非定型Edwardsiella tarda(=Edwardsiella anguillarum)に感染したマダイの好中球の誘導型顆粒

Inducible Granules in Neutrophils from Red Seabream *Pagrus major* Infected with Atypical *Edwardsiella tarda* (=*Edwardsiella anguillarum*)

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Abstract : Numerous alkaline phosphatase (AlP)-positive granules appeared in neutrophils of red seabream *Pagrus major* after infection with pathogenic bacteria, atypical *Edwardsiella tarda* (=*Edwardsiella anguillarum*). This granule consisted of AlP-positive core and its AlP-negative surrounding. Both parts were chromophobic with May-Grünwald・Giemsa stain, and react negatively to several lysosomal enzymes, peroxidase, Sudan black B, etc. This granule type was not found in the neutrophils from non-infected fish. Therefore, the granules may be induced by infection with *E. tarda*. We designate the granule inducible chromophobic granules (iβG).

Key words : granule, neutrophil, *Pagrus major*, red seabream, *Edwardsiella tarda, Edwardsiella anguillarum*

We have revealed that neutrophils in peripheral blood of red seabream *Pagrus major* contain two types of chromophobic granules (βG), namely one without eosinophilic core (EC; βG-1) and the other with EC (βG-2)[1]. The EC contained some lysozomal enzymes. On the other hand, the chromophobic area of both types of granules (the whole βG-1 and surrounding of the EC of βG-2) reacted positively to peroxidase (PO) and Sudan black B[1]. Here, we report novel granules of neutrophils from red seabream infected with atypical *Edwardsiella tarda*.

The fish used in this study were red seabream (mean body weight, 127 g) reared in National Fisheries University. Fish were acclimatized at 25℃ for 7 days prior to the experiment. During the acclimatization period, fish were fed commercial diet (Marine No. 6, Hayashikane Sangyo Co., Ltd) *ad libitum*. Atypical *Edwardsiella tarda* (=*Edwardsiella anguillarum*) HME-1 isolated from disease red seabream in 2007 was used in the experiment. Forty fish were immersed in bacterial suspension (100 L) of 9.8×10^7 CFU/*ml* at 25℃ for 1 h with aeration. After immersion with *E. tarda*, fish were accommodated in four 500 L tanks (10 per tank). Two tanks were monitored without sampling (Mean mortality was 65 % (60 and 70%) until 7 day post-inoculation (dpi)). Blood was collected from four fish in each sampling period (1, 3, 5 and 7 dpi). Smears were stained with May-Grünwald・Giemsa and several cytochemical stains as described previously[1]. Intact and lysed neutrophils were observed under a light microscope.

Many neutrophils with basophilic hyaloplasm and perinuclear halo were observed in the smear from fish sampled at 5 and 7 dpi. (Fig. 1A). The number of βG-2 of these neutrophils was similar to that from non-infected fish. There was no defference in almost all cytochemical tests except for alkaline phosphatase (AlP), periodic acid Schiff reaction (PAS) and toluidine blue (TB), between the neutrophils from infected fish and non-infected fish (Table 1). The neutrophils from infected fish contained many AlP-positive granules (Fig. 1B). These granules consisted of AlP-positive core and negative surrounding (Fig. 1C). The AlP was never detected in the neutrophils from non-infected fish. In PO staining preparations (Fig. 1D & 1E), many PO-negative granules were detected around PO-positive granules. PO-negative granules were not observed in the neutrophils from non-infected fish. These findings strongly suggest that (1) AlP is inducible enzyme and the AlP-positive granule is inducible granule; (2) AlP-positive granule is chromophobic (therefore, this

