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Epithelial sentinels or protozoan parasites? Studies on isolated rodlet cells on the 100th anniversary of an enigma

Centinelas epiteliales o parásitos protozoarios? Estudios en células rodlet aisladas en el centenario de un enigma.

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ABSTRACT

Rodlet cells are an unusual cell type found exclusively in teleost fishes. Their principal characteristics are a fibrous capsule and arrow or club-like structures pointing towards the apex of the cell, which are called rodlets. Rodlet cells were first described by Thelohan (1892) as undetermined sporozoan fish parasites, and soon after named *Rhabdospora thelohani* by Laguesse (1895). In 1906, a presently ongoing controversy started, with Plehn's independent characterization of rodlet cells as endogenous glandular cells, and a prompt refutation by Laguesse (1906). Both maintained their position, and during the following century both views continued to coexist with varying popularity, while additional interpretations of rodlet cell function were proposed. Here I present observations of live rodlet cells from the olfactory epithelium of the marine teleost *Isacia conceptionis*. Rodlet ejection was monitored and the fate of rodlet cells and ejected rodlets was tracked for up to 12 h. While rodlet cells died within a few hours, usually after rodlet expulsion, the rodlets remained stable over the observation period. These results are discussed in the light of the current hypotheses regarding rodlet cell function.

Key words: fish, immune cell, parasite, rodlet cell, teleosts.

RESUMEN

Las células "rodlet" son un tipo celular poco usual que se encuentra exclusivamente en peces teleósteos. Sus características principales son tener una cápsula fibrosa y estructuras en forma de lanza que apuntan hacia el ápice de la célula, denominadas "rodlet". Las células "rodlet" fueron descritas por primera vez por Thelohan (1892) como parásitos esporozoarios no determinados de peces, y poco después bautizados por Laguesse (1895) como *Rhabdospora thelohani*. En 1906, con la caracterización independiente realizada por Plehn de estas células como células glandulares endógenas, y la pronta refutación por Laguesse (1906), comienza una controversia que se ha mantenido hasta hoy. Ambos defendieron su posición, y durante el siglo siguiente ambas visiones continuaron coexistiendo con variada popularidad, al mismo tiempo que se proponían interpretaciones alternativas sobre la función de las células "rodlet". Aquí presento observaciones de células "rodlet" vivas del epitelio olfatorio del teleósteo marino *I. conceptionis*. Se monitoreó la expulsión de los "rodlets", el destino de las células "rodlet", y se siguió la trayectoria de los "rodlets" expulsados durante 12 h. No obstante las células "rodlet" murieron dentro de unas pocas horas, generalmente después de la expulsión de los "rodlets", los "rodlets" siguieron siendo estables durante el período de observación. Se discuten estos resultados teniendo en cuenta las hipótesis actuales sobre la función de estas células.

Palabras clave: pez, parásito, teleósteo, sistema inmune.

INTRODUCTION

The first description of rodlet cells (RCs) dates back to 1892, when Thelohan interpreted these conspicuous cells as sporocysts of an unknown coccidian parasite (Thelohan 1892). Three years later, Laguesse agreed with that view and named the organism *Rhabdospora thelohani* after its discoverer (Laguesse 1895). When Plehn (1906a) published her account of RCs, she concluded that they were endogenous secretory cells and named them

Staebchendruesenzellen, which translates into rodlet glandular cells. Soon Laguesse and Plehn realized that they were dealing with the same object, and engaged in a vivid public discussion (Laguesse 1906, Plehn 1906b), each without giving up his original viewpoint. Amazingly, in spite of well over 100 publications treating the problem during the following century, that controversy still remains unresolved today.

RCs seem to be a general feature of teleosts, as they are found in both wild and cultured species from fresh- and seawater (reviewed in Manera & Dezfuli 2004), but interestingly several reports indicate that they are absent from individual specimen (Mayberry et al. 1979, Iger & Abraham 1997, Fishelson & Becker 1999, Reite 2005). The principal factor influencing the number of RCs seems to be the presence of an infection, especially by parasites (Chaicharn & Bullock 1967, Dezfuli et al. 2000, 2003, Reite 2005, Reite & Evensen 2006), or a generalized irritation (Iger & Abraham 1997, Koponen & Myers 2000), a phenomenon which gave birth to the hypothesis of RCs as part of the non-specific immune system of teleosts.

