When the venerid clam Tapes decussatus is parasitized by the protozoan Perkinsus sp. it synthesizes a defensive polypeptide that is closely related to p225

Juan F. Montes, Mercè Durfort, José García-Valero

Departament de Biologia Cel·lular Animal i Vegetal, Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal, 647, E-08028 Barcelona, Spain

Author responsible for the editing procedure and for offprint requests:

Dr. José García-Valero
Departament de Biologia Cel·lular Animal i Vegetal
Facultat Biologia
Avda. Diagonal, 645
08028 Barcelona
SPAIN

Tel.: 34-3-4021550
FAX: 34-3-4112967

ABSTRACT: Molluscs, like other invertebrates, have primitive defense systems. These are based on chemotaxis, recognition and facultative phagocytosis of foreign elements. Previously, we have described one of these systems: A cellular reaction involving infiltrated granulocytes against Perkinsus sp. parasitizing the Manila clam Tapes semidecussatus, in which the parasites are encapsulated by a defensive host product, the polypeptide p225. The aim of this study is to determine the similarities between the defense mechanisms of two venerid clams, T. semidecussatus and T. decussatus, when they are infected by Perkinsus sp. The hemocytes of both species infiltrate the connective tissue, redifferentiate, and ultimately, express and secrete the polypeptide which constitutes the main product of the capsule that surrounds the parasites. The main secretion product of T. decussatus shows a high degree of homology to that of T. semidecussatus, since it has a similar electrophoretic mobility and the polypeptide is recognized by the polyclonal serum against p225 from T. semidecussatus, as confirmed by Western blotting and immunocytochemistry. In conclusion, we demonstrate the existence of two polypeptides that are closely related at the molecular and functional level, and are specific in the defense of some molluscs against infection by these protozoan parasites.

KEY WORDS: Defense mechanisms · Encapsulation · Granulocytes · Parasitism · Perkinsus sp. · Tapes decussatus · Tapes semidecussatus · Veneridae

INTRODUCTION

Protozoa belonging to the genus Perkinsus (formerly Dermocystidium or Labyrinthomyxa) (Apicomplexa, Perkinsea) (Levine 1978) have been described as disease agents in 63 species of bivalves and 4 species of gastropods (Perkins 1993). In Europe, during the last 10 years, Perkinsus spp. trophozoites have been associated with mass mortalities of commercially important venerid clams of the genus Tapes (=Ruditapes =Venerupis) (Mollusca, Bivalvia), such as the indigenous species T. decussatus (Da Ros & Canzonier 1985; Comps & Chagot 1987; Chagot et al. 1987; González et al. 1987; Villalba & Navas 1988; Azevedo 1989; Figueras et al. 1992; Goggin 1992; Navas et al. 1992) and the introduced species T. semidecussatus (=T. philippinarum =T. japonica) (Villalba & Navas 1988; Sagristà et al. 1991, 1995; Goggin 1992; Navas et al. 1992; Montes et al. 1995a).

Recently, Bachère et al. (1995) have reviewed the defense mechanisms that are present in bivalve molluscs. There is general agreement that the molluscs have cellular effectors and immune mechanisms. However, it is necessary to consider that whereas vertebrates have an immune system, invertebrates have more primitive defense systems, which often rely chiefly on phagocytic cells (Alberts et al. 1994). Those immune mechanisms are based on chemotaxis and recognition of foreign elements by lectin-like molecules, and subsequent phagocytosis. However, Chintala et al. (1994) concluded that serum agglutinins, the putative lectin-like molecules, did not have a role in oyster defense against P. marinus or Haplosporidium nelsoni.

