UNIVERSITAT DE BARCELONA

DEPARTAMENT DE BIOLOGIA CEL·LULAR ANIMAL I VEGETAL

Respuesta defensiva inducida por *Perkinsus atlanticus*. Reacción celular y expresión de p225 en *Tapes* spp.

Juan Francisco Montes Castillo

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Capítulo III

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

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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 2 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 2 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

RESUMEN

Los moluscos, igual que otros invertebrados, presentan sistemas de defensa poco desarrollados. Estos sistemas se basan en la quimiotaxis, reconocimiento y fagocitosis facultativa de los cuerpos extraños. Previamente, hemos descrito uno de estos sistemas: una reacción celular desarrollada por la almeja *Tapes semidecussatus* que origina la encapsulación de *Perkinsus* sp. por un producto defensivo del huésped, el polipéptido p225. El propósito de este estudio ha sido determinar las similitudes entre las respuestas defensivas elaboradas por 2 almejas venéridas, *T. semidecussatus* y *T. decussatus*, frente a la infección de *Perkinsus* sp. Nuestras observaciones indican que los hemocitos granulares de ambas especies acceden al tejido conjuntivo, se rediferencian en un nuevo tipo celular de carácter secretor, y por último, expresan y secretan el polipéptido p225, el componente más abundante de la cápsula. En efecto, el producto de secreción mayoritario de *T. decussatus* muestra gran homología con aquél de *T. semidecussatus*. Así, presenta una movilidad electroforética similar y es inmunolocalizado en los mismos compartimentos intra y extracelulares relacionados con la síntesis, secreción y depósito de p225 que lo fueron en *T. semidecussatus*. En conclusión, demostramos la existencia de 2 polipéptidos que están íntimamente relacionados molecular y funcionalmente, siendo específicos en la defensa de estos moluscos contra la infección de *Perkinsus* sp.

Palabras clave: Encapsulación - Granulocitos - Mecanismos defensivos - Parasitismo - *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 yr, *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; Chagot et al. 1987; Comps & Chagot 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 play a role in oyster defense against *Perkinsus marinus* or *Haplosporidium nelsoni*.

In the venerid clams, *Tapes 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 periodic Acid-Schiff (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 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 *Tapes decussatus* and *T. semidecussatus* were collected in the delta of the River Ebro, Tarragona (NE Spain), at the Mediterranean Sea.

Gill tissue processing

Abscesses from parasitized gills of *Tapes 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 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 performed 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, USA). 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 *Tapes semidecussatus* (diluted 1:800 in TBST with 10% non-fat dried milk) (Montes et al. 1995b) overnight at 4°C. After 3 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, USA) as chromogen, and finally recorded on Technical Pan film (Kodak, Hemel Hempstead, UK).

Immunocytochemistry

Abscesses from parasitized gills (*Tapes 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

Light microscopy of semi-thin sections of gill abscesses from the butterfish clam *Tapes 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 totally 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 *Tapes decussatus*. No apparent differences were observed, between these 2 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 in *Tapes decussatus* 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 2 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 of intermediate maturation stages (Fig. 8). Granulocytes that lay at the periphery of the cellular reaction showed immature 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 $\pm$ 9.4 gold particles μm^{-2}). This LD was nevertheless significantly lower than LD obtained for the mature granules of *Tapes semidecussatus* (116.9 $\pm$ 11.5 gold particles μm^{-2}) (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 gelation of the cytoplasm, leading to 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 a euchromatic nucleus, positioned 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 a capsule (Figs. 13, 14 & 16) which originated from 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 *Tapes 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 the 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 the cellular and molecular level, that 2 venerid clams present against the parasitism by *Perkinsus* sp.

The butterfish clam *Tapes decussatus* is a native species from 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 those of *T. decussatus*. Although these venerid species are phenotypically similar, they constitute 2 distinct 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 *Tapes 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), in 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 *Tapes decussatus* from Portugal, Comps & Chagot (1987) and Chagot et al. (1987) reported an uncommon host reaction in comparison with those described in other 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 recruiting of blood granulocytes, which redifferentiate and constitute a cellular reaction (Montes et al. 1995a). These infiltrated cells synthesize and secrete their 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 *Tapes 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 into 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 *Tapes decussatus* showed a considerable set of endomembranes, such as large mitochondria and round cisternae of the endoplasmic reticulum, that were not seen in the Manila clam. Granules from the butterfish clam did not show the parallel arrays of the internal membranes that are characteristic in *T. semidecussatus*. In some cases, granulocyte granules from *T. decussatus* showed early fusion, giving rise to 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 *Tapes decussatus* were sometimes incomplete, and the parasites were only partially encapsulated. It is interesting to point out that the percentage of dead trophozoites relative to total encapsulated parasites was lower for *T. semidecussatus* than for *T. decussatus* (11 and 36% respectively; data not shown). These data are consistent with the results obtained by Rodríguez et al. (1994), who stated that *Perkinsus atlanticus* spreads more easily into *T. semidecussatus* than *T. decussatus* after infection by zoospores.

The p225 from *Tapes 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 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 2 polypeptides specifically related to clam defense against infection by *Perkinsus* sp. These 2 polypeptides are not only closely related at the molecular and the functional level, but are also a specific product of the reactive connective tissue.

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Figures and table

Figures 1 to 4

Tapes decussatus parasitized by *Perkinsus* sp. trophozoites. Lowicryl semithin sections from gills of the clam.

1 Closely packed granulocytes constitute the only cell type observed in the cellular reaction against the parasites. Cysts (*c*) consist of a variable number of trophozoites and the capsule, which surrounds the parasites totally or partially. x470. *Bar*: 50 μm. **1 inset** Mitotic figure from an infiltrated granulocyte of the cellular reaction (*asterisk*). x1325

2 Cytoplasm gelation (*asterisk*) and lysosomes (*ly*) in some granulocytes. x830. *Bar*: 20 μm

3 Trophozoite proliferation by binary fission in a cyst (arrow). x810. *Bar*: 20 μm

4 Cysts containing a variable number of trophozoites. Some dead trophozoites are seen in several cysts (*asterisks*). x720. *Bar*: 20 μm

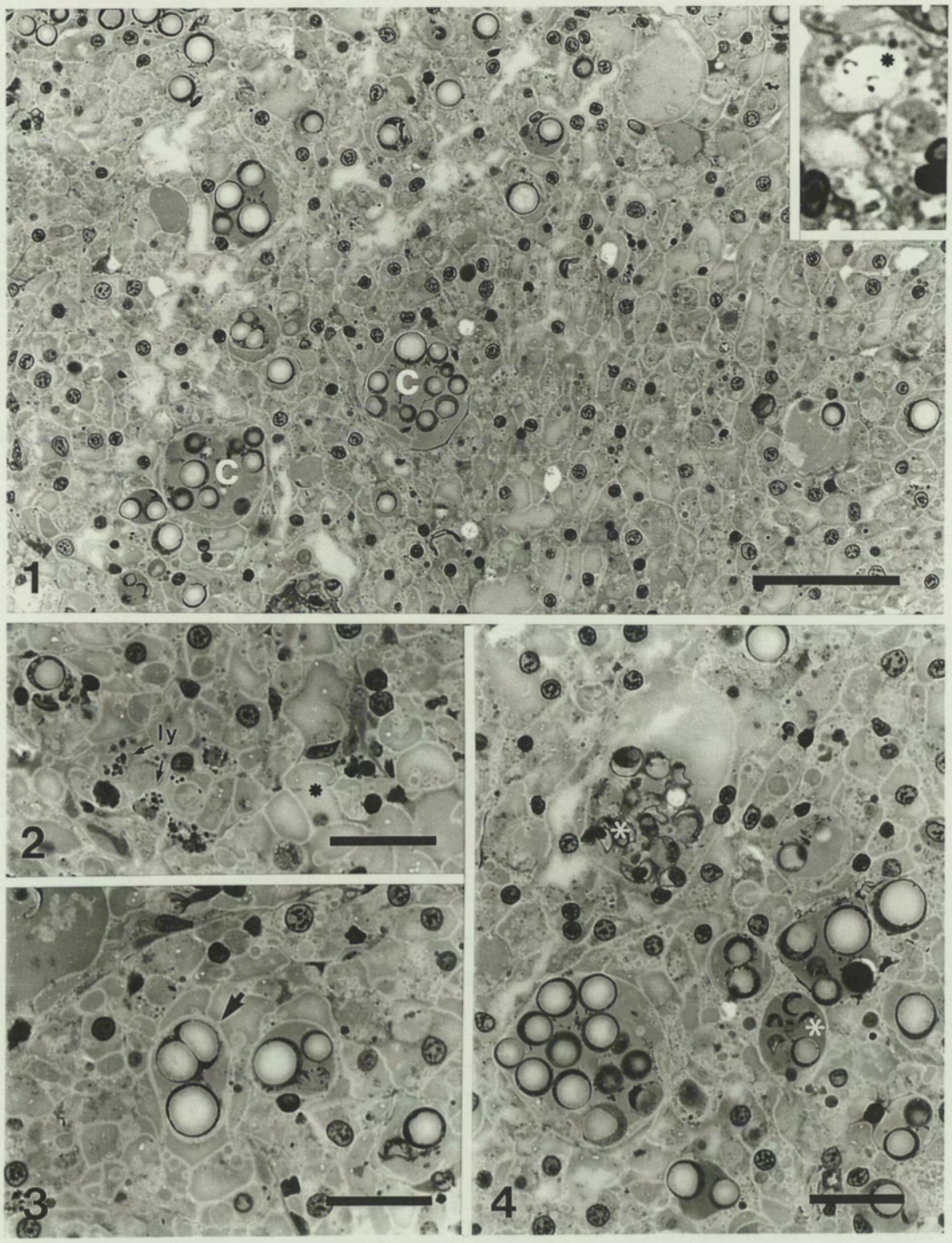
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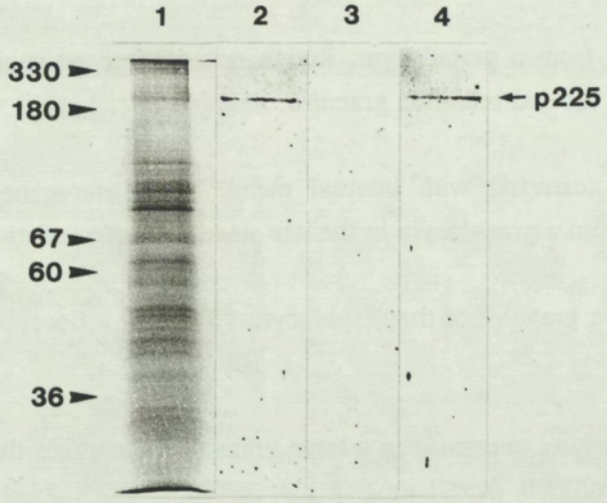
Figure 5

Lane 1 SDS-PAGE of abscesses from *Perkinsus* sp. parasitized gills of *Tapes 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 are indicated in kDa on the left. In addition, the position of band p225 is indicated on the right.



