

Distributive and Phagocytic Characteristics of Hepatic Macrophages in Five Cetaceans belonging to Delphinidae and Ziphiidae

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(Received 15 October 2003/Accepted 19 January 2004)

ABSTRACT. Details of morphology and distribution of hepatic macrophages in cetaceans were investigated using the immunohistochemistry with an antibody (SRA-E5) generated against human macrophage scavenger receptor antigen. Liver samples were obtained from five species of cetaceans (Baird's beaked whales, short-finned pilot whales, Risso's dolphins, bottlenose dolphins, and pantropical spotted dolphins). Except for two species of whales, the number of SRA-E5-positive Kupffer cells was greatest in the perivenous zone (zone 3), followed by the mid-zonal (zone 2) and periportal (zone 1) zones; this distribution pattern was different from that in cattle examined here and previously reported rodents with the highest number in zone 1. The frequency of Kupffer cell in each of zones was significantly different among species, and interestingly, the total mean of the Kupffer cell number in three zones increased as the body-length of species was small. In cetaceans, Kupffer cells in zone 1 appeared larger and more stellate in shape, whereas those in zone 3 were smaller and rounder. All cetaceans but Baird's beaked whales had the black pigment-containing Kupffer cells, with the greatest number in zone 3, and macrophages with the similar pigments were also seen in the hepatic intermediate septa, indicating an active phagocytosis. Most of the black pigments were considered to be lipofuscin and such pigments were not seen in the bovine livers. These results indicate that cetacean hepatic macrophages show differences in the distribution and phagocytosis among hepatic lobular zones, or between cetacean species and terrestrial animals.

KEY WORDS: cetacean, Kupffer cell, lipofuscin, phagocytosis, zonal distribution.

J. Vet. Med. Sci. 66(6): 671–680, 2004

Kupffer cells present in the liver, that is one of major organs of the metabolism, are one of representative resident macrophages, and are known to play important roles in the pathophysiology or the maintenance of the homeostasis [18]. They are located along the hepatic sinusoids, and have a close contact with the blood stream to play active phagocytosis or pinocytosis [14]. The zonal heterogeneity of the distribution, cellular morphology and functions has been reported in Kupffer cells of rodents [1, 5, 22]. Kupffer cells of rats are predominantly present in the periportal zone of the hepatic lobules to monitor the blood entering into the liver, and the periportal Kupffer cells are larger in size and exhibit greater lysosomal enzyme activities as compared with the mid-zonal and perivenous Kupffer cells [22]. Generally, it has been considered in rats that large-sized Kupffer cells in the periportal zone show the greater latex bead phagocytic activity, whereas small-sized cells in the perivenous zone exhibit more active Ia antigen expression and cytokine production such as interleukin-1 in relation to immune response [5].

Cetaceans are the representative mammals that have been entirely adaptive in the aquatic environment. Some investigators have been interested in the immune system of marine mammals, because such animals may be exposed to environmental contaminants and infectious agents [12, 19]. Pre-

viously, we reported that the distribution of resident macrophages of whole body in short-finned pilot whales and Risso's dolphins was similar fundamentally to that in the terrestrial mammals [11]. However, detailed distribution and morphology of hepatic macrophages remained to be investigated. The aim of the present study is to establish the basic information on the distributive and phagocytic characteristics of hepatic macrophages including Kupffer cells under the physiological condition in five species of cetaceans.

MATERIALS AND METHODS

Tissue preparation: Liver samples were obtained from five species in two families of *Odontoceti*, short-finned pilot whale (*Globicephala macrorhynchus*, n=9), Risso's dolphin (*Grampus griseus*, n=10), bottlenose dolphin (*Tursiops truncatus*, n=4), pantropical spotted dolphin (*Stenella attenuata*, n=5) and Baird's beaked whale (*Berardius bairdii*, n=3) (Table 1); these samples were provided for our study by National Research Institute of Far Seas Fisheries, Japan. These animals were caught for the purpose of fisheries by small type whaling or hand harpoon fishery off the coast at Wakayama or Chiba prefecture in 2002, Japan. The age class (adult or immature) of animals was estimated on the basis of standard length measurements [6–10, 13]. Liver samples from all animals were fixed in 10% neutral buffered formalin and embedded in paraffin. For the light-microscopy, 3–5 µm-thick sections were stained with hematoxylin-

