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PHENOLOXIDASE AND CYTOTOXICITY IN THE COMPOUND ASCIDIAN *Botryllus schlosseri*

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□Abstract—The vacuoles of morula cells (MC) of the colonial ascidian *Botryllus schlosseri* contain phenoloxidase (PO). As the release of their vacuolar content at the border of incompatible contacting colonies is associated with the formation of necrotic masses which characterize the rejection reaction, the role of PO in *Botryllus* cytotoxicity was investigated. When hemocytes are incubated with blood plasma from incompatible (heterologous) colonies, MC degranulate and, after 60 min, the cytotoxicity index becomes significantly greater than that observed in controls incubated with autologous plasma. The rise in cell mortality is completely inhibited by the addition of PO inhibitors sodium benzoate, tropolone and phenylthiourea, and serine protease inhibitors phenylmethylsulfonyl fluoride, benzamidine, N-tosyl-L-phenylalanine chloromethyl ketone and N-tosyl-L-lysine chloromethyl ketone. The addition of either reducing agents L-cysteine and ascorbic acid or reactive oxygen species scavenger enzymes superoxide dismutase and catalase has a similar effect. Significant inhibition of cytotoxicity is also observed with the quinone scavenger, 3-methyl-2-benzothiazolinone hydrazone. In the presence of sodium benzoate and phenylthiourea, there is a significant reduction in the number, size and color intensity of necrotic masses along the contact border of incompatible colonies. A significant increase in superoxide anion production, completely inhibited by sodium benzoate, is observed when hemocytes are incubated with heterologous blood plasma. These results indicate that: (i) PO is the enzyme responsible for the cytotoxicity observed in both hemocyte cultures and rejection reactions; (ii) PO is present inside MC vacuoles as a proenzyme which is activated, upon release, by humoral proteases; (iii) cytotoxicity appears to be mainly due to oxidative stress generated by PO during oxidation of polyphenols to quinones without the involvement of other oxidases such as NADPH oxidase and peroxidase. **© 1998 Elsevier Science Ltd. All rights reserved**

□Keywords—Immunity; Ascidians; *Botryllus*; Rejection reaction; Phenoloxidase; Cytotoxicity.

Nomenclature

BAEE:	N-benzoyl-L-arginine ethyl ester;
BP:	blood plasma;
CA:	contacting ampullae;
DMSO:	dimethyl sulfoxide;
L-DOPA:	dihydroxyphenyl-L-alanine;
FSW:	filtered sea water;
MBTH:	3-methyl-2-benzothiazolinone hydrazone;
MC:	morula cells;
NBT:	nitroblue tetrazolium;
PBS:	phosphate buffered saline;
PMSF:	phenylmethylsulfonyl fluoride;
PO:	phenoloxidase;
POR:	points of rejection;
PTU:	phenylthiourea;
ROM:	reactive oxygen metabolites;
RU:	relative units;
SOD:	superoxide dismutase;
TB:	Tris buffer;

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TLCK: N-tosyl-L-lysine chloromethyl ketone;
TPCK: N-tosyl-L-phenylalanine chloromethyl ketone.

Introduction

Phenoloxidase (PO) is a copper enzyme, widely distributed in plants and animals and able to convert mono- and/or diphenols to quinones which, in turn, polymerize to form melanins. Among invertebrates, it has been described in the blood of several species belonging to various phyla (1–6). In arthropods, the enzyme is the end product of a complex cascade of reactions, called the proPO activating system, which is involved in humoral and cellular immune defences (7–9).

As far as ascidians are concerned, PO has been described in both solitary and colonial species and is located inside the morula cells (MC) which are characterized by the presence of several vacuoles, about 2 μm in diameter, conferring a typical mulberry-like shape (5, 6, 10–12).

The role of PO (tyrosinase for vertebrate cytologists) in cytotoxicity is well documented in vertebrates in which quinones may be toxic according to at least two mechanisms: (i) quinones can be reduced to semiquinones by a variety of enzyme systems; semiquinones, in turn, can rapidly react with and damage biological macromolecules or, with oxygen, reducing it to superoxide anions which can give rise to other reactive oxygen metabolites (ROM), such as peroxide, hydroxyl radicals and singlet oxygen, able to generate oxidative stress (13–16); (ii) quinones and semiquinones are able to react rapidly and conjugate with -SH groups on essential molecules (e.g., cysteine residues of proteins or reduced glutathione) thus altering their function (14, 17–19). Conversely, there are few experimental data supporting the hypothesis of the cytotoxic role of PO in invertebrates. However, it has been demonstrated that quinones and melanin generated by crayfish PO inhibit fungal growth (20), that melanogenesis is linked to the generation of cytotoxic molecules in insect immune responses (21), and that locust PO can immobilize and kill the parasite *Trypanosoma rangeli* (Brookman et al., unpublished data, quoted in Ratcliffe (9)). Recently, Cammarata et al. (22) have described PO-linked cytotoxicity in the solitary ascidian *Styela plicata*.

