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Source: Zoological Science, 14(2) : 271-276

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.271

ZOOLOGICAL SCIENCE 14: 271–276 (1997)


# Characterization of Ascidian Plasma Growth Factors Promoting the Proliferation of Mouse Thymocytes

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**ABSTRACT**—The plasma of a solitaly ascidian, *Halocynthia roretzi*, stimulates the proliferation of mouse thymocytes in the presence of a mitogen, phytohemagglutinin P (PHA-P). Eight factors capable of stimulating the proliferation of mouse thymocytes were isolated from *H. roretzi* plasma by procedures including hydrophobic chromatography, gel filtration, and anion-exchange chromatography. Four active fractions with molecular weights of 100-110 K, 75-90 K, 10-30 K, and 5-6 K were first separated by gel filtration. Each of these fractions was then separated into two fractions with different ionic properties by anion-exchange chromatography. The eight factors thus obtained have different heat stabilities, heparin-binding abilities, and PHA-P sensitivities.

## INTRODUCTION

Ascidians occupy a phylogenetically important position between the vertebrates and invertebrates. We have been investigating the defense mechanisms of a solitary ascidian, *Halocynthia roretzi* (Azumi and Yokosawa, 1996), and in the course of studies, we have found that hemocytes of *H. roretzi* have a central role in the biological defense of this animal. They show several cellular defense reactions including phagocytosis against foreign materials (Ohtake *et al.*, 1994), aggregation at a site of an injured tunic (Takahashi *et al.*, 1994), release of metallo-proteases in response to stimuli (Azumi *et al.*, 1991), and release of phenoloxidase in a self and nonself recognition reaction (Akita and Hoshi, 1995). In the hemolymph of *H. roretzi*, there are several types of hemocytes, which are functionally distinguished on the base of cellular reactions or the abilities to produce defense-related substances (Azumi *et al.*, 1993).

Among hemocytes of *H. roretzi*, some types release the metallo-proteases and other types contain a factor that stimulates the release of the metallo-proteases (Azumi *et al.*, 1993), strongly suggesting that the latter hemocytes may possess cytokine-like substances that act on the former hemocytes to cooperate each other. Also in other cellular reactions such as hemocyte aggregation, phagocytosis and the self and nonself recognition reaction, *H. roretzi* plasma can stimulate these reactions *in vitro* (Takahashi *et al.*, 1994). In these cases, certain plasma components, which are assumed to be produced by hemocytes and/or other tissues, have a possibility of interacting with hemocytes to regulate those reactions.

Several cytokine-like molecules have been so far isolated from the hemolymph of invertebrates. For instance, interleukin-1 (IL-1)-like molecules, which can stimulate the proliferation of mouse thymocytes, were isolated from the hemolymph of a starfish (Beck and Habicht, 1986, 1991). An IL-6-like molecule, which can support the growth of an IL-6-sensitive cell line, has also been isolated from the starfish hemolymph (Beck and Habicht, 1994). In ascidians, IL-1-like molecules have been isolated from the hemolymph, which can stimulate [$^3$H]-thymidine uptake by cultured tunicate tissues (Raftos *et al.*, 1991) and mouse thymocytes (Beck *et al.*, 1989). In addition, evidence for the presence of IL-1-, IL-2-, and IL-6-like molecules in the hemolymph of molluscs has been presented (Hughes *et al.*, 1990; Ottaviani *et al.*, 1993).

The above accumulating evidence led us to suppose that the ascidian *H. roretzi* may contain some growth factors which support the growth of hemocytes. In order to elucidate the mechanisms for growth of *H. roretzi* hemocytes, we attempted to isolate the growth factors from the plasma by employing mammalian thymocytes as target cells. Here, we describe the isolation and characterization of several growth factors present in the plasma of *H. roretzi*, which can promote the proliferation of mouse thymocytes in the presence of a mitogen.

