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Partial Biochemical Characterization of Humoral Factors Involved in the Nonfusion Reaction of a Botryllid Ascidian, *Botrylloides simodensis*

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ABSTRACT — By means of an *in vivo* bioassay technique based on the micro-injection of blood plasma, it has been demonstrated that the blood plasma of *Botrylloides simodensis* has a specific activity corresponding to the colony specificity or allogeneic histoincompatibility. When intact blood plasma is exposed to various physico-chemical conditions, the specific activity is easily replaced by a nonspecific activity. The specific and nonspecific activities have some characteristics in common, both being resistant to dialysis and heat-labile and dependent on bivalent cations. These facts suggest that both activities are attributable to the same factor in the blood plasma. Furthermore, the nonspecific activity appears in large molecule fractions either by ammonium sulfate fractionation or by Sephadex G75 gel filtration. The activity is not affected by trypsin, protease or neuraminidase.

INTRODUCTION

In some compound ascidians, a phenomenon analogous to transplantation specificity has been known as "colony specificity" [1-7]. Colony specificity manifests itself among others as fusion or nonfusion between colonies and is governed genetically by multiple alleles at one locus. Two colonies having at least one allele in common are fusible, while two colonies having no allele in common are nonfusible [8-11].

Mukai [12] has reported that in *Botryllus primigenus* blood components of a colony are responsible for its fusibility. Later, Tanaka [13] has suggested in the same species that the allogeneic rejection or nonfusion reaction (NFR) is induced by some humoral factor or factors in the blood.

Recently, Taneda and Watanabe [14] have developed an *in vivo* bioassay technique which demonstrates that an NFR-inducing factor is contained in the blood plasma of *B. primigenus*. This factor should be isolated and purified. Unfortunately, however, the colony of this species is so thin that it is difficult to collect a sufficient volume of blood plasma for use in biochemical approach.

Botrylloides simodensis also has colony specificity, and a remarkable NFR appears when two nonfusible colonies are brought into contact with each other by their artificial cut surfaces [4]. Moreover, the colony of this species is thicker than that of *B. primigenus*, facilitating the collection of blood plasma. Therefore, in this study we used *B. simodensis* and attempted partial characterization of the presumed NFR-inducing factor.

MATERIALS AND METHODS

Animals used A colonial ascidian *Botrylloides simodensis* Saito and Watanabe [15] was used. In a colony, the individual blastozooids are grouped into a ladder system, and are connected with one another by a ramifying network of vascular vessels which terminate in ampullae

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at the periphery of the colony. Many colonies collected at a rocky shore near the Shimoda Marine Research Center, the University of Tsukuba, were fastened to microscopical glass slides or glass plates (8 cm × 11 cm) by means of the method of Oka and Usui [16]. Then, they were cultured in cages immersed in seawater in the Nabeta Bay near the Center where the environment was perfectly natural. After culturing for about two weeks, several colonies were selected and their fusibility was tested by ordinary fusion experiments.

Preparation of blood plasma A colony was stripped off from the glass plate. After removing the debris and hydrozoans adhering to the colony surface, the colony was washed with filtrated seawater (FSW). The seawater remaining on the colony surface and in the branchial sacs of the zooids was removed with filter paper. Then, the colony was cut into stripes about 3–5 mm wide and blood drops exuding through the cut surfaces of the stripes were collected in the cold (about 4°C). The collected blood was centrifuged at 12000 rpm at 4°C for 20 min to remove cellular debris. The resultant clear cell-free supernatant was used as intact blood plasma. The blood plasma thus prepared usually contained 2500–3500 μg/ml protein estimated with Lowry's method. The blood plasma was stored at 4°C and used within three days after preparation.

Heat treatment In order to examine the heat stability of the NFR-inducing activity, blood plasma was heated in a water bath set at a desired temperature (45°, 50°, 55°, 56° or 60°C) for 30 min and then additionally at 100°C for 3 min. Samples of heated blood plasma were used for bioassay.