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Figures 6 to 12

Tapes decussatus parasitized by *Perkinsus* sp. Immunolocalization of granulocyte structures of *decussatus* that react with the serum against p225 from *T. semidecussatus*.

6 Granulocyte cytoplasm filled by membrane-bound secretion granules (*sg*). The granule cont is the only structure labeled. x23000. *Bar*: 0.5 μm

7 Endomembranes from a granulocyte. Rough endoplasmic reticulum (*er*), endosome/go vesicles (*eg*) and labeled secretion granules. x11000. *Bar*: 1 μm

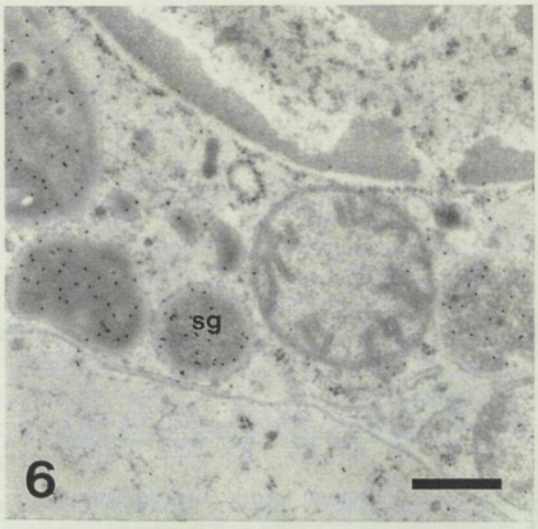
8 Autophagosome (*asterisk*) with internal membranes, heterogeneous content and we scattered label from a granulocyte in the late stages of differentiation. x16000. *Bar*: 1 μn

9 Labeled immature granules of the granulocytes, showing a floccular content. x23000. *B* 0.5 μm

10 Several small granules contained in a large granule. Membranes that surround granules devoid of label. x20000. *Bar*: 1 μm

11 Big granule enclosing all granules of a granulocyte. Label is heterogeneously distribute x13000. *Bar*: 1 μm

12 Strongly labeled granules contained in the gelated cytoplasm from a pre-secreto granulocyte. x20000. Bar: 1 μm



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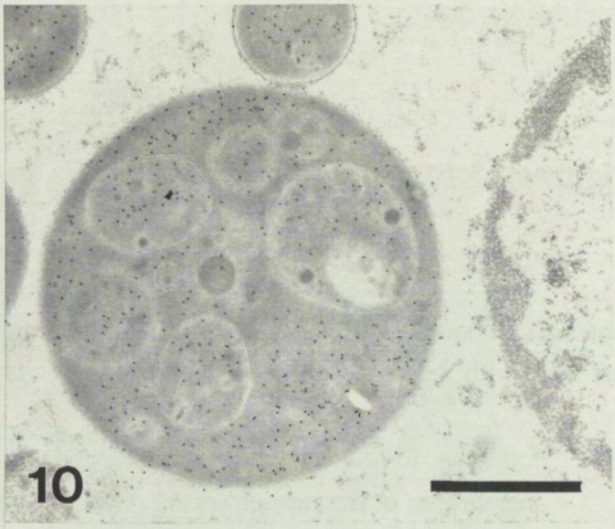

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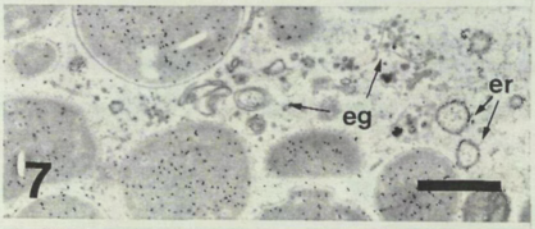

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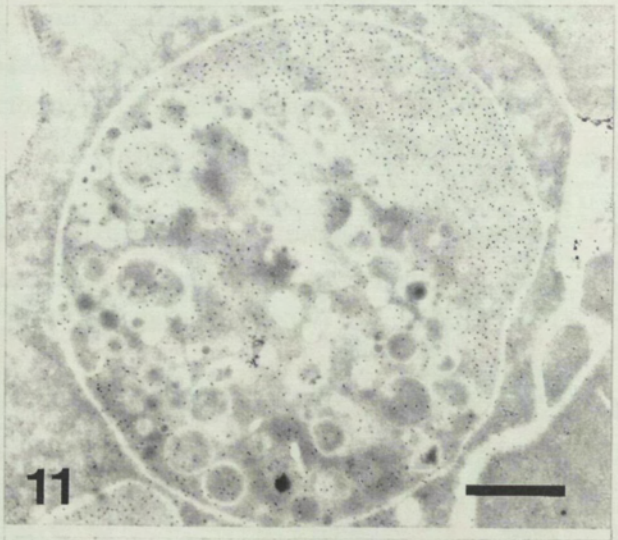

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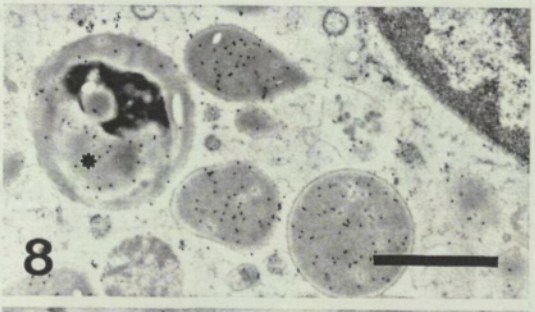

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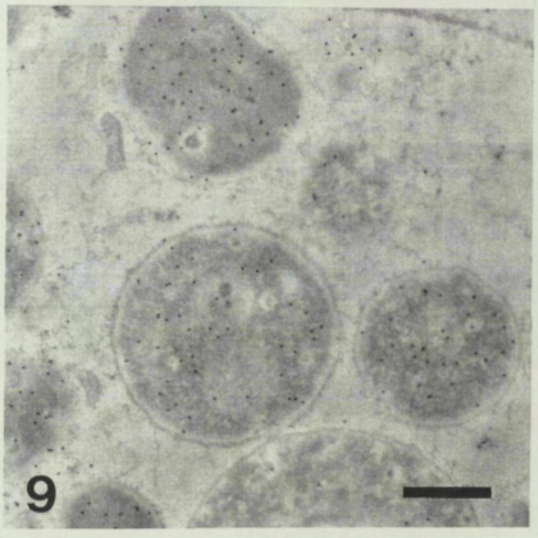

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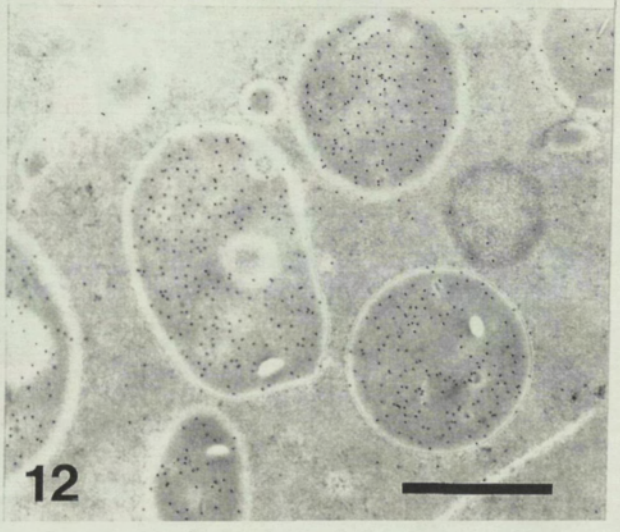

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Figures 13 to 16

Tapes decussatus parasitized by *Perkinsus* sp. Immunolocalization of trophozoite-associated structures that react with the serum against p225.

13 Labeling in the capsule that surrounds a *Perkinsus* sp. trophozoite (*tr*). The internal membranes of the capsule (*c*) are devoid of label. Amount of label in the trophozoite wall (*w*) is low but significant. x20000. *Bar*: 1 μm

14 Labeled unfused granules in the outermost region of the capsule (*c*), adjacent to the trophozoite wall (*w*). x23000. Bar: 0.5 μm

15 Trophozoite in close contact with a granulocyte that shows gelated cytoplasm and that contains labeled residual bodies (*asterisks*) and remnants of rough endoplasmic reticulum (*er*). Trophozoite wall (*w*) was unlabeled. x23000. *Bar*: 0.5 μm

16 Trophozoites at several stages of disorganization, surrounded by a labeled capsule (*c*) that shows internal membranes and paracrystalline structures. x13000. *Bar*: 1 μm