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Table 1. Information of animals and evaluation of black pigments in hepatic macrophages and pathological finding in the liver

Species	No.	Sex	Body- Length (m)	Percentage (%) of Kupffer cells containing black pigments				Area (μm^2) of black pigments in macro- phages of the hepatic intermediate septa	Histopathological findings in the liver
				Zone 1	Zone 2	Zone 3	Mean		
BB	1	M	9.62	0.00	0.00	0.00	0.00	0.00	
	2	F	10.19	0.00	0.00	0.00	0.00	0.00	
	3	M	9.69	0.00	0.00	0.00	0.00	0.00	
GM	1	M	4.75	5.22	16.20	18.52	13.31	29.12	Centrilobular congestion, fibrosis and vacuolar degeneration
	2	M	5.11	5.08	26.60	25.86	19.18	98.91	Centrilobular congestion and vacuolar degeneration, Trematodes in bile ducts
	3	F	3.65	17.53	25.22	51.55	31.43	200.89	Centrilobular congestion and vacuolar degeneration
	4	M	4.99	52.12	50.26	75.56	59.31	82.91	
	5	M	5.20	3.24	10.28	20.37	11.30	15.55	Centrilobular congestion and vacuolar degeneration
	6	M	4.81	1.53	5.36	22.83	9.90	11.21	Centrilobular congestion, fibrosis and vacuolar degeneration, Trematodes in bile ducts
	7	M	5.27	2.68	2.94	16.39	7.34	21.86	
	8	M	4.57	10.53	10.00	36.11	18.88	73.32	
	9	M	4.47	1.65	8.07	36.55	15.42	125.03	
GG	1	M	2.94	5.35	14.72	38.88	19.65	176.89	
	2 ^{a)}	M	2.47	1.90	0.57	1.50	1.32	0.65	
	3	F	2.94	3.81	14.84	42.38	20.34	200.93	
	4	F	2.72	2.61	9.74	6.18	6.18	5.82	
	5	M	2.79	8.75	8.02	36.56	17.78	83.78	Trematodes in bile ducts
	6 ^{a)}	M	2.58	0.00	1.41	10.26	3.89	3.39	Trematodes in bile ducts
	7	M	2.89	4.67	7.93	24.73	12.44	184.51	Trematodes in bile ducts
	8	M	2.67	2.68	3.59	20.00	8.76	122.22	
	9	M	2.83	2.86	8.26	12.94	8.02	83.31	Trematodes in bile ducts
	10	F	2.66	0.00	0.68	6.80	2.49	2.27	Trematodes in bile ducts
TT	1 ^{a)}	M	2.37	0.98	3.18	6.09	3.42	0.60	
	2 ^{a)}	F	2.61	10.64	5.65	21.97	12.76	2.25	Centrilobular fibrosis
	3 ^{a)}	F	2.52	0.00	0.00	0.00	0.00	0.00	
	4	F	3.00	7.73	26.60	43.72	26.02	105.52	
SA	1 ^{a)}	F	1.72	0.00	3.78	5.00	2.93	1.02	
	2 ^{a)}	M	1.82	0.00	2.47	0.56	1.01	0.00	Trematodes in bile ducts
	3	M	1.99	1.89	6.81	23.77	10.82	32.36	Trematodes in bile ducts
	4 ^{a)}	F	1.70	0.00	0.00	11.27	3.76	0.02	Trematodes in bile ducts
	5 ^{a)}	M	1.83	0.00	0.50	2.91	1.14	1.68	Trematodes in bile ducts

BB, Baird's beaked whales (*Berardius bairdii*). GM, short-finned pilot whales (*Globicephala macrorhynchus*). GG, Risso's dolphins (*Grampus griseus*). TT, bottlenose dolphins (*Tursiops truncatus*). SA, pantropical spotted dolphins (*Stenella attenuata*). M, male, F, female. a), sexually immature animal.

eosin (H-E) stain, Berlin blue stain, Schmorl method, Ziehl-Neelsen method and Periodic acid Schiff (PAS) reaction.