In ascidians of the genus *Botryllus*, contact between two incompatible colonies results in the appearance of diffuse cytotoxic foci along the contact border, giving rise to necrotic masses. MC are involved in this rejection reaction: they initially accumulate at the apices of facing ampullae, then cross the ampullar epithelium and infiltrate into the fused tunics where they degranulate, release the content of their vacuoles and degenerate, thus contributing to the formation of the necrotic masses (23–26). Degranulation is triggered by the recognition of humoral factors diffusing from contacting colonies and is easily induced when hemocytes are incubated with blood plasma (BP) from incompatible colonies (27). In the same experimental conditions, a significant increase in PO activity of the incubation medium is observed. High PO activity is also detectable along the border of incompatible contacting colonies even before the appearance of the necrotic regions (27).

The above results suggest that PO is responsible for the cytotoxicity observed in the contact area between incompatible colonies. Therefore, in the present study, we used in vitro hemocyte cultures and in vivo colony fusibility assays for further evidence of the involvement of PO in cytotoxicity in botryllid ascidians.

Materials and Methods

Animals

Hibernating colonies of *Botryllus schlosseri* from the Lagoon of Venice and colonies of defined fusibility genotypes

from our laboratory were used. They were kept in aerated aquaria, attached to glass slides and fed with Liquifry Marine (Liquifry Co., Dorking, England) and algae.

The terms "homologous", "heterologous" and "autologous" refer to fusible and nonfusible colonies, and to subclones of the same colony, respectively. Homologous colonies share at least one allele at the fusibility locus (28, 29).

Blood Plasma, Blood Cell and Hemolysate Preparation

Hibernating colonies of 500–800 zooids were used to obtain high quantities of hemocytes and blood plasma.

Blood was collected with a glass micropipette by puncturing the tunic marginal vessels of colonies previously blotted dry. The supernatant obtained after blood centrifugation at $780 \times g$ for 15 min was referred to as BP.

Hemocytes were obtained from blood collected from colonies previously rinsed in filtered sea water (FSW) containing 10 mM L-cysteine, pH 7.5, to prevent clotting and then centrifuged at $780 \times g$ for 15 min; pellets were finally resuspended in FSW to give a concentration of 15×10^6 cells/ml.

In other experimental conditions, whole blood obtained from untreated colonies, previously blotted dry, was sonicated at 0°C in a Braun Labsonic U sonifier at 50% duty cycles for 5 min and subsequently centrifuged at $12{,}000 \times g$ for 20 min at 4°C. The supernatant, referred to as "hemolysate", was used in spectrophotometric enzyme assays.

Protein concentrations were determined according to Bradford (30), using bovine serum albumin as standard.

Morula Cell Degranulation Assay and in vitro Cytotoxicity Determination

Fifty μl of hemocyte suspension (15×10^6 cells/ml) were placed in the center of the culture chambers prepared as already described (31) and left to adhere to washed coverslips for 30 min. After discarding the FSW, hemocytes were then incubated for 60 min at room temperature with 50 μl of autologous or heterologous BP and washed in FSW. MC morphology was observed under a phase contrast light microscope (Leitz Dialux 22). Cytotoxicity was assessed by trypan blue exclusion; results are expressed as the cytotoxicity index, i.e., the percentage of cells positive for trypan blue staining. At least 800 cells per slide in 10 fields were counted and absolute values were compared using the χ^2 test. The following substances were added to BPs to determine their effects on cytotoxicity: PO inhibitors sodium benzoate (Na-benzoate; Sigma; 10 and 20 mM), tropolone (Fluka; 1 and 2 mM) and phenylthiourea (PTU; Sigma; 0.5 and 1 mM), serine protease inhibitors phenylmethylsulfonyl fluoride (PMSF; Sigma) and benzamidine (Sigma), both at 1 and 2 mM concentrations, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma) and N-tosyl-L-lysine chloromethyl ketone (TLCK; Fluka), both at 10 and 50 μM concentrations, antioxidants, L-cysteine (Sigma) and ascorbic acid (Sigma), both at 1 and 2 mM concentrations, scavenger enzymes superoxide dismutase (SOD; Sigma; 60 and 120 U/ml) and catalase (Sigma; 70 and 140 U/ml) and the quinone scavenger 3-methyl-2-benzothiazolinone hydrazone (MBTH; Fluka; 1 and 2 mM). All compounds were first dissolved in PBS (absolute ethanol for PTU) to a concentration 100 times the working dilution. One percent ethanol was added in the controls of the PTU series.