EDTA treatment This treatment was carried out to examine the effects of bivalent cations on the NFR-inducing activity of blood plasma. Blood plasma was divided into five samples. One was stored at 4°C until use for assay; the others were dialyzed at 4°C for 12 hr against Tris–HCl buffer solution (TBS, 0.05 M Tris–HCl in 0.4 M NaCl, pH 7.9) containing 10 mM ethylendiaminetetraacetic acid disodium salt (EDTA). After the EDTA treatment, three of the four samples were again dialyzed at 4°C for 12 hr; one against TBS with 10 mM $MgCl_2$, another against TBS with 10 mM $CaCl_2$, and the last against TBS with 10 mM $MgCl_2$ and 10 mM $CaCl_2$. These five samples were used for bioassay.

Ammonium sulfate fractionation Four fractions were obtained from 1.5 ml blood plasma by salt fractionation with ammonium sulfate. The four fractions, F1, F2, F3 and F4, were precipitated by ammonium sulfate of 0–20%, 20–40%, 40–60% and 60–100% saturation, respectively. Then, each fraction was dissolved in 0.5 ml FSW. This fractionation was carried out at 4°C.

Gel filtration Sephadex G75 gel filtration was performed at 4°C on a column (43 cm × 1.3 cm ID) equilibrated with TBS. Two milliliters of blood plasma was applied, and about 1.7 ml eluate volumes were collected. The optical density of each tube was read at 280 nm for protein absorption. On the basis of the elution profile by scanning at 280 nm, the tubes were divided into three groups and concentration by negative pressure dialysis was carried out for each group. The three samples thus concentrated were used for assay.

Enzymatic treatment Three enzymes, trypsin (Merck), protease (Type I, Sigma), and neuraminidase (Type V, Sigma), were employed. Solutions of respective enzymes at a concentration of 500 μg/ml in FSW were prepared just before use. The following four samples were used as a set: intact blood plasma stored at 4°C (control); a mixture of 0.5 ml blood plasma and 0.1 ml FSW; a mixture of 0.5 ml blood plasma and 0.1 ml enzyme solution; and a mixture of 0.5 ml FSW and 0.1 ml enzyme solution. For proteolytic enzymes, prior to injection, the latter three samples were incubated for 12 hr at the optimum temperature of the respective enzymes (35.5°C for trypsin and 37°C for protease). For neuraminidase, similarly the latter three samples were first dialyzed against 0.1 M acetate buffer solution, pH 5.0, for 12 hr at 4°C, incubated for 12 hr at 37°C and finally dialyzed against FSW

for 12 hr at 4°C.

Assay for NFR-inducing activity The *in vivo* bioassay system developed by Taneda and Watanabe [14] was employed. For each sample, about 7 μl was injected into each recipient through an ampulla with a micropipette (approximately 50 μm in tip diameter). Recipient colonies were about 5 mm × 5 mm in size, each consisting of about 10 zooids. After the injection, the recipients were kept in the laboratory aquarium with continuously renewed running seawater. Four hours after injection, they were observed under a binocular stereomicroscope to assess the NFR-inducing activity of the injected samples.

In the reactions of recipient colonies, the following four types were distinguished:

(−): No harmful effect was induced except the ampulla was injured by injection.

(+): Slightly harmful effects, such as weak contraction of ampullae and slight increase of opacity in ampullae, were induced.

(++): Contraction of ampullae and increase of opacity in ampullae were distinctly recognized.

(+++): Withdrawal of ampullae from the fringe of the colony, amputation of vessels, disintegration of ampullae, and/or degeneration of zooids were observed.

In the text, the latter three types are referred to as NFR-like responses, or simply as NFRs.

RESULTS

Injection of blood plamsa Generally, injection of blood plasma of a fusible colony did not produce any harmful effect on the recipient. On the other hand, injection of blood plasma derived from a nonfusible colony induced an NFR-like response in the recipient. Both the proportion of recipients showing the NFR-like response and the intensity of the response gradually increased until about 4 hr after injection. This is the reason why the assessment of the NFR-inducing activity was done 4 hr after injection.

The results of injection of freshly collected blood plasma in three different combinations of colonies are shown in Table 1. When blood plasma derived either from a fusible syngeneic colony (Auto BP) or from a fusible allogeneic colony (F-Allo BP) was injected, most of the recipients showed no response. On the other hand, when blood plasma derived from a nonfusible allogeneic colony (NF-Allo BP) was injected, NFR-like responses were induced in the recipients. These results clearly show that blood plasma contains a factor or factors responsible for colony specificity.