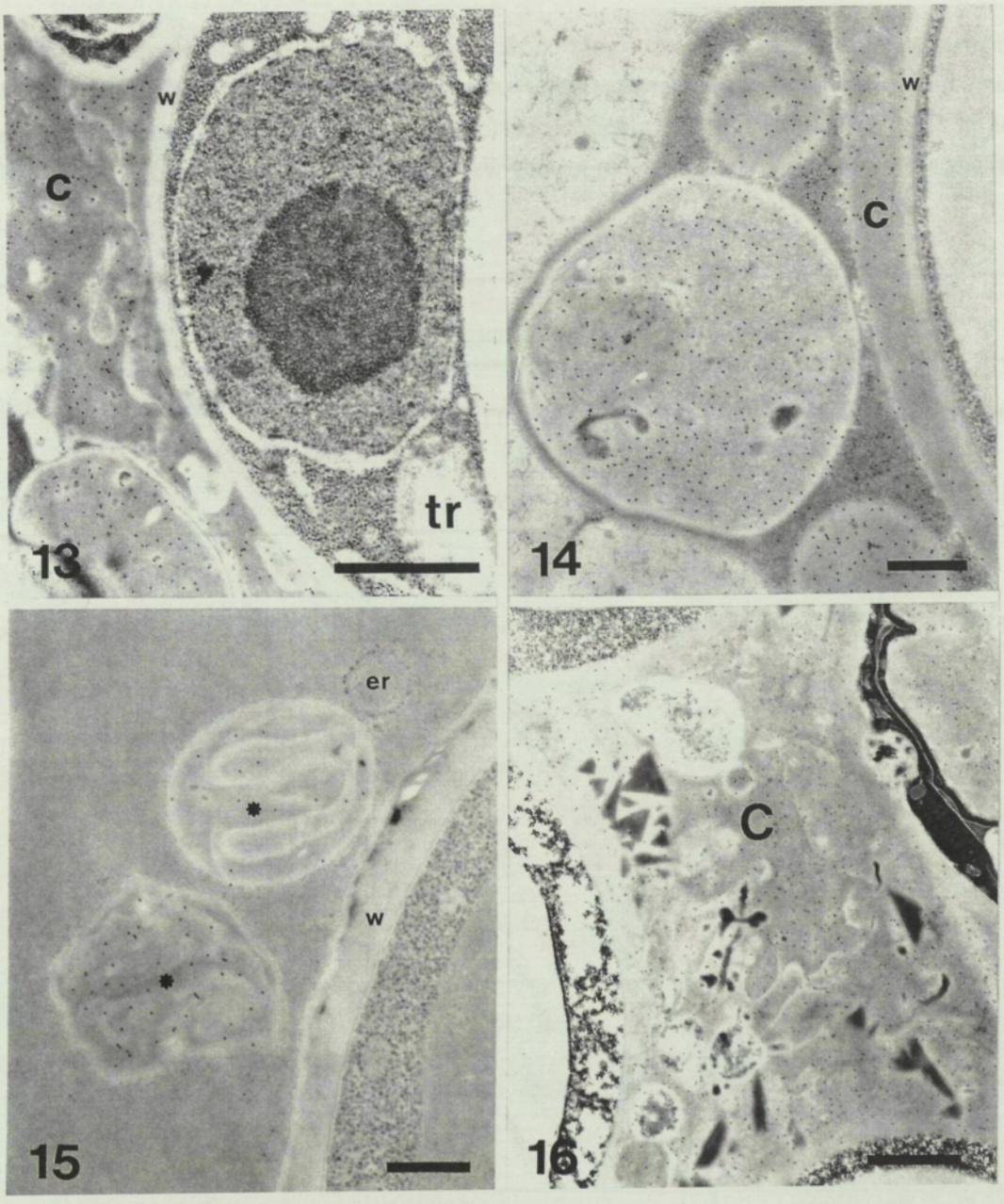
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Table 1

Label density expressed as number of gold particles μm^{-2} labelling p225 in mature granules and in capsules from both *Tapes semidecussatus* and *T. decussatus* parasitized by *Perkinsus* sp. Values are means ± 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; $F = 5.03$

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

**$p < 0.01$ and ***$p < 0.001$ comparing values in homologous compartments from both species

Capítulo IV

The protozoan parasite *Perkinsus atlanticus* elicits a unique defensive response in the clam *Tapes semidecussatus*

Juan F. Montes, José A. Del Río, Mercé Durfort, José García Valero

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ABSTRACT

The venerid clams, *Tapes decussatus* and *T. semidecussatus*, develop a singular defensive response to *Perkinsus atlanticus* infection. This reaction involves the redifferentiation of recruited granulocytes and the expression *de novo* of the polypeptide p225. To determine whether the association of this defensive process with the natural parasitism by *P. atlanticus* is unique, the inflammatory response elicited by inoculations of bacteria, algae and non-viable *P. atlanticus* prezoosporangia in the clam *T. semidecussatus* was shown. Inoculated areas were heavily infiltrated by granulocytes and delimited by myofibroblast-like cells and extracellular matrix. While bacteria and algae were phagocytosed by the infiltrated granulocytes, prezoosporangia were not. After 40 days, neither cell redifferentiation nor the expression of p225 was observed. These findings indicate that both redifferentiation and p225 expression are specifically associated with *P. atlanticus* infection. After 5'-bromodeoxyuridine administration, only a few cells were labelled either in the inoculated zone or in the cellular reaction around *P. atlanticus* meronts. Significant differences between untreated and inoculated groups were observed in the epicardic connective tissue soon after injection. These results suggest that this anatomical region could be the main site of haemocyte proliferation stimulated after inoculation of foreign bodies in *T. semidecussatus*.

Key words: 5'-bromodeoxyuridine - Cell proliferation - Defence mechanisms - Granulocytes - *Perkinsus atlanticus*, *Tapes semidecussatus*.

RESUMEN

Las almejas venéridas, *Tapes decussatus* y *T. semidecussatus*, desarrollan frente a la infección de *Perkinsus atlanticus* una respuesta defensiva de características singulares. Esta reacción conlleva la rediferenciación de los granulocitos reclutados y la expresión *de novo* del polipéptido p225. Con el propósito de determinar si la respuesta defensiva inducida por la infección de *P. atlanticus* es específica, hemos comparado esta reacción defensiva con la respuesta inflamatoria elicitada tras la inoculación de bacterias, algas y prezoosporangios no viables de *P. atlanticus* en *T. semidecussatus*. Las áreas inoculadas fueron infiltradas por granulocitos y delimitadas por células semejantes a miofibroblastos y matriz extracelular. Mientras las bacterias y algas fueron fagocitadas por los granulocitos infiltrados, los prezoosporangios no lo fueron. Después de 40 días, no se observó ni la rediferenciación celular ni la expresión de p225. Tras la administración de 5-bromodeoxiuridina, tan sólo un pequeño número de células de la zona inoculada y de la reacción celular alrededor de los merontes de *P. atlanticus* presentaron marca. Se observaron diferencias significativas entre los grupos control y los inoculados en el tejido conjuntivo epicárdico poco después de la inyección. Estos resultados sugieren que esta región anatómica podría ser el principal lugar de proliferación hemocitaria estimulado tras la inoculación de cuerpos extraños en *T. semidecussatus*.

Palabras clave: 5-bromodeoxiuridina - Granulocitos - Mecanismos defensivos - *Perkinsus atlanticus* - Proliferación celular - *Tapes semidecussatus*

INTRODUCTION

In molluscs, as in all invertebrates, the defence mechanisms are mainly cellular and there are no indications of specific immune responses. Haemocytes, the cellular effectors of these defence systems, are involved in phagocytosis, cytotoxicity and the encapsulation of foreign bodies (Cheng, 1981; Ratcliffe *et al.* 1985; Feng, 1988; Loker, 1994; Bachère *et al.* 1995). The response to infection or wounding is an inflammatory reaction based on the accumulation of haemocytes at the site of injury (Cheng, 1981; Bayne, 1983; Ratcliffe *et al.* 1985; Sparks & Morado, 1988). It has been reported that this defensive reaction is the result of the infiltration of haemocytes, which do not proliferate at the site of infection or damage (Sminia *et al.* 1973; Alvarez *et al.* 1995).

Protozoa of the genus *Perkinsus* (Apicomplexa, Perkinsea) (Levine, 1978) are major disease agents in marine molluscs and have been associated with large-scale mortalities in bivalves and gastropods worldwide (Lauckner, 1983; Perkins, 1993, 1996; Bower *et al.* 1994). In the venerid clams, *Tapes decussatus* and *T. semidecussatus* (Mollusca, Bivalvia), *Perkinsus atlanticus* (Azevedo, 1989) infection induces an inflammatory response involving the infiltration of granule-containing haemocytes, which provokes the encapsulation of the parasite (Chagot *et al.* 1987; Comps & Chagot, 1987; Montes *et al.* 1995*a*, 1996; Sagristà *et al.* 1995). These recruited granulocytes constitute the cellular reaction. Infiltrated granulocytes redifferentiate and synthesize a secretory product, stored in membrane-bound granules, which is released and organized as a capsule around the meronts (Montes *et al.* 1995*a*, 1996). The polypeptide p225, the main component of this defensive product, is not expressed in non-parasitized clams (Montes *et al.* 1995*b*, 1996).

The aim of the present study is two-fold. Firstly, to determine if *P. atlanticus* infection induces a specific defence mechanism in the clam *T. semidecussatus* by comparing this cellular reaction with the inflammatory reaction elicited either by experimental wounding or by inoculations of bacteria, algae and non-viable *P. atlanticus* prezoosporangia. Secondly, to determine whether the redifferentiated granulocytes that lie around *P. atlanticus* meronts or the infiltrated granulocytes proliferate locally.

MATERIALS AND METHODS

Animals

Adult specimens of non-parasitized and *P. atlanticus*-infected clams *T. semidecussatus* (50-60 cm in shell length) were collected from the Delta of the River Ebro, Mediterranean Sea, Tarragona (NE Spain) in early winter. Clams, acclimatized for 10 days before inoculations, were kept in tanks containing circulating, aerated, filtered seawater at a similar salinity to that of the specimens origin, 35 ‰. Animals were fed daily with Liquizell phytoplankton (Hobby; Bonn, Germany).

General strategy and preparation of materials for inoculations

To reach our objectives, we performed 5 series of inoculations, which were followed for 40 days.

Bacteria for the inoculations were obtained from *Escherichia coli* (strain K12) cultures. *E. coli* was grown overnight at 37 °C in Luria Broth medium (Sigma; St. Louis, Mo). Prior to the inoculations, bacterial cells from stationary phase broth cultures were washed by centrifugation and resuspended in sterile seawater. Bacteria were injected together with green algae from Liquizell (Hobby) in order to determine whether the reactive cells showed a preferential phagocytosis or encapsulation, depending on the mean diameter of the foreign bodies. Bacteria and algae-inoculated clams constituted the *BA* experimental group.

The prezoosporangia of *P. atlanticus* were isolated and purified after culture of parasitized gills from *T. semidecussatus* in fluid thioglycollate medium (Difco Laboratories; Detroit, MI) as described elsewhere (Montes *et al.* 1995*b*). The diameter of isolated prezoosporangia ranged from 28 to 79 μm. Prezoosporangia were killed by repeated cycles of freezing-thawing and then stored at -80 °C until the inoculations. *P. atlanticus* prezoosporangia-inoculated specimens constituted the *Pk* experimental group.

The inocula were prepared by dissolving the materials in sterile seawater-diluted ink. The colloidal particles of ink were used in the inoculations to indicate the precise location of the injections, as well as endocytic markers.

Three additional experimental groups were established as controls. The *untreated* group consisted of uninjected animals. The *wounded* and *ink* control groups were injected with sterile seawater or sterile seawater-diluted ink, respectively.