PO and Protease Activity Assay

PO activity was measured according to Winder and Harris (32), using the reagent MBTH, which reacts directly with dopaquinones. This method has the advantage

of measuring the direct product of dihydroxyphenyl-L-alanine (L-DOPA) oxidation, since not all dopaquinones are converted to dopachromes, which are measured when L-DOPA only is used as substrate. Briefly, 20 μl of hemolysate were incubated with 490 μl of phosphate buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, pH 7.2), 290 μl of 20.7 mM MBTH in PBS containing 4% of N,N-dimethylformamide and 200 μl of L-DOPA-saturated PBS. The reaction was read spectrophotometrically at 505 nm, each min for 5 min. PO inhibitors Na-benzoate (10 and 20 mM), tropolone (1 and 2 mM) and PTU (0.5 and 1 mM) were added to the incubation mixture to evaluate their effects on PO activity. SOD (60 and 120 U/ml), catalase (70 and 140 U/ml), L-cysteine and ascorbic acid, both at 1 and 2 mM concentrations, were also investigated for their effects on PO activity.

For protease activity measurement, 125 μl of hemolysate were incubated with 750 μl of Tris buffer (TB: 0.05 M Tris HCl, 20 mM $CaCl_2$, pH 8.0) and 125 μl of the serine and SH-protease substrate N-benzoyl-L-arginine ethyl ester (BAEE) 10 mM in TB (final concentration: 1 mM). The reaction was followed spectrophotometrically at 254 nm for at least 5 min (modified after Yonemura et al. (33)). Protease inhibitors, PMSF and benzamidine, at a final concentration of 1 mM, and TPCK and TLCK, at a final concentration of 10 μM, were added to the incubation mixture to assay their effects on protease activity. The two ketones were first dissolved to yield a 1 mM concentration in dimethyl sulfoxide (DMSO) and absolute ethanol, respectively. Ten μl of the two solvents were added to the incubation mixtures in controls.

Each experiment was replicated three times ($n = 3$). Results are expressed as relative units (RU)/mg of protein and compared with Student's t-test. In both cases, one relative unit of enzyme activity is defined as an increase in absorption of 0.001/min at 25°C in 1 ml of reaction mixture.

In vitro Superoxide Anion Production

Hemocytes (40×10^6) from a single colony were incubated for 60 min in Eppendorf tubes with 500 μl of autologous or heterologous BP in the presence of 0.3% nitroblue tetrazolium (NBT) with or without 20 mM Na-benzoate. The supernatant resulting from the centrifugation at $780 \times g$ for 10 min was collected and 480 μl were added to 640 μl of a solution of 2 M KOH and DMSO (ratio 6:7) to dissolve formazan precipitates (modified after Song and Hsieh (34)). After 5 min, the absorbance at 620 nm was read and compared with that of the control BP, to which 0.3% NBT was added. SOD (120 U/ml) was added to check the specificity of the reaction.

Fusibility Assay

Pairs of laboratory heterologous colonies were used. Two subclones from each colony provided an equal number of control and experimental pairs. Colonies of each pair were juxtaposed on a supporting glass slide and left to attach and then grow in FSW at a temperature of 19°C until the facing marginal ampullae came into contact. Then, half the pairs were placed in a 10 mM Na-benzoate or a 0.5 mM PTU solution in FSW for a period of time not exceeding 48 h. A longer time in the above solutions induced some stress and consequent withdrawal of the facing ampullae; in more concentrated solutions, it was difficult to obtain ampullar contact and cuticle fusion. The number of points of rejection (POR (35)) and the total number of contacting ampullae (CA) were scored and the POR/CA ratio was calculated. The resulting values were compared through Student's paired t-test. SOD and catalase were not used, due to the difficulty of

having a stable enzyme activity for long periods in FSW. All the other compounds, at the concentrations used in the in vitro cytotoxicity assays, were toxic in in vivo experiments.

Results

PO and Protease Activity in Hemolysate and Effects of Inhibitors

The previously shown PO activity of the hemolysate (11) significantly decreased ($p < 0.001$) in the presence of enzyme inhibitors Na-benzoate (10 and 20 mM), tropolone (1 and 2 mM) and PTU (0.5 and 1 mM). The addition of antioxidants L-cysteine or ascorbic acid at 1 and 2 mM concentrations also significantly reduced ($p < 0.001$) the amount of dopaquinone formed. Enzyme activity was not affected when 60 U/ml of SOD were present in the incubation medium, but a significant reduction ($p < 0.05$) was observed with 120 U/ml, whereas catalase, at the two concentrations used, had no effects. The *Botryllus* hemolysate was also endowed with protease activity, detectable using BAEE as a substrate: it was significantly reduced by the addition of 1 mM PMSF, 1 mM benzamidine, 10 μM TPCK or 10 μM TLCK in the reaction mixture ($p < 0.01$ for PMSF; $p < 0.001$ for the other compounds) (Table 1).

