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Mutual and Directional Allogeneic Cytotoxic Reaction of Hemocytes in the Solitary Ascidian *Halocynthia roretzi* Revealed by One-step Quantitative Fluorimetric Assay

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ABSTRACT—A novel one-step microplate cytotoxicity assay using the cytoplasmic fluorescent viability dye calcein AM was established for simple, rapid, sensitive, and quantitative measurements of the allogeneic cytotoxic reaction (ACR) mediated by hemocytes in the ascidian *Halocynthia roretzi.* The mutual and directional ACR was distinguishable by the assay using the hemocytes from pairs of animals with different alloreactivities. The ACR assay may allow more precise genetic analysis of the gene that controls alloreactivity of hemocytes, since the mutual and directional ACR may be related to levels of expression or numbers of the gene product or products on the target cells. The directional ACR will be useful in elucidating the cellular and molecular mechanisms of self-recognition in *H. roretzi*, since it allowed us to equate hemocytes from one animal with "effector cells" and those from the other animal of the pair with "target cells". In addition, the quantitative ACR assay in a large number of samples is possible and it will allow production of monoclonal antibodies that may recognize receptors or ligands functioning in self-recognition processes by the *H. roretzi* hemocytes.

Key words: quantitative assay, allogeneic cytotoxic reaction, self-recognition, hemocytes, ascidian

INTRODUCTION

The hemocytes (coelomocytes or blood cells) of the solitary ascidian *Halocynthia roretzi* display striking allogeneic or xenogeneic recognition reactions *in vitro*, which are referred to as the contact reaction (Fuke, 1980). When the hemolymph (coelomic fluid) of two different individuals is mixed together, the hemocytes undergo a cytotoxic reaction within 90 seconds of initial contact. This extremely rapid recognition process in *H. roretzi* is based on polymorphic alleles at one or two histocompatibility-determining loci (Fuke and Nakamura, 1985), the same as in the fusion/non-fusion histocompatibility locus of the colonial ascidian *Botryllus primigenus* (Oka and Watanabe, 1957; see review by Saito *et al*., 1994) and the well-studied species *Botryllus schlosseri* (Scofield *et al*., 1982; see review by Magor *et al*., 1999). Previous attempts to determine the gene and the gene product or products of the locus or loci have been unsuccessful, and the entities of the gene and the receptors and the ligands involved in these self-recognition processes remain unknown.

The contact reaction can be observed under a phase-contrast microscope (Fuke, 1980), but it is quite difficult to quantitatively analyze the reactions in a large number of samples by this method. Development of a method for quantitative detection of the reaction in a large number of samples is essential to determining the entities of the gene and screening for receptors and ligands involved in the reaction. Contact reactions are similar to cytotoxic reactions by NK cells in mammalians in term of the reaction occurring immediately upon contact and without prior sensitization (Humphreys and Reinhertz, 1994). Thus, in principle, the reactions can be analyzed by any standard assay for measuring the cytotoxicity mediated by mammalian NK cells. At present, however, by far the most widely used method is measurement of release into the supernatant of ^{51}Cr from

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cells prelabeled with $^{51}Cr_2O_7^{2-}$ (Brunner *et al.*, 1968). However, we found that the ^{51}Cr-release assay cannot be applied to the contact reaction, because freshly collected *H. roretzi* hemocytes do not take up sufficient amounts of $^{51}Cr_2O_7^{2-}$ (unpublished observation).

In this report, we describe the development of a quantitative one-step assay to analyze contact reactions *in vitro*. We refer to the contact reaction determined by the fluorimetric measurement after 30 to 60-min incubation of allogeneic hemocytes as the "allogeneic cytotoxic reaction (ACR)" to distinguish it from the original contact reactions observed by the phase-contrast microscopy, and we call the fluorimetric assay to determine the contact reaction the "ACR assay". We also describe the mutual and directional ACR distinguished by the assay, which will be useful in genetic studies on alloreactivity as well as in elucidating the cellular and molecular mechanisms of self-recognition in *H. roretzi*.