RCs are migratory cells principally found in epithelial tissues from every organ investigated, notably skin, gills and intestine (e.g., Bielek 2002, Kramer et al. 2004). They are also present in high quantity in the bulbus arteriosus (Leknes 2001, Reite 2005), but at low density or absent in the blood, in connective tissues and the brain (Manera & Dezfuli 2004). RCs are considered to mature on their way from the basal to the apical zone of the epithelium, a process during which the rodlets gradually form (Leino 1974). This suggests that the actual function of RCs is executed at the epithelial surface. Here I describe the presence of RCs in the olfactory organ of a marine bentho-pelagic teleost, demonstrate a method to isolate RCs and present image data of live isolated RCs and ejected rodlets monitored over a period of up to 12 h.

MATERIAL AND METHODS

Cabinza grunts, *Isacia conceptionis* (Family: Haemulidae) of 18-22 cm total length were obtained from a local fishery in the bay of Valparaiso. They were maintained for up to 10 days without receiving food in a laboratory tank of aerated seawater (80 L for up to 4 animals) at 15-18 ºC, filtered mechanically and through activated carbon at a rate of approximately 10 liters per minute. The fish were sacrificed by fast decapitation on ice, pithed and the olfactory organs were removed with fine scissors. These procedures were approved by the Ethics Committee of the Universidad de Valparaíso.

Isolated olfactory rosettes were transferred to Ringer's solution containing (mM): 150 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 5 HEPES, 10 glucose, pH 7.4. On a few occasions, the fish Ringer was replaced by Leibovitz L-15 culture medium (Invitrogen, Carlsbad, California, USA), supplemented with 10 % blood serum from the sacrificed fish. After a visual examination to detect infection, olfactory lamellas were separated and cut into pieces of about 0.5-1 mm^2. The tissue was stored at 4 ºC with the addition of gentamycin (80 $\mu g\ mL^{-1}$, Sigma-Aldrich, St. Louis, Missouri, USA) and used only on the same day. Several pieces of olfactory epithelium were triturated with a fire-polished Pasteur pipette in 200 µL saline, allowed to settle for two minutes, and the upper half of the solution was transferred to the poly-L-lysine-coated recording chamber. The recording chamber was cooled down to about 15-18 ºC with a custom-built refrigeration device, to match the temperature of the sea water. After resting for 15 min, the chamber was perfused gently to remove cell debris. Cells were viewed with a 40x phase contrast or a 100 x oil immersion objective on an inverted Olympus IMT-2 video microscope. Video images and time lapse movies were digitalized and stored on a computer hard drive.

Live whole olfactory epithelium was analyzed under Nomarski optics with a Nikon Eclipse 2000 microscope and photographed with a Sensicam QE (Cooke Corp., Romulus, Minnesota, USA). For transmission electron microscopy, olfactory organs were excised under sea water and instantly transferred to the fixation solution containing 2.5 % glutaraldehyde and 1 % formaldehyde in sodium cacodylate buffer, pH 7.4. After overnight fixation at 4 ºC, the tissue was postfixed in reduced osmium (1:1 mixture of 2 % osmium tetroxide and 3 % potassium ferrocyanide) for 2 h, dehydrated in a graded series of ethanol and acetone and embedded in Eponate (Pelco, Redding, California, USA). Semi-thin sections (1 µm) were cut in an

ultramicrotome (Reichert, Austria) and stained with 1 % toluidine blue (Sigma-Aldrich, St. Louis, Missouri, USA) for 2 min. Light microscopical preparations were inspected with an Olympus BX-51 microscope and photographed with a CoolSNAP-Pro digital camera. General image parameters were adjusted with Adobe Photoshop 8. Ultra-thin sections (70 nm) were cut with a diamond knife (Pelco, Redding, California, USA) on a Reichert ultramicrotome, stained with uranyl acetate and lead citrate and analyzed with a Zeiss EM 900 electron microscope.

