Cytochemical Characteristics of Neutrophil Granules from Red Seabream *Pagrus major*

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Abstract：Cytochemical characteristics of two types of neutrophil granules, chromophobic granules (βG-1 and βG-2), in red seabream *Pagrus major* were examined. The βG-1 reacted positively to peroxidase and to Sudan black B. Both positive reactions were also observed in the chromophobic area which surrounds eosinophilic core of the βG-2. Lysosomal enzymes such as acid phosphatase, β-glucuronidase, and non-specific and specific esterases were detected in the core of the βG-2. A curious phenomenon, spot formation, was found in/above the βG-2 stained with peroxidase.

Key words：granule, neutrophil, *Pagrus major*, red seabream

Introduction

In previous reports[1-7], only eosinophilic granules have been recognized in the neutrophils of red seabream *Pagrus major* (eosinophil described in Ikeda et al.[1], eosinophilic cell in Watanabe et al.[2], heterophilic granulocyte (heterophil) in Watanabe et al.[3,5], granulocyte in Watanabe et al.[4] and granulocyte in Toida et al.[7] correspond to neutrophils). We have found two types of granules in red seabream neutrophils: Eosinophilic granules (αG) and chromophobic granules (βG)[8]. The former was round to oval (≤0.4 μm in diameter) and contained lysozomal enzymes, whereas the latter was also round to oval (≤0.5 μm in diameter) but react positively to peroxidase (PO) and Sudan black B (SBB)[8].

Recently, it became apparent that the αG was surrounded by a chromophobic area; this finding suggests that the αG itself is not a granule but is instead a core part (eosinophilic core, EC) of βG[9]. We also observed βG without EC. These results indicate that red seabream neutrophils contain two types of βG, namely one without EC (βG-1) and the other with EC (βG-2)[9]. Both types of granules react positively to peroxidase PO, but EC of βG-2 was negative[9]. Here, we report cytochemical characteristics of neutrophil granules in red seabream.

Materials and Methods

The fish used in this study were one-year-old red seabream (approx. 200 g body weight) and reared in National Fisheries University, at 25℃ and fed commercial diet (Marine No. 6, Hayashikane Sangyo Co., Ltd) *ad libitum*. Blood smears on slides were stained with May-Grünwald・Giemsa, acid phosphatase (AcP), β-glucuronidase (β-Glu), α-naphtyl acetate esterase (α-NAE), α-naphtyl butyrate esterase (α-NBE), naphthol AS-D chloroacetate esterase (NASDCAE), PO and SBB, as described previously[10]. Intact and lysed neutrophils were observed under a light microscope.

Results and Discussion

All tested lysosomal enzymes except for α-NAE, showed a granular positive reaction in intact neutrophil (Fig. 1, Table 1). The activity of α-NAE was observed in the cytoplasm with diffuse and granular reaction. This diffuse positive site corresponds to hyaloplasm. The number of positive granules was different among tested enzymes (many: AcP, α-NBE, NASDCAE. some: α-NAE. a few: β-Glu). However, positive granules were similar to EC of βG-2 in shape (round or oval) and size (≤0.4 μm). Furthermore, negative area was detected around the

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positive granules in the lysed neutrophil. These findings indicate that the core of βG-2 contains lysosomal enzymes.

PO-positive reaction was limited to the granules in intact and lysed neutrophils (Fig. 1, Table 1). The positive granules were classified into two types as described by Kondo et al.[9]; one showed positive reaction in the entire granule, and other in part: Surrounding of the negative core (hereafter called surrounding) was PO-positive. These positive granules may correspond to βG-1 and βG-2, respectively. PO-negative granule was not found in lysed neutrophils. A curious phenomenon, spot formation, was observed in/above the PO-stained βG-2: The βG-2 was out of focus in that focus. Strong positive (dark brown) and round spot was overlapped in PO-negative core of βG-2 in intact and lysed neutrophils. The diameter of the spot was smaller than that of the core. This phenomenon may be explained by lens-like action of the core; namely, light passed through PO-positive surrounding could be condensed by the core into spot. If the spot is an entity, it would be found not only above but also beside the core. However, the spot was never detected beside the core. This result suggests that the spot could not be entity, but an image optically generated.