MATERIALS AND METHODS

Reagents and buffer solutions

The fluorogenic dye calcein AM was purchased from Molecular Probes (Eugen, OR, USA). A stock solution of calcein AM (1.0 mM) was prepared in anhydrous DMSO, and it was diluted with saline immediately prior to target cell labeling. Pantin's artificial seawater (ASW; 402.3 mM NaCl, 9.7 mM KCl, 52.6 mM $MgCl_2$, 10.1mM $CaCl_2$, 27.7 mM Na_2SO_4) buffered with 20 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) at pH 7.2 (neutral ASW) was used as the saline for *H. roretzi* hemocytes (Ohtake *et al.*, 1994). Two kinds of Ca^{2+}- and Mg^{2+}-free saline, having different pH values, were prepared: Ca^{2+}- and Mg^{2+}-free ASW buffered with 20 mM MES ([N-morpholino] ethanesulfonic acid) at pH 5.6 (acidic Ca^{2+}- and Mg^{2+}-free ASW), and Ca^{2+}- and Mg^{2+}-free ASW buffered with 50 mM MES at pH 9.0 (alkaline Ca^{2+}- and Mg^{2+}-free ASW). Concentration of NaCl in these Ca^{2+}- and Mg^{2+}-free ASW was raised to 465.1 mM to keep osmolarity of ASW constant.

Animals

Samples of *H. roretzi* cultured at Mutsu Bay, Aomori Prefecture, were purchased from a farm. They were kept in circulating seawater in a laboratory fish tank at 7°C, without feeding, and were used for experiments within a month.

Mixed-hemocyte-incubation (MHI) assay and the contact reactions

The MHI-assay, which conveniently determines the alloreactivity of pairs of *H. roretzi* samples, was performed as described by Sawada and Ohtake (1994). In brief, a portion (1 to 5 ml) of the hemolymph was withdrawn with a syringe from the space just beneath the tunicate papilla without any anticoagulant. Hemolymph (100 μl) from different individuals was mixed and incubated in U-bottomed 96-well plates for 18 hr at room temperature. The alloreactivity of a pair of animals was determined by the MHI-assay prior to fluorescence microscopic observations of the contact reactions or fluorescence measurements of the ACR with a multiwell plate scanner. A pair of individuals that reacted positively in the MHI-assay was selected as an alloreactive combination, and a pair of individuals that reacted negatively was selected as a non-alloreactive combination.

The contact reactions were tested according to Fuke (1980), with slight modification (Sawada and Ohtake, 1994).

Labeling of hemocytes with calcein AM

All hemocytes were labeled with calcein AM just before testing for various assays. The following protocol was adopted for standard calcein AM loading. The hemolymph was collected by amputation at the bottom of the body. In order to prevent hemocyte aggregation, about 45 ml of the hemolymph that flowed out was placed into a 50-ml centrifuge tube containing 5 ml of ice-cold alkaline Ca^{2+}- and Mg^{2+}-free ASW containing 0.1 M EDTA. After immediate and gentle mixing of the tube by inversion, the acidity of hemolymph was pH 5.6. Under these conditions, minimal or virtually no aggregation or activation of the hemocytes occurred as previously described (Sawada and Ohtake, 1994). When small numbers of the hemocytes were required, the hemolymph was withdrawn into a syringe containing 1/10 volume of ice-cold alkaline Ca^{2+}- and Mg^{2+}-free ASW containing 0.1 M EDTA. The hemocytes were collected by centrifugation and then washed twice with ice-cold acidic Ca^{2+}- and Mg^{2+}-free ASW containing 0.54 mM EDTA (washing buffer). The hemocytes were counted with an eosinophil counter. For labeling with calcein AM, the hemocytes (approximately 4 to 8×10^7 cells) were resuspended in 1 ml of the washing buffer containing 12.5 μM calcein AM, and after labeling for 30 min at room temperature, they were washed twice with the washing buffer and suspended in 1 ml of neutral ASW.

Fluorescence microscopic observation of the contact reactions

The washed alloreactive hemocytes (approximately 2×10^7 cells) prepared from the pair of individuals with the alloreactive combination in the MHI-assay were separately loaded with calcein AM. The labeled hemocytes of the two member of the pair (2×10^6 cells) in 100 μl of neutral ASW were mixed and incubated for two hours at room temperature on a slide glass and the labeled non-alloreactive hemocytes prepared from the pair of individuals with the non-alloreactive combination, were reacted in the same manner. As a negative control, labeled hemocytes from each of the above individuals were mixed with labeled autologous hemocytes and incubated under the same conditions. The contact reactions were observed under a fluorescence microscope.