## MATERIALS AND METHODS

### *Animals and chemicals*

BALB/c mice were obtained from Kurea Co. (Japan). RPMI 1640 was purchased from Nissui Seiyaku Co. (Japan). Fetal calf serum (FCS) was from Boehringer Mannheim (Germany). Penicillin G, streptomycin sulfate, and bovine serum albumin were from Sigma Chemical Co. (USA). Fungizone, phytohemagglutinin P (PHA-P), and mouse recombinant IL-1 β (mrIL-1 β) were from GIBCO (USA), Waco

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Junyaku Co. (Japan), and R&D Systems (USA), respectively. [$^3$H]-thymidine was from New England Nuclear (USA). Mono Q, phenyl-Sepharose, Sephadex G-100, and blue dextran were obtained from Pharmacia Fine Chemicals AB (Sweden). Heparin-5pw was from Toso (Japan). Chymotrypsinogen A and ovalbumin were from Worthington Biochemical Corp. (USA) and Miles Laboratories (USA), respectively.

*Collection of hemolymph and preparation of ammonium sulfate precipitate fraction*

Solitary ascidians, *H. roretzi*, type C (Numakunai and Hoshino, 1974), were harvested in Mutsu Bay, Japan. The tunic matrix was extensively washed with seawater and cut without injuring the internal organs. The hemolymph was collected from individual animals and subjected to centrifugation (1000 × g, 5 min) to remove hemocytes. This procedure was carried out separately with individual ascidians, because mixing hemolymph from different individuals before centrifugation results in lysis of hemocytes due to contact reaction. The plasma thus obtained was pooled and subjected to ammonium sulfate precipitation in 75% saturation. The resulting precipitates were collected by centrifugation, suspended in 20 mM Tris-HCl (pH 8.0) in a volume corresponding to one-tenth the volume of the pooled hemolymph, and stored at -40°C until use.

*Thymocyte proliferation assay*

The thymus was obtained from a 4-8-week-old BALB/c mouse. A single cell suspension of thymocytes was prepared by gently grinding thymus with sterile stainless steel mesh and the piston of a syringe. The cells were washed twice in Hanks' balanced salt solution (HBSS) and suspended at a density of $1 \times 10^7$ cells/ml in RPMI 1640 containing 10% FCS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.25 μg/ml fungizone, and 2 μg/ml PHA-P. Fifty microliters of thymocyte suspension were added to each well of a Falcon 96-well, flat-bottomed tissue culture plate ($5 \times 10^5$ cells/well) and subsequently added 50 μl of the test samples that had been obtained from each chromatography followed by dialysis against phosphate-buffered saline (PBS). The plate was incubated at 37°C under 7% $CO_2$ for 24 hr, and then for 12 hr in the presence of 10 μCi of [$^3$H]-thymidine. The cells were harvested on glass fiber filter papers using an automatic cell-harvester. Each paper was put into a vial containing 5 ml of scintillation cocktail, and the incorporation of [$^3$H]-thymidine into thymocytes was measured using a scintillation counter. PBS and mrIL-1 β (5 pg/ml) were used as negative and positive controls, respectively.

*Heparin-5pw fast protein liquid chromatography (FPLC)*

Each of growth factor-containing fractions (M1-M8) obtained from the Mono Q column in a volume of 500 μl was applied on a column (0.75 × 7.5 cm) of heparin-5pw previously equilibrated with 20 mM Tris-HCl (pH 7.6) using the FPLC system (Pharmacia, Sweden) at a flow rate of 0.5 ml/min. Following washing the column with the same buffer, elution with equilibration buffer containing 0.5 M NaCl was carried out. The non-bound and NaCl-eluted fractions were pooled separately, dialyzed against PBS overnight, and then tested in thymocyte proliferation assay.

*Isolation of growth factors from* H. roretzi *plasma*

The purification procedure was performed at 4°C as follows:

Step 1. Phenyl-Sepharose chromatography: The frozen suspension of ammonium sulfate precipitates obtained from *H. roretzi* plasma (5.3 L, 105 individuals) was thawed and dialyzed against 20 mM Tris-HCl (pH 7.6) containing 2 M $NH_2SO_4$. After insoluble materials were removed by centrifugation, the resulting supernatant (182 ml) was applied to a column (3.5 × 10 cm) of phenyl-Sepharose previously equilibrated with 20 mM Tris-HCl (pH 7.6) containing 2 M $NH_2SO_4$. The column was washed with the equilibration buffer, and the adsorbed materials were then eluted with 20 mM Tris-HCl (pH 7.6). The effluent was collected in 10-ml fractions at a flow rate of 1.6 ml/min. Each fraction was dialyzed against PBS and its thymocyte stimulating activity was assayed.