MC Degranulate in the Presence of BP from Heterologous Colonies

MC degranulate when incubated with heterologous BP and consequently change their morphology, becoming irregular as their vacuoles are reduced to small, pale

Table 1. Phenoloxidase and protease activity of *B. schlosseri* hemolysate in various experimental conditions.

Enzyme	Treatment	Enzyme activity (RU/mg protein ±SD)	% inhibition
PO			
	PBS (controls)	32.8±0.8	—
	Na-benzoate 10 mM	11.9±0.8***	63.7
	Na-benzoate 20 mM	6.6±0.2***	80.0
	Tropolone 1 mM	12.6±0.2***	61.5
	Tropolone 2 mM	1.7±0.1***	94.8
	PTU 0.5 mM	1.7±0.1***	94.2
	PTU 1 mM	1.9±0.1***	94.8
	SOD 60 U/ml	34.0±0.5	—
	SOD 120 U/ml	24.7±0.2*	24.7
	Catalase 70 U/ml	33.1±0.6	—
	Catalase 140 U/ml	33.6±0.9	—
	L-cysteine 1 mM	7.3±0.8***	77.7
	L-cysteine 2 mM	0.3±0.0***	98.9
	Ascorbic acid 1 mM	6.9±0.6***	78.9
	Ascorbic acid 2 mM	0.5±0.0***	98.5
Protease			
	TB (controls)	16.3±5.3	—
	PMSF 1 mM	5.1±0.8**	68.8
	Benzamidine 1 mM	0.2±0.0***	98.8
	TPCK 10 μM	0.1±0.0***	99.4
	TLCK 10 μM	0.1±0.0***	99.5

Differences with respect to controls indicated by asterisks. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

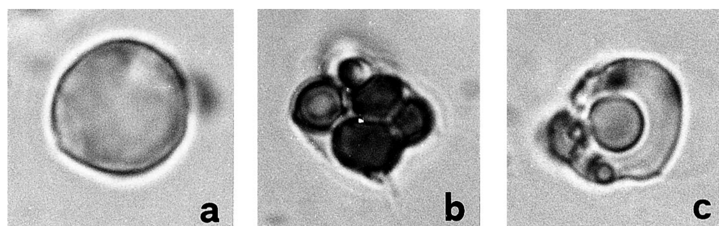

Figure 1. Living morula cells from hemocyte monolayers incubated for 60 min in autologous blood plasma (a), heterologous blood plasma (b) and heterologous blood plasma in presence of 0.5 M PTU (c). Magnification: 1200 ×.

brown vesicles [Fig. 1(b)]. This response to BP from heterologous incompatible colonies could already be observed after 30 min of incubation and was not prevented by the presence in the incubation medium of any of the above-listed drugs, with the exception of 2 mM tropolone which inhibits degranulation. In the presence of the drugs, MC vacuoles appear pale and transparent [Fig. 1(c)], with the exception of cells treated with Na-benzoate 10 mM, L-cysteine (at both concentrations used), SOD 60 U/ml and catalase 70 U/ml, which did not appear to be different from the degranulated MC of the controls and with MBTH (at both concentrations), which presented dark pink collapsed vacuoles. Degranulation was not observed when MC were incubated in BP from fusible colonies (Table 2).

Table 2. Effects of various treatments on MC degranulation and pigmentation of residual vacuoles.

Treatment	MC degranulation	Pigmentation of collapsed vacuoles
Autologous BP	—	
Heterologous BP (Het BP)	+	+
Het BP + Na-benzoate 10 mM	+	+
Het BP + Na-benzoate 20 mM	+	—
Het BP + Tropolone 1 mM	+	—
Het BP + Tropolone 2 mM	—	
Het BP + PTU 0.5 mM	+	—
Het BP + PTU 1 mM	+	—
Het BP + PMSF 1 mM	+	—
Het BP + PMSF 2 mM	+	—
Het BP + Benzamidine 1 mM	+	—
Het BP Benzamidine 2 mM	+	—
Het BP + TPCK 10 μM	+	—
Het BP TPCK 50 μM	+	—
Het BP + TLCK 10 μM	+	—
Het BP + TLCK 50 μM	+	—
Het BP + SOD 60 U/ml	+	+
Het BP + SOD 120 U/ml	+	—
Het BP + Catalase 70 U/ml	+	+
Het BP + Catalase 140 U/ml	+	—
Het BP + L-cysteine 1 mM	+	+
Het BP + L-cysteine 2 mM	+	+
Het BP + Ascorbic acid 1 mM	+	—
Het BP + Ascorbic acid 2 mM	+	—
Het BP + MBTH 1 mM	+	+ (dark pink)
Het BP + MBTH 2 mM	+	+ (dark pink)

In vitro Cytotoxicity of Heterologous BP and Effects of Added Inhibitors

As shown in Table 3, the cytotoxicity index in hemocyte monolayers differs significantly depending on whether they were incubated with autologous (control) or heterologous BP. However, the cytotoxicity was decreased when PO inhibitors (except for Na-benzoate at the lower concentration), serine protease inhibitors, reducing agents, ROM scavengers and quinone scavengers were added to the heterologous BP. The cytotoxicity index of autologous BP did not change in the presence of the above-mentioned compounds.