Fluorescence measurements of the ACR using with a multiwell plate scanner

The following protocol was adopted for an ACR assay. Hemocytes prepared from a pair of individuals were separately labeled with calcein AM as described above. Labeled hemocytes (2×10^6) from each animal suspended in 50 μl of neutral ASW were added to duplicated wells on the white microplate to which 200 μl of neutral ASW had been added. The average fluorescence in the wells is abbreviated as F_{Allo} in the following equation. To measure the fluorescence in a sample without alloreaction (F_{Auto} in the equation), in which all (or nearly all) cells are alive, 50 μl of each cell suspension was added to separate duplicated wells containing 250 μl of neutral ASW. To measure the fluorescence in a sample in which all (or nearly all) cells had died (F_W in the equation), 50 μl of each cell suspension was added to duplicated wells containing 250 μl of sterilized ultra pure water. The reaction was started by gentle mixing of cell suspensions with a plate shaker, and the fluorescence in the wells was measured with the fluorescence multiwell plate scanner, Fluoroskan II (Flow Lab. Inc.), after 60-min incubation at room temperature. All measurements were carried out with excitation at 485 nm and emission at 538 nm.

The extent of the contact reactions was expressed as cytotoxicity in the form of percentage of dead cells. Cytotoxicity was calculated from the following equation:

$$\% \text{ cytotoxicity} = (F_{Auto} - F_{Allo}) / (F_{Auto} - F_W) \times 100$$

where F_{Auto} represents the average of the fluorescence without alloreaction, F_{Allo} represents the fluorescence after allogeneic

reactions, and *Fw* represents maximal fluorescence release by water.

The kinetics of the fluorescence measurements of the ACR was analyzed using hemocytes prepared from 10 pairs of individuals with the alloreactive combination and 9 pairs with the non-alloreactive combination. The fluorescence was measured at 5 and 10 min after mixing the cell suspensions and every 10-min afterward over a 60-min period.

Effect of divalent cations on the ACR

The effect of Ca^{2+} on the ACR was analyzed as follows. Hemocytes from 4 pairs of individuals with the alloreactive combination were loaded with calcein AM and resuspended in Ca^{2+}- and Mg^{2+}-free neutral ASW containing different concentrations (0 to 20 mM) of $CaCl_2$, and their cytotoxicity was determined every 15 min for a 60-min period. The effect of Mg^{2+} on the ACR was analyzed in the same way. Labeled hemocytes from 4 pairs of individuals with the alloreactive combination were resuspended in Mg^{2+}-free neutral ASW containing different concentrations (1.6 to 52.6 mM) of $MgCl_2$.

Detection of mutual and directional cytotoxic reactions by the ACR assay

The alloreactivity of the hemocytes from one particular animal (No.1) was analyzed by the ACR assay with hemocytes from 4 pairs of animals that showed strong (No.5), intermediate (No.2 and No.3), and non-reactive (No.4) alloreactivities to No.1 animal in the MHI-assay. Hemocytes from five different individuals (No.1–5) were separately labeled with calcein AM. Non-labeled hemocytes from No.1 were reacted with equal numbers of labeled hemocytes from No.1–5. In the opposite direction, non-labeled hemocytes from No.1–5 were reacted with equal numbers of labeled hemocytes from No.1.

ACR assays using hemocytes with different "effector":"target" cell (*"E":"T"*) ratios

Four pairs of animals that showed directional ACR were selected first. Then, unlabeled hemocytes from one of the animals with cytotoxicity for the other were reacted as "effector cells" with labeled hemocytes of the latter that had not been cytotoxic to the former as "target cells". The ACR assays were carried out at different *"E":"T"* ratios, 1:1, 3:1 and 5:1, with "target cell" numbers maintained at 2×10^6 cells.

As a negative control, hemocytes from four pairs of non-alloreactive combination were selected, and the ACR assays were carried out as described above.

RESULTS

Fluorescence microscopic observation of allogeneic cytotoxic reactions

To determine whether the calcein retained in the cytoplasm of hemocytes is released from the cells by contact reactions, the reaction was observed on a glass slide under a fluorescence microscope. The impartial staining of hemocytes by calcein AM was confirmed by the flow cytometric analysis (data not shown) and the microscopic observations (Fig. 1a). No obvious decrease in green fluorescence was observed during 2 hours incubation for observation of the contact reaction after incubation of autologous cells alone (Fig. 1a). Similarly, virtually no decrease in fluorescence occurred as a result of incubation of hemocytes from a pair of non-alloreactive animals (Fig. 1b). By contrast, a significant decrease in fluorescence of all cells was observed after reaction of hemocytes from a pair of alloreactive animals (Fig. 1c). Many fragments and cell debris with faint green fluorescence were noted after the reaction.