Immunohistochemistry: A monoclonal antibody (SRA-E5; CD204) against human macrophage scavenger receptor (MSR) type I protein antigen was used to detect cetacean hepatic macrophages. Immunoglobulin subclass of SRA-E5 was IgG1, kappa, and culture supernatant of the hybridoma was used in this study [26, 27, 29]. Non-immunized mouse serum served as negative control. After pre-treatment with autoclave in 10 mM citrate buffer, tissue sections were immersed in 3% H_2O_2 to quench endogenous peroxidase and incubated with 5% skimmed milk for 40 min at room temperature to inhibit nonspecific reactions. Then, sections were incubated with the primary antibody for 14–18 hr at 4°C and immunolabeled by the Avidin-Biotin Complex (ABC) method (VECTASTAIN UNIVERSAL Elite ABC KIT; Vector Laboratories., Burlingame, CA, U.S.A.). Positive reactions were visualized brown with 3,3'-diaminoben-

zidine (DAB). Sections were counterstained lightly with hematoxylin. Liver samples collected from necropsied cases of cattle (four adult Holsteins) in our laboratory were also examined as a representative of terrestrial mammals, because the cetaceans branched off the ancestor common to the ruminant (such as cattle) and returned to the ocean about 50–60 million years ago [23].

Kupffer cell distribution and statistical analyses: SRA-E5 positive nucleated cells were counted in 30 randomly selected fields (0.21×0.16 mm) of different 3 zones of hepatic lobules: the periportal (zone 1), mid-zonal (zone 2) and the perivenous (zone 3) zones. The mean \pm standard error (S.E.) was calculated in each zone or totally in zones 1, 2, and 3. Statistically, after population variances were recognized to be equal by the Bartlett test, the one-factor analyses of variance (ANOVA) were used to test the difference of values among cetacean species or three zones. Additionally, after the equality of population variances was made

sure by F test, Student's *t*-test was performed to compare values between cetaceans and cattle. To evaluate the correlation between the total mean of Kupffer cell number in three zones and the mean body-length of each species, the correlation coefficient was calculated by Pearson's correlation coefficient test. Values of $p < 0.05$ were considered significant.

Hepatic macrophages-englobing black pigments and statistical analyses: Since black pigments-engulfing macrophages have been observed in the liver of the cetaceans [20, 21], the distribution of Kupffer cells having such pigments in the cytoplasm was evaluated in 10 randomly selected fields (0.21×0.16 mm) of each zone of the hepatic lobules. The evaluation was regarded as an indicator of phagocytic functions of hepatic macrophages. The mean \pm S.E. of percentage of pigment-containing Kupffer cells was calculated in each animal or each zone of each species. Kruskal-Wallis test was used to examine the difference of values among zones. For the evaluation of amount of black pigments, the areas (μm^2) of the pigments in macrophages of the hepatic intermediate septa were measured with a Color Image Analyzer (Mac SCOPE; Mitani Inc., Japan) in 10 randomly selected intermediate septa. The mean \pm S.E. was calculated in each animal. To examine the relationship of black pigment-containing Kupffer cells with the amount of black pigments in interlobular macrophages, the correlation coefficient was calculated between the percentage of Kupffer cells-containing the pigments and pigment areas (μm^2) in interlobular macrophages. Statistic used was either Pearson's correlation coefficient test or Spearman's correlation coefficient by rank test. Values of $p < 0.05$ were considered significant.

Transmission electron microscopic and fluorescent

microscopic observations of Kupffer cells: The formalin-fixed liver tissue pieces were post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin. One μm -thick sections were stained with toluidine blue for detail light microscopic observation. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with an electron microscope (Hitachi, H7500).

Sections stained with H-E to be examined for the autofluorescence of lipofuscin by fluorescent microscopy were used.

RESULTS

Pathological findings in livers: There were no marked gross findings in livers of animals examined here. Some animals showed several histopathological findings, as shown in Table 1, such as trematode infection in larger bile ducts with slight pericholangitis, fibrosis and vacuolar degeneration of hepatocytes, but there were not significant findings.