Table 3. Hemocyte cytotoxicity: effects of PO inhibitors Na-benzoate, tropolone and PTU, protease inhibitors PMSF, benzamidine, TPCK and TLCK, reactive oxygen species scavenger enzymes SOD and catalase, reducing agents L-cysteine and ascorbic acid, and quinone scavenger MBTH.

Treatment	Cytotoxicity index#
Autologous BP	1.83 ± 0.22
Heterologous BP (HetBP)	6.85 ± 1.50***
HetBP + Na-benzoate 10 mM	6.52 ± 0.82***
HetBP + Na-benzoate 20 mM	2.15 ± 0.57
HetBP + Tropolone 1 mM	2.32 ± 0.22
HetBP + Tropolone 2 mM	2.07 ± 0.23
HetBP + PTU 0.5 mM	3.59 ± 0.80
HetBP + PTU 1 mM	2.08 ± 0.45
HetBP + PMSF 1 mM	1.91 ± 0.39
HetBP + PMSF 2 mM	1.32 ± 0.76
HetBP + Benzamidine 1 mM	1.86 ± 0.53
HetBP + Benzamidine 2 mM	1.10 ± 0.26
HetBP + TPCK 10 mM	1.64 ± 0.43
HetBP + TPCK 50 mM	1.49 ± 0.42
HetBP + TLCK 10 mM	1.41 ± 0.35
HetBP + TLCK 50 mM	1.38 ± 0.39
HetBP + SOD 60 U/ml	1.39 ± 0.43
HetBP + SOD 120 U/ml	1.11 ± 0.21
HetBP + Catalase 70 U/ml	1.93 ± 0.16
HetBP + Catalase 140 U/ml	1.79 ± 0.18
HetBP + L-cysteine 1 mM	1.13 ± 0.25
HetBP + L-cysteine 2 mM	1.01 ± 0.10
HetBP + ascorbic acid 1 mM	1.44 ± 0.30
HetBP + ascorbic acid 2 mM	1.20 ± 0.11
HetBP + MBTH 1 mM	1.33 ± 0.14
HetBP + MBTH 2 mM	1.32 ± 0.23

Percentage of cells positive for trypan blue staining. Asterisks indicate significant differences with respect to autologous BP. ***: $p < 0.001$.

In vitro Production of Superoxide Anion

When hemocytes were incubated with autologous BP, a certain degree of NBT reduction was observed. When they were incubated with heterologous BP, the amount of formazan formed, as measured spectrophotometrically after its solubilization, significantly increased ($p < 0.01$). In the presence of SOD, the increase in absorbance was not significantly different from that obtained with autologous BP, whereas the presence of Na-benzoate completely inhibited the NBT reduction. (Table 4).

Na-benzoate and PTU affect the rejection reaction

The rejection reaction between heterologous colonies incubated in 10 mM Na-benzoate and 0.5 mM PTU solutions were compared with the rejections of the same colonies in FSW. In both controls and experimental series POR appeared within 15–20 h after facing ampullae made contact (Fig. 2). Their number varied according to both the number of CA and the contact period which in the controls, therefore, was never allowed to exceed that of the corresponding treated pairs of colonies. As Table 5 shows, the POR/CA ratio was significantly higher in the controls than in the experimental series ($p < 0.001$). Moreover, the necrotic spots in the experimental series were smaller and lighter in color.

Discussion

PO (tyrosinase for vertebrate cytologists) is the key enzyme in melanization

Table 4. Production of superoxide anion by *Botryllus* hemocytes from same colony incubated with autologous and heterologous BP. Results expressed as difference between absorbance of BP after incubation with hemocytes and absorbance of BP in absence of cells.

Treatment	ΔA_{620}
Autologous BP	0.182 ± 0.021
Autologous BP + SOD 120 U/ml	0.179 ± 0.032
Autologous BP + Na-benzoate 20 mM	0.002 ± 0.001***
Heterologous BP	0.281 ± 0.013**
Heterologous BP + SOD 120 U/ml	0.173 ± 0.040
Heterologous BP + Na-benzoate 20 mM	0.003 ± 0.001***

Difference with respect to autologous BP are marked with asterisks. **: $p < 0.01$; ***: $p < 0.001$.