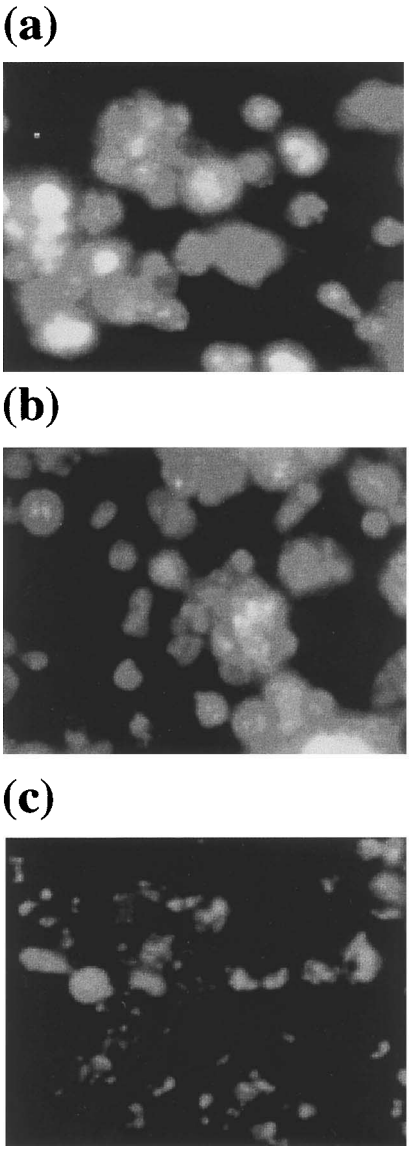

Fig. 1. Fluorescence microscopic observation of the contact reaction by calcein AM-labeled hemocytes. The negative reactions by autologous hemocytes (a) and by hemocytes from a pair of non-alloreactive combination (b) and the strong cytotoxic reaction by the hemocytes from a pair of individuals with an alloreactive combination (c) are shown. Note the retention of the fluorescence after the negative reactions shown in (a) and (b), and the significant decrease in the green fluorescence after the positive reaction shown in (c).

The ACR assay using the multiwell plate scanner

Since the decrease in retained calcein in the hemocytes was so significant after the contact reactions, we attempted to identify the optimal conditions for determining the decrease in fluorescence in a well on a 96-well white microplate

by using the fluorescence scanner. Significant decreases in fluorescence, which were expressed as cytotoxicity, were detected when more than 2×10^6 alloreactive hemocytes were incubated at room temperature for 60 min. Based on practical considerations for a more reliable reaction by a minimal number of cells, a standard cell number of 4×10^6 cells was selected for this assay.

The kinetics of the fluorescence decrease or cytotoxicity by the contact reaction with either alloreactive hemocytes or non-alloreactive hemocytes was monitored for 60 min (Fig. 2). Statistically significant cytotoxicity was observed 10 min after the start of the reaction when alloreactive hemocytes were mixed, and the reaction peaked at 60 min. No significant cytotoxic reaction was observed with non-alloreactive hemocytes throughout the 60-min incubation period.

The ACR was entirely dependent on Ca^{2+} in the saline, and no cytotoxic reaction occurred in the absence of Ca^{2+}. The optimal concentration of $CaCl_2$ was 10 mM (Fig. 3a), which was exactly the same concentration as in the ASW. The presence of Mg^{2+} in the saline had an inhibitory effect on the ACR (Fig. 3b). The maximal ACR in the presence of 10 mM $CaCl_2$ was observed when Mg^{2+} was omitted from the saline. Inhibition by Mg^{2+} was evident when more than 26.3 mM MgCl2, which was the half concentration as in the ASW, was present in the saline. In fact, a stronger ACR was observed when labeled hemocytes were reacted in Mg^{2+}-free neutral saline than in standard neutral ASW (data not shown).

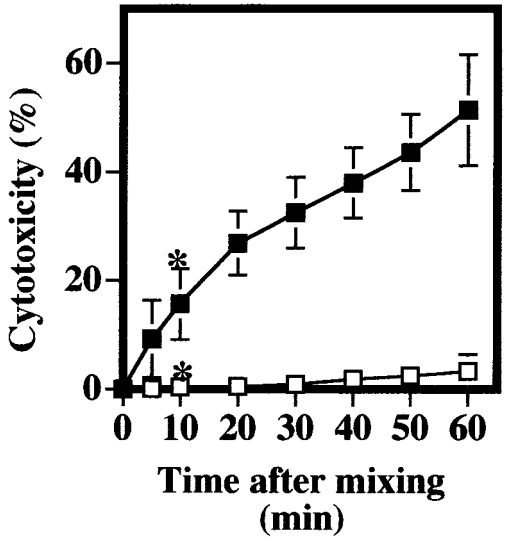

Fig. 2. Allogeneic cytotoxicity assay using the multiwell plate scanner. The cytotoxicity of allogeneic hemocytes was determined by the decrease in the retained calcein fluorescence in hemocytes measured with the multiwell plate scanner (see details in Materials and Methods). In the kinetics assay, the ACR mediated by hemocytes with alloreactive combination (■) and non-alloreactive combination (□) were monitored. The data points are means±SD (alloreactive combination, n=10; non-alloreactive combination, n=9). *: Significant at $p<0.05$ in *t*-test.

