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SPHINGOMYELIN INHIBITION OF *Ciona intestinalis* (TUNICATA) CYTOTOXIC HEMOCYTES ASSAYED AGAINST SHEEP ERYTHROCYTES

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□Abstract—Hemocytes from the ascidian, *Ciona intestinalis*, are capable of lysing erythrocytes in vitro following cell membrane contact. With the aim of examining the mechanism of cytotoxicity, we performed inhibition experiments with lipid components of erythrocyte membranes. Cholesterol is not an inhibitor, whereas, among the phospholipids tested, (sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine) sphingomyelin inhibits the hemolytic activity of hemocytes. However, thin layer chromatography showed that sphingomyelinase activity was not contained in the chloroform-methanol extracts from hemocyte debris. The inhibition capacity of the components ceramide and phosphorylcholine suggests that the entire sphingomyelin molecule is involved in binding cytolysins. The lysin-lipid interactions probably cause changes in erythrocyte membrane permeability, leading to lysis.

□Keywords—Hemocytes; Cytotoxicity; Hemolysis inhibition; Sphingomyelin inhibition; Invertebrate; Tunicate; *Ciona intestinalis;* Sheep erythrocytes.

Introduction

Natural cytotoxicity directed against allogeneic and xenogeneic cells including mammalian tumor cells has been demonstrated using effector cells from several invertebrate groups. The cell-killing mechanism can involve soluble cytotoxic mediators or it may require contact between effector and target cells (see reviews: 1–3). Cytotoxic cells from annelids, echinoderms, and molluscs have been compared to vertebrate natural killer cells (4–9); in particular hemocytes from the mollusc, *Planorbarius corneus*, have been found to possess certain NK plasma membrane markers common to mammals using FACS analysis (9).

In tunicates, hemocytes from solitary ascidians display cytotoxic activity in vitro when examined in xenogeneic or allogeneic combinations (10,11). In vivo, they have been implicated in tunic inflammatory responses (12,13) and rejection of allografts (14,15). Moreover, they are involved in non-fusion reactions between allogeneic colonies of *Botryllus* (16).

The mechanisms of hemocyte cytotoxic activity have been little investigated. We have reported (17) that hemocytes from the ascidian *Ciona intestinalis* are able to lyse rabbit, human, guinea pig, and sheep erythrocytes in vitro. Hemocyte cytotoxic activity (HCA), assayed against sheep erythrocytes (SE), is a calcium-dependent, cell-mediated reaction with occurs rapidly (15–30 min) at 25–37°C, and was maximal at a 1:3 effector/target cell ratio. Apparently, the cytotoxic mechanism is mediated by contact between cell membranes and

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could be enhanced by release of agglutinins. Recently, Peddie and Smith (18) reported that hemocytes of *C. intestinalis* also lyse in vitro mammalian tumor cell lines. Cytotoxic activity occurred within 15 min at 20°C, at 35:1 effector/target cell ratio.

Erythrocytes are well known in their membrane structure and lipid distribution (19–21), and represent useful cell targets for examining the cytolytic mechanisms of effector hemocytes. The lipidic components of erythrocyte membranes are not symmetrically distributed between the two halves of the bilayer. The neutral phospholipid phosphatidylcholine, and sphingomyelin, are restricted mainly to the outer monolayer, whereas the inner leaflet contains predominantly the anionic phospholipids, phosphatidylethanolamine and phosphatodylserine. In erythrocytes with low phosphatidylcholine content (such as those of sheep), the outer layer is composed primarily of sphingomyelin (choline-containing lipid). Cholesterol, abundant in erythrocytes, is probably arranged in clusters distributed among the other lipid molecules (22,23).

With the aim of examining the interaction between erythrocyte membrane lipids and hemocyte lysins, we performed inhibition studies of HCA with phospholipids. In the present paper, two points emerge: We confirm that HCA occurs in media optimal for erythrocyte targets (17) as well as for *C. intestinalis* hemocytes; 2) we show that sphingomyelin is a target of cytolysins.