TABLE 1. Effects of injection of intact blood plasma

Injected blood plasma	Number of recipients				Total
	+++	++	+	−	
Auto BP[a)]	0	3	24	70	97
F-Allo BP[b)]	0	2	3	30	35
NF-Allo BP[c)]	35	70	27	8	140

a) Blood plasma derived from syngeneic colonies.
b) Blood plasma derived from fusible allogeneic colonies.
c) Blood plasma derived from nonfusible allogeneic colonies.

When intact blood plasma was frozen, or left at room temperature for a day, or stored at 4°C for more than three days after preparation, the above-mentioned colony specific activity became nonspecific. In other words, the blood plasma thus treated induced NFR-like responses not only in nonfusible colonies but also in originally fusible colonies. Therefore, until otherwise described, in the following experiments blood plasma stored at 4°C for less than three days was used.

Effect of dialysis The pH range of blood plasma was from 7.6 to 7.8, slightly lower than that of seawater (pH 8.0–8.1). After dialysis of blood plasma against FSW for 12 hr at 4°C, the NFR-inducing activity was preserved as before. Injection of FSW alone produced no harmful effect. The nonspecific activity in long-stored blood plasma was also not affected by dialysis.

Effect of heat treatment When blood plasma was heated, its transparency was reduced due to the sedimentation of some blood

plasma components. The turbidity increased with elevation of temperature, and many particles were found in the sediment of blood plasma heated at 60°C or 100°C. These turbid samples of blood plasma were used for the injection.

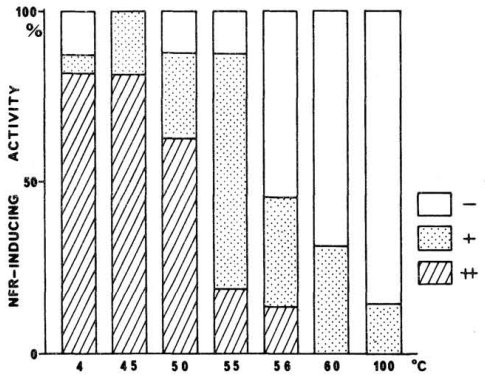

FIG. 1. Histograms showing the effect of heat treatment on the NFR-inducing activity of blood plasma in *B. simodensis*. The first histogram shows the activity of intact blood plasma stored at 4°C (control). Blood plasma was heated for 30 min at all temperatures except 100°C, where it was heated for 3 min. Recipient colonies used here were nonfusible to the colonies from which blood plasma was derived.

As shown in Figure 1, the NFR-inducing activity of blood plasma was nearly fully retained up to 50°C. However, the activity began to reduce distinctly at 55°C and was mostly lost by heating at 60°C for 30 min. When long-stored blood plasma was heated, its nonspecific NFR-inducing activity was reduced in a similar fashion.

Effect of EDTA treatment As shown in Table 2, the NFR-inducing activity of blood plasma was mostly removed by dialysis against TBS with 10 mM EDTA. When the EDTA treated blood plasma was secondarily dialyzed against TBS containing both 10 mM $MgCl_2$ and 10 mM $CaCl_2$, the activity recovered to the original level. On the other hand, when the secondary dialysis was made against TBS containing neither Ca^{2+} nor Mg^{2+}, the blood plasma failed to recover the activity (data not shown). The secondary dialysis against TBS with 10 mM $CaCl_2$ was much more effective in restoring the activity than against TBS with $MgCl_2$. The nonspecific NFR-inducing activity of long-stored blood plasma was also dependent on bivalent cations.

TABLE 2. Effects of EDTA, calcium, and magnesium on the NFR-inducing activity

Injection	Number of recipients[a]			Total
	++	+	−	
Intact blood plasma (control)	32	7	3	42
Blood plasma with 10 mM EDTA	0	7	37	44
Blood plasma with 10 mM $MgCl_2$	9	13	22	44
Blood plasma with 10 mM $CaCl_2$	21	13	13	47
Blood plasma with 10 mM $MgCl_2$ and 10 mM $CaCl_2$	30	12	5	47

a) Recipient colonies were nonfusible to the colonies from which blood plasma was derived.