RESULTS

Light and electron microscopical analysis was performed to assess the occurrence and possible role of RCs in the olfactory epithelium of *I. conceptionis*. In whole olfactory epithelium and transverse tissue sections, RCs were oriented nearly always perpendicular to the surface, with their apical side up (Fig. 1C and 1E), as previously reported (Iger & Abraham 1997, Koponen & Myers 2000). The rodlets were clearly visible under light microscopy, and stained intensely with toluidine blue. Under electron microscopy, RCs from *I. conceptionis* displayed the characteristic morphological features exhaustively described in the literature (e.g., Bielek & Viehberger 1983, Bielek 2005a), (Fig. 1A, and 1H). In summary, mature RCs are elongated and polarized, oval to cigar-shaped cells with a conspicuous fibrous capsule of up to 1 μm thickness and a basal condensed nucleus. The average size of RCs from *I. conceptionis* was 7 x 20 mm. Apart from the nucleus, the basal part of the cell is filled with endoplasmic reticulum, whereas mitochondria and other organelles are displaced to the apical segment of the cell. Rodlets are arrow- to club-like structures involved in an individual membrane, forming the rodlet sac. Three distinct rodlet zones can be discerned: A rod-shaped electron-opaque core with a sharp tip, an intermediate zone and an electron-transparent outer area lined by the rodlet sac. The rodlets, generally between 8 and 15, point towards the apex of the cell, where the fibrous capsule is thinned or open. Sometimes a cytoplasmic bleb protrudes through this opening. The general fate of the rodlets seems to be their ejection, after which the RC dies (Fig. 2B-F). An active contraction of the fibrous capsule, proposed as the ejection mechanism (Leino 1974), could not be discerned. Free rodlets may remain attached to the RC as a bundle or distribute independently by passive diffusion. Alternatively, rodlets may be injected into other cells (Bielek 2002; Fig. 1F and 2D).

To investigate the physiology of live RCs, a straightforward dissociation procedure was applied to the olfactory epithelium. RCs were always present and easily identifiable in preparations of dissociated olfactory epithelium, in a sample of about 150 fish over a period of three years. However, their relative quantity with respect to all other isolated cells varied greatly and was positively correlated to general symptoms of infection, such as swollen blood vessels, excess secretion of mucus and tissue damage. The nature of the infectious agents was not investigated. Initially after dissociation, most RCs appeared intact and no expelled rodlets were seen. A significant number of RCs remained apically attached to other living or dead cells (Desser & Lester 1975). During the following 5 h an increasing fraction of RCs expelled their rodlets and degenerated, until virtually all RCs were spent (Fig. 2). Free rodlets did not dissolve and remained visually unchanged for up to 12 h, although the rodlet sac burst occasionally. The storage of RCs in L-15 culture medium supplemented with fish serum did not yield any different results.

Since RCs are frequently found in outer layers of fish skin, they may be subjected to osmotic stress from seawater, possibly entering through microscopic injuries in the course of an infection. To investigate whether the fibrous capsule serves to protect the RC against osmotic stress, RCs were superfused with sea water or distilled water. However, RCs displayed swelling and shrinking comparable to other cells in the vicinity (Fig. 2G), suggesting that this is not the function of the capsule.

To examine the kinetics of rodlet ejection, the ejection process was filmed and subjected to image analysis. As shown in Fig. 2H-2M, the rodlet ejection process started with intracellular transformations and terminated in a sub-second expulsion of the first rodlet. Most cells ejected all rodlets in a rapid sequence completed within 5-10 s (not shown).

