ANTITUMOR ACTIVITY OF AQUEOUS EXTRACTS OF MARINE ANIMALS

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Aqueous extracts (macromolecule fractions in particular) of 25 species of well-known marine animals were tested for their antitumor activity against transplanted sarcoma 180 solid form in ICR mice. All of the macromolecule fractions from the extracts of animals in the shellfish category except that of *Mytilus edulis* inhibited the growth of sarcoma 180. The use of aqueous extracts from *Strongylocentrotus nudus*, *Halocynthia hilgendorfi* f. *ritteri*, *Styela plicata*, *Ecteinascidia turbinata*, and *Megabalanus rosa* also revealed relatively high inhibition ratios of over 60%.

Keywords — sarcoma 180; ICR mouse; antitumor activity; marine animal extract; macromolecule

INTRODUCTION

There are numerous reports on the pharmacological and toxicological effects elicited by extracts of marine organisms.¹⁾ However, most of them are cytotoxic in their action and are also toxic to the host. With increasing attention to the importance of immunotherapy, it would be desirable to search not only for chemotherapeutic agents that show cytotoxicity on cancer cells but also for agents which can stimulate the host immunological defense against neoplastic growth. Such considerations prompted us to begin evaluating marine organisms, especially biopolymers in marine invertebrates, as new sources of potentially useful cancer chemotherapeutic drugs. In the previous study, we found that the high molecular weight substances from *Patinopekten yessoensis*, an edible cultured scallop found in Japanese coastal waters, showed a strong antitumor activity.²⁾ The suggestion was made that the antitumor activity was due to indirect host-mediated effect.

The studies reported here examine the antitumor effect of the macromolecule fractions from aqueous extracts of 25 species of marine animals found in Japanese coastal waters or the East coast of the U.S.A.

MATERIALS AND METHODS

Collection of Marine Animals — *Haliotis discus hannai*, *Haliotis diversicolor aquatilis*, *Batillus cornutus*, *Strongylocentrotus intermedius*, *Strongylocentrotus nudus*, *Halocynthia hilgendorfi* f. *ritteri*, *Styela plicata*, *Ciona intestinalis*, *Megabalanus rosa*, *Urotrygon daviesi*, *Dasyatis akajei*, and *Theragra chalcogramma* were collected from the Pacific coast of Japan. Other species in this study were collected off the East coast of the U.S.A.

Preparation of the Samples for Bioassay — Tissue extracts of fresh specimens of shellfish were prepared by removing the animals from their valves. Each species (100 g) was separately homogenized with 1 l of saline for 5 min and

then centrifuged at 10000 rpm for 10 min. The supernatant fluid was successively fractionated with the use of Diaflo ultrafiltration membranes (Amicon Corp., Lexington, MA). Each fraction was dialyzed, lyophilized, dissolved in an appropriate volume of saline and submitted to bioassay.

Measurement of Total Carbohydrate and Protein — Total carbohydrate was measured as anhydroglucose by the phenol-sulfuric acid method using D-glucose as the standard.³⁾ Total protein was determined by the Lowry method with egg albumin used as the standard.⁴⁾ Their values are summarized in Tables I and II.

Mice — Female ICR mice, weighing about 23 g, were obtained from CLEA Japan, Inc. They were maintained on standard diet and water throughout the experiments.

Tumor — Sarcoma 180, initially obtained from the Memorial Sloan-Kettering Cancer Center, New York, NY, has been maintained in the National Cancer Center Research Institute in an ascites form. A volume of 0.05 ml (6×10^6 cells) of 7-day-old ascites sarcoma 180 tumor was transplanted subcutaneously into the right groin of the ICR mice. We have never observed any spontaneous regression of this tumor.

Assay of Antitumor Activity — Test samples at a dose of 400 mg/kg in 0.1 ml of saline were injected into the tumor site on days 5, 7, and 9 after tumor inoculation, by which time the tumors had reached an average diameter of 4 mm. Mice receiving similar treatment with saline were served as controls. At the end of week 5, the mice were killed and the tumors dissected and weighed. The inhibition ratios were calculated by use of the following formula: Inhibition ratio (%) = $[(A - B)/A] \times 100$, where A is the average tumor weight of the control group and B is that of the treated group. Complete regression indicates the ratio of the number of mice showing complete tumor regression to the total number of mice tested.