Step 2. Gel filtration on Sephadex G-100: Active fractions (148 ml) from the phenyl-Sepharose column were pooled, concentrated into a volume of 7 ml using an Amicon PM-10 membrane, and applied to a Sephadex G-100 column (2 × 50 cm) previously equilibrated with PBS. The eluent was collected in 2-ml fractions at a flow rate of 14.7 ml/hr, and the proliferative activity of each fraction was assayed. The molecular weights of active fractions were estimated using blue dextran (void volume), bovine serum albumin (68 K), ovalbumin (45 K), and chymotrypsinogen A (26 K) as standards.

Step 3. Mono Q FPLC: Each of four active fractions, S1 (15 ml), S2 (12.5 ml), S3 (24 ml) or S4 (12.5 ml), from the Sephadex G-100 column was dialyzed against 4 L of 20 mM Tris-HCl (pH 7.6) overnight and applied to a Mono Q column (0.5 × 5 cm) previously equilibrated with the same buffer, using the FPLC system. After washing the column with the same buffer, elution with a linear gradient of increasing NaCl concentrations (0-1 M) was carried out. The eluent was collected in 1-ml fractions at a flow rate of 0.5 ml/min.

## RESULTS

*Isolation of factors having proliferative activity from plasma of* H. roretzi

Growth factors stimulating the proliferation of mouse thymocytes in the presence of PHA-P were isolated from *H. roretzi* plasma by phenyl-Sepharose chromatography, gel filtration and Mono Q FPLC. The proliferative activity was detected in the Tris-eluted fraction in the first phenyl-Sepharose chromatography, and in four fractions, designated as S1, S2, S3 and S4, the molecular weights of which were estimated to be 100-110 K, 75-90 K, 10-30 K, and 5-6 K, respectively, in the subsequent purification step of gel filtration (Fig. 1). Additionally, Mono Q FPLC demonstrated that two peaks of thymocyte proliferative activity were detected in each of S1, S2, S3, or S4 fraction. The eight active fractions isolated from

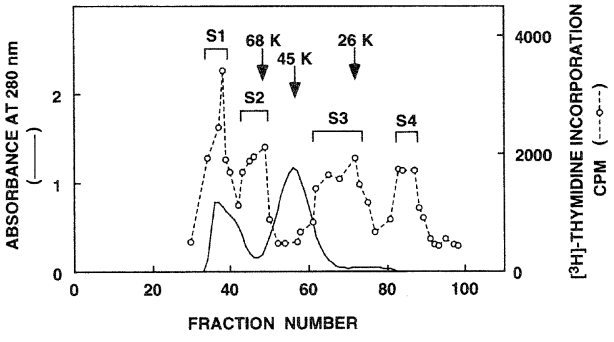


Fig. 1. Sephadex G-100 gel filtration of growth factors from *H. roretzi* plasma. Active fractions obtained from the phenyl-Sepharose column were concentrated and applied to a Sephadex G-100 column (2 × 50 cm) previously equilibrated with PBS. The effluent was collected in 2-ml fractions at a flow rate of 14.7 ml/hr. The thymocyte proliferating activity assayed in the presence of PHA-P was detected in four fractions, S1, S2, S3 and S4. Blue dextran (void volume), bovine serum albumin (68 K), ovalbumin (45 K), and chymotrypsinogen A (26 K) were used as standards for measurement of molecular weights. Absorbance at 280 nm (—); [$^3$H]-thymidine incorporation (–○–). Each point of cpm represents the mean value calculated from the data of triplicated measurements.

the Mono Q column were designated as M1-M8, respectively (Fig. 2).

*Characterization of growth factors*

The eight active fractions from the Mono Q column were characterized, and their properties including molecular weight, heat stability (90°C, 10 min), and heparin binding ability are summarized in Table 1. The activities of M1 and M5 fractions are heat-stable, while those of other fractions are heat-labile. The M3, M5, and M8 fractions can bind strongly to heparin-Sepharose column. In the cases of M6 and M7 fractions, each activity was separated into two fractions (adsorbed and non-bound fractions) by heparin-Sepharose chromatography, suggesting that these fractions may consist of at least two