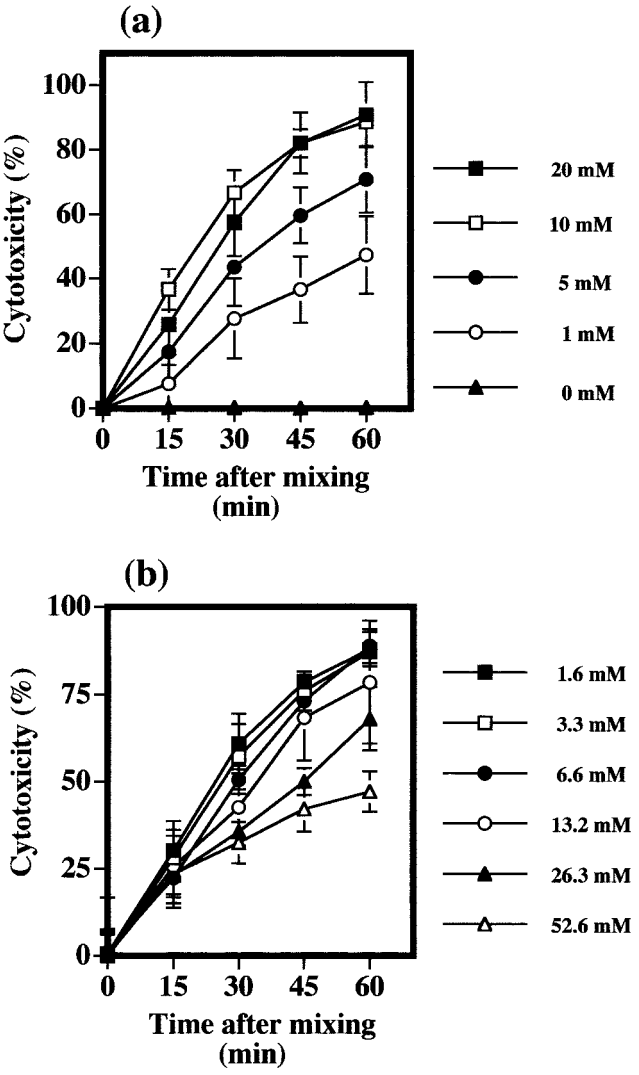

Fig. 3. Effects of divalent cations on the ACR. (a) The effect of Ca^{2+} on the ACR was tested by monitoring the ACR in Ca^{2+}- and Mg^{2+}-free neutral ASW containing different concentrations of $CaCl_2$. The data points are means±SD (n=4). (b) The effect of Mg^{2+}on the ACR was examined by monitoring the ACR in Mg^{2+}-free neutral ASW containing different concentrations of $MgCl_2$. The data points are means±SD (n=4).

Correlation between specificity and intensity in the MHI- and ACR assays

Reciprocal alloreactivities predetermined by the MHI-assay among 6 individuals (Fig.4a) were compared to reciprocal reactivities determined by the ACR assay (Fig. 4b). The pattern of alloreactivities determined by the ACR assay was consistent with that predetermined by the MHI-assay. In addition, the cytotoxicities (%) by the ACR corresponded to the extent of alloreaction in the MHI-assay judged by the cell-aggregation and the formation of brown pigmentation. Correlations of higher cytotoxicities with strong alloreactivities and with less high cytotoxicities and intermediate alloreactivities were noted in both assays. In a separate experiment, a correlation between the intensity of brown pigmentation