In Vitro Cytotoxicity Testing — The ascites tumor cells (10^5) of sarcoma 180 were incubated in a CO₂ gas incubator at 37 °C for 24 h in 1 ml

of medium containing various concentrations of test samples. Viability of tumor cells, estimated by staining with 0.17% Trypan blue, was then compared with that of control cells incubated in the same medium without samples. Eagle's minimum essential medium with 10% fetal calf serum was used for suspension cultures.

RESULTS AND DISCUSSION

The present investigations concern the antitumor activity of the macromolecule fractions from aqueous extracts of well-known marine animals. The effect of the fractions was tested on sarcoma 180 transplanted subcutaneously into the ICR mice.

As shown in the two Tables, the fractions showed various antitumor effects when given into the tumor site in a dose of 400 mg/kg on days 5, 7, and 9 after tumor inoculation. There was no detectable toxicity at the dose used in the present study in which the animals were absolutely healthy and gained weight.

The bioassay results of *H. discus hannai*, *H. diversicolor aquatilis*, *B. cornutus*, *P. magellanicus*, *A. irradians*, *M. mercenaria*, *M. arenaria*, *M. edulis*, and *C. virginica* in the shellfish category are summarized in Table I. All of the aqueous extracts except that of *M. edulis* (mussel) highly inhibited the growth of the transplanted tumor (inhibition ratio >60%). *H. discus hannai* (abalone) resulted in tumor inhibition ratio of 78.6% in the fraction of molecular weight (M_r) above 50000. *H. diversicolor aquatilis* (ear shell) and *B. cornutus* (top shell) showed substantial inhibition ratios (83.7 and 96.7%, respectively) with complete regression in three and one of six mice tested, respectively, in the fraction of M_r ranging from 100000 to 300000. The fractions of M_r ranging from 100000 to 300000 from the Atlantic sea scallop (*P. magellanicus*) and common bay scallop (*A. irradians*) also gave high antitumor activities (inhibition ratios of 81.5 and 76.2%, respectively) and caused complete disappearance of the tumor in some mice. These results are very similar to those found in a Japanese scallop, *P. yessoensis*.²⁾ The three clams have the

same scientific name, *M. mercenaria*. However, they are sold under different names according to their age. The youngest clam (which is the cherrystone) showed the major activity in the fraction of M_r ranging from 100000 to 300000. The older clams (littleneck clam and quahog) showed major activity in the larger molecular range of above 300000. Each fraction in M_r ranging from 100000 to 300000 from *M. arenaria* (steamer clam) and *C. virginica* (oyster) demonstrated relatively high antitumor activity (inhibition ratios of 77.6 and 82.2%, respective-

ly) but did not cause tumor regression. Schmeer and Huala reported that the clams (cherrystone and quahog) showed the greatest amount of anti-neoplastic activity in comparison to squid and oyster.⁵⁾ Our present results from marine clams are almost similar to those of Schmeer, but our result from oyster is different from that of Schmeer. A possible reason for this discrepancy is due to the different bioassay method, system and sample species.

The bioassay results of the macromolecule fractions of the aqueous extracts from Echino-

TABLE I. Local Treatment of Sarcoma 180 in ICR Mice with Macromolecule Fractions of Aqueous Extracts from the Shellfishes