Experimental procedure

Fifty μl of the corresponding inoculum were injected into the foot of the experimental clams. Specimens were killed and foot tissues, including the inoculated area, were obtained at 1, 3, 7, 15 and 40 days post-inoculation. On each occasion, the foot samples from 3 clams in each of the 5 experimental groups were processed for transmission electron microscopy (TEM) and immunocytochemical techniques, as described below, to characterize the cellular and tissue host reaction and the possible expression of p225.

In addition, 3 specimens from both the BA and Pk experimental groups were used to assess the proliferative activity at 3, 15 and 40 days post-inoculation, as described below.

Tissue processing for TEM

For TEM, the samples were immediately fixed after their isolation in 2 % paraformaldehyde, 2·5 % glutaraldehyde in 0·1 M phosphate-buffered saline (PBS) for 2 h at room temperature. Primary fixation was followed by post-fixation with 1·0 % osmium tetroxide in 0·1 M PBS for 1 h. The samples were dehydrated in acetone and embedded in Spurr resin. Semithin sections were stained with methylene blue and ultrathin sections were stained with uranyl acetate and lead citrate. Semithin sections were observed with a Reichert-Jung Polyvar 2 optical microscope and the ultrathin sections with a Hitachi H-600 AB transmission electron microscope.

Immunocytochemistry

For immunocytochemical techniques, foot samples from each of the experimental groups and foot abscesses from *P. atlanticus*-infected clams were fixed in 4 % paraformaldehyde, 0·1 % glutaraldehyde in 0·1 M PBS (pH 7·4) for 2 h at 4 °C. Samples were processed for Lowicryl K4M resin embedding (Chemische Werke Lowi; Waldkraiburg, Germany) following the method described by Carlemalm *et al.* (1982). Immunogold labelling for p225 was performed as described (Montes *et al.* 1995*b*). Briefly, the grids were incubated with the serum against p225 (diluted 1:3000) (Montes *et al.* 1995*b*). Bound polyclonal antibody was visualized following incubation with 10 nm protein A gold (Sigma). Finally, sections were stained with uranyl acetate and lead citrate.

Cell proliferation

Three specimens from each of the untreated, BA and Pk experimental groups at 3, 15 and 40 days post-inoculation, and several *P. atlanticus*-infected clams were injected with a 8 h-separated double pulse of 5'-bromodeoxyuridine (BrdU; Sigma) (50 mg/kg bw, 10 mg/ml dissolved in sterile seawater) in the posterior adductor muscle sinus.

BrdU immunocytochemistry. After 4 h of the second pulse, experimental clams were carefully removed from their shells and immersed in Carnoy's fixative solution for 5 days. Specimens were embedded in paraffin and sectioned at a nominal thickness of 10 μm. After dewaxing, BrdU detection was carried out essentially as described previously (Del Río & Soriano, 1989; Soriano & Del Río, 1991). Briefly, after endogenous peroxidase inhibition with 10 % methanol, 3 % H_2O_2 in 0·1 M PBS for 30 min, the sections were deproteinized with cold 0·1 N HCl for 10 min, treated with 2 N HCl in 0·05 M PBS at 37 °C for 12 min to denature the DNA, and then neutralized with 0·1 M borate buffer (pH 8·5). After permeabilization with 0·2 % Triton X-100 in 0·1 M PBS, the sections were incubated in a blocking solution containing 10 % normal goat serum (Sigma), Fab fragment goat anti-mouse IgG (1:150 dilution) (Jackson Immunoresearch Laboratories; West Grove, PA), 0·2 % gelatine, 0·2 M glycine, 0·2 % Triton X-100 in 0·1 M PBS for 2 h. Afterwards, the sections were sequentially incubated with a monoclonal anti-BrdU antibody (1:100 dilution) (Clone Bu20a; Dako; Glostrup, Denmark) overnight, goat anti-mouse IgG secondary antibody (1:75 dilution) (Dako) for

2 h and peroxidase-antiperoxidase complex (1:100 dilution) (Sternberger-Meyer; Jarretsville, MD) for 2 h. Immunoreagents were diluted in 0·1 M PBS containing 5 % normal goat serum, 0·2 % gelatine and 0·1 % Triton X-100. Peroxidase activity was developed with 0·03 % diaminobenzidine tetrahydrochloride (Sigma), 0·2 % nickel ammonium sulphate and 0·005 % H_2O_2 (DAB-Ni) (Hancock, 1982) in 0·1 M Tris-HCl buffer (pH 7·6). Finally, sections were slightly counter-stained with haematoxylin and coverslipped with DPX (Serva; Heidelberg, Germany).

RESULTS

Experimental inoculations and infiltrated cells

During the sampling period, the survival of the specimens, irrespective of the experimental group, was greater than 95% in comparison with the uninjected animals. Inoculated clams showed normal behaviour as regards light responses (siphonal retraction and valve adduction), foot extension and feeding.

Inoculated clams showed a local disorganization at the site of the injection with regression of foot muscular tissue. Extensive necrotic zones and oedematous areas were observed at the periphery of the lesion. Damaged striated muscle fibres lost their filamentous aspect, as a consequence of cytoskeleton disorganization, showing nuclear pyknosis and a severe reduction of the endomembranes. This necrotic muscular tissue and the oedematous areas were progressively replaced by newly organized connective tissue (Fig. 1A-F).

By light microscopy, at 72 h after inoculation, the BA and Pk experimental groups showed a massive cellular infiltration in the site of injury, especially around the inoculum (Fig. 1A and B). The boundaries of the infiltration were continuous with the peripheral connective tissue and the adjacent necrosed areas. The infiltrated cells were characterized by their small size and heavily stained nuclei. Cells containing elongated nuclei became fusiform, arranged parallel to one another and with their major axis perpendicularly orientated to the trajectory of the injection (Fig. 1A and B). The inoculated material followed a different evolution. Thus, inoculated algae were identified as uniform masses having suffered the loss of cellular organization and the collapse of the cell wall (Fig. 1A). The irregular shape shown by the prezoosporangia (Fig. 1B) was the consequence of the freezing-thawing cycles performed prior to the inoculation.

At 15 days post-injection, inocula were surrounded by infiltrated cells (Fig. 1C and D). The area occupied by these reactive cells was smaller than that observed at 72 h post-injury (Fig. 1A and B). Moreover, inoculated feet showed a partial reorganization of the muscular tissue with an absence of necrotic areas and a reduction of the healing connective tissue. In the Pk but not BA group, the recruited cells surrounding the inoculated prezoosporangia were delimited by a multi-layered sheath

of myofibroblast-like cells, which constituted a histological border between the infiltrated area and the regenerating muscular tissue (Fig. 1D). On day 40, the infiltrated area was reduced and criss-crossed by new striated muscle fibres (Fig. 1E and F).

Myofibroblast-like and phagocytic cells were the two cell types observed in the inoculated areas (Fig. 2A-C). Myofibroblast-like cells were typified as agranular fusiform cells organized in a parallel array (Figs 1D and 2B). Phagocytic cells showed a heterogeneous size and variable profile with numerous granules, mainly lysosomes, which contained abundant cell debris from inocula (Fig. 2A and C).

Although the cellular reaction observed in foot abscesses from clams parasitized by *P. atlanticus* meronts generally consisted of a reduced number of granulocytes (Fig. 2D), these reactive cells were homologous to the redifferentiated granulocytes previously described in gills (Fig. 2E). Moreover, these granulocytes showed a different pattern to that of the recruited phagocytic cells from the inoculated area (Fig. 2A and C). The granulocytes of the cellular reaction exhibited a secretory pattern with the cytoplasm being filled by granules of different sizes, which were exclusively secreted around the meronts provoking their encapsulation (Fig. 2D).

By electron microscopy, myofibroblast-like cells showed a fusiform shape with an elongated nucleus (Fig. 3A). Heterochromatin had a pale aspect and was distributed in several clots. The endomembranous compartment was well-developed, basically composed by mitochondria and endoplasmic reticulum. The second cell type, the phagocytic cells, were infiltrated granulocytes which were characterized by numerous membrane-bound granules with a floccular content (Fig. 3B-D). Granule-containing haemocytes that were located in the vicinity of the inoculated material showed high phagocytic activity. Thus, degraded cell walls from bacteria and algae or remnants of the encapsulating material from inoculated prezoosporangia were seen in vesicles and granules of these infiltrated granulocytes (Figs 2A, C and 3B, E).

After incubations with the serum against p225, labelling was absent from all compartments of the mobilized cells at the injury site at all experimental times (Fig. 3E). Therefore, neither of the 2 cell types, the myofibroblast-like cells and the phagocytotic cells, expresses p225, the specific polypeptide of the cellular reaction against *P. atlanticus* meronts (Fig. 3F).

Cell proliferation

The results obtained after BrdU administration showed the lack of significant labelling in the infiltrated region at all experimental times; thus, BrdU-positive nuclei were only observed in a few striated muscle fibres at the injury site (Fig. 4A and B). No differences were seen between the BA

and Pk experimental groups. Similar results were obtained in *P. atlanticus*-infected clams, since non-immunoreactive nuclei were found in the cellular reaction against *P. atlanticus* meronts (Fig. 4C and D). Moreover, all experimental groups showed numerous BrdU-containing nuclei in epithelial cells from gill filaments (Fig. 4E and F) and marginal tubules of the digestive gland (Fig. 4G).

However, BrdU-labelled cells were located in the connective tissue from both the BA and Pk experimental groups, but not in that of the control specimens. At 72 h post-inoculation, numerous BrdU-positive nuclei were observed in the epicardic connective tissue, where mantle and gill lamellae are inserted to the visceral mass (Fig. 5A and B), and in the connective tissue of the mantle (Fig. 4H). On day 15, the number of BrdU-immunoreactive nuclei declined (Fig. 5C) and non-significant labelling was observed on day 40 (Fig. 5D). This epicardic zone was composed of loose connective tissue of reticular type, which showed a spongy aspect in control clams (Fig. 5E and F), but exhibited high cellularity in inoculated specimens (Fig. 5A-C). These cells were mainly BrdU-labelled haemocytes. Brown or serous cells were also associated with the reticular fibres of this connective tissue (Fig. 5F).

DISCUSSION

Parasitism by *P. atlanticus* of the venerid clams, *T. decussatus* and *T. semidecussatus*, elicits a singular host reaction, quite unlike those described in other molluscs against several infectious agents (Comps & Chagot, 1987; Montes *et al.* 1995*a*, 1996; Sagristà *et al.* 1995). In the present study, we have characterized the inflammatory defensive response caused by inoculations of bacteria, algae and non-viable *P. atlanticus* prezoosporangia in the clam *T. semidecussatus* in order to compare it with the cellular reaction against natural parasitism by *P. atlanticus*.