Immunohistochemical reactivities for SRA-E5: Hepatic macrophages of all species examined here were immunolabeled with SRA-E5 (Figs. 1a-c). The positive cells were located along the sinusoids of the hepatic lobules and in the hepatic interlobular septa (Fig. 1a). The cells in the hepatic lobules showed various configurations such as round or stellate, indicative of Kupffer cells. The positive reactions to SRA-E5 in cetacean Kupffer cells were stronger all over in the cytoplasm (Fig. 2a), as compared with bovine Kupffer cells showing punctuated reactions (Fig. 2b).

Morphological characteristics and phagocytosis of hepatic macrophages: The majority of Kupffer cells in the

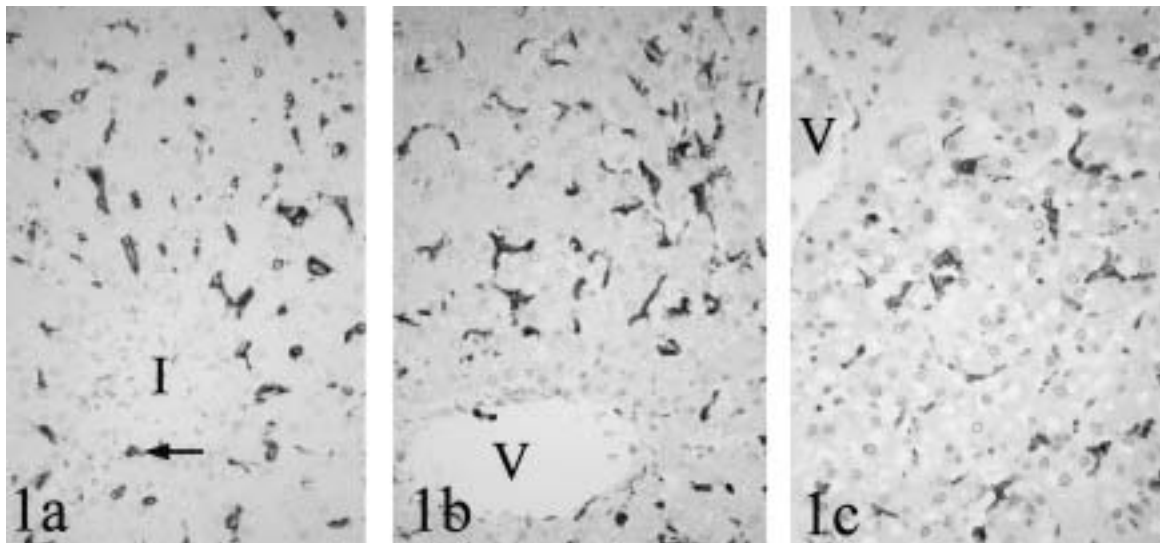


Fig. 1. Immunohistochemistry for SRA-E5 in the livers of a bottlenose dolphin (a), pantropical spotted dolphin (b) and Baird's beaked whale (c). Hepatic macrophages of these species are immunolabeled by SRA-E5. Most positive cells are located along the sinusoids of the hepatic lobules and show various configurations such as round or stellate, indicative of Kupffer cells. Positive cells are also located in the hepatic intermediate septa (I) (a, arrow). The frequency of Kupffer cells in dolphins (b) is higher than that in whales (c). V, the central vein. a, b and c, $\times 200$. Counterstained with hematoxylin.