Common name	Scientific name	Molecular weight range ($\times 10^3$)	% contents		Average tumor weight (g) (treated/control mice)	Tumor inhibition ratio (%)	Complete regression ^{a)}
			Protein	Carbohydrate			
Abalone	<i>Haliotis discus hannai</i>	$M_r > 10$	33.1	16.9	$5.6 \pm 1.6/14.6 \pm 4.9$ ^{b)}	61.6	0/6
		$M_r < 10$	4.3	1.4	$13.5 \pm 3.0/14.6 \pm 4.9$	7.5	0/6
		$M_r > 50$	26.8	12.6	$2.2 \pm 1.4/10.3 \pm 4.7$	78.6	0/6
		$50 > M_r > 10$	13.0	5.9	$4.4 \pm 1.6/10.3 \pm 4.7$	57.3	0/6
Ear shell	<i>Haliotis diversicolor aquatilis</i>	$300 > M_r > 100$	54.9	9.3	$1.7 \pm 2.1/10.3 \pm 4.7$	83.5	3/6
Top shell	<i>Batillus cornutus</i>	$300 > M_r > 100$	27.0	30.8	$0.4 \pm 0.3/10.3 \pm 4.7$	96.7	1/6
Sea scallop	<i>Placopecten magellanicus</i>	$M_r > 300$	43.4	10.5	$5.4 \pm 3.3/10.3 \pm 4.7$	47.6	1/6
		$300 > M_r > 100$	33.6	15.2	$1.9 \pm 1.1/10.3 \pm 4.7$	81.6	1/6
Bay scallop	<i>Aequipecten irradians</i>	$M_r > 300$	29.2	10.7	$4.5 \pm 3.3/10.3 \pm 4.7$	56.3	2/6
		$300 > M_r > 100$	38.4	21.5	$2.4 \pm 2.4/10.3 \pm 4.7$	76.7	3/6
Quahog	<i>Mercenaria mercenaria</i>	$M_r > 300$	30.0	48.4	$4.1 \pm 1.7/10.7 \pm 6.5$	61.7	0/6
		$300 > M_r > 100$	4.7	13.7	$8.2 \pm 1.8/10.3 \pm 4.7$	20.4	0/6
Little neck clam	<i>Mercenaria mercenaria</i>	$M_r > 300$	20.7	23.2	$1.5 \pm 2.5/10.7 \pm 6.5$	86.0	1/6
		$300 > M_r > 100$	23.0	10.9	$7.8 \pm 3.8/10.7 \pm 6.5$	27.1	0/6
Cherry stone clam	<i>Mercenaria mercenaria</i>	$M_r > 300$	38.3	16.7	$5.3 \pm 2.5/10.7 \pm 6.5$	50.5	0/6
		$300 > M_r > 100$	16.6	7.8	$2.6 \pm 1.7/10.7 \pm 6.5$	75.7	0/6
Steamer clam	<i>Mya arenaria</i>	$M_r > 300$	35.9	12.2	$8.2 \pm 3.5/10.7 \pm 6.5$	23.4	0/6
		$300 > M_r > 100$	14.5	12.3	$2.4 \pm 3.8/10.7 \pm 6.5$	77.6	0/6
Mussel	<i>Mytilus edulis</i>	$M_r > 300$	24.3	15.4	$9.4 \pm 2.8/10.7 \pm 6.5$	12.1	0/6
		$300 > M_r > 100$	21.5	8.5	$10.2 \pm 1.4/10.7 \pm 6.5$	4.7	0/6
Oyster	<i>Crassostrea virginica</i>	$M_r > 300$	34.7	22.7	$7.6 \pm 4.7/10.7 \pm 6.5$	29.0	0/6
		$300 > M_r > 100$	48.3	44.3	$1.9 \pm 1.3/10.7 \pm 6.5$	82.2	0/6

a) No. of tumor free mice/ No. of mice tested.

b) Values are means $\pm$ S.D.

derms, Ascidians, and others are summarized in Table II.

In Echinoderms, *S. intermedius* (sea urchin) exhibited only mild inhibition ratio. However,

TABLE II. *Local Treatment of Sarcoma 180 in ICR Mice with Macromolecule Fractions of Aqueous Extracts from Echinoderms, Ascidians, and Others*