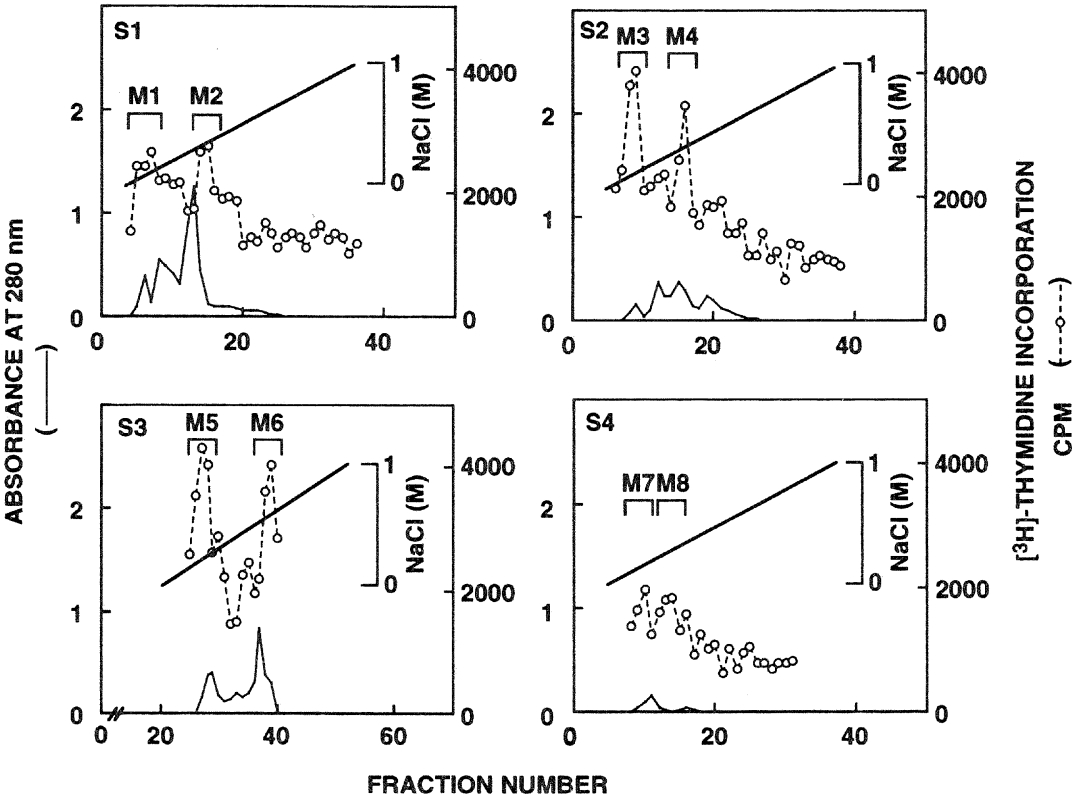


Fig. 2. Mono Q FPLC of growth factors from *H. roretzi* plasma. Four active fractions (S1, S2, S3 and S4) obtained from the Sephadex G-100 column were separately dialyzed against 4L of 20 mM Tris-HCl, pH 7.6, overnight and each sample was applied on a column (0.5 × 5 cm) of Mono Q previously equilibrated with the same buffer. After washing the column with the same buffer, elution with a linear gradient of increasing NaCl concentrations (0-1 M) was carried out. The effluent was collected in 1-ml fractions at a flow rate of 0.5 ml/min. In each chromatography (S1, S2, S3, or S4), the thymocyte proliferating activity assayed in the presence of PHA-P was detected in two fractions, and the eight fractions isolated were named as M1-M8, respectively. Absorbance at 280 nm (—); [$^3$H]-thymidine incorporation (–○–). Each point of cpm represents the mean value calculated from the data of triplicated measurements.

Table 1. Growth factors in *H. roretzi* plasma

| Gel filtration | | Mono Q FPLC | | Heat stability | Heparin binding |
|---|---|---|---|---|---|
| Fraction | MW | Fraction | NaCl[a] | (%)[b] | |
| S 1 | 100-110 K | M 1 | 0.17 M | 100 | weak |
| | | M 2 | 0.45 M | 50 | weak |
| S 2 | 75-90 K | M 3 | 0.18 M | 25 | strong |
| | | M 4 | 0.42 M | 10 | weak |
| S 3 | 10-30 K | M 5 | 0.13 M | 178 | strong |
| | | M 6 | 0.53 M | 15 | weak/strong[c] |
| S 4 | 5-6 K | M 7 | 0.20 M | 4 | weak/strong[c] |
| | | M 8 | 0.30 M | 3 | strong |

[a] The concentration where each active fraction was eluted.
[b] Remaining activity after heat treatment (90°C, 10 min).
[c] The activity was detected in two fractions, pass-through and adsorbed fractions of heparin-Sepharose chromatography.