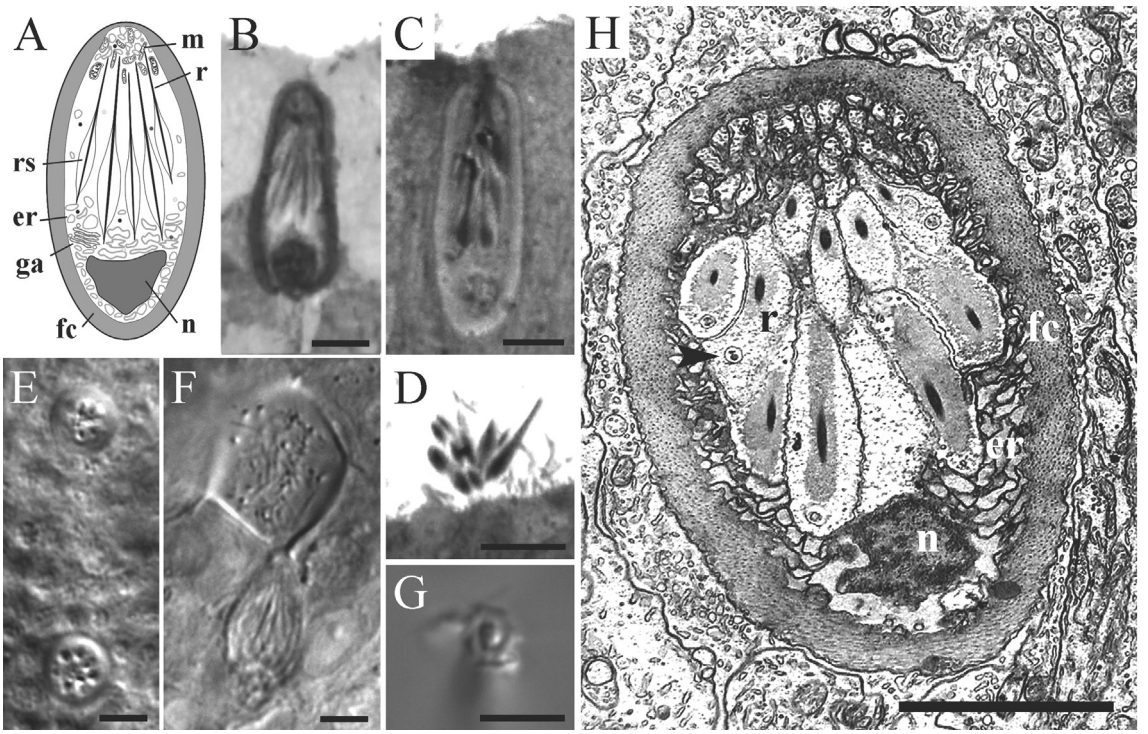

Fig. 1: RCs in whole tissue. (A) Schematic drawing of a RC. M = mitochondria; r = rodlet; rs =, rodlet sac; er = endoplasmic reticulum; ga = golgi apparatus; fc = fibrous capsule; n = nucleus. (B-D) Toluidine blue-stained semithin sections of olfactory epithelium. (B,C) Intensely stained rodlet cells in the upper middle zone of the epithelium. (D) Free rodlet bundle on the epithelial surface. (E-G) Nomarski contrast images of unstained live olfactory epithelium, seen from above. (E) Most RCs are oriented perpendicular to the surface in the outer layer of the epithelium. (F) RC coupled with its apical tip to a nonsensory ciliated cell, prior to rodlet ejection. (G) The apical pore of a RC, with a rodlet in the middle. (H) Electron microscopical image of an obliquely sectioned RC. Note the membrane-delimited inclusions in the rodlet sacs (arrowhead). r, rodlet; fc, fibrous capsule; er, endoplasmic reticulum; n, nucleus. Scale bars: 5 μm.

Células "rodlet" (RCs) en un tejido completo. (A) Dibujo esquemático de una RC. m = mitocondria; r = rodlet; rs = saco del rodlet; er = retículo endoplasmático; ga = aparato de golgi; fc = cápsula fibrosa; n = núcleo. (B-D) Secciones semi-delgadas del epitelio olfatorio teñidas con azul de toluidina. (B,C) Células "rodlet" teñidas intensamente en la zona media superior del epitelio. (D) Paquete libre de "rodlets" en la superficie epitelial. (E-G) Imágenes del epitelio olfatorio en vivo no teñido, visto desde arriba. (E) La mayoría de las RCs están perpendicularmente orientadas en la superficie de la capa externa del epitelio. (F) Una RC acoplada mediante su extremo apical a una célula no sensorial ciliada, antes de la expulsión del rodlet. (G) El poro apical de una RC, con un rodlet en el centro. (H) Micrografía electrónica de una RC oblicuamente seccionada. Observar las inclusiones delimitadas por membranas en los sacos del "rodlet" (punta de flecha). r = rodlet; fc = cápsula fibrosa; er = retículo endoplasmático; n = núcleo. Barras = 5 μm.

