Hemocytes Release Phenoloxidase upon Contact Reaction, an Allogeneic Interaction, in the Ascidian Halocynthia roretzi

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ABSTRACT. Contact reaction is the morphological expression of allogeneic recognition by hemocytes in the ascidian, Halocynthia roretzi; namely they undergo an exocytotic burst upon contact with non-self (allogeneic) hemocytes in vitro. We have found that contact reaction is accompanied by a burst of cyanide-insensitive oxygen consumption that, unlike exocytotic events of mammalian phagocytes, is Ca2+-dependent and does not culminate in the production of superoxide anions or H2O2. Instead, the burst is due to the release of phenoloxidase from hemocytes as known for the self-defense systems in insects and crustaceans. The activity of phenoloxidase released from hemocytes corresponds well to the degree of contact reaction observed under the microscope. Therefore, it is possible to quantify the contact reaction simply by measuring the activity of phenoloxidase released from hemocytes into the medium.

A significant feature of the vertebrates is the acquisition of highly elaborate immune systems. Although somehow similar systems such as insect self-defense mechanisms are reported in various invertebrates, the development of such systems is greatly limited in most invertebrates (for reviews see Ref 7). It is expected from a phylogenetic point of view that the ascidians may provide an essential clue to understand the origin and evolution of immune systems in vertebrates. Besides xenogeneic recognition by a self-defense system, the ascidians are capable of expressing allogeneic recognition in at least two different systems, namely, colony fusion expressed by somatic cells of compound ascidians and self-incompatibility expressed by gametes in many ascidians including compound species (23, 25, 27, 31, 32). It is suggested that two types of allogeneic recognition in the ascidians are regulated by a mechanism similar to cellular immunity in mammals and controlled by a single or a few closely related genes (11, 18, 19, 27, 31).

A solitary ascidian, Halocynthia roretzi, is hermaphroditic yet strictly self-incompatible in fertilization. Fuke found in this species that hemocytes have a capacity for allogeneic recognition (10). When hemocytes from two individuals are mixed in vitro, they immediately start to agglutinate each other and end up with cellular evacuation. She named this remarkable phenomenon “contact reaction” and found also that the allogeneic recognition by hemocytes is quite strict, yet less than that by gametes (11).

We attempted to elucidate the molecular mechanisms underlying the self-incompatibility in fertilization of H. roretzi and other ascidians. Suppose the gametes and hemocytes share a similar mechanism, or even similar molecules, for allogeneic recognition, the direct comparison of the two systems may provide a clue for understanding the mechanism of allogeneic recognition in the ascidians. For such an approach to the problem, quantitative analyses are very important. Although self-sterility is not difficult to quantify, the contact reaction is rather difficult to quantify since it is defined as a morphological change in the whole population of hemocytes. We have therefore searched for biochemical changes accompanying the contact reaction. As a result of contact reaction, the medium gradually becomes pale brown, but the color is not due to the absorption at any specific wavelength and the intensity is too faint to be quantitatively measured.

In this paper, we present evidence that the contact reaction is accompanied by a respiratory burst supported by a surge of phenoloxidase release from the hemocytes. We also show that contact reaction can be quantified simply by measuring the activity of phenoloxidase released into the medium.

MATERIALS AND METHODS

Animals. Halocynthia roretzi were collected on the coast of northern Honshu Island (Mutsu Bay, Aomori Prefecture and Ohtsuchi Bay, Iwate Prefecture), Japan. Only type C
animals (21) were used throughout the following experiments. 