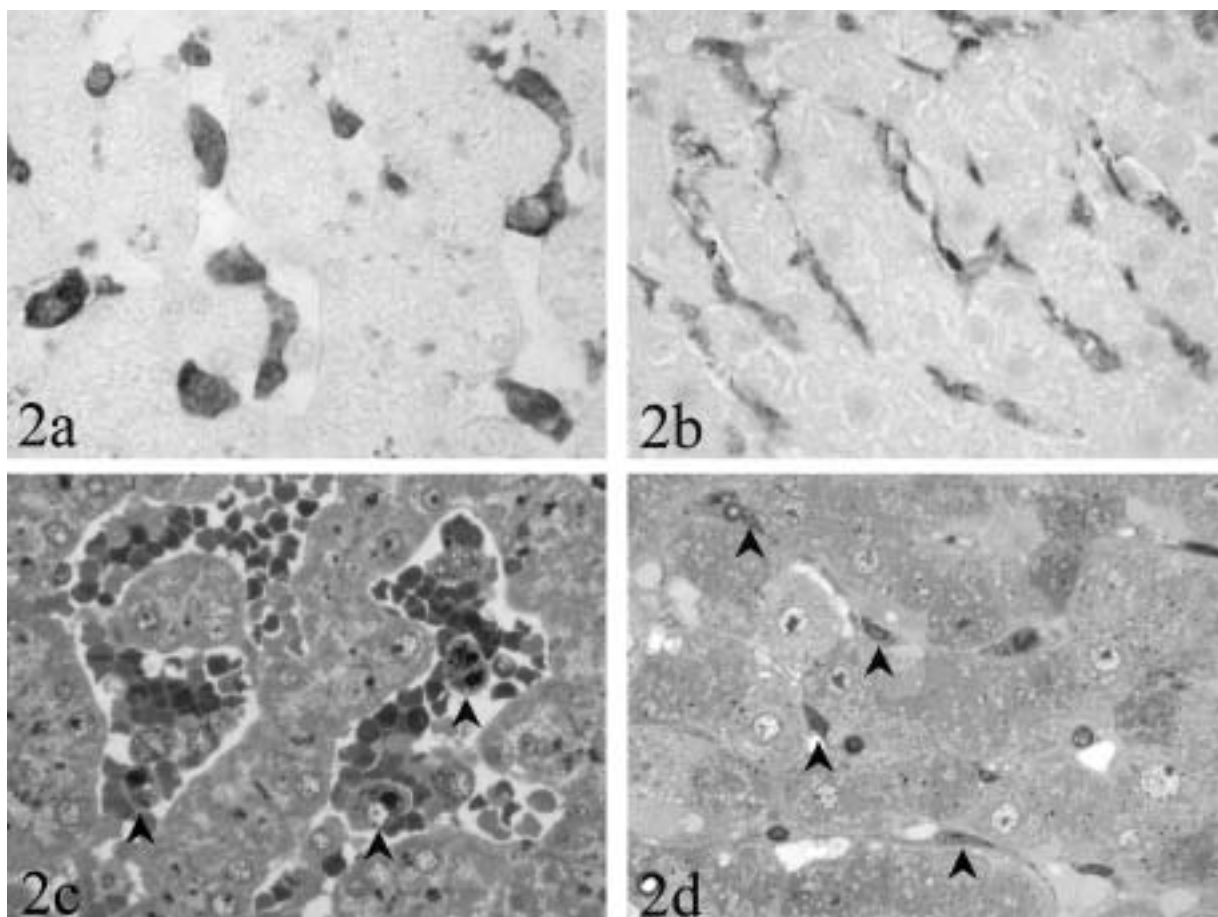


Fig. 2. Cellular morphology of Kupffer cells in the perivenous zone (zone 3) of a short-finned pilot whale (a, c) and cattle (b, d). Kupffer cells of short-finned pilot whales exhibit round or stellate shapes with abundant cytoplasm (a, c), comparing to bovine Kupffer cells showing spindle or stellate in shape (b, d), and the SRA-E5 positive reactions in cetacean Kupffer cells are seen all over in the cytoplasm (a), whereas those in bovine Kupffer cells is punctuated (b). Rounder Kupffer cells in zone 3 of short-finned pilot whales (c, arrowheads) show active phagocytosis, as indicated by containing black pigments or erythrocytes in cytoplasm. Spindle Kupffer cells in zone 3 of cattle (d, arrowheads) contain no black pigments. a, b, c and d, $\times 400$. a and b, counterstained with hematoxylin. c and d, toluidine blue stain.

cetaceans exhibited round-shape and had abundant cytoplasm (Fig. 2c), whereas those in cattle were spindle or stellate in shape with scanty cytoplasm (Fig. 2d). In cetaceans examined, generally, Kupffer cells in zone 1 appeared to be larger and more stellate (Fig. 3a), as opposed to those in zone 3 showing small-round in shape (Fig. 3b); especially, such morphological characteristics were prominent in short-finned pilot whales and bottlenose dolphins.