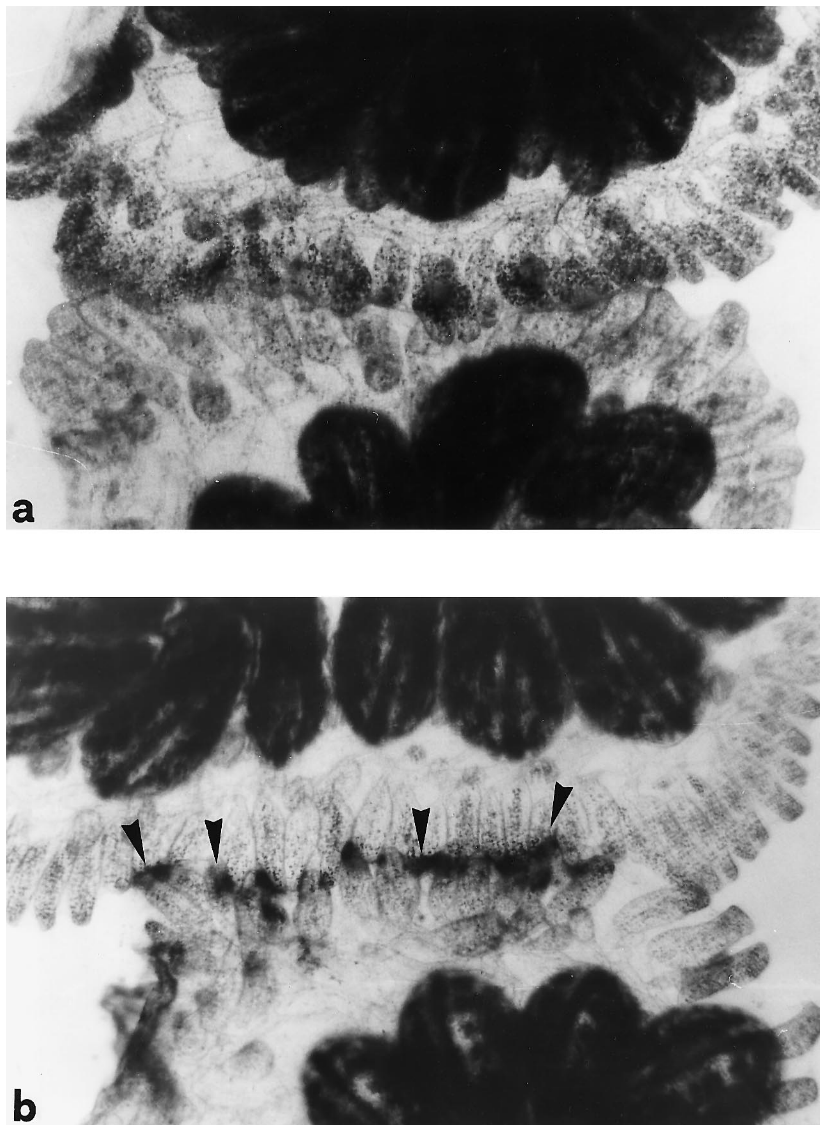

Figure 2. Contact region between fusible colonies (a) and incompatible colonies showing necrotic spots marked by arrowheads (b). Magnification: 25×.

Table 5. Ratio of number of points of rejection (POR) to number of contacting ampullae (CA) in a series of pairs of incompatible colonies, each pair subdivided into two couples of subclones serving respectively as controls and experimental series (treatment with 10 mM Na-benzoate or 0.5 mM PTU).

	FSW (control)			Na-benzoate 10 mM			$\Delta_{POR/CA}$
# Pair	#CA	#POR	POR/CA	#CA	#POR	POR/CA	
I	24	11	0.45	16	2	0.13	0.32
II	24	7	0.29	26	4	0.15	0.14
III	20	10	0.50	26	2	0.08	0.42
IV	14	3	0.21	21	3	0.14	0.07
V	16	5	0.31	10	2	0.20	0.11
VI	12	5	0.42	24	0	0.00	0.42
VII	13	7	0.54	12	1	0.08	0.46
VIII	16	2	0.13	12	4	0.33	−0.20
IX	24	11	0.46	13	4	0.31	0.15
X	16	9	0.56	20	6	0.30	0.26
						mean	0.21
						S.D.	0.21
						t	3.16
						p	<0.001

