and applied to the column (5.6 × 80 cm). All eluate was collected and pooled. The pool of eluate, approximately 1000 ml, was then lyophilized at —30°C and stored at —70°C until rehydrated for use. The required amounts of partially purified lyophilized powder were rehydrated in sterile normal saline containing 100 units penicillin G and 100 μg streptomycin sulfate per ml.

Mice. Pregnant BALB/c mice were obtained from a breeding farm† and male offspring utilized for the Moloney virus experiments at 5 weeks of age. This was done to insure that all animals were less than 6 weeks of age at time of inoculation. DBA/2J female mice obtained from a breeder‡ at 6 weeks of age were utilized for the Friend virus experiments.

Viruses. Moloney virus was obtained from the American Type Culture Collection and passaged in BALB/c mice 4 times prior to experimental use. Homogenized spleen-lymph node suspensions from infected animals were used for passage of virus. After being reduced to liquid consistency in a cooled (4°C) Waring blendor, a 40% (by volume) suspension of these tissues was made in sterile normal saline. This material was then injected intraperitoneally into mice for passage. The transplantable Moloney virus (MV) model system containing malignant cells was chosen because previous reports(7,8) indicated that it produced death from leukemia consistently and within a relatively short period of time in contrast to experiments in which virus alone was inoculated.

Friend virus was also obtained from the American Type Culture Collection and passaged in Swiss mice 6 times prior to experimental use. In the preparation of Friend virus material, cell-free filtrates of infected splenic tissue homogenates were used for passage. The filtrates used in these experiments were quick frozen and stored at —70°C until used.

Procedures and results. To determine the effect of clam extracts on longevity of mice using the MV system with BALB/c mice, 200 BALB/c male mice 5 weeks old were inoculated intraperitoneally with 0.1 ml of MV preparation. These mice were separated randomly into two groups and initial therapy was started on one group 24 hours later. Portions of the partially purified lyophilized clam extract were rehydrated daily so that 2 mg were contained in 0.2 ml fluid. Each treated animal received the extract, intraperitoneally, on 7 consecutive days after virus inoculation. Control animals received injections of diluent on the same schedule.

Eight weeks following virus inoculation, a week of similar daily intraperitoneal injections of an increased dose of 2.5 mg of extract in 0.2 ml diluent was given to the treatment group of animals. Control group animals were simultaneously injected with diluent.

As shown in Fig. 1, the first deaths in control animals began 35 days after MV inoculation. Fifty per cent of the control animals were dead by the 84th day and all were dead by the 108th day after virus inoculation. In contrast, no deaths occurred among the treated animals until the 105th day following virus inoculation and approximately 50% were still alive on the 160th day. By the 180th day, however, all treated animals had died. Thus, the data show that the extracts doubled the longevity of the treated group

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‡ Jackson Laboratories, Bar Harbor, Maine.

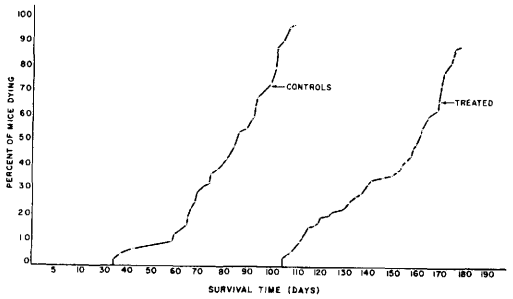

FIG. 1. Cumulative deaths in *BALB/c* mice following inoculation with Moloney virus.

and corroborate previous findings of others that Mercenaria tissue extracts contain a principle active against neoplastic growth.

All animals succumbed to leukemia, however, as evidenced by gross and histologic examinations at time of death. None of the treated animals manifested localized tumors but all revealed pathology suggestive of virus-induced leukemia, *i.e.*, thymic, splenic and generalized lymphatic enlargement which on histological examination showed almost complete malignant infiltration. In contrast, many of the control animals, particularly those dying early in the course of the experiment, did not show evidence of marked thymic or splenic enlargement but did reveal manifestations of localized intra-abdominal tumors as well as histologic evidence of generalized leukemia.

In separate experiments, another system was chosen to study the effect of Mercenaria extracts on splenomegaly (an accepted index of Friend disease) as well as longevity. The Friend virus (FV) model system using DBA/2J female mice was utilized in these studies.