As reported in the terrestrial mammals [14], some Kupffer cells contained vacuoles and erythrocytes within cytoplasm, indicating phagocytic activity. Most characteristic bodies within hepatic macrophages of cetaceans were black pigments (Figs. 4a, 4b). The frequency of pigment-possessing Kupffer cells varied among zones of hepatic lobules, individuals and species (Table 1). Short-finned pilot whales showed the highest frequency (21%) in the total of the pigment-containing Kupffer cells (Fig. 5a); an animal (GM No. 4) exhibited greatest frequency at 59% (Table 1).

Risso's dolphins and bottlenose dolphins had pigments-containing Kupffer cells at 10% frequency, and in pantropical spotted dolphins it was about 4% (Table 1). The frequency of these Kupffer cells in each species was greatest in zone 3 among three zones (Fig. 6), with statistically significant difference in short-finned pilot whales, Risso's dolphins, and pantropical spotted dolphins. Aggregations of macrophages with black pigments were often observed in the hepatic intermediate septa (Fig. 4b), and the amount of pigments in this area was most abundant in Risso's dolphins ($86 \mu\text{m}^2$), and then short-finned pilot whales ($73 \mu\text{m}^2$) (Fig. 5b). Interestingly, in Risso's dolphins, the aggregations of macrophages swelled with pigments were also present around the central veins (Fig. 4a). Macrophages with black pigments sometimes floated in the central veins. As shown in Fig. 7, there was a significant positive correlation in three species (short-finned pilot whales (Fig. 7a), Risso's dolphins (Fig. 7b), and pantropical spotted dolphins (Fig. 7d)) between the

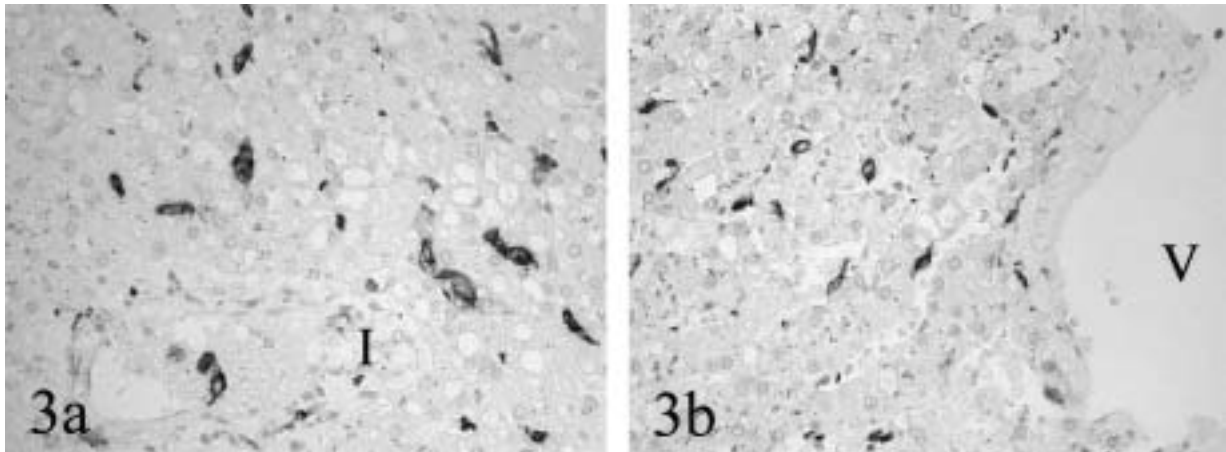


Fig. 3. Immunohistochemistry for SRA-E5 in the livers of a short-finned pilot whale. Kupffer cells in the periportal zone (zone 1) appear to be larger and more stellate (a), while those in the perivenous zone (zone 3) are smaller and rounder in shape (b). I, the hepatic intermediate septa. V, the central vein. a and b, $\times 200$. Counterstained with hematoxylin.