Materials and Methods

Animals, Bleeding, Media, Hemocyte Preparation and Quantification

Ciona intestinalis specimens were collected from the Gulf of Palermo. Hemolymph was withdrawn from the heart with a syringe. Artificial sea water without $CaCl_2$ and $MgCl_2$ but enriched with 10 m*M* EDTA (FSW-EDTA, pH 7.0) or marine anticoagulant (0.1 *M* glucose; 15 m*M* trisodium citrate; 13 m*M* citric acid; 10 m*M* EDTA; 0.45 *M* NaCl) was used (v/v) as diluent. To obtain a suitable number of hemocytes for each experiment, hemolymph from about 100 specimens was pooled, and centrifuged at 1500 × *g* for 15 min at 4°C. The pelleted hemocytes were washed three times in marine solution (MS) (12 m*M* $CaCl_2 \cdot 6H_2O$; 11 m*M* KCl; 26 m*M* $MgCl_2 \cdot 6H_2O$; 43 m*M* Tris; 38 m*M* HCl; 0.4 M NaCl; pH 7.4) and finally suspended in MS, isosmotic with hemocytes (1090 mOsm kg^{-1}), or Tris-Buffered Saline (TBS) (50 m*M* Tris-HCl, 150 m*M* NaCl, pH 8) isosmotic with erythrocytes to obtain a 1–1.5 × 10^7/mL cell concentration. TBS for the final hemocyte suspension contained 10 m*M* $CaCl_2$ (TBS-Ca, 450 mOsm kg^{-1}). Total cell counts were determined using an improved Neubauer hemocytometer.

Hemocyte Viability

Allogeneic interactions have not been observed among hemocytes from *Ciona intestinalis* (10). However, the possibility exists that reactions occur among different populations. Therefore experiments were performed using animals collected from the same locale. Hemocyte mortality in each experimental procedure was evaluated by the eosin-y dye (0.5% in suitable medium) exclusion test.

Preparation of Erythrocyte Targets

Sheep erythrocytes (SE) were provided by the Zooprophylaxis Institute (Palermo, Italy), rabbit erythrocytes (RE) were from Sclavo (Siena, Italy). Erythrocytes were washed three times in

phosphate buffered saline (PBS), then in MS or TBS-Ca and finally suspended at the required concentration. Erythrocytes were also equilibrated to 1090 mOsm kg^{-1}: prior to use, the cells were washed in PBS and centrifuged for 5 min at 400 × *g*, resuspended in 10 mL low salt marine solution (LMS) (MS containing 0.3 *M* NaCl; 740 mOsm kg^{-1}), incubated for 30 min at 20°C, and resuspended in MS (1090 mOsm kg^{-1}).

Experimental Cytotoxic Assay of Hemocytes (HCA)

As previously described (17), 200 μL of hemocyte suspension (2–3 × 10^6 cells) in TBS-Ca or MS was mixed with an equal volume of a suspension of SE (8 × 10^6 cells) freshly prepared in the same medium. Hemocyte numbers were determined in the final volume of the reaction mixture, and incubated with continuous and moderate shaking at 37°C for 1 h. At the end of incubation, the supernatant was separated and the amount of released hemoglobin (Hb) was estimated by reading the absorbance at 541 nm. The degree of hemolysis was determined according to the equation: Percent hemolysis = (Measured release − spontaneous release/complete release − spontaneous release) × 100. Complete hemoglobin release was obtained by preparing an erythrocyte suspension in distilled water at room temperature. Reaction media isosmotic with effector cells (MS) or target cells (TBS) were used. Mortality of hemocytes was evaluated in different media, isosmotic with effector cells (MS) or target erythrocytes (TBS-Ca), under the experimental conditions. Control SE or RE suspensions were prepared in MS or TBS-Ca and incubated as the reaction mixtures: The spontaneous hemoglobin release never exceeded 5% of complete release. All experiments were performed at least three times using different hemocyte pools. For each experiment three samples were prepared, the values repesenting the mean ± SD.