The SBB-positive granules in intact neutrophil were classified into two types based on the presence or absence of negative core. Each SBB-positive granule seems to be βG-1 and βG-2, respectively (Fig. 1, Table 1). Spot formation was not recognized in SBB-positive granules. Unfortunately, no positive granules were detected in lysed neutrophils. In process of SBB stain, overstained preparation was rinsed with ethanol to wash off surplus SBB dye. The granules of lysed neutrophil were localized out of cell membrane. Therefore, SBB dye in the granules of lysed neutrophil would have disappeared more rapidly than that in the granules of intact neutrophil.

Watanabe et al.[2-4] also reported that the neutrophil granules were PO- and SBB- positive, but negative core in the these positive granule and spot formation described above were not observed. Furthermore, they failed to detect the esterase activity in the neutrophil[4].

It became evident from the present study that the βG-1 and the surrounding of βG-2 were PO- and SBB-positive, and the core of βG-2 contained several lysosomal enzymes. It has not escaped our notice that the cytochemical similarity between βG-1 and surrounding of βG-2 suggests a granule maturation from βG-1 to βG-2: The βG-1 appear first; later, lysosomal enzymes add to βG-1; finally, lysosomal enzymes would have spontaneously condensed in the βG-1 into core. If this is the actual case, almost all granules of mature neutrophils from red seabream should be βG-2.

Table 1. Summary of reactions of red seabream neutrophil to cytochemical tests (modified from Kondo et al.[8])

Test	Type of granules and reaction			Other positive site (shape, number and size)
	βG-1	βG-2		
		Core	Surrounding	
Periodic acid Schiff reaction (PAS)	−	−	−	G (round or oval, many, ø≤0.3 μm)*; H
PAS after digestion with α-amylase	−	−	−	−
Alcian blue (pH1.0)	−	−	−	−
Alcian blue (pH2.5)	−	−	−	−
Toluidine blue (distilled water)	−	−	−	G (amorphous, a few, eq Yb); N
Sudan black B	+	−	+	−
SudanⅢ	−	−	−	−
Oil red O	−	−	−	−
Alkaline phosphatase	−	−	−	−
Acid phosphatase	−	+	−	−
β-Glucuronidase	−	+ (a few)	−	−
α-Naphtyl acetate esterase	−	+ (some)	−	H
α-Naphtyl butyrate esterase	−	+	−	−
Naphthol AS-D chloroacetate esterase	−	+	−	−
Peroxidase	+	−	+	−

βG-1, chromophobic granule type 1; βG-2, chromophobic granule type 2 (consisted with eosinophilic core and chromophobic surrounding); G, granular; H, hyaloplasm; N, nucleus; Yb, Yasumoto body; eq, equivalent to; +, positive; −, negative (non-detection).

*PAS-positive granule was accumulation of glycogen particles because the positive reaction of the granule disappeared after digestion with α-amylase.