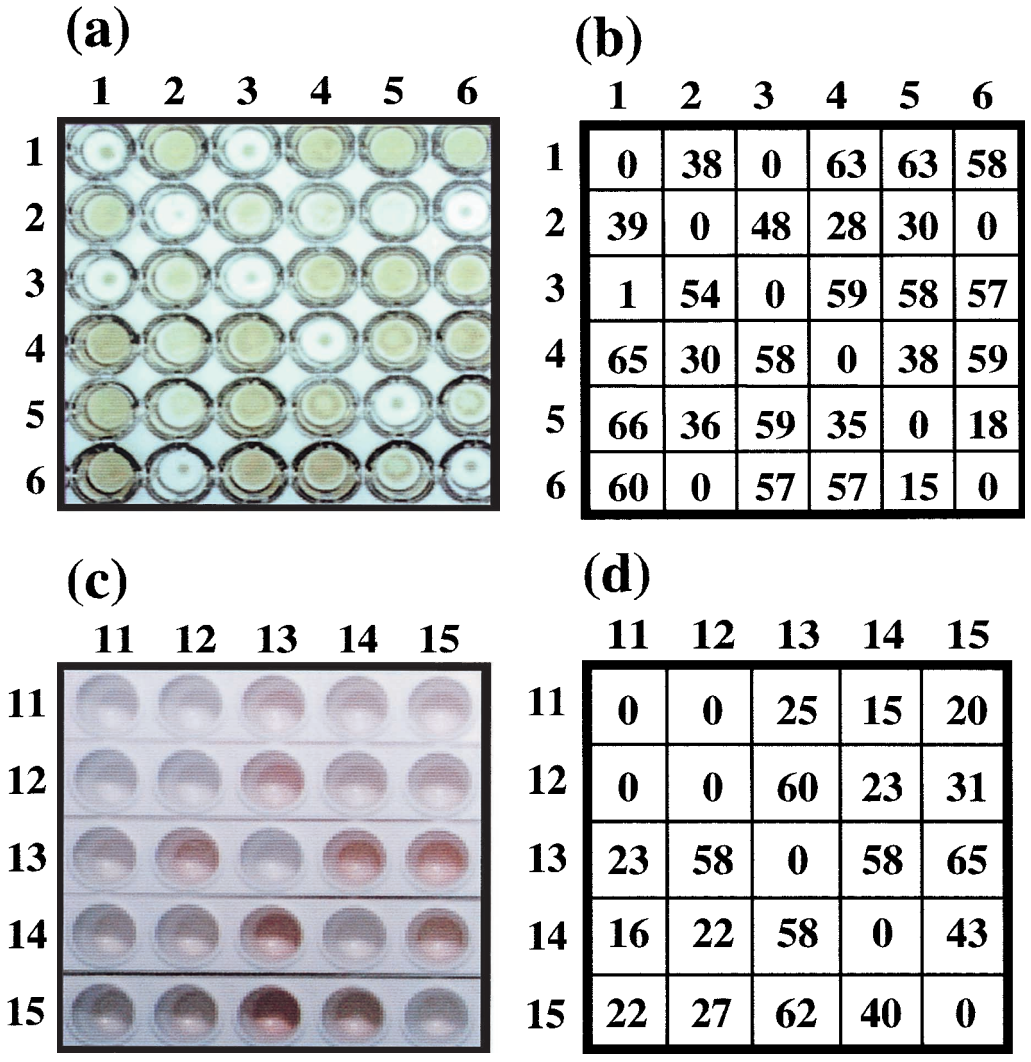

Fig. 4. Correlation between the specificity and intensity of the allogeneiccontact reaction determined by the MHI- and ACR assays. The allogeneic contact reaction was analyzed by the MHI-assay (a) and the ACR assay (b) using six individuals (No. 1–6) with different alloreactivity. Note the exact correlation between the alloreactivity patterns of six individuals in both assays. Also note the correlation between the intensity of the reaction: degrees of the brown pigmentation in (a) and % cytotoxicity in (b). In a separate experiment using hemocytes from five individuals (No. 11–15) with different alloreactivity, correlation between the intensity of brown pigmentation (c) and % cytotoxicity (d) was demonstrated on a 96-well white plate.

(Fig. 4c) and cytotoxicity (%) was demonstrated on a 96-well white microplate (Fig. 4d) using hemocytes from individuals with strong and intermediate alloreactivities.

Detection of mutual and directional ACR

To test the possibility that the different levels of alloreactivities observed above are related to levels of the expression of a still undefined self-marker or –markers, the hemocytes from pairs of animals with different alloreactivities were divided into two test tubes and separately labeled with calcein AM. Non-labeled and labeled hemocytes from each individuals were tentatively equated with effector cells and target cells, and the standard ACR assay was carried out (Table 1). There were clear correlations between the mutual cytotoxic reaction in the ACR assay and strong alloreactivity in the MHI-assay (No.1 *vs.* No.5) and between the directional cytotoxic reaction in the ACR assay and intermediate alloreactivity in the MHI-assay (No. 1 *vs.* No.2 and No.1 *vs.* No.3).