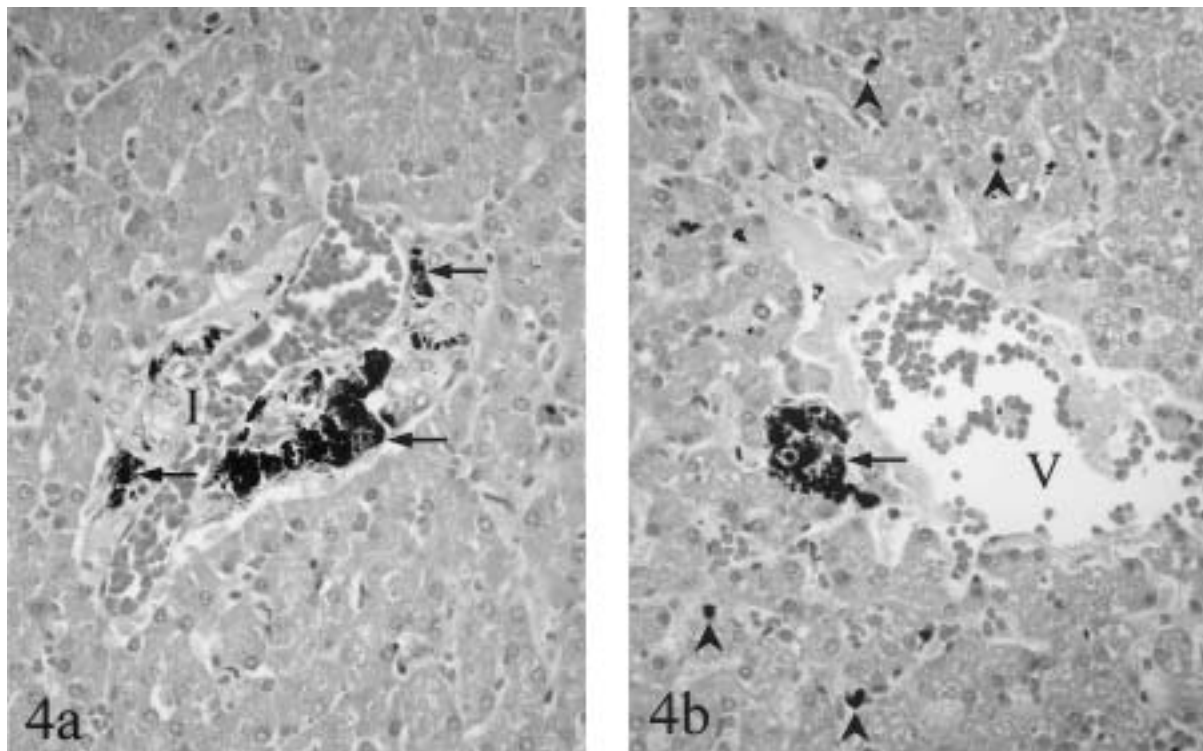


Fig. 4. The deposition of black pigments in hepatic macrophages in a Risso's dolphin. (a) Black pigments are not seen in Kupffer cells in the periportal zone (zone 1), but interlobular macrophages with black pigments are scattered or aggregated in the hepatic intermediate septum (I) (arrows). (b) Black pigment-containing Kupffer cells are located in the hepatic sinusoids of the perivenous zone (zone 3) (arrowheads); aggregations of macrophages englobed black pigments are also present around the central vein (V) (arrows). a and b, $\times 300$. Hematoxylin-eosin stain.

percentage of Kupffer cells with the pigments and the amount of pigments in interlobular macrophages. Because younger cases of Risso's dolphin, bottlenose dolphin and pantropical spotted dolphin showed small accumulation of pigments within macrophages, the individual difference of black pigment's deposition might be related with the age of animals (Table 1). No hepatic macrophages

with black pigments were seen in the liver of Baird's beaked whales (Table 1).