DISCUSSION

The parasite hypothesis

At a first glance, RCs strongly resemble certain developmental stages of Apicomplexa (Sporozoa) parasites, a phylum of Protozoa, and several authors concluded that they indeed belong to this group (Laguesse 1906, Gruenberg & Hager 1978, Mayberry et al. 1979, Richards et al. 1994). The rodlets are reminiscent of rhoptries, characteristic cytoplasmic inclusions of the motile stages of sporozoans, the RC capsule looks like a parasitic pellicle or cyst, and the apical pore suggests the presence of a proteinacious apical complex that is characteristic of this group of sporozoa. The general association of RCs with infections could be interpreted as an opportunistic invasion of tissues by RCs in animals with debilitated immune system, and is therefore not an argument against the parasite

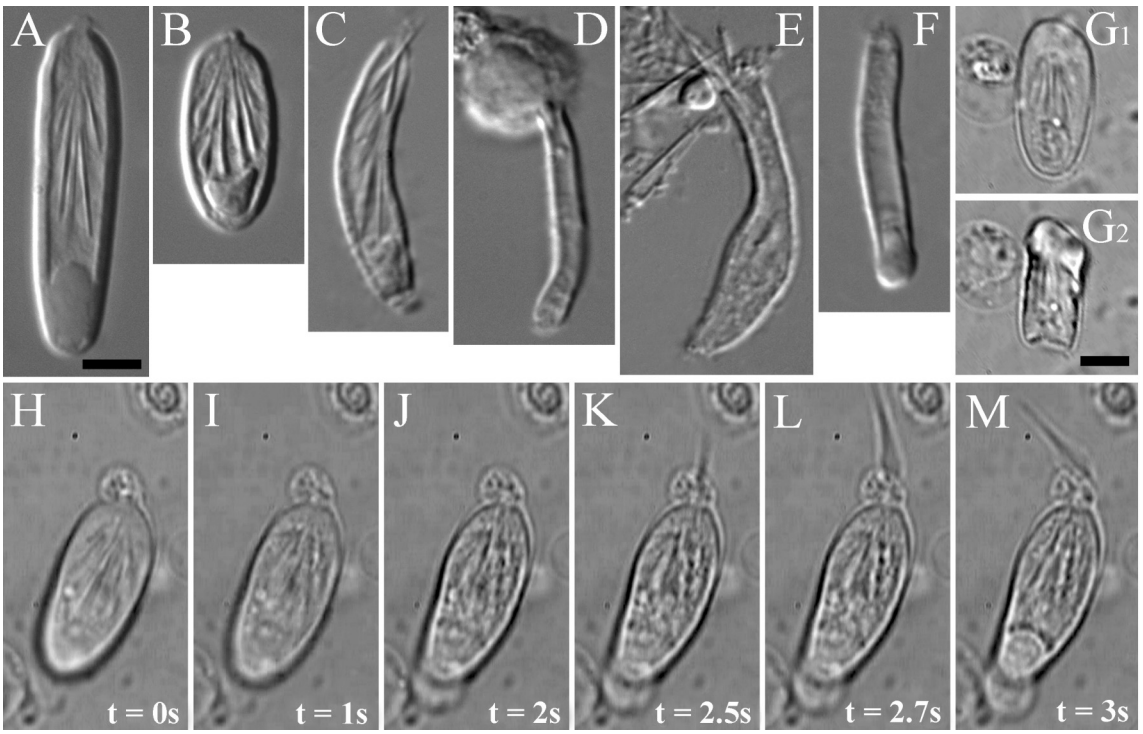

Fig. 2: RCs from dissociated olfactory epithelium of *I. conceptionis*. (A,B) Mature RCs have well-defined rodlets, a condensed nucleus and an apical pore. (C) Rodlets are ejected through the apical pore. (D,E) Rodlets may be injected into cells attached to the apex of the RC, but free rodlets, often associated with cell debris, are also regularly seen. (F) The final stage: Empty carcass of a RC. (G_1,G_2) RC prior and during perfusion with seawater. The fibrous capsule does not resist osmotic pressure and the cell shrinks. (H-M) The ejection process of rodlets starts with intracellular transformations (I,J) and terminates with sub-second rodlet expulsion through the apical cytoplasmic tuft (K-M). Note the cytoplasmic bleb that appears on the basal part of the cell, possibly resulting from increased intracellular pressure. Scale bar = 5 μm.