Tentative assignment of effector and target hemocytes in the ACR assay

Based on the directional ACR observed above, it was possible to equate hemocytes from one animal with “effector cells” and those from the other animal of the pair with “target cells”. A typical combination in Table 1 was No.3 hemocytes as “effector cells” and No.1 hemocytes as “target cells”. To confirm this possibility, we carried out ACR assays using hemocytes from this type of combination and non-alloreactive combination at different “effector”:“target” cell (*“E”:“T”*) ratios. This ACR assay clearly indicated *“E”:“T”*-ratio-dependent increases in cytotoxicity with hemocytes with the direc-

Table 1. Detection of mutual and directional allogeneic cytotoxic reactions by hemocytes from pairs of animals with different alloreactivity.

MHI-assay		ACR assay			
Combination	Alloreactivity[a]	Hemocytes		Cytotoxicity (%)	ACR[b]
		Non-labeled	Labeled		
No. 1 *vs* No. 1	–	No. 1	No. 1	0.1	–
		No. 1	No. 2	10.1	+
No. 1 *vs* No. 2	Intermediate	No. 1	No. 3	2.9	–
		No. 1	No. 4	0.2	–
No. 1 *vs* No. 3	Intermediate	No. 1	No. 5	37.7	++
		No. 1	No. 1	0.1	–
No. 1 *vs* No. 4	None	No. 2	No. 1	64.4	+++
		No. 3	No. 1	22.3	++
No. 1 *vs* No. 5	Strong	No. 4	No. 1	0.1	–
		No. 5	No. 1	38.0	++

[a] Intensity of the alloreaction: None, no reaction; Intermediate, aggregation with weak brown pigmentation; Strong, aggregation with strong brown pigmentation.
[b] Scores of –, +, ++, and +++, denote cytotoxicity less than 10%, 10 to 20%, 20 to 50%, and more than 50%, respectively. Note the correlation between the mutual cytotoxic reaction in the ACR and strong alloreactivity in the MHI-assay (No. 1 *vs.* No. 5), and between the directional cytotoxic reaction in the ACR and intermediate alloreactivity in the MHI-assay (No. 1 *vs.* No. 2 and No. 1 *vs.* No. 3).

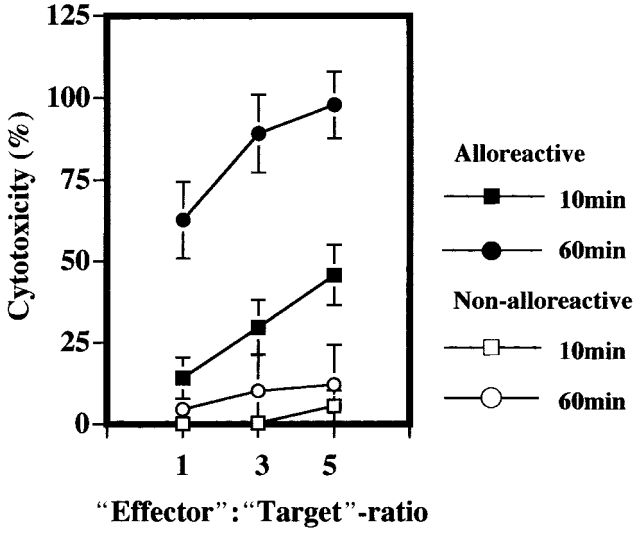

Fig. 5. Tentative assignment of effector and target cells in the ACR. A pair of animals with directional alloreactivity was selected, and hemocytes from one animal that had shown cytotoxicity were reacted as "effector cells" with calcein AM-labeled hemocytes from another animal as "target cells". The ACR assay was carried out at different "effector":"target" cell (*"E":"T"*) ratios. The data points are means±SD (n=4). As a negative control the same experiments were carried out using hemocytes from a pair of animals with non-alloreactive combination. The data points are means±SD (n=4).

tional alloreactivity, and virtually no *"E":"T"*-ratio-dependent increase in cytotoxicity with non-alloreactive hemocytes (Fig. 5).

DISCUSSION

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by enzymatic conversion of the virtually non-fluorescent and cell-membrane permeable calcein AM to the intensely fluorescent calcein. Polyanionic calcein is retained well within living cells, producing an intense uniform green fluorescence in them. Calcein AM has many advantages over other available fluorescein-based cytoplasmic dyes (Weston and Parish, 1990) and is considered the optimal dye for viability and cytotoxicity tests in vertebrates (Haugland, 1992). In the present study, we examined various conditions for application of calcein AM to determining the ACR mediated by alloreactive *H. roretzi* hemocytes. No application of calcein AM to testing cell viability or the cytotoxicity of marine invertebrates has ever been reported, however a cytotoxicity assay using 5-carboxyfluorescein diacetate (CFA) is used to determine the xenogeneic cytotoxic activity of *Ciona intestinalis* hemocytes (Peddie and Smith, 1994). We did not compare CFA with calcein AM in this study, since it is clear that calcein AM has the most favorable properties for use as a viability indicator, and can easily be substituted for CFA and other dyes in all applications (Haugland, 1992).