Inhibition Experiments

Preparation of chemicals. All chemicals (Sigma chemical Co. St. Louis) tested for inhibitory activity (sphingomyelin types, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine cholesterol, ceramide and the headgroup phosphorylcholine) were dissolved in MS or TBS-Ca to obtain concentrations of 0.025, 0.25, 2.5, 25.0, and 250.0 μg/mL in the reaction mixture. Phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and cholesterol were relatively insoluble in TBS. For these compounds, stock solutions were briefly sonicated (Vibra-Cell, Sonics & Materials Inc., Dambury, CT).

Inhibition assay. Hemocyte suspensions were prepared in the medium containing the compounds at various concentrations. Since TBS, isosmotic with erythrocytes, caused the highest degree of hemolytic activity against SEs, this medium was used primarily for inhibition experiments. After 20 min preincubation, the erythrocyte suspension (prepared in TBS-Ca containing the same amount of inhibitor), were added at various E/T ratios for cytotoxic assays. Control lysis, due to chemicals alone, was measured. Sphingomyelin inhibition was also performed in MS. Different experiments were performed as follows: 1) hemocytes pretreated with drugs were washed with TBS-Ca before hemolytic assays. 2) RBC were incubated for 30 min with the indicated concentrations of inhibitors, subjected to one centrifugation; the pelleted erythrocytes were resuspended in a suitable volume of TBS-Ca, and incubated with hemocytes in a hemolytic assay.

Evaluation of inhibitory capacity. In each experiment, inhibitory capacity

was evaluated by comparison with a control of HCA in the absence of inhibitor. All experiments to test inhibition were performed at least three times, using pools of different hemocytes. The values presented in the figures or listed in the tables, unless otherwise reported, are the means of three samples ± SD of typical experiments. Some results are expressed as percentage inhibition of cytotoxicity: 1 − (% hemoglobin release in experimental/%hemoglobin release in control) × 100.

Sphingomyelinase Activity

Hemocytes (6×10^7/mL) were homogenized in TBS-Ca by using a Potter homogenizer (Kontes, Vineland, New Jersey) kept in an ice bath. The cellular debris, separated from the homogenate by centrifuging at 27,000 × *g* at 4°C, was washed twice and finally suspended in the same volume of TBS-Ca. A sample of this suspension was assayed for hemolytic activity. In order to assay sphingomyelinase activity of the hemocyte debris, a mixture of 0.4 mL suspension of cellular debris and 1 mg sphingomyelin in 0.6 mL of TBS-Ca was allowed to stand for 60 min at 37°C. The solution was extracted with two volumes of chloroform-methanol 2:1 (v/v) and the extract was subjected to thin layer, bidimensional chromatography on silica gel plates (E. Merck, Darmstadt) according to Broekhuyse (24). A control reaction mixture was prepared using 1 mg sphingomyelin in 0.4 mL solution and 2.0 units of *Staphylococcus aureus* sphingomyelinase (Sigma chemical Co. St. Louis). In addition, the following controls were prepared in the same way for chromatography: 1) 0.4 mL suspension of cellular debris; 2) 1 mg sphingomyelin in 0.6 mL medium. The spots on the chromatograms were stained with iodine vapor.

Results

Cytotoxic Activity Is Found in Media of Increasing Osmolality

Experiments designed to examine the effects of osmolality on HCA (E/T ratio 1:3), showed that effector cells exerted cytotoxic activity either in medium of 1090 mOsm kg^{-1}, optimal for hemocytes, or of 450 mOsm kg^{-1} optimal for erythrocytes.

Figure 1 shows that a significant degree of hemolysis was found at 1090 mOsm kg^{-1} (about 40%). In this medium, hemocyte mortality was low (<4%) after 1 h incubation at 37°C. The hemolytic activity of hemocytes against SE increased at a lower osmolality, reaching a maximum at 450 mOsm kg^{-1} despite the fact that hemocyte mortality was 20–30%.