TABLE 3. NFR-inducing activity of fractions obtained by ammonium sulfate fractionation

Injection	Number of recipients[a]				Total
	+++	++	+	−	
F1 (0–20% saturation)	1	36	1	1	39
F2 (20–40% saturation)	15	21	0	1	37
F3 (40–60% saturation)	0	29	10	2	41
F4 (60–100% saturation)	0	2	11	28	41

a) Recipient colonies were nonfusible to the colonies from which blood plasma was derived.

Ammonium sulfate fractionation The NFR-inducing activity was found in three fractions, F1, F2 and F3, with the highest activity in F2 (Table 3). Importantly, however, the activity of these fractions had turned nonspecific. That is, these fractions induced NFR-like responses even in originally fusible colonies (not shown in Table 3).

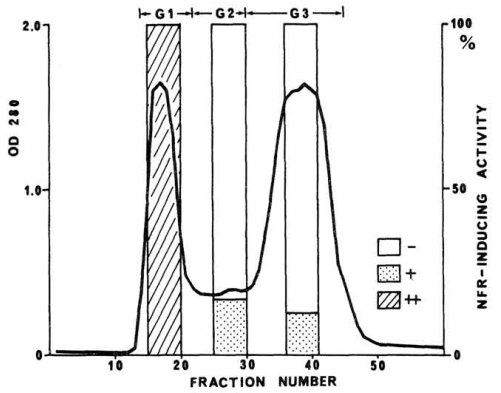

FIG. 2. Elution profile by scanning at 280 nm of the *B. simodensis* blood plasma eluted from Sephadex G75 column. The first peak was eluted with void volume. The fractions were divided into three groups (G1, G2 and G3) corresponding to the observed three peaks. Histograms show the NFR-inducing activities of these groups against non-fusible colonies.

Gel filtration Elution profile by scanning at 280 nm of the blood plasma eluted from Sephadex G75 column showed two large peaks and a small one (Fig. 2). The fractions obtained from this column were divided into three groups (G1, G2 and G3) corresponding to these three peaks. Then, the NFR-inducing activity in each peak was examined. The activity was found only in G1, although it was again nonspecific.

Effect of enzymatic treatment When blood plasma alone was incubated at an optimum temperature for enzyme activity (35.5°C or 37°C), the blood plasma induced nonspecifically NFR-like responses in the recipient colonies. Specific activity completely disappeared from the blood plasma within 2 hr of incubation. In parallel with this change, filamentary sediment appeared in the blood plasma. Therefore, we could study