Hemocytes. Coelomic fluid was collected separately from each animal by cutting the tunic without injuring the internal organs, and filtrated through two layers of cheese cloth. Hemocytes were recovered by centrifugation in a swing rotor at 100 × g for 10 min at 4°C, and then washed once with artificial sea water (ASW) consisting of 420 mM NaCl, 9 mM KCl, 10 mM CaCl₂, 24.5 mM MgCl₂, 24.5 mM MgSO₄, 2.15 mM NaHCO₃, and 10 mM N-[2-hydroxyethyl]piprazine-N'-[3-propanesulfonic acid] (EPPS), pH 8.0, and then re-suspended in fresh ASW. For examination of bivalent cation requirements, hemocytes were suspended in Ca²⁺-free ASW (380 mM NaCl, 50 mM MgCl₂, 10 mM KCl, 30 mM Na₂SO₄, 2.5 mM EGTA, 2 mM NaHCO₃, 10 mM EPPS, pH 8.0). In some experiments, Ca²⁺-, Mg²⁺-free ASW (380 mM NaCl, 10 mM KCl, 30 mM Na₂SO₄, 2.5 mM EDTA, 2 mM NaHCO₃, 10 mM EPPS, pH 8.0) was also used. The number of collected hemocytes was counted by using a Bürkèl-Türk hematocytometer.

Measurement of oxygen consumption. For standard assays, two hemocyte suspensions containing 1 × 10⁷ cells/ml from different animals were mixed in a total volume of 1 ml, and the oxygen consumption was recorded by using a Clark-type oxygen electrode (YSI 4004, Shinsei-Rikagakukikai) at 23°C according to the method of Estabrook (8). For phenoloxidase assays, oxygen consumption was similarly monitored except that the medium and samples were suitably modified as described below.

Measurement of superoxide anion. Superoxide anion (O₂⁻) was measured according to the modification by Babior et al. (5) of the cytochrome c reducing method by Fridovich (9). Differential absorbance at 550 nm and 540 nm was monitored with a model 556 Hitachi dual-wavelength spectrophotometer. Reference wavelength (540 nm) is the isosbestic point where cytochrome c (Fe³⁺) switches over to cytochrome c (Fe²⁺). For recording, hemocyte suspensions were adjusted to 1 × 10⁷ cells/ml. Hemocyte suspension (1 ml) from one animal and 20 µl of 1.9 mM cytochrome c (Sigma Chemical Co.) in distilled water were mixed in both reference and sample cuvettes, and the basal line was recorded until it stabilized. The reaction was started by adding another 1 ml hemocyte suspension of the same (reference) or a different animal (sample). Reaction mixtures were continuously stirred by a windmill cell mixer unless the spectra were scanned (16). Spectra were also recorded in the presence or absence of superoxide dismutase (SOD; Sigma Chemical Co.).

Rate of H₂O₂ generation. Production of H₂O₂ was assessed at the room temperature by the H₂O₂-peroxidase complex method of Boveris et al. (6). Horseradish peroxidase (HRP; Sigma Chemical Co., 0.125 mg) and 1 × 10⁷ hemocytes were mixed in a final volume of 1 ml in both the reference and sample cuvettes. After stabilization of the absorbance at 417–403 nm, 1 ml of a suspension containing the same number of hemocytes from either the same (reference) or a different animal (sample) was added. Changes in absorbance were recorded with constant stirring as used for superoxide measurement. Mixtures of two different suspensions of hemocytes were scanned from 400 to 450 nm to detect a possible peak-shift due to the formation of HRP complex II. Scanning was repeated several times for a sample, because hemocytes precipitated significantly during each run of scanning.

Measurement of phenoloxidase activity. Contact reaction was induced in u-bottom wells of a multi-well dish (Corning) by mixing 100 µl each of hemocyte suspensions in ASW (2 × 10⁷ cells/ml) prepared from two individuals. After 30-min incubation at room temperature, the plate was centrifuged with a swing rotor at 1,000 rpm for 5 min. The supernatant was assayed at room temperature for phenoloxidase activity by the method of Ashida et al. (2) and Pye (24) with slight modifications. Briefly, a 20 µl aliquot of the supernatant was diluted into 0.14 ml with 0.1 M phosphate buffer (pH 6.0). The reaction was initiated by adding 20 µl of 7 mM 4-methylcatechol in the buffer and stopped after incubation at room temperature for 2 min by adding 20 µl of 2 mM tropolone in the buffer. The medium gradually became pink after the addition of 20 µl of 14 mM 4-hydroxyproline ethyl ester in the buffer. The pigmentation almost reached a plateau after incubation at room temperature for 15 min, when the absorption at 520 nm was measured with a micro-plate reader (MPRA4, Tosoh).