To examine whether hemolytic values are dependent on the status of erythrocytes or hemocytes, hemocytes were assayed in MS with equilibrated SE or with a different type of erythrocyte (RE). In the medium isosmotic for hemocytes (MS) the degree of hemolysis was 70% with RE (Fig. 1), whereas, SE, adjusted to high osmolality, reduced their susceptibility to lysis (Fig. 1).

Cytolysins Are Not Present in the Reaction Medium

To ascertain if cytotoxic soluble factors are released in isosmotic medium (MS) or during the effector-target cell interactions (1:3 E/T ratio) as an effect of hemocyte activation in TBS, the supernatant obtained after centrifuging hemocyte suspensions or effector-target reaction mixtures was assayed for cytotoxic activity against SE. The supernatant from hemocytes suspended in MS was inactive in lysing SE. It is known that no release of lytic factors occurs when

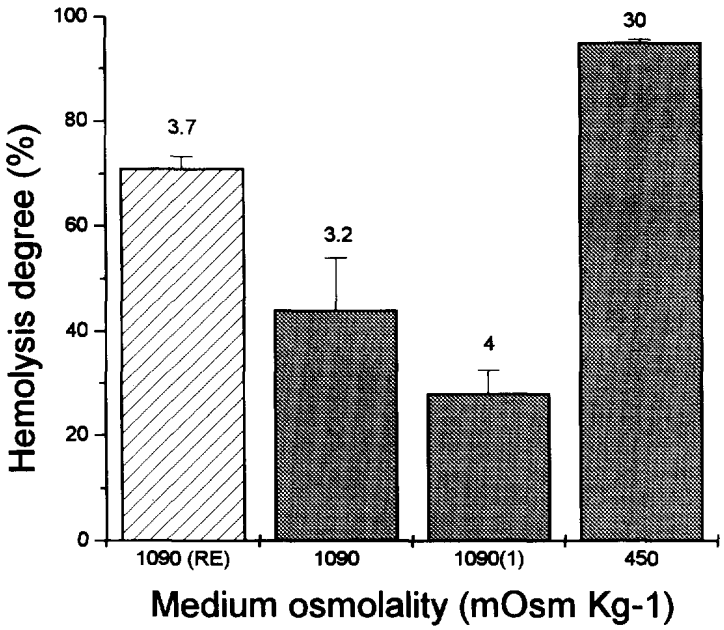

Figure 1. Hemolytic activity of *Ciona intestinalis* hemocytes assayed (2–3 × 10^6 cells) with sheep erythrocytes (8 × 10^6 cells) in media at various osmolalities. The hemolysis of rabbit erythrocytes (RE) at 1090 mOsm kg^{-1} is also reported (first bar). Values on top of the bars indicate the mortalities (%) of hemocytes. (1) Sheep erythrocytes equilibrated to 1090 mOsm kg^{-1}.

hemocytes were held in TBS-Ca (17). Therefore, to examine the possible release following erythrocyte-effector contacts, the difference between degrees of hemolysis after incubation of SE with hemocytes and the subsequent incubation of supernatants with SE was evaluated. In order to calculate residual hemolytic activity, supernatants from reaction mixtures, in which hemoglobin release in TBS-Ca was less than 50% degree of hemolysis, were used. No significant differences were found (Table 1) in eight separate experiments.

Cytotoxic Activity at Various Effector/Target Cell Ratios

Cell-cell interaction mechanisms and the cytotoxic capacity of effector cells may be elucidated by examining the effector/target cell ratio. The curves, obtained by plotting the numbers of hemocytes vs. hemolysis, are sigmoidal in shape either in low or high osmolality media (Fig. 2). A significant rise in hemolysis was found when 4 × 10^6/mL

Table 1. Hemolytic Activity of Supernatants (TBS-Ca) Obtained From Reaction Mixture in Which *Ciona intestinalis* Hemocytes had Reacted Against Sheep Erythrocytes.