	FSW (control)			PTU 0.5 mM			$\Delta_{POR/CA}$
# Pair	#CA	#POR	POR/CA	#CA	#POR	POR/CA	
I	27	41	0.66	6	12	0.50	0.16
II	16	34	0.47	5	21	0.24	0.23
III	15	24	0.63	5	10	0.50	0.13
IV	6	14	0.43	2	8	0.25	0.18
V	14	26	0.54	4	14	0.29	0.25
VI	4	10	0.40	2	11	0.18	0.22
VII	11	20	0.55	4	11	0.36	0.19
VIII	10	28	0.36	3	10	0.30	0.06
IX	5	15	0.33	4	14	0.29	0.04
X	8	14	0.57	6	12	0.50	0.07
XI	6	19	0.32	3	11	0.27	0.05
XII	12	24	0.50	4	10	0.40	0.10
XIII	13	23	0.57	2	10	0.20	0.37
XIV	8	15	0.53	9	21	0.43	0.10
XV	4	13	0.31	4	15	0.27	0.04
						mean	0.15
						S.D.	0.09
						t	5.98
						p	<0.001

as it converts phenols to *o*-quinones which then polymerize to form melanins. In the course of the reaction, semiquinones and ROM may be produced, causing membrane lipid peroxidation and/or protein inactivation (13, 16, 36). Quinones and semiquinones may also react rapidly with sulfydryl groups of cysteines, thus leading to loss of protein functions (14, 15, 17–19). These cytotoxic effects of PO have been exploited in the therapy of mammalian malignant melanomas, in which phenols are frequently used as specific antitumoral agents (13, 15, 19). The enzyme is present

in several species of invertebrates and plays a role in humoral and cellular immunity (7–9). Nevertheless, its role in cytotoxicity has been poorly investigated (7–9, 20–22).

Ascidian MC contain PO inside their vacuoles (5, 6, 10–12) and the presence of polyphenol substrata has been revealed in *B. schlosseri* and *S. plicata* MC (22, 27). Although the exact function of this enzyme in ascidian biology is still debated, there is an increasing amount of data suggesting the direct role of PO in cytotoxic immune defence reactions. In *S. plicata*, the rejection of allogeneic tunic transplants and their subsequent necrosis is coupled to the accumulation of hemocytes, particularly MC, under the transplanted tunic ((37), rediscussed in Parrinello (38)). Moreover, in *Halocynthia roretzi*, the "contact reaction" leading to cytolysis of contacting hemocytes from incompatible donors occurs in parallel with the release of PO into the incubation medium (39). In addition, the cytotoxicity of *Ciona intestinalis* and *S. plicata* hemocytes towards rabbit erythrocytes appears to be mediated by MC (36); in the latter case, they exert their cytotoxic activity through the release of PO (22).

In ascidians of the genus *Botryllus*, the contact between heterologous colonies, sharing no alleles at the fusibility locus, triggers a cytotoxic rejection reaction characterized by the appearance of necrotic masses along the contact border (24, 26, 35, 40–43).

In the present study, we demonstrate that MC are the effector cells of this reaction, which is initiated after the fusion of the contacting tunics by the infiltration of blood cells, primarily MC, in the contact area, where they degranulate and release PO and its polyphenol substrata. It is the oxidation, by PO, of these compounds which causes the death of cells and tunic tissue. The intense PO activity revealed histochemically in the contact area between incompatible colonies and preceding necrosis (27) strongly favors the hypothesis of a role played by PO in POR cytoxicity.

The following points support our assumption:

(a) recognition factors able to trigger MC degranulation, the subsequent release of PO into the incubation medium (27) and the increase in in vitro cytotoxicity are present in the heterologous BP. The exocytosis of MC vacuolar content upon recognition of heterologous humoral factors leads to the shrinkage of MC vacuoles into small vesicles and a general change in cell morphology.

(b) the cytotoxicity of heterologous BP is blocked if PO inhibitors Na-benzoate (44) at 20 mM concentration, tropolone (45) at 1 mM concentration and PTU (46) at 0.5 and 1 mM concentrations, are added to the hemocyte monolayers, although MC regularly degranulate. In their presence, the collapsed vacuoles are unpigmented, while they are pale brown in their absence, probably due to PO oxidation of residual polyphenols in the vacuoles and incipient melanization; this also holds true in the presence of 10 mM Na-benzoate. At 1 mM tropolone concentration, the degree of PO inhibition is no different from that observed in the presence of 10 mM Na-benzoate, although the latter does not produce any significant decrease in cytotoxicity. This probably reflects the different mechanisms of action of the two compounds, tropolone having metal ion chelation abilities (46, 47) and, therefore, acting as an non-competitive inhibitor, whereas Na-benzoate mimics the substrate and produces reversible competitive inhibition. At 2 mM tropolone concentration, inhibition of cytotoxicity is probably due to the observed inhibition of degranulation which, in turn, is related to the disassembly of microtubule organization caused by tropolone and related molecules (48, 49).

(c) both Na-benzoate and PTU significantly reduce the formation of the

necrotic masses along the contact border between heterologous colonies.