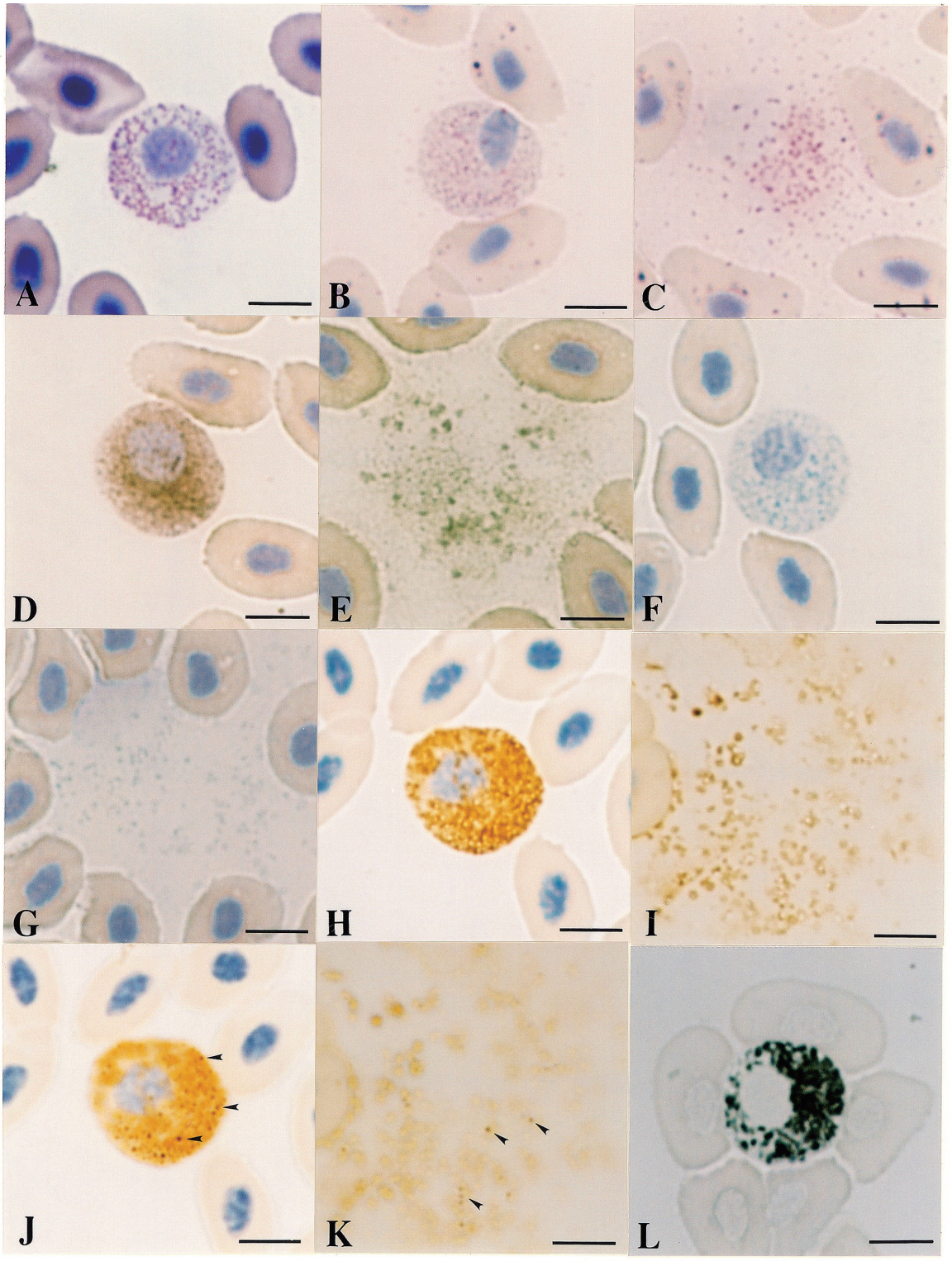

Fig. 1. Cytochemistry of red seabream neutrophil. A, May-Grünwald・Giemsa (intact cell); B & C, acid phosphatase (B, intact cell; C, lysed cell); D & E, α-naphtyl acetate esterase (α-NAE; D, intact cell; E, lysed cell); F & G, naphthol AS-D chloroacetate esterase (F, intact cell; G, lysed cell); H & I, peroxidase (H, intact cell; I, lysed cell); J & K, peroxidase (same cells in H & I with different focus); L, Sudan black B (intact cell). All enzymes except for α-NAE and peroxidase, were detected in the core of βG-2 only. Activity of α-NAE was localized not only in the core of βG-2 but also in the hyaloplasm. The βG-1 and surrounding of βG-2 were positively to peroxidase and Sudan black B. Note spot formation in J & K (arrowheads). Bars=5 μm.

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マダイの好中球顆粒の細胞化学的特徴

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マダイの好中球に存在する２種類の難染性顆粒の細胞化学的特徴を調べた。エオシン好性の芯を欠く顆粒（βG-1）と芯を有する顆粒（βG-2）のどちらも難染性領域（βG-1では顆粒全体，βG-2では芯の周囲）がペルオキシダーゼおよびSudan black B陽性であった。種々のライソゾーム酵素はβG-2の芯に検出された。顕微鏡の焦点をペルオキシダーゼ染色されたβG-2からその上方に移すと，芯に重なるように強陽性を示す斑が形成された。斑形成は芯のレンズ様作用によるものであり，斑に実体はなく，このレンズ様構造で見える光学的な像であると考えられた。