	Hemolysis Degree (% ± SD; n = 3)	
Exp. n.	Reaction Mixture: Hemocytes With SE*	Supernatant of the Reaction Mixture Tested With SE†
1	26.0 ± 2.3	27.6 ± 0.8
2	26.4 ± 7.1	33.6 ± 3.6
3	33.6 ± 3.4	36.2 ± 9.6
4	32.2 ± 3.1	31.3 ± 7.4
5	40.0 ± 2.8	39.6 ± 2.2
6	42.2 ± 4.0	40.7 ± 3.3
7	43.3 ± 3.9	44.0 ± 5.9
8	42.8 ± 5.7	50.2 ± 4.8

* To enable the calculation of the residual hemolytic activity of the supernatant, reaction mixture which showed hemolysis less than 50% were used.
† 200 μL of supernatant assayed with 1% sheep erythrocytes suspension; the effect of 1:1 dilution was adjusted by multiply each value of absorbance at 541 nm by two, and then the hemolysis degree was determined.

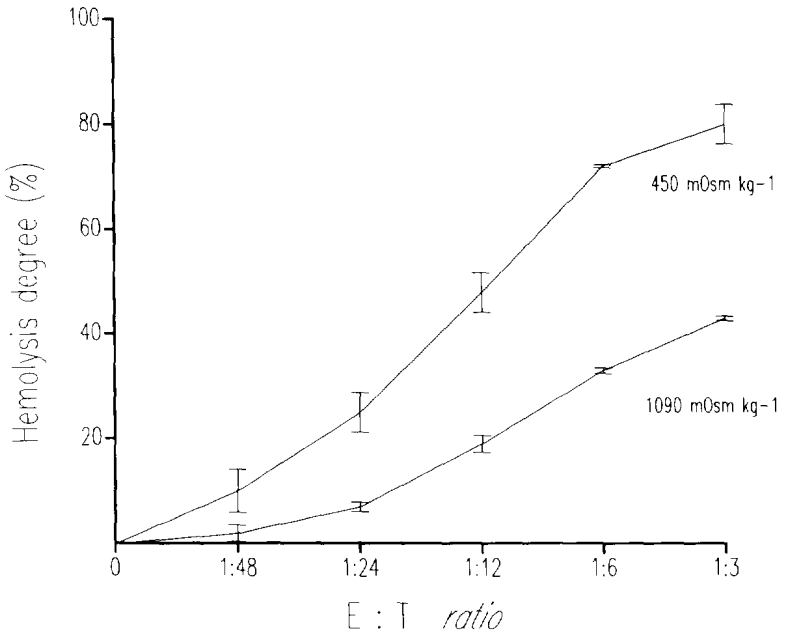

Figure 2. Hemolytic activity of *Ciona intestinalis* hemocytes assayed with sheep erythrocytes at various effector/target cell ratios. Assays were performed in media of 450 mOsm kg^{-1} (TBS) or 1090 mOsm kg^{-1} (MS). The number of targets (8×10^6 cells) were held constant.

hemocytes reacted against 4×10^7mL target cells (E/T ratio 1:10), the highest value (hemolysis about 80%) was reached using 15×10^6 hemocytes (E/T ratio 1:3). More numerous target cells at lower E/T proportions did not yield an increased degree of hemolysis, whereas they produced an inhibitory effect (data not shown). Unless otherwise reported the E/T ratio 1:3 was used in the assays.

Significant Inhibition of Lysis by Sphingomyelin

To reveal the possible inhibitory effect of various phospholipid concentrations on HCA, SE were assayed in TBS-Ca in which the highest degree of SE hemolysis was observed. Sphingomyelin inhibited HCA in a dose-dependent fashion (Fig. 3A) at various effector/target cell ratios. The inhibitory capacity, by comparison with the profile of non-inhibited HCA, is evident at optimal E/T ratios (1:3–1:12). At those values, the inhibitory capacity of SM was about 40–80%, depending on doses of the inhibitors. Degrees of inhibition increased in the range of SM concentrations above 0.25 μg/mL; 250 μg/mL completely abolished cytotoxic activity of hemocytes. Sphingomyelins from various sources, characterized by different fatty acids (bovine brain: primarily stearic acid and nervonic acid; bovine erythrocytes: primarily linoleic acid; chicken egg yolk: primarily palmitic acid), act as inhibitors. In Figure 3A, the inhibitory activity of 25 μg/mL bovine eritrocyte SM assayed in marine solution (MS) is shown at various E/T ratios, (Fig. 3). Also in this medium, isosmotic for hemocytes, a significant inhibition of HCA was found, i.e., hemolysis decreased from 45.0% to 10.0% (Fig. 3A). An equivalent inhibitory capacity was observed when sphingomyelins from bovine brain and chicken egg yolk were assayed (data not shown).