In *T. semidecussatus* the tissue damage caused by the experimental injections, such as local disorganization, regression and necrosis of the muscular tissue, led to the infiltration of circulating haemocytes at the injury site within 14 h (Cheney, 1969). This haemocytic mobilization, accompanied by oedema, constituted the inflammatory defence response induced in this clam after experimental wounding, and is in line with earlier descriptions of other bivalves (DesVoigne & Sparks, 1968; Ruddell, 1971; Sparks & Morado, 1988; Suzuki *et al.* 1991). Although agranulocytes have been described as being involved in these post-wounding reactions (Ruddell, 1971; Suzuki *et al.* 1991), the inflammatory process seen here was mediated by granulocytes. Thus, these infiltrated cells, as circulating granulocytes (Auffret, 1988), showed membrane-bound granules with a floccular content, a peripheral lucid halo and an absence of internal membranes.

In molluscs, as in all animal groups, the termination of these inflammatory reactions corresponds to wound repair, which results in the replacement of dead or damaged cells and the extracellular matrix (Sparks & Morado, 1988). In *T. semidecussatus*, the healing tissue mainly

consisted of infiltrated granulocytes and myofibroblast-like cells. In molluscs, it has been suggested that the fibroblasts involved in wound healing mechanisms are in fact modified haemocytes (Rifkin *et al.* 1969; Rudell, 1971; Sminia *et al.* 1974). However, we did not observe any redifferentiation of infiltrated granulocytes. Thus, our results suggest that the myofibroblast-like cells involved in this reparative process are resident cells which migrate from the adjacent connective tissue.

The main feature of wound repair is the substitution of injured tissues following the removal of inflammatory exudate, cellular debris and the inoculated foreign bodies by phagocytic cells (Sparks & Morado, 1988). Our results are in agreement with these findings, since cellular debris and inoculated bacteria and algae were progressively phagocytosed by the infiltrated granulocytes. However, *P. atlanticus* prezoosporangia were not phagocytosed. This failure could be due to the size of the prezoosporangia, since the phagocytic cells from molluscs encapsulate the foreign objects when the latter are too large to be phagocytosed by a single cell (Cheng & Rifkin, 1970; Bayne, 1990). Effectively, the arrangement of the granulocytes and the myofibroblast-like cells around non-viable inoculated prezoosporangia suggests the development of an encapsulation process, similar to that described for natural parasitism by *Tylocephalum* metacestodes in this clam (Cheng & Rifkin, 1968).

In *P. atlanticus*-infected feet, the cellular reaction to meronts involved the redifferentiated granulocytes, as we described previously in gill filaments (Montes *et al.* 1995*a*, 1996). The cytoplasm of these secretory cells, but not that of the infiltrated granulocytes, was filled by membrane-bound granules of homogeneous content and internal membranes in a parallel array. Moreover, whereas *P. atlanticus* meronts were encapsulated by the polarized secretion of the redifferentiated granulocytes (Montes et al. 1995*a*, 1996), secretion from the infiltrated granulocytes was not recorded at any point in the experiment. However, these recruited granulocytes were involved in phagocytosis, as the abundant cell debris and ink particles seen in the granules indicate. In any case, these phagocytic granulocytes did not express p225, the main capsule component that is released by the redifferentiated granulocytes of the cellular reaction (Montes *et al.* 1995*b*, 1996), as shown by the results obtained with the specific serum.

Therefore, our findings demonstrate that parasitism by *P. atlanticus* specifically induces the expression of p225 and the subsequent redifferentiation of recruited granulocytes in secretory granulocytes; neither experimental wounds, nor inoculations with biotic materials of varying diameter, such as bacteria, algae or non-viable *P. atlanticus* prezoosporangia, led to this specific cellular reaction.

Results from BrdU injection strongly suggest that the inflammatory reaction involved the infiltration of granular haemocytes from haemolymph without cell division of these granulocytes during initial accumulation. Likewise, secretory granulocytes of the cellular reaction against *P. atlanticus* meronts did not proliferate, given that BrdU labelling was not observed in these

redifferentiated granulocytes in any parasitized organ examined nor in any developmental stage of infection observed.

In agreement with the general opinion that haematopoiesis in bivalves occurs in the connective tissue (Cheng, 1981; Suzuki & Awaji, 1995), significant differences were observed in this tissue between inoculated specimens and control clams. Interestingly, most BrdU-labelled haemocytes were observed in the epicardic connective tissue, where mantle and gill lamellae are inserted to the visceral mass. This anatomical position and the presence of brown or serous cells suggest that this region might be homologous to Kleber's gland (Cheng, 1981).

In conclusion, the variation in haemocyte number and the proliferative response observed in the epicardic connective tissue suggest that this anatomical region is the main site of haemocyte proliferation stimulated after inoculation of foreign bodies in this clam. Furthermore, the results obtained from the inoculation experiments allow us to conclude that *P. atlanticus* infection elicits a unique defensive response in the clam *T. semidecussatus*.

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Figures

Figure 1

A Massive cellular infiltration around inoculated algae (*a*) at 72 h post-injection. *m* Muscular bundles. x210. *Bar*: 50 μm

B *P. atlanticus* prezoosporangia (*pk*)-inoculated foot at 72 h post-injection. Fusiform cells of the inflammatory reaction are organized in a parallel array. m Muscular bundles. x235. *Bar*: 50 μm

C At 15 days post-inoculation, remnants of the algae (*a*) are surrounded by a reduced inflammatory area. *m* Muscular bundles. x210. *Bar*: 50 μm

D Prezoosporangia (*pk*)-inoculated foot at 15 days post-injection. A multi-layered sheath of myofibroblast-like cells (*mfb*) delimits the infiltrated area and the regenerating muscular tissue. x210. *Bar*: 50 μm

E On day 40, the reduced inflammatory area is criss-crossed by newly formed muscular bundles. *a* Algae remnants. *m* Muscular bundles. x180. *Bar*: 50 μm

F Prezoosporangia (*pk*)-inoculated foot at 40 days post-inoculation. *m* Muscular bundles. x 290. *Bar*: 50 μm

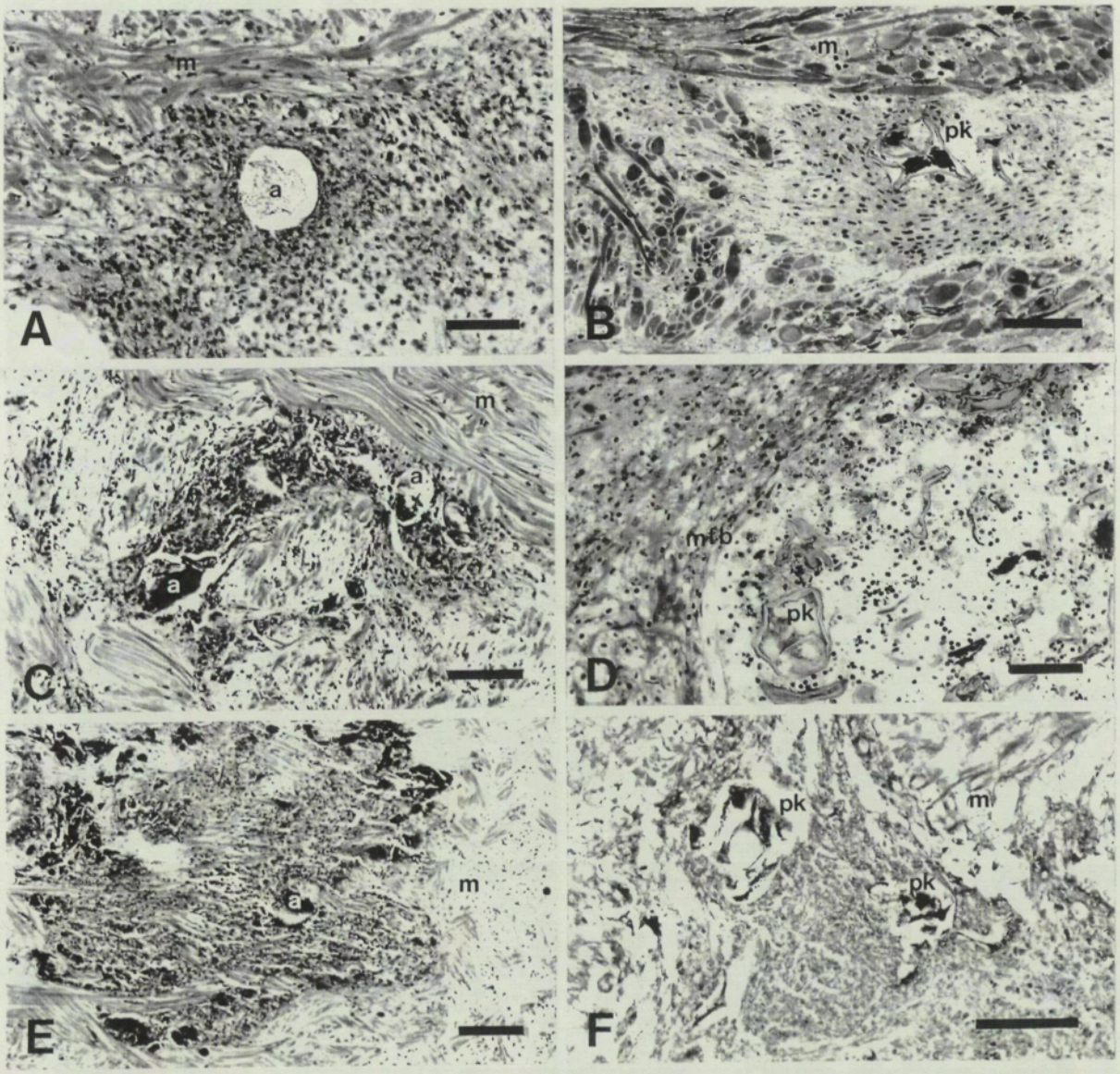
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Figure 2

A Infiltrated cells exhibit phagocytic activity in the BA group. Numerous cell debris (*arrows*) from inocula are seen within these cells. x810. *Bar*: 15 μm

B A layer of parallel arranged myofibroblast-like cells (*mfb*) forms a histological border between the infiltrated cells that surround 2 inoculated prezoosporangia and the healthy muscular tissue at 15 days post-injection. x610. *Bar*: 15 μm