RCs del epitelio olfatorio disociado de *I. conceptionis*. (A,B) Las RCs maduras tienen "rodlets" bien definidos, un núcleo condensado y un poro apical. (C) Los rodlets son expulsados a través del poro apical. (D,E) Los "rodlets" se pueden inyectar en células que están unidas al ápice de la RC, pero los "rodlets" libres, a menudo asociados con restos celulares, también se ven regularmente. (F) La etapa final: caparazón vacía de una RC. (G_1,G_2) La RC antes y durante la perfusión con agua de mar. La cápsula fibrosa no resiste la presión osmótica y la célula se contrae. (H-M) El proceso de expulsión de los "rodlets" comienza con transformaciones intracelulares (I,J) y termina con la expulsión del "rodlet" en menos de un segundo a través del penacho citoplásmico apical (K-M). Observar la protuberancia citoplasmática que aparece en la parte basal de la célula, posiblemente como resultado de la aumentada presión intracelular. Barras = 5 μm.

hypothesis, as alleged by Manera & Dezfuli (2004). The occasional finding of rodlets phagocytosed by leukocytes (Bielek 2002) and the observation that individual animals lack RCs (Mayberry et al. 1979, Fishelson & Becker 1999, Reite 2005) also support the parasite hypothesis, because rodlets are more likely to be subject to phagocytosis if they represent an exogenous object than a natural secretory component of the host organism, and as endogenous cells RCs should be generally present in all individuals of an animal group.

Maybe the strongest argument in favor of the parasite hypothesis is based upon the inexistence of any related cell type in vertebrates. Although RCs, especially at immature stages, were reported to have a comparable distribution and morphological similarities with certain leukocytes (Duthie 1939, Cenini 1984, Leknes 2001, Reite 2005, Bielek 2005a), the mature RC is clearly distinct of any known kind of vertebrate cell. Since natural evolution is essentially conservative and the number of vertebrate cell types limited to about 200

(Alberts et al. 1994), it is hard to explain why a unique kind of cell should appear in teleosts and be absent from all higher vertebrates.

In a variation of the parasite hypothesis, Barber & Westermann (1986a,b) concluded from DNA labeling and in situ hybridization studies that only the rodlets represent a virus-like parasitic element, whereas the RC would be of endogenous origin. This interpretation was recently supported by Fishelson & Becker (1999) based on an ultrastructural survey.

Among the principal arguments against the parasite hypothesis are the observation of significant structural and ultrastructural differences between RCs and the Apicomplexa (Paterson & Desser 1981, Manera & Dezfuli 2004), notably the absence of the characteristic apical complex, the obvious ultrastructural dissimilarity between a parasitic cyst and the fibrous capsule of RCs and the formation of the latter inside the cell membrane. In addition, RCs lack the typical life cycle stages of sporozoan parasites, and are never found within host cells as opposed to these obligatory intracellular parasites. Although some Apicomplexa species display little host specificity, a worldwide universal distribution without significant tissue discrimination, as observed in the case of RCs, is highly unusual for a parasite. It is also difficult to explain how RCs should have invaded embryos and neonates of viviparous teleosts, in which they were detected prior to and around birth in the posterior intestine (Kramer & Potter 2003).

Furthermore, the directional migration from basal to apical parts of epithelia and the general orientation of RCs perpendicular to the surface facing outwards cannot be explained readily with the parasite hypothesis. Other common arguments against the parasite hypothesis include the universal lack of inflammatory responses to RCs, and the occasional observation of desmosomes between RCs and their surrounding cells (Iger & Abraham 1997, Dezfuli et al. 2000, Bielek 2005a). However, both phenomena are also known from some actual parasites, and should not be overrated.

The immune cell hypothesis

As mentioned above, RC density rises in response to diverse types of pathogens and also as consequence of other kinds of exogenous stressors. Iger & Abraham (1997) found an increased, although not statistically analyzed expression of epidermal RCs in response to treatment with distilled water, heavy metals and wounding. Seasonal changes in RC occurrence were also reported to correlate with parasitic prevalence in a Finnish lake (Koponen & Myers 2000). The immune cell hypothesis is sustained by this generally accepted association of RCs with infection or irritation, and by the arguments against the parasite hypothesis. A role of RCs within the immune system was first proposed by Duthie (1939) in an account of blood cells of marine teleosts. During the following decades, several authors described the analogous attributes of RCs and leucocytes, notably their migration and tissue distribution, and concluded a functional and/or developmental relationship between them (reviewed by Manera & Dezfuli 2004, Reite & Evensen 2006).