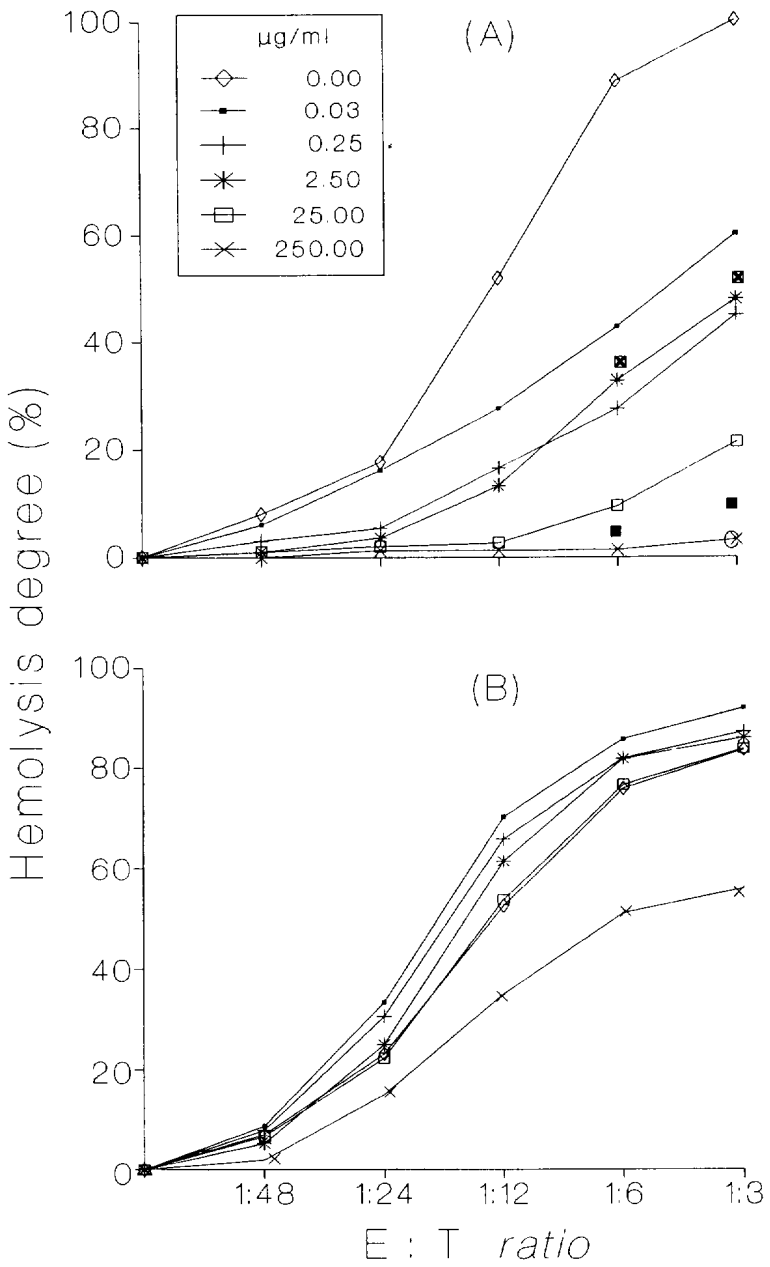

Figure 3. Effect of bovine erythrocyte sphingomyelin at various concentrations on hemolytic activity of *Ciona intestinalis* hemocytes in TBS, at various effector/target ratios. Target cells were sheep erythrocytes. (A) Sphingomyelin present in reaction mixture. Assays were also performed in MS (1090 mOsm kg^{-1}) in the presence (■) or absence (☒) of sphingomyelin (250 µg/mL). (B) Sphingomyelin removed after a preincubation period of 20 min. SD < ±5%.