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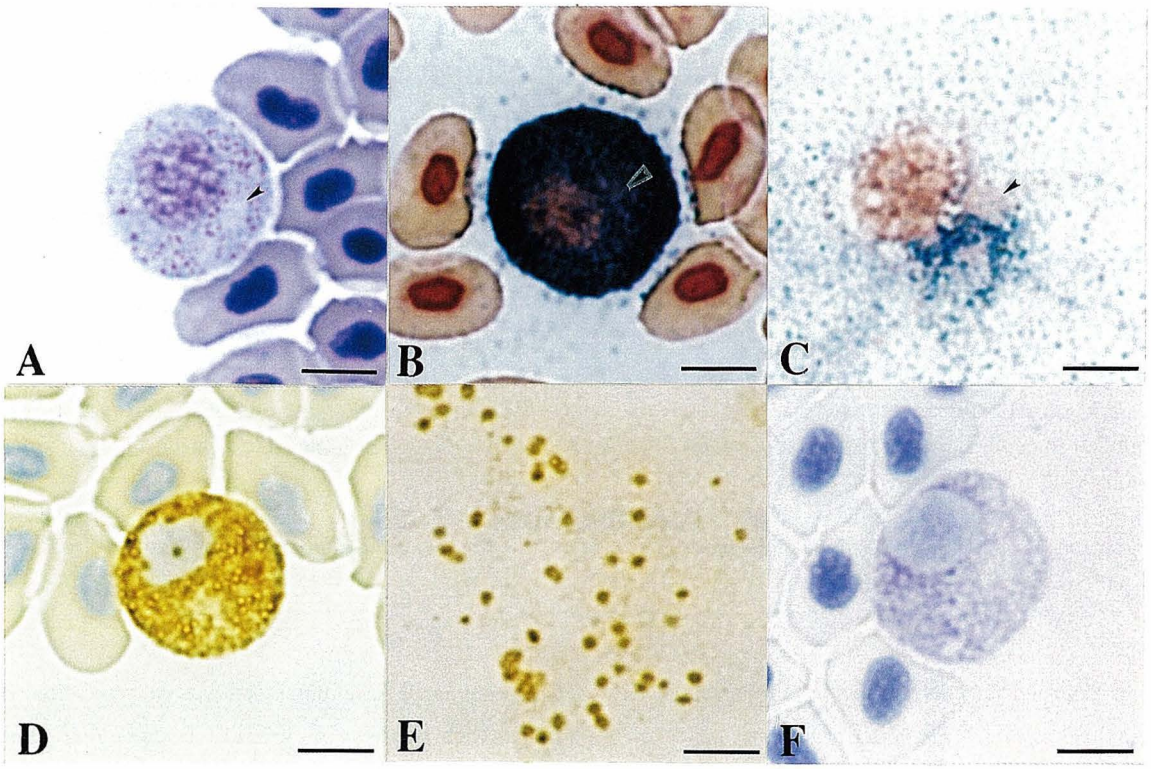

Fig. 1. Cytochemistry of neutrophil from red seabream infected with atypical *Edwardsiella tarda* (=*Edwardsiella anguillarum*). A, May-Grünwald・Giemsa stain (intact cell; arrowhead, perinuclear halo); B & C, alkaline phosphatase (B, intact cell; C, lysed cell); D & E, peroxidase (D, intact cell; E, lysed cell. Note many negative granules in E.); F, toluidine blue in distilled water (intact cell). Perinuclear halo (correspond to Golgi apparatus) is stained with safranin O (counter stain for alkaline phosphatase) (arrowheads in B and C). Bars=5 μm.

Table 1. Comparison of neutrophil granules from red seabream *Pagrus major* infected with atypical *Edwardsiella tarda* (=*Edwardsiella anguillarum*)

Staining[*1]	Type of granules and reaction[*2]					Other positive site (shape, number and size)[*2,*5]
	oβG-1[*3]	oβG-2[*3]		iβG[*4]		
		Core	Surrounding	Core	Surrounding	
MGG	Chromophobic	Eosinophilic	Chromophobic	Chromophobic	Chromophobic	
PAS	−	−	−	−	−	G (round or oval, some, ø≤0.3 μm)[*6]; H
PAS-αA	−	−	−	−	−	−
AB (pH1.0)	−	−	−	−	−	−
AB (pH2.5)	−	−	−	−	−	−
TB	−	−	−	−	−	G (amorphous, a few, eq Yb); N; H[*7]
SBB	+	−	+	−	−	−
Sudan III	−	−	−	−	−	−
Oil red O	−	−	−	−	−	−
AlP	−	−	−	+	−	−
AcP	−	+	−	−	−	−
β-Glu	−	+	−	−	−	−
α-NAE	−	+	−	−	−	H
α-NBE	−	+	−	−	−	−
NASDCAE	−	+	−	−	−	−
Peroxidase	+	−	+	−	−	−