In some experiments, the activity of phenoloxidase was also measured by oxygen consumption. For this, the supernatant was added to 7 mM 4-methylcatechol in the buffer, and oxygen consumption was monitored by an oxygen electrode as described above.

RESULTS

Respiratory burst upon contact reaction. If hemocytes from two individuals were mixed, they underwent contact reaction in most, but not necessarily all, combinations. Contact reaction was not observed in about 6% of random combinations so far tried within Mutsu Bay and Ohtsuchi Bay populations. Whenever the mixing resulted in contact reaction, a burst of oxygen consumption was observed; and vice versa (Fig. 1). Allogeneric, or homologous, combinations of hemocytes consumed oxygen on the average ten fold faster than did autologous combinations (Table I). When fixed hemocytes were added to fresh ones, neither the contact reaction nor respiratory burst was observed even in allogeneric combinations. Naturally, the contact reaction and respiratory burst were absent when hemocytes from a single individual were mixed.

Hemocytes out of contact with allogeneric cells consumed oxygen at a low yet detectable rate (Figs. 1 and 2). Oxygen consumption of such a basic level by not-stimulated hemocytes was independent of external Ca²⁺ and slightly inhibited by 1 mM cyanide, whereas the increased oxygen consumption after allogeneric contact...
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Table I. Respiratory burst upon contact reaction in *H. roretzi* hemocytes.

<table>
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<tr>
<th>Combination</th>
<th>Respiratory Rate (nmol/min/10^7 cells)</th>
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<tr>
<td>Autologous</td>
<td>0.71 ± 1.09 (51)</td>
</tr>
<tr>
<td>Homologous</td>
<td>8.72 ± 4.92 (57)</td>
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Data represent the mean ± SD (experiment number).

was dependent on external Ca\(^{2+}\) and highly resistant to potassium cyanide (Fig. 2) and sodium azide (data not shown). Thus the respiratory burst upon contact reaction was not attributable, as in the one after phagocytosis in mammalian blood cells, to an increase in mitochondrial respiration.

Measurement of superoxide anion. In mammals, phagocytosis of pathogenic microbes and other foreign particles by blood cells (phagocytes) is generally followed by a cyanide-insensitive respiratory burst and the production of superoxide anion (O\(_2^-\)) that destroys phagocytosed microbes. Thus we examined whether the respiratory burst upon contact reaction was accompanied by the production of superoxide anion. So far as superoxide anion was measured by the reduction of cytochrome c, no evidence for the production of super-

![Figure 1](image1.png)

**Fig. 1.** Respiratory burst of hemocytes upon encountering allogeneic (homologous) ones in *H. roretzi*. O\(_2\) consumption by 1 x 10^7 hemocytes from one animal designated A was recorded by using a Clark-type oxygen electrode at 23°C. At the time indicated by the second arrow, the same number of hemocytes from the same animal (a) or a different one designated B (b) was added in a final volume of 1 ml.

![Figure 2](image2.png)

**Fig. 2.** Effects of external Ca\(^{2+}\) (a) and potassium cyanide (b) on the respiratory burst. Experimental design was the same as applied for Fig. 1 except for replacement of ASW with Ca\(^{2+}\)-free ASW (a, broken line) or the presence of 1 mM potassium cyanide (b). (a) Allogeneic hemocytes were mixed in normal ASW (solid line) or in Ca\(^{2+}\)-free ASW (broken line). At the point indicated by the open arrow, 10 \(\mu\)l of 1 M CaCl\(_2\) was added to restore calcium concentration to the normal level, 10 mM. (b) Together with hemocytes B, 10 \(\mu\)l of 100 mM KCN (1 mM as a final concentration) was added to hemocytes A.
oxide anion was obtained; namely, the absorbance gap at $E_{550-540}$ did not increase with time (Fig. 3a) and the absorption peak of cytochrome c ($Fe^{2+}$) at 550 nm did not appear in the absence or presence of superoxide dismutase (Fig. 3b).