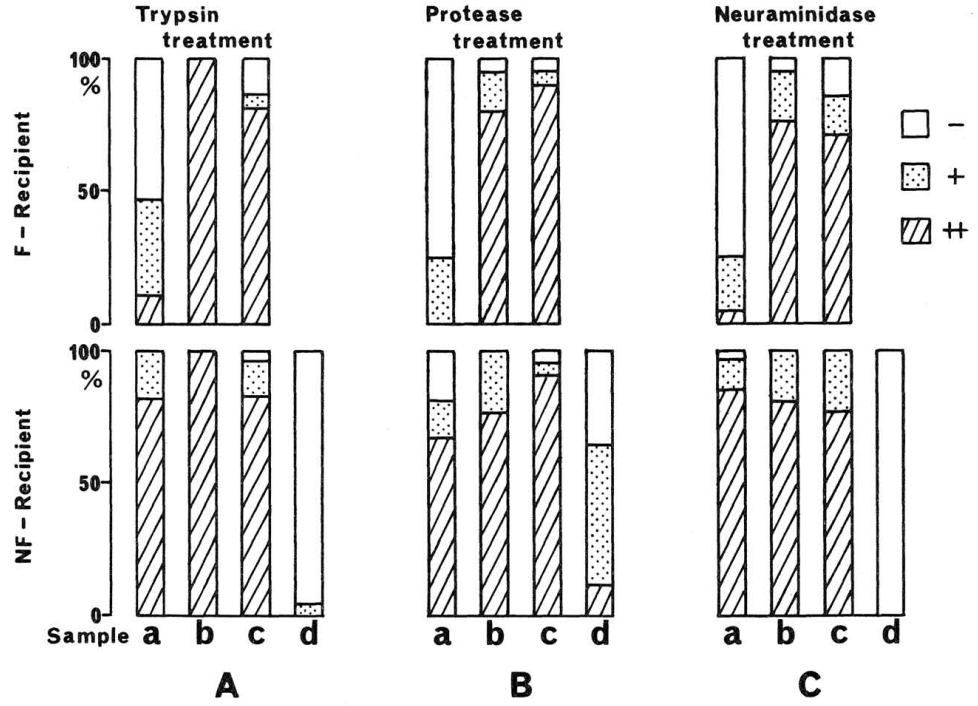

FIG. 3. Histograms showing the effect of enzymatic treatment on the NFR-inducing activity of blood plasma in *B. simodensis*. In all treatments (A, B and C), sample a is an intact blood plasma stored at 4°C; sample b is a blood plasma passed through the same procedure as enzymatic treatment but without enzyme; sample c is a blood plasma treated with enzyme; and sample d is an enzyme solution. The upper row shows the results when fusible colonies were used as recipients, and the lower row shows the results when nonfusible colonies were used as recipients. Tryspin treatment was performed for 12 hr at 35.5°C at pH 7.6–7.8, protease treatment was performed for 12 hr at 37°C at pH 7.6–7.8, and neuraminidase treatment was performed for 12 hr at 37°C at pH 5.0.

the effects of enzymes only on this nonspecific activity.

The trypsin solution did not induce any harmful effect in the recipients. Trypsin treatment did not reduce the nonspecific activity of blood plasma, which contained filamentary sediment (Fig. 3A). In another series of experiments, the incubated turbid samples were centrifuged at 12000 rpm for 20 min to remove the sediment, and the supernatants were injected into the recipients. They retained the same activity as before.

When the protease solution was injected singly, a weak response very similar to the NFR occurred in the recipients. Protease did not reduce the nonspecific activity of blood plasma (Fig. 3B).

The neuraminidase solution did not bring any harmful effect to the recipients. Neuraminidase treatment did not reduce the nonspecific NFR-inducing activity of blood plasma (Fig. 3C). Next, we prepared the supernatant of each sample by removing the filamentary sediment through centrifugation, and tested its activity. The activity was unchanged. Further, when neuraminidase treatment was carried out at pH 7.6–7.8 in a way similar to protease treatment, the nonspecific activity of blood plasma was not affected.

DISCUSSION

This is the first approach to the biochemical characterization of humoral factors responsible for the colony specificity in ascidians.

Blood plasma from a colony of *Botrylloides simodensis* induces, when injected, an NFR-like response in a nonfusible colony; while it induces no response in a fusible colony. Thus, the blood plasma of this species has an activity corresponding to colony specificity. This specific activity is retained after dialysis of blood plasma against FSW, but is mostly lost by heating at temperatures higher than 55°C. Further, the activity is dependent on bivalent cations, such as Ca^{2+} and Mg^{2+}, especially the former.

The specific activity in the blood plasma easily turns nonspecific by various gentle treatments, e.g., long-term storage at 4°C, freezing and thawing, incubation at a moderate temperature, and other physico-chemical treatments. The blood plasma thus treated invariably induces NFR-like responses in fusible as well as nonfusible colonies.

The above-mentioned nonspecific activity is rather stable, remaining unchanged for more than one year in the cold. Like the specific activity, the nonspecific activity is resistant against dialysis, heat-labile, and dependent on bivalent cations. Furthermore, the nonspecific activity is found in large molecule fractions either by ammonium sulfate fractionation or by Sephadex G75 gel filtration. The activity is not affected by trypsin, protease or neuraminidase.

At present, it is not known whether the observed specific and nonspecific activities are carried by the same factor or by different factors in the blood plasma. The fact that both activities share some characteristics, e.g., stability to dialysis, lability to heating, and dependency on bivalent cations, suggests that the former alternative is more probable. If this is the case, the factor might be a conjugate protein. To clarify these points, however, further study is needed.

In some ascidians, naturally occurring hemagglutinins have been reported; and such hemagglutinins have often been considered to function as humoral recognition factors [17–19]. In future, our hypothetical factor may be related in some way or other to these hemagglutinins. However, little information is currently available.

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