In the venerid clams, T. semidecussatus and T. decussatus, Perkinsus spp. parasitism activates the host defense mechanisms and provokes an inflammatory response based on the infiltration of hemocytes, the cellular effectors. In the butterfish (also named carpet-shell) clam T. decussatus from Portugal, Comps & Chagot (1987) and Chagot et al. (1987) reported an inflammatory reaction constituted by granulocytes causing the encapsulation of the trophozoites by a PAS-positive substance. Recently, we have described a similar cellular reaction, involving infiltrated granulocytes, against this pathogen in the Manila (also named Japanese-littleneck) clam T. semidecussatus from the Spanish Mediterranean coast. This cellular reaction is functionally

polarized. The trophozoites are encapsulated by the secretory product which is released after the death of the granulocytes that are closest to the parasites (Montes et al. 1995a). The main component of this specific defense product is a slightly glycosylated polypeptide of about 225 kDa (p225). Moreover, the absence of this polypeptide in non-parasitized Manila clam indicates the exclusive association of p225 with the parasitosis (Montes et al. 1995b).

The aim of the present study is to determine the similarities between two Tapes species (T. semidecussatus and T. decussatus) in relation to their defense mechanisms. Our purpose is to correlate the main component of the cellular reaction at both cellular (infiltrated granulocytes) and molecular (secretory product) levels. The results allow us to establish a high homology between the cellular reactions of these closely related molluscan species against Perkinsus sp. parasitism.

MATERIALS AND METHODS

Animals. Specimens of parasitized and non-parasitized clams T. decussatus and T. semidecussatus were collected in the delta of the River Ebro, Mediterranean Sea, Tarragona (NE Spain).

Gill tissue processing. Abscesses from parasitized gills of T. decussatus and T. semidecussatus, isolated under a stereoscopic microscope, and non-parasitized gills of T. decussatus were prepared for SDS-PAGE and Western blot as previously described (Montes et al. 1995b).

SDS-PAGE and Western blot. SDS-PAGE was performed as described by Laemmli (1970) in 4%-9% acrylamide gels under reducing conditions. Polypeptides were resolved by silver staining according to the method of Merril et al. (1981). Western blot analysis was achieved following Towbin et al. (1979). After SDS-PAGE, polypeptides were transferred to nitrocellulose membranes. Transfer was realized at 15 V for 2 h in a Trans-blot semi-dry transfer cell (Bio Rad, Richmond, CA). Membranes were blocked with 10% non-fat dried milk in TBST (10 mM Tris-HCl (pH = 8), 150 mM NaCl, 0.05% Tween 20) for 30 min and then incubated with the polyclonal serum against p225 from T. semidecussatus (diluted 1:800 in TBST with 10% non-fat dried milk) (Montes et al. 1995b) overnight at 4°C. After three washes in TBST the membranes were incubated with peroxidase-conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark) for 2 h at room temperature. After washing, the membranes were developed in a substrate solution of 3,3'diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co, St. Louis, Mo) as chromogen, and finally recorded on Technical Pan film (Kodak, Hemel Hempstead, UK).

Immunocytochemistry. Abscesses from parasitized gills (T. decussatus and T. semidecussatus) were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS). Samples were prepared for Lowicryl K4M resin embedding (Chemische Werke Lowi, Waldkraiburg, Germany) following Carlemalm et al. (1982). Immunolabeling for p225 was achieved as previously described (Montes et al. 1995b). In brief, the grids were incubated with serum against p225 (1:3000 dilution), and then bound polyclonal

antibodies were visualized following incubation with 10-nm or 15-nm protein A gold (pAg; Sigma). Finally, thin sections were observed with a Reichert-Jung Polyvar 2 optical microscope and the ultrathin sections were examined on a Hitachi H-600 AB transmission electron microscope.

Quantitative evaluation. The label density (LD) was estimated as the number of gold particles per sectioned area of granules and capsule profiles. The area parameter was estimated by stereological methods (Weibel 1979) and significance of mean differences between experimental groups was tested by ANOVA.