Ceramide, a component of the sphingomyelin molecule, is a less active inhibitor than the whole molecule and its inhibitory capacity was about 51% at an amount of 250 µg/mL. Moreover, the headgroup phosphorylcholine showed a 35% inhibitory activity that was proportional to increasing (0.25–25.0 µg/mL) concentrations (Table 2). Phosphatidylcholine, phosphatidylserine, phosphaty-

Table 2. Inhibitory Effect of Phospholipids, Cholesterol, and Phospholipids Components on Hemolytic Activity of *Ciona intestinalis* Hemocytes (1.0–1.5 × 10^7/mL) Assayed With Sheep Erythrocytes in TBS-Ca

	% of Inhibition (n = 3 ± SD)				
	Inhibitor Concentration (μg/mL)				
	0.025	0.25	2.5	25.0	250.0
Ceramide	—	—	18 ± 4	30 ± 2	51 ± 3
Phosphorylcholine	7 ± 2	20 ± 1	20 ± 3	25 ± 4	35 ± 2
Phosphatydilserine	0	4 ± 3	10 ± 7	0	0
Phosphatidylcholine	0	9 ± 2	0	0	9 ± 3
Cholesterol	0	6 ± 6	8 ± 3	0	0
Phosphatydilethanolamine	—	—	—	—	4 ± 1

— Not detected.

dilethanolamine, and cholesterol were inactive even when assayed at their highest amounts (Table 2).

Preincubation with Sphingomyelin does not Affect Lytic Activity of Effector Hemocytes and Susceptibility of Target Cells

To rule out the possibility that inhibition of cytotoxic activity may be due to a cytotoxic effect of SM on effector cells, the mortality of hemocytes incubated in MS with 250 μg/mL SM for 30 min was assessed. In three different assays, no significant difference was observed in the mortality of treated (0.1–0.3% dead cells) and untreated (0.2–0.3% dead cells) hemocytes.

To determine whether SM was acting on effector or target cells, hemocytes or erythrocytes were preincubated separately with each of the inhibitor concentrations, then washed and assayed for HCA. After preincubation, when SM was removed, hemocytes maintained their hemolytic activity at the level of untreated cells. A small decrease was found only in hemocytes pretreated with 250 μg/mL SM (Fig. 3B). No changes in the susceptibility of erythrocytes to lysis occurred, even if they were treated with 250 μg/mL of inhibitor.

Apparent Absence of Sphingomyelinase Activity in Cellular Debris

Hemocyte debris, containing 50% hemolytic activity, was assayed for sphingomyelinase activity. The stained chromatograms revealed the following patterns: 1) a single spot, having the *ratio frontis* (Rf) of uncleaved sphingomyelin, from the reaction mixture containing hemocyte debris; 2) in control reaction mixtures containing sphingomyelinase, the sphingomyelin spot almost disappeared and a new spot appeared near the solvent front; 3) spots were not observed in chromatograms containing samples from reaction mixtures in which cellular debris alone was incubated.

Discussion

In accord with previously reported results (17), the cytotoxic activity (HCA) of *Ciona intestinalis* hemocytes appears to be a cell-mediated reaction requiring cell membrane contact. The effector cells functioned in a medium at an osmolality optimal either for the effector hemocytes (1090 mOsm kg^{-1}), or for erythrocyte targets (450 mOsm kg^{-1}). Concerning HCA, the osmolality of the reaction medium affected mostly erythrocyte susceptibility to lysis as shown by

high hemolysis of RE in MS medium, and by low hemolysis of equilibrated SE (pretreatment at 740 mOsm kg^{-1}).