Common name	Scientific name	Molecular weight range ($\times 10^3$)	% contents		Average tumor weight (g) (treated/control mice)	Tumor inhibition ratio (%)	Complete regression ^{a)}
			Protein	Carbohydrate			
Sea urchin	<i>Strongylocentrotus intermedius</i>	$M_r > 10$	30.4	4.0	$8.2 \pm 4.8/14.6 \pm 4.9$ ^{b)}	43.8	0/6
		$M_r < 10$	5.5	<2.6	$9.5 \pm 4.0/14.6 \pm 4.9$	34.9	0/6
Sea urchin	<i>Strongylocentrotus nudus</i>	$M_r > 10$	ND ^{c)}	ND	$3.8 \pm 1.2/14.6 \pm 4.9$	74.0	0/6
		$M_r > 300$	50.3	11.8	$4.5 \pm 2.5/10.3 \pm 4.7$	56.3	0/6
		$300 > M_r > 50$	56.5	12.5	$2.4 \pm 2.4/10.3 \pm 4.7$	76.7	1/6
		$50 > M_r > 10$	13.6	13.3	$8.2 \pm 1.8/10.3 \pm 4.7$	20.4	0/6
Sea squirt	<i>Halocynthia hilgendorfi</i> f. <i>ritteri</i>	$M_r > 10$	22.5	29.4	$2.6 \pm 1.1/10.3 \pm 4.7$	74.8	0/6
		$10 > M_r > 0.5$	11.3	22.6	$4.5 \pm 1.6/10.3 \pm 4.7$	56.3	0/6
		50% EtOH extract	5.0	3.5	$4.7 \pm 1.8/10.3 \pm 4.7$	54.4	0/6
Sea squirt	<i>Styela plicata</i>	$M_r > 10$	36.7	34.5	$4.0 \pm 2.3/10.3 \pm 4.7$	61.2	0/6
Sea squirt	<i>Ciona intestinalis</i>	$10 > M_r > 0.5$	7.0	<2.6	$8.5 \pm 2.1/10.3 \pm 4.7$	17.5	0/6
		50% EtOH extract	2.9	<2.6	$5.6 \pm 2.5/10.3 \pm 4.7$	45.6	0/6
Sea squirt	<i>Ecteinascidia turbinata</i>	$M_r > 10$	17.8	<2.6	$0.8 \pm 1.3/10.3 \pm 4.7$	92.2	3/6
Acorn barnacle	<i>Megabalanus rosa</i>	Coelomic fluid	1.5	<2.6	$8.6 \pm 2.2/14.6 \pm 4.9$	41.1	0/6
		$M_r > 50$	35.4	9.0	$5.4 \pm 2.6/14.6 \pm 4.9$	63.0	0/6
		$50 > M_r > 10$	ND	ND	$5.1 \pm 3.0/14.6 \pm 4.9$	65.1	0/6
Tube worm	<i>Pseudopotamilla ocellata</i>	$M_r > 10$	14.0	3.0	$10.5 \pm 4.2/14.6 \pm 4.9$	28.1	0/6
Red crab	<i>Geryon quinquedens</i>	$M_r > 300$	25.1	2.9	$5.6 \pm 2.5/10.7 \pm 6.5$	47.7	0/6
		$300 > M_r > 100$	15.6	2.9	$5.6 \pm 1.9/10.7 \pm 6.5$	47.7	0/6
Rock crab	<i>Cancerueta irroratus</i>	$M_r > 300$	44.4	3.2	$4.7 \pm 4.4/10.7 \pm 6.5$	56.1	0/6
		$300 > M_r > 100$	15.8	3.3	$5.1 \pm 4.4/10.7 \pm 6.5$	52.3	0/6
Ray	<i>Urotrygon daviesi</i>	Serum					
Stingray	<i>Dasyatis akajei</i>	$M_r > 10$	34.6	5.5	$15.7 \pm 4.9/14.6 \pm 4.9$	-7.5	0/6
		Serum					
Alaska pollack	<i>Theragra chalcogramma</i>	$M_r > 10$	45.2	<2.6	$9.9 \pm 4.3/14.6 \pm 4.9$	32.2	0/6
		Chalone fraction	39.3	<2.6	$7.6 \pm 1.6/10.3 \pm 4.7$	26.2	0/6
		70% EtOH ppt	39.4	<2.6	$4.9 \pm 1.7/10.3 \pm 4.7$	52.4	0/6
Squid	<i>Loligo paelici</i>	70% EtOH sup	31.0	2.8	$6.4 \pm 5.0/10.3 \pm 4.7$	37.9	0/6
		$M_r > 300$	53.2	<2.6	$6.9 \pm 2.6/10.7 \pm 6.5$	35.5	0/6
		$300 > M_r > 100$	34.1	<2.6	$5.3 \pm 1.7/10.7 \pm 6.5$	50.5	0/6

a) No. of tumor free mice/No. of mice tested.

b) Values are means $\pm$ S.D.

c) Not determined.

administration of *S. nudus* in the fraction of M_r ranging from 500000 to 300000 led to a more substantial inhibition ratio of 76.7% with complete regression in one of six mice tested. Pettit *et al.* have reported that *S. drobachiensis* (green sea urchin) has been found to contain a new glycoprotein antineoplastic agent.⁶⁾ Its M_r was suggested to be over 40 million. In the present study, the fraction of M_r over 300000 showed a moderate inhibition ratio of 56.3%. Thus, the active fraction obtained here is physically different from that of Pettit *et al.*⁶⁾