RESULTS

Histological examination

By light microscopy, semi-thin sections of gill abscesses from the butterfish clam T. decussatus revealed the presence of trophozoites of Perkinsus sp. in the connective tissue. Parasites, isolated or grouped, were surrounded by closely packed granulocytes, which constituted the cellular reaction of this organism against Perkinsus sp. trophozoites. Parasites were seen total or partially encapsulated by a dense homogeneous substance, giving rise to the formation of cysts (Fig. 1).

Infiltrated granulocytes constituted the only cell type observed in the cellular reaction, which was organized as a single cellular mass without cells or fibers from connective tissue. Moreover, processes of cell division were occasionally observed in these infiltrated cells (Fig. 1 inset). Granulocytes showed a variable profile and diameter, with the cytoplasm filled by granules. Lysosomes were recognized as dense granules (Fig. 2). Furthermore, cytoplasm condensation in a single, undifferentiated mass was observed in some granulocytes (Figs. 2, 3, 4). The nuclei were circular in section and often situated eccentrically. Abundant clots of heterochromatin were located in the nucleoplasm, whereas peripheral heterochromatin was scarce (Figs. 1, 2, 3, 4).

Cysts contained one to several trophozoites surrounded by a non-cellular, non-fibrillar capsule. Uninucleated trophozoites were characterized by the presence of a vacuole that occupied up to 90% of the cell volume. Parasites were circular in section with a diameter ranging between 5 and 12 μm (Fig. 1). In some instances, trophozoite division by binary fission was noticed (Fig. 3). On the other hand, dead trophozoites were also seen in the cysts, which appeared as damaged cells with dense cytoplasm, shrunken aspect and spindle shape (Fig. 4).

Western blotting

Incubation with the serum against p225 revealed a band of about 225 kDa in the Western

blots from gill abscesses from T. decussatus. No apparent differences were observed, between these two clams species, in molecular weight or in antibody recognition of the polypeptide. The band was not detected in the lane corresponding to the non-parasitized gills.

Immunolocalization

By electron microscopy, granulocytes of the cellular reaction were characterized by the presence of numerous secretory membrane-bound granules with a homogeneous content (Figs. 6, 7, 8) and large mitochondria with tubular cristae and an extended matrix (Fig. 6). The granulocyte cytoplasm also showed a well developed endomembranous compartment distributed in two populations; round cisternae and vesicular-tubular saccules, the former related with the rough endoplasmic reticulum and the latter with the Golgi apparatus and the endosomal pathway (Fig. 7). Moreover, autophagosomes derived from granules were occasionally seen, which showed a heterogeneous content with internal membranes and residual bodies (Fig. 8).

In the infiltrated granulocytes, labeling by the serum against p225 was restricted to granules. Ultrastructural characteristics and LD of the granules were variable according to the maturation stage of the granulocytes. Granulocytes in early and medium stages of maturation showed the cytoplasm filled by granules with a similar size, homogeneous content and devoid of internal membranes. The LD for these granules was high and the label was uniformly distributed (Figs. 6, 7). Autophagosomes with low LD were characteristic at intermediate maturation stages (Fig. 8). Granulocytes that lay at the periphery of the cellular reaction showed non-mature granules and were similar to the circulating granulocytes. These granules were identified by their uniform size, floccular content, absence of internal membranes and low LD (Fig. 9).

Mature granulocytes, located in the inner regions of the cellular reaction, were typified by a regression of the endomembranous compartment and the presence of granules with a variable size, dense content, internal membranes and high LD (70.6 ± 9.4). This LD was nevertheless significantly lower than LD obtained for the mature granules of T. semidecussatus (116.9 ± 11.5) (Table 1). Giant granules resulting from fusion were occasionally observed in these mature

granulocytes (Fig. 10). The LD for these giant granules was lower than the LD for unfused mature granules. On the other hand, some mature granulocytes were observed with a single granule that enclosed all granules in the cell. These single granules, which showed a heterogeneous content and labeling (Fig. 11), constituted intermediate stages between giant granules and autophagosomes, and were never secreted around the trophozoites or the capsule. Finally, late stages of granulocyte maturation were sometimes characterized by a cytoplasm gelation, provoking the formation of a matrix around the granules, which showed high LD (Fig. 12).