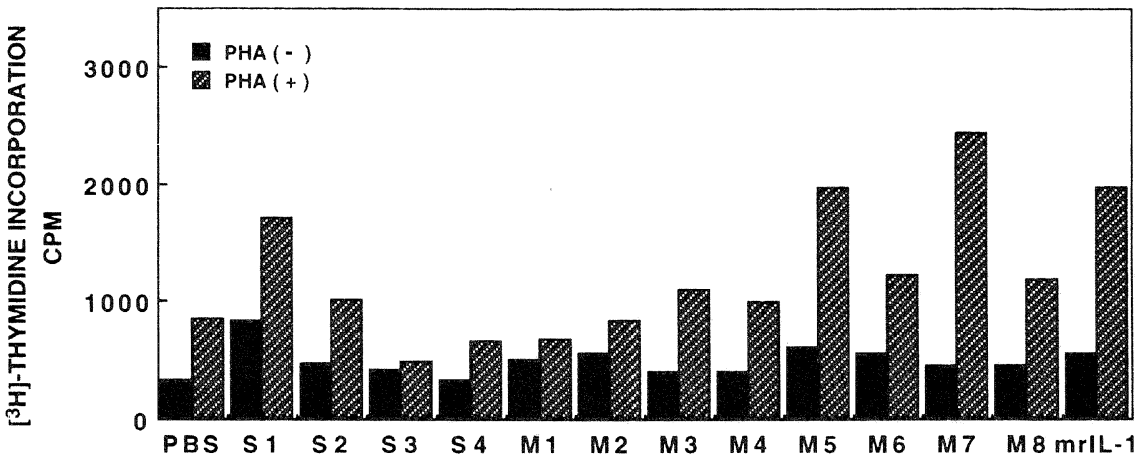


Fig. 3. Effects of PHA-P on eight growth factors of *H. roretzi* plasma. The thymocyte proliferating activities of eight active fractions obtained from the Mono Q column (M1-M8) and mrIL-1 β (5 pg/ml) were assayed in the presence or absence of 2 μg/ml PHA-P. Each point of cpm represents the mean value calculated from the data of triplicated measurements.

active components. Among isolated eight fractions, the activities of M5 and M7 fractions were enhanced by the addition of PHA-P as strongly as that of mrIL-1 β was (Fig. 3).

## DISCUSSION

It has been reported that IL-1-like activity is detected in the hemolymph of invertebrates such as the starfish (Beck and Habicht, 1986, 1991), the tunicate (Raftos *et al.*, 1991; Beck *et al.*, 1989) and the mollusc (Hughes *et al.*, 1990). The molecular weights of invertebrate IL-1-like molecules were estimated to be about 20 K, similar to that of mammalian IL-1. To define whether the hemolymph of *H. roretzi* also has a factor that stimulates the proliferation of murine thymocytes, we first checked the activity of ammonium sulfate-concentrated plasma in the presence of the mitogen, PHA-P, and found that it has a PHA-P-dependent thymocyte-proliferating activity: The activity in the presence of PHA-P was much higher than that in the absence of PHA-P. Although we also tried to detect the activity in the extract of *H. roretzi* hemocytes, we could not find any activity in the whole extract. In connection with this, we found that the whole extract was cytotoxic, probably because some *H. roretzi* hemocytes would contain substances cytotoxic to mammalian cells. It is necessary to measure the activity in each type of hemocytes separated from each other. Through gel filtration and anion-exchange chromatography, at least eight active fractions that can stimulate the proliferation of mouse thymocytes in the presence of PHA-P were identified. The subsequent purifica-tion of each of the active fractions has been attempted, but thymocyte-proliferating activity was undetectable in each fraction after further purification due to a low recovery of the activity. In this study, we used mammalian thymocytes as target cells. We also tried to detect the incorporation of [$^3$H]-thymidine into *H. roretzi* hemocytes in the presence of each of the eight growth factors, but, however, we could not detect any proliferating activity.