C Infiltrated cells from the inflammatory reaction against inoculated prezoosporangia show numerous lysosomes filled with cellular debris (*arrows*). x775. *Bar*: 15 μm

D *P. atlanticus*-infected foot. The cellular reaction (*cr*) against meronts (*pk*) involves secretory granulocytes. x850. *Bar*: 15 μm

E *P. atlanticus*-infected gills. Several meronts (*pk*) are surrounded by the same redifferentiated granulocytes seen in the foot (*cr*). x875. *Bar*: 15 μm

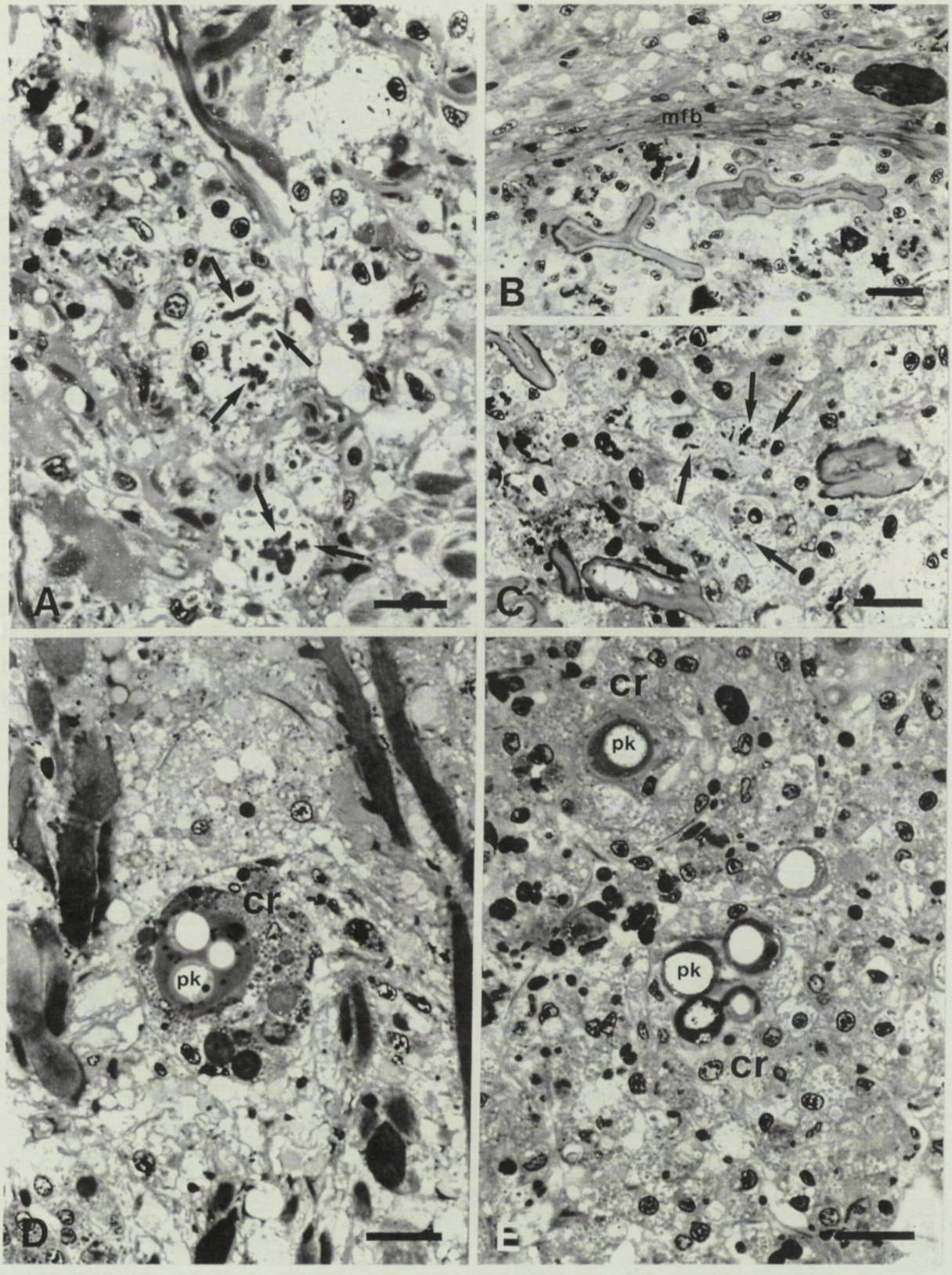
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Figure 3

A Myofibroblast-like cells in the vicinity of an inoculated prezoosporangium. *cw* Prezoosporangium cell wall. x8000. *Bar*: 2 μm

B Infiltrated granulocyte in the proximity of an inoculated prezoosporangium, showing cellular debris within the lysosomes. Capsule remnants with ink particles (*arrows*) are seen in close contact with the prezoosporangium cell wall (*cw*). x8000. *Bar*: 2 μm

C Low-power electron micrograph of an infiltrated granulocyte. x6600. *Bar*: 2 μm

D Membrane-bound granules from a circulating granulocyte. x16000. *Bar*: 1 μm

E, F p225 immunolocalization. **(E)** The granules (*gr*) from the infiltrated granulocytes are devoid of label. x30000. *Bar*: 0.5 μm.**(F)** Strongly labelled granule (*gr*) from a redifferentiated granulocyte of the cellular reaction against *P. atlanticus* meronts from foot. x33000. *Bar*: 0.5 μm

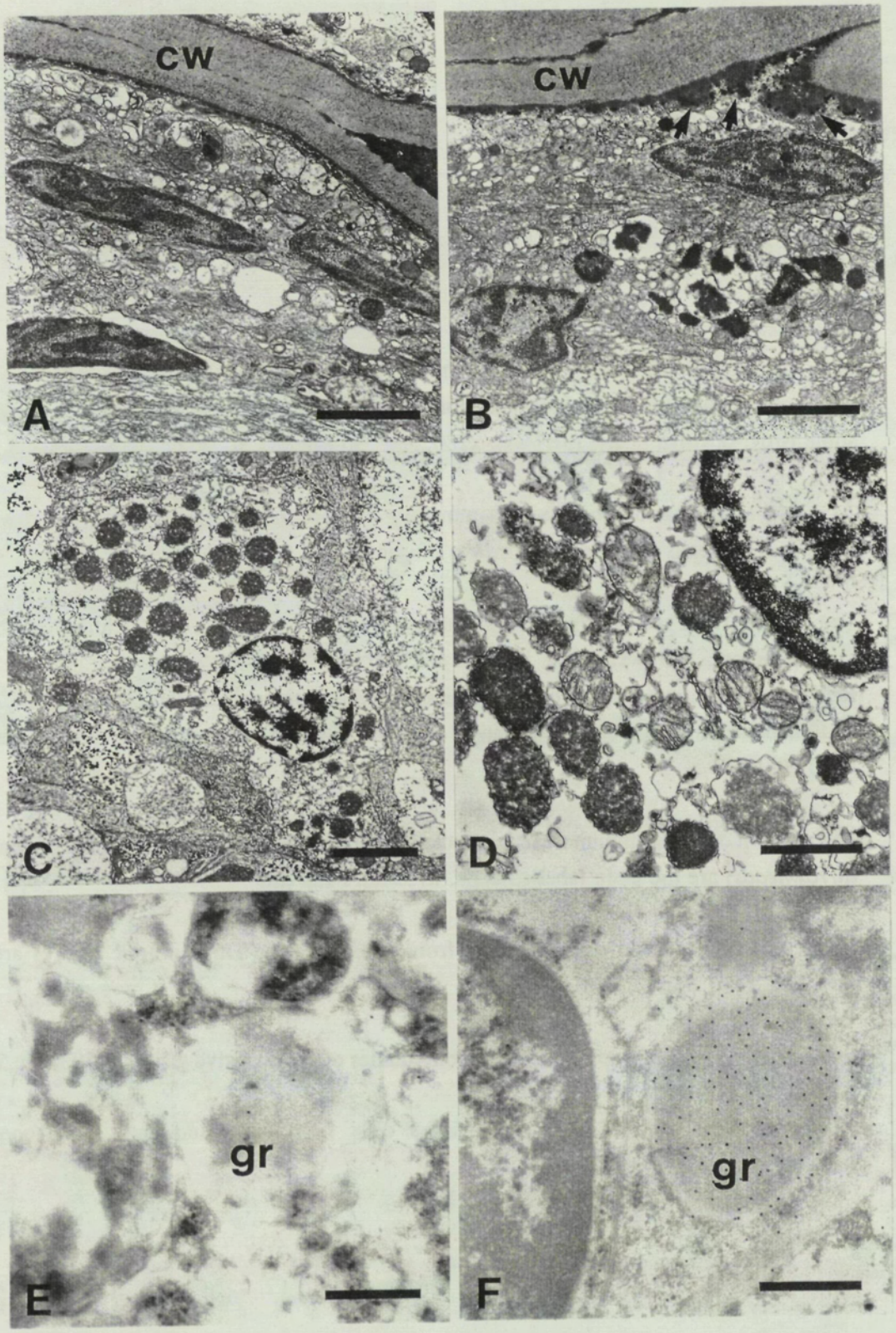
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Figure 4

Cell proliferation

A Non-immunoreactive nuclei are seen in the infiltrated area that surrounds algae masses (*a*) at 72 h post-inoculation. Some muscular fibres at the periphery of the inflammatory zone show some BrdU-positive nuclei (*arrows*). x280. *Bar*: 50 μm

B At 40 days post-inoculation, the inflammatory area around prezoosporangia (*pk*) show few BrdU-labelled cells (*arrows*). x290. *Bar*: 50 μm

C, D Ten μm-separated sections from *P. atlanticus*-infected labial palps. (**C**) Cellular reaction (*cr*) around meronts (*arrows*). x250. *Bar*: 40 μm. (**D**) BrdU-containing nuclei are not seen in the redifferentiated granulocytes of the cellular reaction (*cr*) against meronts (*arrows*). A few BrdU-labelled muscular and connective cells (*arrowheads*) surround the cellular reaction. x230. *Bar*: 40 μm

E Distribution of BrdU-immunoreactive nuclei in gill filaments. Epithelial and endothelial cells (*arrowheads*) are the main labelled cells. x75. *Bar*: 125 μm

F Detail of BrdU-labelled epithelial cells from gill filaments. Three mitotic figures are seen (*arrows*). x520. *Bar*: 25 μm

G BrdU-positive nuclei in epithelial cells from tubules of the digestive gland. x250. *Bar*: 50 μm