By electron microscopy, the trophozoites were characterized by the presence of an euchromatic nucleus, placed eccentrically, which contained a large nucleolus; in addition, the vacuolar compartment was constituted by a voluminous vacuole and some smaller elements placed in the peripheral cytoplasm (Figs. 13, 14, 15). Furthermore, mitochondria with an expanded matrix, cisternae of endoplasmic reticulum, polysomes, an endomembranous network and lipid droplets were recognized. Trophozoites were surrounded by a homogeneous thin wall (Fig. 13).

Trophozoites were frequently enclosed by the capsule (Figs. 13, 14, 16), which was originated by the holocrine secretion of the infiltrated granulocytes. As a consequence of this, the outermost regions of the capsule were characterized by the presence of unfused granules and remnants of granulocyte membranes (Fig. 14). On the other hand, the innermost regions of the capsule showed an amorphous content with remnants of granule membranes and, frequently, paracrystalline inclusions (Fig. 16). Finally, dead trophozoites were observed in advanced stages of encapsulation (Fig. 16). The capsule and the trophozoite wall were labeled by the serum (Figs. 13, 14, 16). The LD for the capsule was similar to that observed in the granules of both species (Table 1). Moreover, the LD obtained for T. semidecussatus was higher than that obtained for T. decussatus (Table 1).

Finally, trophozoites were observed, in some instances, surrounded by a dense matrix lined by the plasma membrane of the granulocytes (Fig. 15). This matrix contained round cisternae of endoplasmic reticulum and residual bodies, which were labeled by the serum. In these cases, the trophozoite wall was devoid of label (Fig. 15), thus indicating that the labeling frequently observed in the trophozoite wall (Figs. 13, 14, 16) was a consequence of diffusion processes of

this polypeptide.

DISCUSSION

Several species belonging to genus Perkinsus have been described as disease agents in infectious processes affecting several molluscan species (Lauckner 1983, Perkins 1988, 1993). These infections are characterized by high mortalities of the host species. In the present study, we show the similarities between the defensive responses, at both cellular and molecular level, that two venerid clams present against the parasitism by Perkinsus sp.

The butterfish clam T. decussatus is a native species from the European coasts and closely related with the Manila clam T. semidecussatus. The Manila clam was introduced into Europe from Indo-Pacific natural populations for commercial purposes, since its size and growth rate are greater than T. decussatus. Although these venerid species are phenotypically similar, they constitute two specific entities with a reproductive mating barrier (Gérard 1978), striking karyological differences and high genetic distance (Borsa & Thiriot-Quiévreux 1990).

Azevedo (1989) described the trophozoites parasitizing T. decussatus from Portugal as a new species of the genus Perkinsus, P. atlanticus. This differs from the other known species, P. marinus (Mackin et al. 1950), P. olseni (Lester & Davis 1981) and P. karlssoni (McGladdery et al. 1991), by the zoospore ultrastructure, host identity and host response. The structural characteristics of the trophozoites and defensive responses noted in T. decussatus and T. semidecussatus (Montes et al. 1995a) from Spanish Mediterranean coast indicate that this parasite is homologous to P. atlanticus.

In Perkinsus sp.-parasitized T. decussatus from Portugal, Comps & Chagot (1987) and Chagot et al. (1987) reported an uncommon host reaction in comparison with those described in molluscs against infectious agents. The host reaction consisted of an inflammatory response by infiltrated granulocytes with PAS-positive granules, in which the trophozoites were frequently encysted by a PAS-positive substance. Recently, we have described a similar host reaction in T. semidecussatus from the Spanish Mediterranean coast. The Manila clam reacts against parasitism by Perkinsus sp. trophozoites by the recruitment of blood granulocytes, which redifferentiate and constitute a cellular reaction (Montes et al. 1995a). These infiltrated cells synthesize and secrete a

main product, a slightly glycosylated polypeptide of about 225 kDa (p225), which encapsulates the parasites (Montes et al. 1995b). The cellular reaction is functionally polarized and its secretion is exclusively located around the trophozoites.