**Measurement of H$_2$O$_2$.** It is well established that
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H₂O₂ is produced by an inproportionation reaction of superoxide anion. Thus we further examined whether hemocytes release H₂O₂ to any extent upon contact reaction. No evidence for the production of H₂O₂ was obtained by using the HRP complex method; namely, the absorbance gap at E₄₁₇₋₄₀₇ did not increase with time (Fig. 4a) and the absorbance peak of the H₂O₂-HRP complex (compound II) at 417 nm was not observed (Fig. 4b).

**Coloration of the medium after contact reaction.**

As the contact reaction proceeded, the medium gradually became pale brown (Fig. 5a). All autologous combinations were negative as shown in the diagonal row. In this particular case, two homologous combinations (A–E and D–E) were reciprocally negative both in contact reaction and coloration. The color was diminished by the addition of reducing agents like sodium hydrosulfite. In mammals, such coloration is mostly due to respiratory pigments and hence commonly shows an absorption spectrum of heme (Fe³⁺). However, the supernatant after the contact reaction did not show the spectral evidence for the presence of heme (Fe³⁺) or any other specific absorbance (data not shown).

**Release of phenoloxidase upon contact reaction.**

Phenoloxidase plays a pivotal role in self-defense mechanisms of insects and crustaceans (30). The enzyme does not produce H₂O₂ and is involved in melanin formation. Thus it is a good candidate for the molecule responsible for both respiratory burst and coloration. Indeed, upon contact reaction, the activity of phenoloxidase toward 4-methylcatechol appeared in the medium in parallel with the degree of spontaneous coloration (Fig. 5). The activity assayed by the colorimetric method was inhibited by 0.4 mM tropolone, a potent chelator of Cu²⁺ and thus a specific inhibitor of phenoloxidase (14). Since homogenates of not-stimulated hemocytes showed the enzyme activity comparable to the one appeared in the medium upon contact reaction (data not shown), it was concluded that phenoloxidase was released from the hemocytes upon contact reaction.

When assayed by oxygen consumption in the presence of a substrate, 4-methylcatechol, the activity of phenoloxidase in the supernatant of hemocyte suspension was detected even if hemocytes from a single individual were used. The released activity per cell did not change after being mixed with autologous hemocytes (Fig. 6a). However, as predicted, the activity was much higher in the supernatant of allogeneic combinations (Fig. 6b). The oxygen consumption by the supernatant was completely inhibited by 0.4 mM tropolone (Fig.

**Fig. 5. Colorimetric assays of contact reaction.** Reciprocal reactivities of the hemocytes from six animals (A–F) were examined by spontaneous coloration (a) and phenoloxidase assay (b). Hemocyte suspensions in ASW (1 x 10⁷ cells/ml) were separately prepared from six animals. A 100 μl-aliquot of each suspension was dispensed into six u-bottom micro-wells in a horizontal row of a 96-well dish, and then similarly into six wells in a vertical row to form all possible combinations. After 30-min incubation at room temperature, the dish was centrifuged at 1,000 rpm for 5 min. The supernatant (20 μl) of each well was assayed for phenoloxidase activity as described in the text. In this particular case, the dish was photographed 3 hr after the mixture of hemocytes (a), and 15 min after the addition of 4-hydroxyproline ethyl ester (b). All autologous combinations expressed in the diagonal row of wells and two homologous combinations (A–E and D–E) were reciprocally negative by both methods.