The eight growth factors of *H. roretzi* plasma have different molecular weights, heat stabilities, and abilities to bind to heparin-Sepharose. Although their structural information is not available at present, we can consider candidate molecules for the ascidian growth factors. The factors of M1 and M2 have high molecular weights (100-110 K) and are moderately heat-stable. It is possible that these molecules are mucopolysaccharide-like molecules or lipoproteins which act as growth factors. Alternatively, a heat-stable low molecular weight growth factor complexed with binding proteins may function as a growth factor. In this context, it has been reported that insulin-like growth factors circulate as components of a 125 to 150 K complex in mammalian plasma (Baxter *et al.*, 1989). A large lectin-like protein is also a candidate for the high molecular weight activity. PHA is a plant lectin that has mitogenic activity toward mammalian cells (Gery *et al.*, 1972). From *H. roretzi* plasma, we have already isolated two high molecular weight galactose-specific and *N*-acetyl galactosamine-specific lectins (Yokosawa *et al.*, 1982; Harada-Azumi *et al.*, 1987), but their molecular weights (600 K and 500 K, respectively) are higher than those of M1 and M2. The recovery of the activity in M1 or M2 was lower than that in S1 (Fig. 3). It can be thought that purified M1 and M2 are unstable after isolation. Alternatively, putative factors in S1 may strongly bound to the resin and cannot be eluted under the conditions used.

In the cases of M3 and M4 factors with molecular weights of around 80 K, transferrin (80 K), which is an iron-transport protein in mammalian plasma, is one of candidate proteins. It has been reported that transferrin can stimulate cell growth by supplying iron, an essential metal for maintenance of cell growth (Barnes and Sato, 1980). In connection with this possibility, we obtained preliminary evidence for the presence of a transferrin-like protein in *H. roretzi* plasma. Vitronectin (75 K), an adhesion and spreading molecule present in mammalian plasma, is also a candidate. This protein can

stimulate the proliferation of NK cells (Rabinowich *et al.*, 1995) and can bind to heparin in a similar fashion to the case of M3 factor (Hayashi *et al.*, 1985).

The molecular weights (10-30 K) of M5 and M6 factors are almost the same as that of mammalian IL-1 (Mizel, 1982). In connection with our results, it should be noted that there are two species of IL-1 with the same molecular weight of 20 K with acidic and neutral pIs, respectively, in starfish as same as the case of mammalian IL-1 molecules (Beck and Habicht, 1986). In addition, it was found that blood and whole body extracts from eight species of tunicates had two species of IL-1-like molecules of 20 K (Beck *et al.*, 1989). These tunicate IL-1 activities were inhibited by an antibody to human IL-1, suggesting that tunicate IL-1 can act on IL-1 receptors in the mammalian cells. It is interesting whether the actions of M5 and M6 would be inhibited by anti IL-1 antibody. In mammals, IL-1 is produced by macrophages after stimulation by lipopolysaccharides (LPS) (Oppenheim *et al.*, 1986). We previously reported that *H. roretzi* hemocytes release metallo-protease in response to stimuli such as LPS or phorbol myristate acetate (Azumi *et al.*, 1993). Taken together, it can be inferred that *H. roretzi* hemocytes release growth factors in response to stimuli. Alternatively, it is possible that such growth factors are released from several organs.

The molecular weights (5-6 K) of M7 and M8 factors are very similar to that of the mammalian insulin-like growth factor (Rinderknecht and Humbel, 1976). In a colonial ascidian, an insulin-like bioactive substance has been reported to be present in the hemolymph and has been found to have a role in the budding formation (Nakauchi and Kawamura, 1990).

In our preliminary studies, we found factors in *H. roretzi* plasma that can promote the proliferating of IL-6-sensitive mammalian cell line. The molecular weights of the factors were estimated to be more than 300 K, much higher than that of mammalian IL-6.

In summary, we found several growth factors in the plasma of the ascidian, *H. roretzi*, which can stimulate the proliferation of mouse thymocytes in the presence of PHA-P. These factors may have roles in the biological defense of *H. roretzi* by maintaining the growth of hemocytes functioning in the defense mechanisms of this animal. Further study is needed to obtain structural information of these factors.

## ACKNOWLEDGMENTS

We are grateful to Dr. T. Numakunai of the Asamushi Marine Biological Station, Tohoku University, Japan, for his cooperation in collecting ascidians. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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(Received November 18, 1996 / Accepted January 27, 1997)