The cellular reaction of T. decussatus against Perkinsus sp. trophozoites showed the same characteristics described for T. semidecussatus (Montes et al. 1995a). The reactive cells are infiltrated granulocytes that redifferentiate and synthesize a secretory product, stored in membrane-bound granules, which encapsulates the parasites. However, the cellular reaction of T. semidecussatus was organized in individualized areas separated by connective tissue, consisting of specific cells (spindle-shaped connective granulocytes) and intermingled fibres, whereas in T. decussatus, the cellular reaction was formed by a single cell mass, without elements from the connective tissue.

Infiltrated granulocytes of T. decussatus showed a considerable set of endomembranes, such as large mitochondria and round cisternae of the endoplasmic reticulum, that were not seen with these ultrastructural characteristics in the Manila clam. Granules from the butterfish clam did not show the parallel arrays of the internal membranes that are characteristic in those from T. semidecussatus. In some cases, granulocyte granules from T. decussatus showed early fusion, giving rise to the formation of a single, giant, secretory granule. This fusion was concurrent with the loss of water, which led to the gelation of the cytoplasm.

The organization of the capsule around the trophozoites showed the same characteristics in both clams. However, those from T. decussatus are sometimes incomplete, and the parasites were only partially encapsulated. It is interesting to point out that the percentage of dead trophozoites vs. total encapsulated parasites was lower for T. semidecussatus than for T. decussatus (11% and 36% respectively, data not shown). These data could be related to the results obtained by Rodríguez et al. (1994), who stated that P. atlanticus spreads easier into T. semidecussatus than T. decussatus after infection by zoospores.

The p225 from T. semidecussatus and the polypeptide from T. decussatus exhibit strong homology. First, both polypeptides have a similar apparent molecular mass and that from T. decussatus cross-reacts with the serum against p225 from T. semidecussatus. Second, at the

cellular level, both are synthesized by infiltrated granulocytes and show the same pattern of synthesis, storage and secretion. Third, at the physiological level, both synthesis products are organized as a capsule around the trophozoites. Finally, the non-parasitized specimens were devoid of the respective polypeptides (Montes et al. 1995b).

Moreover, regardless of the nature and mechanism of the clam infection by Perkinsus sp., these results allow us to speculate about the organization of the cellular reaction and the encapsulation of the trophozoites (Montes et al. 1995a). Once the parasites have reached the connective tissue of these clam species, hemocytes are recruited. Under the parasite induction these hemocytes redifferentiate, giving rise to secretory granulocytes. These constitute the cellular reaction around the trophozoites, which are encapsulated by the holocrine secretion of granulocytes. This encapsulation could block trophozoite dissemination (Rodríguez & Navas 1992, 1995; Montes et al 1995a), even though this cellular reaction could obliterate the blood sinuses of the clam and, thus, be the eventual cause of the host death (Montes et al. 1995a).

In conclusion, we demonstrate for the first time two polypeptides specifically related with clam defense against infection by Perkinsus sp. These two polypeptides are not only closely related at the molecular and the functional level, but they are also a specific product of the reactive connective tissue.

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FIGURES

Figs. 1 to 4. Lowicryl semithin sections from gills of the clam T. decussatus parasitized by Perkinsus sp. trophozoites. Fig. 1. Closely packed granulocytes constitute the only cell type observed in the cellular reaction against the parasites. Cysts (c) are constituted by a variable number of trophozoites and the capsule, which surround the parasites totally or partially. x 470. Scale bar = 50 μm. Fig. 1 inset. Mitotic figure from an infiltrated granulocyte of the cellular reaction (asterisk). x 1325. Fig. 2. Cytoplasm gelation (asterisk) and lysosomes (ly) in some granulocytes. x 830. Scale bar = 20 μm. Fig. 3. Trophozoite proliferation by binary fission in a cyst (arrow). x 810. Scale bar = 20 μm. Fig. 4. Cysts containing a variable number of trophozoites. Some dead trophozoites are seen in several cysts (asterisks). x 720. Scale bar = 20 μm.