Four species of ascidians were tested for antitumor activity. Among them, *E. turbinata* gave the best inhibition ratio of 92% with complete tumor regression in three of six mice tested. Also, the fractions in the M_r of over 10000 from *H. hilgendorfi* f. *ritteri* and *S. plicata* showed relatively high inhibition ratios of 74 and 61%, respectively, without complete tumor regression. However, 50% ethanol extracts from *H. hilgendorfi* f. *ritteri* and *C. intestinalis* gave mild inhibition ratios of 54 and 45%, respectively. Although the antitumor activity of *E. turbinata* has been known for more than 10 years,⁷⁾ we feel that there is some confusion about the nature of the active components. The present results revealed that the antitumor active fraction of *E. turbinata* was extracted with saline and its M_r was over 10000.

In Crustacea, two crabs were tested for antitumor activity. Both *G. quinque-dens* (red crab) and *C. irrorantus* (rock crab) showed inhibition ratios of approximately 50%. On the other hand, the coelomic fluid from *M. rosa* (acorn barnacle) has a strong agglutinating activity in the presence of Ca^{2+} ions and showed a tumor inhibition ratio of 41%. The fractions of M_r over 50000 and M_r ranging from 10000 to 50000 from *M. rosa* gave substantial inhibition ratios of 63 and 65%, respectively. *P. ocellata* (tube worm) showed a very mild inhibition ratio of 28%.

There is very limited information on the antitumor activity of macromolecules from marine lower vertebrates.¹⁾ Accordingly, we studied the

antitumor effects of four species of marine vertebrates. The sera from *U. daviesi* (ray) and *D. akajei* (stingray) gave only mild and negligible inhibition ratios. Bullough *et al.* reported that extracts of vertebrate epidermis contain an anti-mitotic chemical called epidermal chalone.^{8,9)} They also extracted fresh cod skin with saline and fractionated the extract by precipitation with ethanol. Epidermal chalone from *G. morhua* (codfish) inhibited mitoses in *in vitro* and *in vivo*, and it also showed antitumor activity. Most of the activity remained in the 81% ethanol supernatant fluid. The chalone fraction from *T. chalcogramma* (Alaska pollack) exhibited a mild inhibition ratio of 26%. The fraction which was precipitated with the addition of 70% ethanol showed an inhibition ratio of 52% and its supernatant fluid gave an inhibition ratio of 37%. The fraction in M_r ranging from 10000 to 30000 from *L. paelici* (squid) showed an inhibition ratio of 50%. Our data indicated that the antitumor activity of squid was less than that of oyster which corresponded fairly well to that of the clam, whereas Schmeer and Huala reported that squid and oyster gave approximately the same weak antitumor effects on sarcoma 180 in Swiss albino mice.⁵⁾

To examine whether the extracts of marine animals had a direct cytotoxic effect on tumor cells, ascites cells of sarcoma 180 were cultured in a medium containing 0.1–0.5 mg of each extract/ml. The viability of the tumor cells was examined by Trypan blue staining and the results indicated a viability of over 93%, which did not differ from that of the cells cultured without the addition of the extracts. Furthermore, 6×10^6 cells incubated the same way as above, with medium containing 0.5 mg of fractions *H. discus hannai* ($M_r > 5000$), *H. diversicolor aquatilis* ($300000 > M_r > 100000$), *B. cornutus* ($300000 > M_r > 100000$), and *S. nudus* ($300000 > M_r > 100000$), were inoculated *s.c.* into the right flank of ICR mice. After 5 weeks, the weights of the solid tumors averaged 8.4 ± 2.3 g, compared with 8.7 ± 1.6 g which resulted from transplantation of control cells in-

cubated in a medium without the fractions. This result indicates that the cultivation of the cells with the fractions had almost no inhibitory effect on tumor growth.

Since various doses, schedules, and administration-routes were not tested relative to the use of each fraction, the fractions may not have been employed under optimal conditions in this study. Nevertheless, it can be concluded that the macromolecule fractions from extracts of some marine animals revealed surprisingly strong antitumor effects on sarcoma 180 in ICR mice. The purpose of this study was to focus on the relatively few materials worthy of a more detailed study. Additional chemical and biologic studies on the active principles of each marine animal extract will be presented in a subsequent publication.

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