In arthropods, PO is present as a proenzyme inside granular cells and is activated by serine proteases which, in turn, are activated by foreign surface carbohydrates (7–9). In ascidians, although PO activity is already detectable in collected blood, a similar activation mechanism is suggested by the increase in PO activity observed in several species after preincubation of blood or hemocyte lysates with serine proteases (5, 6, 11, 12). In the present study, we show detectable levels of protease activity in the hemolysate of *B. schlosseri*, inhibited by PMSF, benzamidine, TPCK and TLCK. Moreover, the observation that the addition to incompatible BP of protease inhibitors at a concentration which significantly reduces protease activity, does not inhibit in vitro MC degranulation but completely suppresses in vitro cytotoxicity, clearly indicates that, upon recognition of humoral incompatible factors, PO is released by MC as an inactive proenzyme which is subsequently activated by humoral proteases present in the BP. In all the above cases, no pigmentation is observed inside the collapsed MC vacuoles, thus indirectly confirming the inhibition of PO activity.

As regards the mechanism of cytotoxicity, our results indicate that it is closely related to the oxidation of phenols to *o*-quinones by PO. Quinones are reduced to semiquinones which react with molecular oxygen to form superoxide anions. The latter, by dismutation, give rise to hydrogen peroxide which, through either a Fenton reaction in the presence of ferrous iron (abundant inside *Botryllus* MC (27)) or a Haber–Weiss reaction in the presence of superoxide anion, generate hydroxyl radicals which are probably responsible for most of the cytotoxicity observed (14). This assumption is supported by the observation that MBTH, which traps quinones in dark pink stable compounds (32), does inhibit cytotoxicity although it has no effects on PO activity, as demonstrated by the dark pink color of MC residual vacuoles. Moreover, the antioxidant ascorbic acid, which significantly decreases DOPA oxidation by reducing quinones and semiquinones to hydroquinones, also decreases cytotoxicity, through its inhibition of ROM production and the scavenging effect on those eventually produced. The absence of pigmentation of collapsed vacuoles in the presence of ascorbic acid is in good agreement with the above-reported inhibition of polyphenol oxidation.

The inhibition of cytotoxicity by L-cysteine is probably due to the antioxidant effects of thiols which counteract the oxidative potential of ROM (14). The dark pigmentation observed inside collapsed MC vacuoles may be phaeomelanin forming, according to Nicolaus (17), upon the reaction of the amino acid with quinones deriving from the PO-driven oxidation of MC vacuolar polyphenols by PO. The reduction of quinones available for the reaction with MBTH can also explain the decrease in the absorbance at 505 nm observed in the PO activity assay in the presence of L-cysteine.

Cytotoxicity is also decreased by the addition of SOD and catalase, the first converting semiquinones and superoxide anions to non-toxic and stable chemical species, the latter scavenging peroxides. Neither SOD nor catalase influences MC degranulation, although they have different effects on the pigmentation of collapsed vacuoles. In the presence of 60 U/ml SOD, which does not affect PO activity, a pale brown color is usually observed. The absence of pigmentation at 120 U/ml SOD is probably due to the prevailing scavenging action of the enzyme, which subtracts semiquinones and decreases the amount of quinones available for melanization: this may also explain the moderate inhibition of PO activity observed in the spectrophotometric assay (50). Catalase does not inhibit PO activity, but no pigmentation is observed inside MC col-

lapsed vacuoles in the presence of catalase 140 U/ml, probably as a result of delayed melanin formation by the scavenger, as ROM may modulate the course of melanogenesis (51).

In conclusion, morula cell PO appears to be the enzyme responsible for the cytotoxicity observed in both in vitro hemocyte cultures and rejection reactions, exerting its toxic activity mainly though the generation of oxidative stress. The significant increase (> 50%) in superoxide anion production, as revealed by formazan formation when hemocytes are incubated with heterologous BP and its complete inhibition in the presence of Na-benzoate, support the above statement and suggest that no other oxidases are involved. The observed, although limited, increase in NBT reduction in the presence of autologous BP is likely to be related to the presence of active PO in the incubation medium, released by MC injured during blood collection, as suggested by its inhibition by Na-benzoate and it is probably due to the production of reducing compounds different from superoxide anions.

Cammarata et al. (22) recently reported a PO-linked cytotoxicity in *S. plicata* which is not related to the generation of ROM. This can reflect a different strategy in nonself recognition between solitary and compound ascidians. In the former case, a direct contact of MC with foreign particles appears to be required, whereas they recognize humoral factors in the latter: no MC degranulation is actually observed when washed hemocytes from incompatible *Botryllus* colonies are co-cultured together (our unpublished observations). This turns into a different mechanism of cytotoxicity, also evidenced by the PO-mediated erythrolytic ability in *Styela* observed in plaque forming cell assays which is missing in *Botryllus* (our unpublished observations).

Future efforts will be directed towards better clarification of the nature of the humoral factor(s) recognized by MC and of events leading to the degranulation response.

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