The ACR assay seems to be superior to a colorimetric microplate assay (Akita and Hoshi, 1995), which measures phenoloxidase activity released into reaction medium from vacuolated cells after the contact reactions. The ACR assay described here is a simple one-step assay but the colorimetric assay is required for sampling steps by centrifugation at each reaction time. The ACR assay monitors viability of all kind of hemocytes during the contact reactions but the colorimetric assay measures the enzyme activity released from a part of hemocytes, vacuolated cells, after the contact reactions.

The ACR assay established in this study is simple, sen-

sitive, and fast. However, quantitative and reproducible measurements of the ACR are of the greatest advantage for this assay. From this standpoint, it seems important to point out that mutual and directional ACR of hemocytes was distinguishable by this assay. The gene that controls the alloreactivity of *H. roretzi* has been analyzed genetically, and the two-gene control hypothesis was proposed long before (Fuke and Nakamura, 1985). The mutual and directional ACR may be related to levels of expression or numbers of the gene product or products on the target cells, and the ACR assay may allow more precise genetic analysis of the gene. The directional ACR distinguished firstly by this assay is remarkable from another point of view. In the case when hemocytes from 'A' are cytotoxic to 'B' but hemocytes from 'B' are not cytotoxic to 'A', we can equate hemocytes from animal 'A' with "effector cells" and those from 'B' labeled with calcein with "target cells". In the ACR assay with this kind of combination, it is possible to determine separately functions of effector and target cells in the ACR. This finding may partly overcome a limitation in studies on self-recognition of hemocytes in *H. roretzi*, *i.e.,* there is no available hemocyte or any kind of somatic cell line of *H. roretzi* for standard allogeneic target cells. The quantitative ACR assay using hemocytes at different *"E":"T"*-ratios may contribute to analysis of the precise mechanisms of the ACR at cellular and molecular levels, since the mechanisms of cytotoxicity by NK cells and cytotoxic T cells in the mouse and humans have been analyzed by such a quantitative cytotoxicity assay (see review by Trinchieri, 1989).

The ACR assay was sensitive enough to perform on large numbers of samples. Since significant ACR was detected by mixing 2×10^6 hemocytes from a pair of animals and since approximately 10^9 hemocytes are available from a single animal, ACR assay with approximately 500 wells in 5 microplates is possible by using single pair of animals. The ACR assay of large numbers of samples will allow production of monoclonal antibodies that block the ACR through binding to receptors or ligands that are involved in ACR. Such monoclonal antibodies will be useful in identifying and cloning the gene or genes that control or controls the alloreactivity of *H. roretzi.*

Nine cell types have been recognized in hemolymph of *H. roretzi* (Fuke and Fukumoto, 1993). Since contact reactions occur in almost all types of hemocytes (Fuke, 1980; Fuke, 2001), impartial staining of freshly isolated hemocytes with fluorogenic dyes and decrease in fluorescence of all the stained cells are essential for quantitative measurements of contact reactions with fluorimetric assay. In the ACR assay, loading of the hemocytes with calcein AM resulted in strong and impartial staining of almost all types of hemocytes, and decrease in fluorescence from almost all of the cells after contact reactions. Meanwhile, loss of plasma membrane integrity, another recognized parameter of cell viability, can be determined by ethidium homodimer that enters cells with damaged plasma membranes and undergoes an enhancement of fluorescence upon binding of to nucleic acids, thereby, producing a bright red fluorescence in dead cells (Haugland, 1992). Ethidium homodimer is excluded by the intact plasma membrane of live cells. Loss of plasma membrane integrity of almost all types of hemocytes after cytotoxic reactions is recently observed by this assay with the fluorescence microscope (Fuke, 2001). A two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells with the two fluorescent dyes, calcein AM and ethidium homodimer, is possible and may improve the ACR assay developed in this report.

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Note added in proof. Recently, we published an article (M. Arai, M. Suzuki-Koike, S. Ohtake, H. Ohba, K. Tanaka, and J. Chiba, *Microbiol. Immunol*. **45**, 857–866, 2001) describing production of monoclonal antibodies that recognize common cell-surface antigens functioning in self-recognition reactions by both somatic cells and gametes in *Halocynthia roretzi*. They were selected from thousanda of hybridomas by use of the ACR assay described in this paper.

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