[*1]MGG, May-Grünwald·Giemsa; PAS, periodic acid Schiff reaction; PAS-αA, PAS after digestion with α-amylase; AB, alcian blue; TB, toluidine blue in distilled water; SBB, Sudan black B; AlP, alkaline phosphatase; AcP, acid phosphatase; β-Glu, β-glucuronidase; α-NAE, α-naphtyl acetate esterase; α-NBE, α-naphtyl butyrate esterase; NASDCAE, naphthol AS-D chloroacetate esterase.

[*2]oβG-1, ordinary chromophobic granule type 1; oβG-2, ordinary chromophobic granule type 2; iβG, inducible chromophobic granule (induced after infection with atypical *Edwardsiella tarda* (=*Edwardsiella anguillarum*)); G, granular; H, hyaloplasm; N, nucleus; Yb, Yasumoto body; eq, equivalent to; +, positive; −, negative (non-detection).

[*3]There was no difference in the reaction of oβG-1 and oβG-2 between infected and non-infected (control) fishes.

[*4]iβG were found in infected fish only

[*5]No difference in the reaction between infected and control fishes except for PAS and TB.

[*6]The number of PAS-positive granule decreased in infected fish (PAS-positive granule was accumulation of glycogen particles as similar to control because the positive reaction of the granule disappeared after digestion with α-amylase).

[*7]In control fish, hyaloplasm was negative.

granule is chromophobic granule, βG) and has a stratified structure (AlP-positive core and AlP-negative surrounding); (3) AlP-positive granule lacks SBB-positive materials, PO and lysosomal enzymes (acid phosphatase, β-glucuronidase, non-specific esterase (α-naphtyl acetate esterase, α-naphtyl butyrate esterase), specific esterase (naphthol AS-D chloroacetate esterase)) detected in non-infected red seabream neutrophils.

The neutrophils containing AlP-positive granules had small number of PAS-positive granules (representing accumulation of glycogen particles) and those hyaloplasm were TB-positive (Fig. 1F). However, AlP-positive granules were PAS- and TB-negative. The perinuclear halo was stained with safranine O (Fig. 1B & 1C). This structure generally indicates existence of developed Golgi apparatus (GA). The GA will participate to formation of AlP-positive granules.

Toida et al.[2] reported appearance of 'immature leukocytes' in the peripheral blood from red seabream infected with atypical *E. tarda*. The leukocytes had basophilic cytoplasm, perinuclear halo and pale peach granules[2]. The cells likely correspond to the neutrophils with AlP-positive granules described in present report (cytoplasm and pale peach granules may correspond to hyaloplasm and EC of βG-2, respectively). Thus the cells should be regarded as 'activated leukocyte (or activated neutrophil)' rather than 'immature leukocytes'.

We propose to call generally the neutrophil granules observed in non-infected fish 'ordinary granules' (o), in contrast to 'inducible granules' (i): Ordinary granules of red seabream are oβG-1 and oβG. The AlP-positive granules observed in neutrophils from infected fish are called iβG.

References

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病原細菌である非定型*Edwardsiella tarda*（=*Edwardsiella anguillarum*）に感染させたマダイの好中球に，未感染魚では認められない顆粒が観察された。ほとんど全ての好中球にはアルカリ性フォスファターゼ陽性の芯とその周囲の陰性領域を有する顆粒が多数観察された。この顆粒の芯とその周囲はMay-Grünwald・Giemsa染色によって難染性を示すと考えられる。