H Numerous BrdU-labelled connective tissue cells from the mantle are seen soon after inoculations. x225. *Bar*: 50 μm

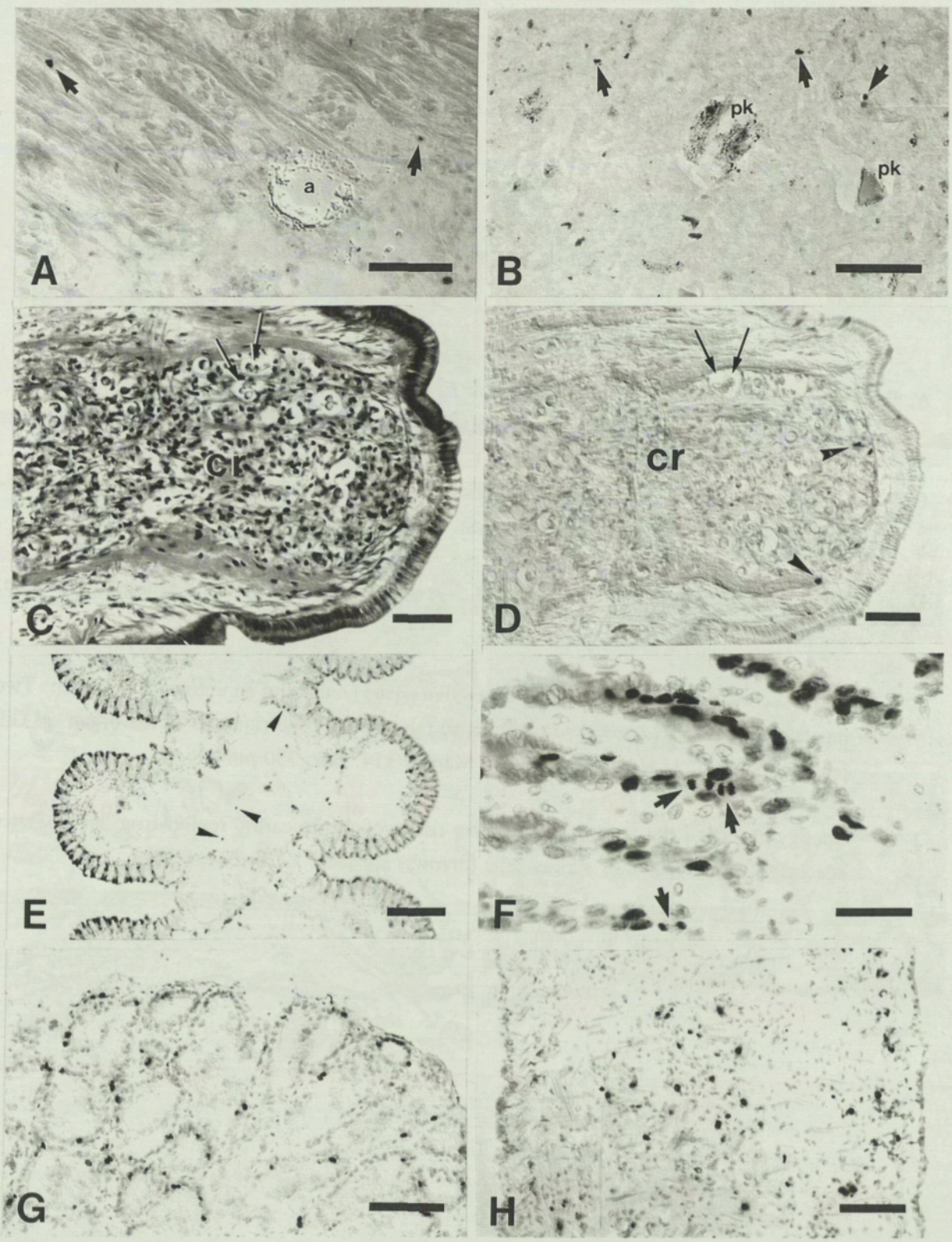
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Figure 5

BrdU immunocytochemistry from connective tissue of the epicardic region at different times post-inoculation.

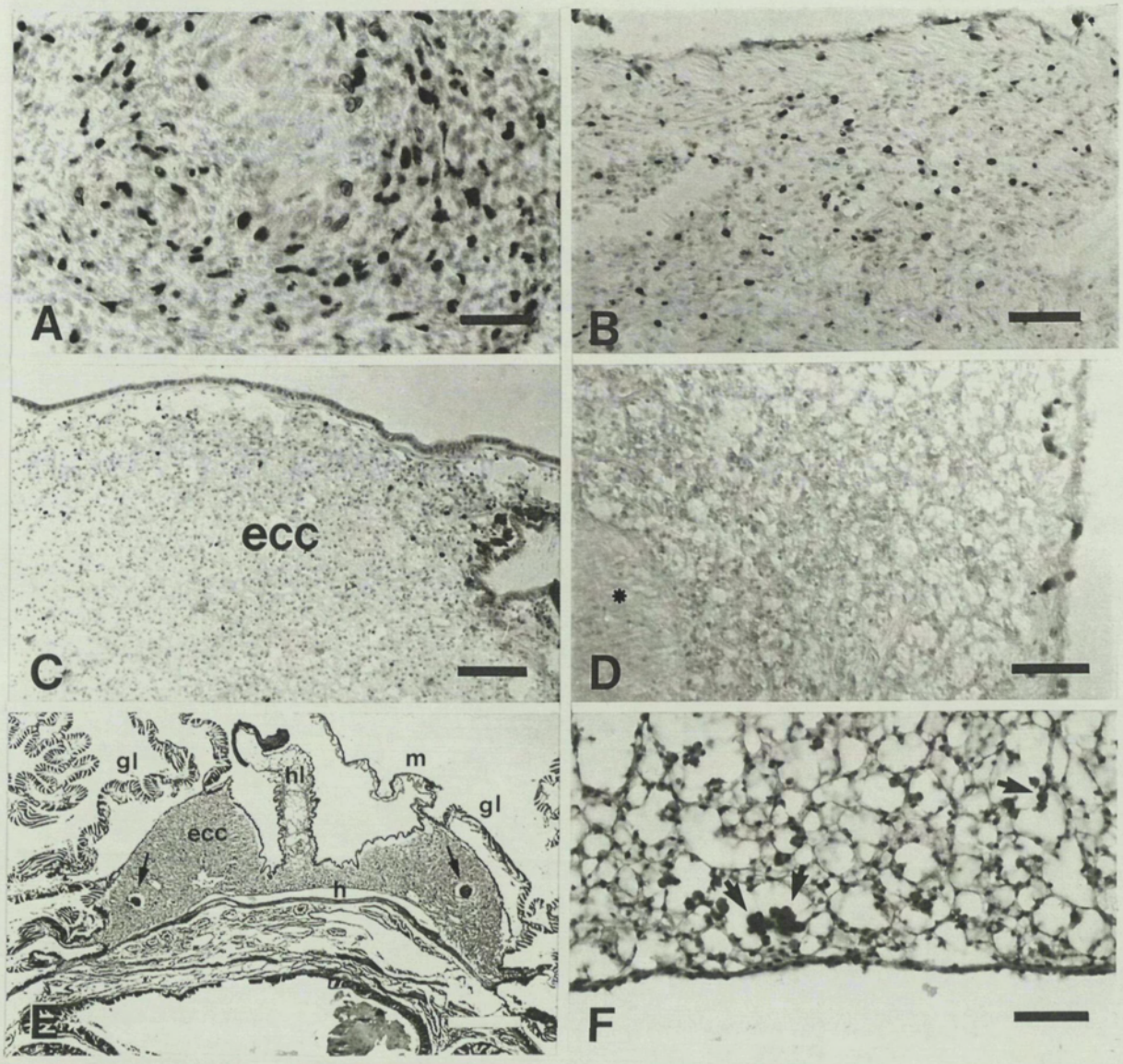
A, B Numerous BrdU-labelled cells are seen at 72 h post-inoculation. **(A)** x340. *Bar*: 30 μm. **(B)** x215. *Bar*: 50 μm

C The epicardic connective tissue (*ecc*) show a massive cellular aspect with various BrdU-positive nuclei at 15 days post-inoculation. x135. *Bar*: 75 μm

D Non-immunoreactive nuclei are seen 40 days post-inoculation. *asterisk* Muscular fascicule. x210. *Bar*: 50 μm

E Cross-section throughout epicardic connective tissue (*ecc*) from an untreated specimen. Two muscular fascicules (*arrows*) run parallel to the anterior-posterior axis of the organ. *gl* Gill lamella. *h* Heart. *hl* Hinge ligament. *m* Mantle. x14. *Bar*: 750 μm

F Spongy aspect of the reticular connective tissue of the epicardic region from an untreated specimen. Some brown or serous cells (*arrows*) are seen. x210. *Bar*: 50 μm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Discusión general

El parásito pertenece a la especie *Perkinsus atlanticus*

Los protozoos presentes en las almejas *T. semidecussatus* (Capítulo I) y *T. decussatus* (Capítulo III) procedentes del Delta del Ebro se caracterizan por la presencia de una gran vacuola, un núcleo voluminoso de localización excéntrica y una pared celular de naturaleza fibrogranular. Estas características estructurales coinciden con las descritas para los trofozoitos del género *Perkinsus* (Perkins, 1988, 1996). Por otra parte, tanto la identidad de las especies parasitadas como la respuesta defensiva desarrollada contra la infección (Capítulos I y III) indican que estos protozoos pertenecen a la especie *Perkinsus atlanticus* (Azevedo, 1989; Sagristà y cols., 1996).

Los trofozoitos de *P. atlanticus* se localizan en el tejido conjuntivo del huésped

El ciclo vital conocido de los protozoos del género *Perkinsus* lo componen trofozoitos, hipnosporas y zoosporas, siendo los trofozoitos el único estadio observado en los huéspedes (Perkins, 1988, 1993, 1996). Nuestras observaciones estructurales (Capítulos I y III) y, especialmente, los resultados obtenidos mediante el suero contra p225 evidencian que los trofozoitos de *P. atlanticus* se localizan en el tejido conjuntivo, siendo las branquias, manto y hepatopáncreas los órganos más susceptibles de invasión. Aunque se desconoce cuál es la fase o fases infectantes que suceden de modo natural, diferentes ensayos comparativos revelan que los trofozoitos y las zoosporas pueden ser los estadios más eficientes en la transmisión de la infección (Perkins, 1993; Volety y Chu, 1994; Chu, 1996).