The experiments performed with varying ratios of effector and target cells confirm that the optimal E/T ratios vary from 1:3 to 1:12. We had previously ruled out the possibility that soluble factor/s could be released from hemocytes in TBS-Ca under the experimental conditions (17). We now show that no soluble lytic factors are left in the medium as a consequence of effector cell activation by target cells. The high cell mortality in TBS-Ca (about 30%) does not cause any release of soluble lytic factors. Moreover, anti-SE lytic factors were not released from hemocytes even if they were held in isosmotic medium (MS). The low E/T ratios we found as optimal suggest a mechanism by which one effector cell acts on several erythrocytes. Therefore, it cannot be excluded that released cytolysins bind to the membranes of erythrocytes. The high number of erythrocytes in the reaction mixture probably sequesters all soluble molecules that were not found in the supernatant of the reaction mixture.

Phospholipids have been proposed as being involved in the regulation of mammalian cytotoxic activities exerted by NK cells and other cells such as cytotoxic T lymphocytes (25,26). These findings are based on cytotoxic inhibition experiments. Tschopp et al. (27) reported that large, multilamellar vesicles containing lipids with phosphorylcholine headgroups such as sphingomyelin and phosphatidylcholine compete with sheep erythrocyte membranes for the binding and insertion of perforin and reduce the hemolytic activity of perforin purified from large granular lymphocytes and cytolytic T lymphocytes. In addition, inhibition with drugs showed that macrophages (28,29) may recognize a phospholipid moiety on the outer membrane leaflet of target cells that may be taken up later by phagocytosis.

Of interest is the finding that, among the phospholipids tested, only sphingomyelin inhibits hemolytic activity of *Ciona* hemocytes. This inhibition is dose-dependent, but is apparently not due to toxic effects on effector cells, as shown by assays in MS medium, isosmotic for hemocytes. The presence of SM throughout the reaction resulted in a strong inhibition of HCA whereas the separate pretreatment of effector or target cells was ineffective and no significant changes in hemocyte and erythrocyte reactivity were observed. These results may be explained by invoking the competition between such compounds and target cell membranes for interaction with the effector cell surfaces, which bind preferentially the SM dissolved in reaction media. An excess of sphingomyelin in pretreatment experiments reduced the lytic capacity of the effector cells, probably because a lipidic film remained on the hemocyte, reducing its activity. Our present data also show that ceramide, a component of this sphingomyelin molecule, and phosphorylcholine, which is the sphingomyelin head group, are less active inhibitors. The whole sphingomyelin molecule appears to be involved in binding to cytolysins. However, membrane bound sphingomyelinase was not shown by thin layer chromatography analysis.

Although we are not able to propose a model that explains the lytic mechanism, we postulate that: 1) experimental conditions (37°C temperature, low osmolality medium) could activate cytotoxic hemocytes or increase SE susceptibility; 2) the hemocyte lysin of *Ciona* may be active with the inner part of the sphingomyelin molecule owing to its arrangement in the membrane outer layer; 3) interactions of hemocyte lysin with this lipid cause changes in erythrocyte membrane permeability leading to lysis; 4) effector/target ratios suggest that hemocytes which lyse SE (E/T = 1:3) are different from those (phagocytic and

nonphagocytic amoebocytes, Ref. 18) that lyse tumor cells (E/T = 35:1). The presence of distinct cytotoxic hemocytes is also supported by density gradient separation experiments (Cammarata et al., in preparation) that show anti-SE effector cells enriched in the populations of vacuolar granulocytes (terminology according to De Leo; 30).

It is known also that the reaction of some soluble invertebrate hemolysins, present in the cell-free coelomic fluid, may be mediated by interactions with membrane targets through lipids. In *Holothuria polii* (31) and *Marthasterias glacialis* (32), sphingomyelin is an inhibitor of hemolytic reactions, whereas cholesterol, phosphatidyl-inositol and phosphatidylethanolamine were effective in inhibiting hemolysin from *Paracentrotus lividus* (33).

The lipid-lysin interaction, in conjunction with mammalian models, suggests that an unrestricted, relatively simple mechanism, involving target membrane lipids may also be important in the expression of tunicata natural cytotoxicity, a process that has endured in the evolutionary development of immunity.

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