A 1:4 saline dilution of cell-free filtrate containing FV was made just prior to use. One hundred DBA/2J female mice 6 weeks old were inoculated intraperitoneally with 0.1 ml of this material. The animals were then randomly separated into 2 groups of 50 each. Seventy-two hours later 1 group was begun on daily intraperitoneal injections of 2.5 mg clam extract in 0.2 ml fluid for 7 days. Control animals received similar amounts of diluent. On day 21 following virus inoculation, 50% of control and treated animals were selected at random and sacrificed for splenic weight measurement and postmortem examination. The remaining 50% of each group were retained for measurement of longevity.

As seen in Table I, 4 separate experiments showed marked reduction in splenomegalic response to FV in treated animals as compared to control animals. Using spleen weight as an index of pathologic response, treatment with Mercenaria extract significantly diminished neoplastic response in these animals in the order of 100%. Only in the last experiment was there a mean splenic weight difference smaller than 1 gram and the t test for statistical significance of each experiment was $<.001$. In contrast the longevity studies revealed no significant difference between control and treated groups.

Discussion. These studies show that some entity of clam tissue, presumably an intracellular component, apparently inhibits neoplastic cellular response to infection with both MV and FV but does not eliminate such infections.

In the longevity studies with MV, it is assumed from the method of preparation that neoplastic cells as well as virus were inoculated into test animals. That the treated animals survived longer than control animals only to succumb to the generalized effects of leukemia suggests that the initial cellular implants were destroyed by the Mercenaria extracts but viruses were not markedly affected. Thus, treated animals outlived control animals as a result of protection against the early lethal effects engendered by transplanted tumor cells, but died later from neoplasia induced by the replicating virus.

This hypothesis is supported by data from

TABLE I. DBA Mice Infected with Friend Virus.*

Exp No.	Group	No. of animals	Mean splenic wt (g)	P value
I	Control	25	1.70	$<.001$
	Treated	25	.64	
II	Control	24	1.60	$<.001$
	Treated	23	.61	
III	Control	22	1.65	$<.001$
	Treated	22	.62	
IV	Control	25	1.34	$<.001$
	Treated	24	.61	

* Sacrificed 21 days after virus inoculation.

the FV experiments in which neoplasia, measured by increased splenic weight, was retarded by treatment with the Mercenaria extract but longevity, relatively long in Friend disease, was not altered. This is again in accord with the premise of late death from neoplasia induced by a replicating virus.

The data obtained support the premise that the inhibiting effect of the Mercenaria extracts is mediated primarily against transformed or neoplastic cells. The partial inhibition of neoplastic cell response in the spleens of the treated mice infected with FV and the absence of localized tumors in treated mice infected with MV point to an oncostatic or oncocidal mechanism of action of these extracts.

Szent-Gyorgyi's studies on normal regulation of mitotic activity in multicellular organisms indicate that certain cell constituents which he terms "autobiotics" markedly affect mitosis in neoplastic cells. There are apparently 2 closely related but separate substances which can cause either proliferation or inhibition of malignant cells and these substances seem to be present in approximately equal amounts in most cells studied. The inhibiting substance is, however, present in greater abundance in molluscs as has been demonstrated in the experiments of Schmeer and Li.

In Li's experiments in which onset of adenovirus-induced tumors in hamsters was delayed by treatment with Mercenaria extract, the results were interpreted as being caused by diminution of virus titer. In the present experiments there may also have been a diminution of virus titer after extract treatment. Dawson *et al*(9) have demonstrated previously, however, that reduction of splenomegalic response by chemotherapy in the FV system does not necessarily imply a concomitant reduction in virus titer. The latter interpretation is more consistent with the data derived from our experiments.

In comparison with currently available antileukemia chemotherapeutic drugs, these crude Mercenaria extracts are relatively ineffectual. The great promise of such cellular components lies in the fact that they are natural components of living cells and that further investigations involving isolation and purification of the active principle may add to fundamental knowledge of mitosis, and, in turn, to the control of neoplasia in man.

Summary. Ammonium sulfate extracts of *Mercenaria mercenaria* were prepared and partially purified by Sephadex® G-25 column chromatography. This extract was tested for inhibition of murine leukemias induced by Moloney and Friend viruses. The extract prolonged the mean survival time in animals inoculated with Moloney virus transplantable leukemia. The extract also inhibited the splenomegalic response in animals infected with Friend virus, but did not extend longevity of this latter group.

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