La respuesta defensiva de *Tapes* spp. se basa en una reacción celular que origina la encapsulación de los trofozoitos

Las almejas *T. semidecussatus* y *T. decussatus* desarrollan frente a la infección de *P. atlanticus* una reacción inflamatoria, mediada por hemocitos, que causa la encapsulación de este parásito (Capítulos I y III). Si bien la encapsulación es una estrategia defensiva habitual tanto en moluscos como en otros grupos de invertebrados (Ratcliffe y Rowley, 1981; Ratcliffe y cols., 1985; Bayne, 1990), la desarrollada por estas almejas contra los trofozoitos de *P. atlanticus* exhibe características singulares (Chagot y cols., 1987; Comps y Chagot, 1987; Sagristà y cols., 1995; Capítulos I y III). Así, mientras las cápsulas de moluscos suelen estar compuestas por hemocitos, células semejantes a fibroblastos y fibras del tejido conjuntivo (Cheng, 1981; Cowden y Curtis, 1981; Sminia, 1981; Bayne, 1983; Ratcliffe y cols., 1985), en estas almejas venéridas, los trofozoitos de *P. atlanticus* son encapsulados por un material de naturaleza no fibrilar secretado por los hemocitos infiltrados en la vecindad de los parásitos (Capítulos I, II y III).

Los hemocitos que constituyen la reacción celular son granulocitos rediferenciados

Los hemocitos granulares son las células implicadas en la respuesta defensiva que *Tapes* spp. desarrolla contra los trofozoitos de *P. atlanticus* (Chagot y cols., 1987; Comps y Chagot, 1987; Azevedo y cols., 1990b; Figueras y cols., 1992; Capítulos I y III), coincidiendo, de este modo, con el principal tipo hemocitario identificado en las cápsulas de moluscos (Ratcliffe y cols., 1985). No obstante, los granulocitos que componen esta reacción celular exhiben características diferenciales respecto a los circulantes. En efecto, mientras los granulocitos circulantes de *T. semidecussatus* muestran gránulos de contenido flocular con un halo lúcido periférico (Auffret, 1988; Capítulo IV), los granulocitos localizados en las proximidades de los trofozoitos se caracterizan por la presencia de numerosos gránulos de contenido homogéneo con abundantes membranas internas (Capítulos I y III).

Las observaciones estructurales (Capítulos I y III) y los resultados obtenidos mediante el suero contra p225 (Capítulos II y III) indican que una vez que los hemocitos granulares han accedido al tejido conjuntivo de los órganos parasitados, sufren un proceso de rediferenciación dando lugar a un nuevo tipo celular de carácter secretor. La secreción ocurre en la estricta inmediación de los trofozoitos e implica la muerte de los granulocitos (Capítulos I y III). Estos resultados revelan un proceso de encapsulación en invertebrados que conlleva no sólo la rediferenciación de los hemocitos infiltrados, sino la secreción holocrina de un producto especializado.

Las reacciones celulares de *T. semidecussatus* y *T. decussatus* exhiben características diferenciales

Aunque tanto la respuesta defensiva elaborada por *T. semidecussatus*, como la desarrollada por *T. decussatus* conllevan la encapsulación de los trofozoitos de *P. atlanticus*, estas respuestas exhiben características diferenciales. Así, mientras la reacción celular de *T. semidecussatus* está organizada en áreas individualizadas, separadas por elementos del tejido conjuntivo (Capítulo I), aquélla de *T. decussatus* está compuesta por una única masa hemocitaria, sin fibras ni células de origen conjuntivo (Capítulo III). Además, los granulocitos rediferenciados de *T. decussatus* (Capítulo III) exhiben un compartimento endomembranoso (mitocondrias y retículo endoplasmático) más desarrollado que el de los granulocitos de *T. semidecussatus* (Capítulo I). Asimismo, los gránulos de *T. decussatus* (Capítulo III) muestran menos membranas internas y, además, sin la típica ordenación en paralelo de *T. semidecussatus* (Capítulo I). Por último, aunque mediante el suero contra p225 se observó inmunoreactividad en *T. decussatus* (Capítulo III) en los mismos compartimentos intra y extracelulares relacionados con la síntesis, secreción y depósito de p225 que lo fueron en *T. semidecussatus* (Capítulo II), las estimaciones de la densidad de marca (LD), para cada uno de los compartimentos, señalan una disminución relativa de la LD en *T. decussatus* (Capítulo III). Estos resultados podrían ser debidos a diferencias bien en el reconocimiento de ambos determinantes, bien en el almacenamiento de los productos (Capítulo III).

La respuesta defensiva de *T. decussatus* es más eficaz

Aunque la organización de la cápsula muestra las mismas características en ambas especies, aquélla observada en *T. decussatus* es, en ocasiones, incompleta (Capítulo III). No obstante, la eficacia de la respuesta defensiva de *T. decussatus* parece ser mayor, dado que nuestros resultados evidencian que el porcentaje de trofozoitos muertos respecto del total de encapsulados es mayor en *T. decussatus* que en *T. semidecussatus* (Capítulo III). Este dato es consistente con los resultados obtenidos por Rodríguez y cols. (1994), quienes mostraron que las zoosporas de *P. atlanticus* se propagan con mayor dificultad en *T. decussatus* que en *T. semidecussatus*. Por otra parte, esta aparente mayor eficacia de los mecanismos defensivos de *T. decussatus* también se ha constatado frente a otras infecciones. Así, Maes y Paillard (1992) indicaron que *Vibrio* P1, el agente causal del anillo marrón, es menos virulento para *T. decussatus* que para *T. semidecussatus*.

p225 es almacenado en los granulos de los granulocitos rediferenciados

El componente mayoritario de la cápsula es un polipéptido (p225) de un peso molecular aproximado de 225 kDa. La inmunolocalización de este polipéptido (Capítulo II), mediante el suero específico, indica que p225 es almacenado en los gránulos de los granulocitos rediferenciados, siendo secretado y organizado como una cápsula alrededor de los trofozoitos. Por otra parte, los ensayos de deglucosilación (Capítulo II) revelan que p225 no está glucosilado, dado que ni la incubación con Endo H ni la ß-eliminación alcalina mostraron un efecto detectable en su peso molecular. Estos resultados se ajustan a los obtenidos tras identificar, mediante lectinas, los restos glucídicos mayoritarios de los componentes de la reacción celular (Capítulo I). Así, tan sólo fueron localizados restos GalNAcα1,3GalNAc en las membranas internas de los gránulos y de la cápsula, estructuras que no colocalizan con p225.

p225 está unívocamente relacionado con la parasitosis

La aplicación de las técnicas inmunocitoquímicas y de Western-blot (Capítulo II) revelan la ausencia de p225 en todos aquellos órganos o tejidos libres de la infección, detectándose, tan sólo, en aquellos tejidos parasitados por *P. atlanticus*. Estos resultados nos permiten afirmar que p225 es un producto de nueva expresión asociado con esta parasitosis. De este modo, el suero contra p225 puede ser utilizado para el desarrollo de un método que permita la detección precoz de la infección.

P. atlanticus elicita una respuesta defensiva específica en *T. semidecussatus*

Los resultados obtenidos tras la inoculación de cuerpos extraños en *T. semidecussatus* (Capítulo IV) demuestran que el parasitismo de *P. atlanticus* induce, específicamente, la rediferenciación de los granulocitos infiltrados y la subsiguiente expresión de p225; ni las heridas experimentales, ni la inoculación de material biológico de diferente tamaño, como bacterias, algas o hipnosporas no viables de *P. atlanticus*, elicita esta reacción celular específica. Así, aunque los hemocitos granulares asociados a las áreas inoculadas exhibieron actividad fagocítica, en ningún punto del experimento se observaron procesos de secreción por parte de estas células. En cualquier caso, estos hemocitos granulares no expresaron p225, el componente mayoritario de la cápsula secretado por los granulocitos rediferenciados de la reacción celular (Capítulo II).

Mientras que bacterias y algas fueron fagocitadas por los granulocitos asociados a la inflamación, las hipnosporas de *P. atlanticus* no lo fueron. Este fracaso aparente de la respuesta fagocitaria puede ser debido al tamaño de las hipnosporas, dado que las células fagocíticas de moluscos encapsulan a los cuerpos extraños demasiado grandes para ser endocitados por un sola célula (Cheng y Rifkin, 1970; Ratcliffe y cols., 1985; Bayne, 1990). En efecto, la organización de los granulocitos inflamatorios y de los miofibroblastos alrededor de las hipnosporas sugiere el inicio de un proceso de encapsulación, similar al descrito contra los metacestodos de *Tylocephalum* en esta almeja (Cheng y Rifkin, 1968).

Los hemocitos de *T. semidecussatus* no proliferan en las áreas inflamatorias

Los resultados obtenidos mediante la técnica de la BrdU (Capítulo IV) evidencian que ni los granulocitos inflamatorios ni los granulocitos rediferenciados proliferan en las áreas inoculadas o en la reacción celular, respectivamente. De este modo, nuestros resultados corroboran la hipótesis de que las reacciones defensivas de moluscos, basadas en una respuesta inflamatoria, son consecuencia del reclutamiento de hemocitos circulantes sin que se produzca la proliferación de estas células en las áreas parasitas o traumatizadas (Sminia y cols., 1973; Alvarez y cols., 1995).

El tejido conjuntivo epicárdico es el principal lugar de proliferación hemocitaria de *T.semidecussatus*

A pesar de la ausencia de evidencias experimentales convincentes, se ha sugerido que en bivalvos la hemopoiesis tiene lugar en el tejido conjuntivo (Cheng, 1981; Suzuki y Awaji, 1995). Nuestros resultados (Capítulo IV) no tan sólo corroboran esta hipótesis, sino que además revelan que es el tejido conjuntivo epicárdico la principal región anatómica de proliferación hemocitaria estimulada en *T. semidecussatus* como consecuencia de un proceso inflamatorio.

Conclusiones

Primera

La respuesta defensiva desarrollada por las almejas del género *Tapes* frente a la infección de *Perkinsus atlanticus* es específica. Este parásito induce la rediferenciación de los hemocitos granulares infiltrados y la subsiguiente expresión de p225.

Segunda

p225 es almacenado en los gránulos de los granulocitos rediferenciados. Tras su secreción constituye el principal componente de la cápsula.

Tercera

En *Tapes semidecussatus*, el tejido conjuntivo epicárdico es el principal lugar de proliferación hemocitaria estimulado como consecuencia de un proceso inflamatorio.

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