Fig. 5. Lane 1 SDS-PAGE of abscesses from parasitized gills of T. decussatus. Lane 2 to 4 Western blotting with the serum against p225 from T. semidecussatus. Lane 2 abscesses from parasitized gills of T. decussatus, lane 3 non-parasitized gills of T. decussatus, lane 4 abscesses from parasitized gills of T. semidecussatus. The migration positions of molecular mass standards in kDa are indicated at the left. In addition, the position of band p225 is indicated at the right.

Figs. 6 to 12. Immunolocalization of granulocyte structures of T. decussatus that react with the serum against p225 from the T. semidecussatus. Fig. 6. Granulocyte cytoplasm filled by membrane-bound secretion granules (sg). The granule content is the only structure labeled. x 23000. Scale bar = 0.5 μm. Fig. 7. Endomembranes from a granulocyte. Rough endoplasmic reticulum (er), endosome/golgi vesicles (eg) and labeled secretion granules. x 11000. Scale bar = 1 μm. Fig. 8. Autophagosome (asterisk) with internal membranes, heterogeneous content and weak, scattered label from a granulocyte in the late stages of differentiation. x 16000. Scale bar = 1 μm. Fig. 9. Labeled non-mature granules of the granulocytes which show a floccular content. x

23000. Scale bar = 0.5 μm. Fig. 10. Several small granules contained in a large granule. Membranes that bind granules are devoid of label. x 20000. Scale bar = 1 μm. Fig. 11. Big granule enclosing all granules of a granulocyte. Label is heterogeneously distributed. x 13000. Scale bar = 1 μm. Fig. 12. Strongly labeled granules contained in the gelated cytoplasm from a pre-secretory granulocyte. x 20000. Scale bar = 1 μm.

Figs. 13 to 16. Immunolocalization of trophozoite-associated structures that react with the serum against p225. Fig. 13. Labeling in the capsule that surrounds a Perkinsus sp. trophozoite (tr). The internal membranes of the capsule (c) are devoid of label. Label in the trophozoite wall (w) is low but significant. x 20000. Scale bar = 1 μm. Fig. 14. Labeled unfused granules in the outermost regions of the capsule (c). Innermost regions of the capsule. Trophozoite wall (w). x 23000. Scale bar = 0.5 μm. Fig. 15. Trophozoite in close contact with a granulocyte that shows gelated cytoplasm, which contains labeled residual bodies (asterisk) and remnants of rough endoplasmic reticulum (er). Trophozoite wall (w) was unlabeled. x 23000. Scale bar = 0.5 μm. Fig. 16. Trophozoites at several stages of disorganization, surrounded by a labeled capsule (c) that shows internal membranes and paracrystalline structures. x 13000. Scale bar = 1 μm.

TABLE

Table 1. Label density (LD) expressed as number of gold particles per unit area (gp/μm^2) for p225 from both T. semidecussatus and T. decussatus in mature granules and capsule

	T. semidecussatus	T. decussatus
Mature granules	116.9 ± 11.5	70.6 ± 9.4 ***
Capsule	107.1 ± 9.1	51.5 ± 11.8 **

Values are mean ± SEM from at least 30 measurements performed on 3 specimens for each clam species. The significance of mean differences between groups was tested by Anova method.
F= 5.03 when LD for T. semidecussatus and T. decussatus are compared.
** $P < 0.01$ and *** $P < 0.001$ comparing values in homologous compartments from both species.

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