Currently, most supporters of the immune cell hypothesis believe that RCs are developmentally related to granulocytes, to which their immature stages bear a resemblance (Duthie 1939, Cenini 1984, Smith et al. 1995, Leknes 2001, Reite 2005). Besides their association with infection or exogenous stressors, the strongest argument in favor of the immune cell hypothesis is the omnipresence of RCs in teleost species over the world. Conversely, their apparent absence from individual specimen constitutes the principal argument against this hypothesis (Mayberry et al. 1979, Fishelson & Becker 1999, Reite 2005). However, it is conceivable that the expression of RCs is tightly regulated and dependent on specific stimuli, and there is also the possibility that very low numbers of RCs were overlooked in individual specimen. On the other hand, despite numerous efforts to tie RCs to granulocytes or other blood cells, mature RCs do not resemble any known type of vertebrate cell with respect to morphology and its basic secretory function. As mentioned earlier, the development of a new cell type is a rare event in evolution, and it is unclear why there should not be any related cells in amphibians or higher vertebrates.

Alternative hypotheses

It is intriguing to note that the arguments against both the parasite and the immune cell

hypothesis are generally stronger than those that support them. This notorious lack of positive evidence led some investigators to speculate about alternative explanations for RC identity and function. Thus, RCs were proposed to participate in osmoregulation and ion transport (e.g., Mattey et al. 1979). Other researchers considered their role as that of secretory cells without defensive functions (Plehn 1906a, Flood et al. 1975). However, the growing amount of data showing an association of RCs with infection and inflammation, and the failure of the glandular hypotheses to explain the role of the rodlets have virtually eradicated their support in recent years.

A new hypothesis was proposed by Bielek in recent studies (Bielek 2002, 2005a, 2005b). After initially supporting the parasite hypothesis in her early works (Bielek & Viehberger 1983), the author presently seeks to explain the rare morphology of RCs and especially the rodlets with an abnormal and chronically elevated protein synthesis possibly accompanied by protein misfolding, a view that is supported by the unusual conformation of the endoplasmatic reticulum in RCs (Bielek 2005a). Accordingly, the rodlets might represent crystallized deposits of some excess protein. While massive protein misfolding is involved in various diseases (Gregersen et al. 2005), it appears highly unlikely that such pathological conditions should occur as universally as is the presence of RCs across fishes, without apparent preference for age groups, species or geographical areas.

CONCLUSIONS

From a general point of view, the solid structure of the rodlets, their sharp tips and their vigorous ejection, either by contraction of the fibrous capsule (Leino 1974), or by a build-up of intracellular pressure (Bielek 2005a), suggest that a forceful mechanical penetration of other, possibly armored cells may be a central part of rodlet cell function. Indeed, rodlets were found within living and apoptotic cells in several electron microscopical studies (e.g., Bielek 2002). Since the rodlets did not dissolve over 12 h in Ringer's solution, as observed in the present study, it is possible that rodlet break up only occurs in the intracellular milieu, over a longer time span, or never.

It is tempting to speculate that RCs were initially Apicomplexa parasites that were assimilated and transformed into an endogenous defensive weapon against other invasive organisms at some early point in teleost evolution. While this would explain several of the features of RCs, it implies the incorporation of at least part of the parasite genome into the fish genome, otherwise the universal RC distribution and their presence in embryos would remain without explanation. A similar idea was proposed by Fishelson & Becker (1999), who suggested that the rodlets might be parasitic elements in symbiosis with fish leucocytes. This hypothesis implies that the rodlets grow and multiply as independent genetic elements, which is currently not supported by any evidence.

The present study sought to follow the fate of rodlets after their ejection, and to observe any possible transformation or incorporation into other dissociated fish cells. Yet, rodlets appeared as passive, stable elements outside the RC, and no evidence was found for a putative parasitic role. It is definitely time to perform molecular biological studies on RCs, to examine whether their genome is identical to that of the host fish cells. With the dissociation method shown here, it should be feasible to perform single-cell PCR on RCs. Alternatively, ejected rodlets might be collected and probed for the presence of DNA with PCR using fish- and parasite-specific primers. While experimentally challenging, these experiments would undoubtedly contribute to finally resolve the century-old mystery of the teleost RC.

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