**Fig. 6. Phenoloxidase assay by O₂ consumption.** The supernatant (20 μl) of a hemocyte mixture prepared as described in Fig. 2 was added to 880 μl of 0.1 M sodium phosphate buffer, pH 6.0. The reaction was initiated by adding 100 μl of 5 mM 4-methylcatechol as the substrate into the mixture at the time indicated by the arrow, and the following O₂ consumption was monitored by using a Clark-type oxygen electrode at 23°C. (a) Autologous combinations (self). (b) Homologous combinations (nonself). The open arrow indicates the addition of 100 μl of 4 mM tropolone to inhibit the enzyme activity.
be exocytosed during massive evacuolation of the hemocytes during preparation. We predict that not-respiration shown in Figs. 1, 2 and 6 may reflect damage not shown). Thus cyanide-insensitive fraction of resting vacuolated cells (13). When hemocytes are harshly managed for the moment is that the enzyme is present in so-called gametes and colony fusion between compound ascidians. In this study, we have found that contact reaction, an experimental allogeneic recognition by hemocytes in the ascidian H. roretzi, is accompanied by a cyanide-insensitive and Ca^{2+}-dependent respiratory burst. Unlike the respiratory burst of mammalian phagocytes (26), the one accompanying the contact reaction does not result in the production of superoxide anion and H_{2}O_{2}, suggesting that contact reaction is not a phagocytic event. Fixed hemocytes are victims of phagocytosis by fresh hemocytes in H. roretzi (22), but intact hemocytes did not undergo contact reaction with fixed ones. Contact reaction is indeed suggested not to be a phagocytic event from a morphological observation (12). Respiration burst upon contact reaction is caused, instead, by a surge of phenoloxidase from hemocytes. Phenoloxidase serves as a pivot in self-defense systems in insects, crustaceans and presumably other arthropods. Hemocytes of these animals have prophenoloxidase and a cascade system of prophenoloxidase-activating enzymes. This cascade is provoked by microbial glycans and eventually triggers a surge of active phenoloxidase (1, 17, 28). Recently the activity of phenoloxidase has been detected in the hemocytes of some other marine invertebrates including several ascidians (15, 29), although the biological significance of the enzyme has yet to be clarified.

This paper presents evidence that phenoloxidase plays a crucial role in allogeneic recognition in the ascidians, at least in the contact reaction of H. roretzi. Although we have yet to determine the location and form of the enzyme in the resting hemocytes, our supposition for the moment is that the enzyme is present in so-called vacuolated cells (13). When hemocytes are harshly managed, the rate of oxygen consumption increased (data not shown). Thus cyanide-insensitive fraction of resting respiration shown in Figs. 1, 2 and 6 may reflect damage to hemocytes during preparation. We predict that not-stimulated hemocytes have an extremely low level, if any, of phenoloxidase activity. The enzyme appears to be exocytosed during massive evacuation of the hemocytes. One possible explanation for Ca^{2+}-dependency of the respiration burst is Ca^{2+}-requirement for exocytosis, which would be clarified by future experiments. The mechanisms underlying the allogeneic recognition by hemocytes and resulting secretion of phenoloxidase are the major questions that remain to be answered.

Phenoloxidase plays a central role in self-defense mechanisms at least in insects and crustaceans. It is suggested in H. roretzi that antimicrobial compounds and an agglutinin against bacteria found in the hemocytes serve as a part of self-defense systems (3, 4). Although phenoloxidase is found in ascidian hemocytes (15, 29), it is not yet known whether phenoloxidase is involved in self-defense mechanisms in H. roretzi or any other ascidians. Hence, the relationship between the contact reaction and self-defense mechanisms is another important issue to be clarified.

It will also be important to identify the physiological substrate(s) for hemocyte phenoloxidase. Coloration upon contact reaction seems to be a result of oxidation by the enzyme, because it was in good accordance with the enzyme activity in the medium (Fig. 5) and because the color was bleached by reducing agents. The color of body fluid is often related to respiratory pigments containing transition metals such as iron. Although H. roretzi contains rather high levels of various metals in hemocytes (20), the color appeared upon contact reaction was not due to heme compounds. Another candidate for the substrate is a DOPA (3,4-dihydroxyphenylalanyl)-like substance that has recently been found in the hemocytes (3). This hypothesis is not contradictory to the fact that the color generated during contact reaction did not have any specific absorption.

This paper opens the way for the quantitative analysis of the contact reaction simply by measuring the activity of phenoloxidase secreted from the hemocytes. By using such a simple assay, the molecular mechanisms underlying allogeneic recognition and the following signal transduction in hemocytes can be studied.

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