Besides black pigments, hepatic macrophages also contained yellow pigments. The black pigments were stained blue or dark blue by Schmorl method and not stained by Ziehl-Neelsen method. The yellow pigments were stained blue or blue-green by Schmorl method, and some parts of

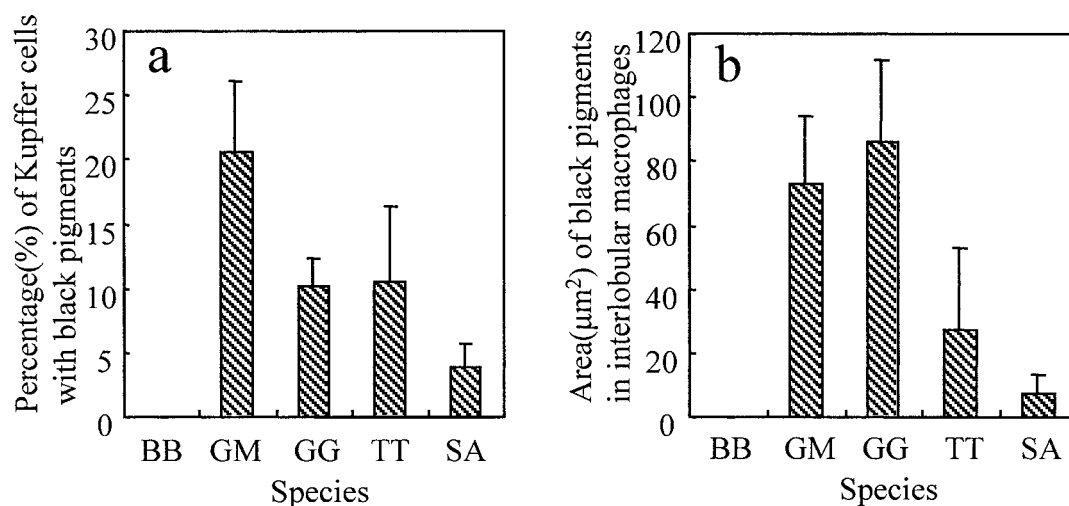


Fig. 5. Evaluation of black pigments in hepatic macrophages of each species of the cetaceans examined. (a) The percentage (%) of Kupffer cells with black pigments in three zones of the hepatic lobules. (b) The area (μm^2) of black pigments in interlobular macrophages of the hepatic intermediate septa. Values are means \pm S.E. BB, Baird's beaked whales. GM, short-finned pilot whales. GG, Risso's dolphins. TT, bottlenose dolphins. SA, pantropical spotted dolphins.

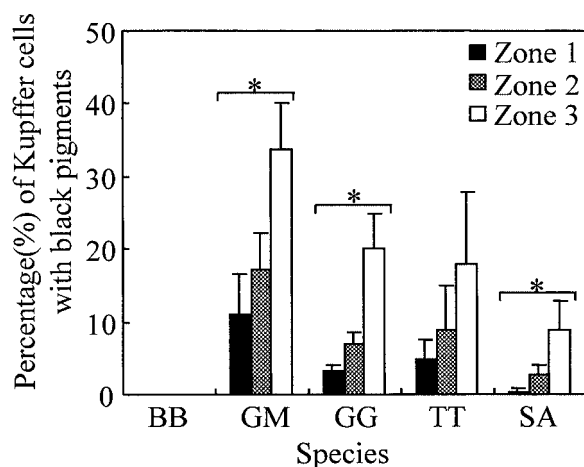


Fig. 6. The percentage (%) of black pigment-englobing Kupffer cells in each zone of the hepatic lobules of cetacean species. The frequency of pigment-containing Kupffer cells in each species is greatest in zone 3. Zone 1, periportal zone; Zone 2, mid-zonal zone; Zone 3, perivenous zone. * significantly different among zones by the Kruskal-Wallis test ($p < 0.05$). Values are mean \pm S.E. BB, Baird's beaked whales. GM, short-finned pilot whales. GG, Risso's dolphins. TT, bottlenose dolphins. SA, pantropical spotted dolphins.

which were stained dark red with Ziehl-Neelsen method and red with PAS reaction, indicating the presence of lipofuscin. Yellow lipofuscin deposits were also commonly observed in hepatocytes, especially in zone 3. In animals showing the high frequency of black pigments-containing macrophages, yellow lipofuscin tended to also deposit in the surrounding hepatocytes. The Berlin blue stain failed to react to the black pigments in hepatic macrophages, denying the pres-

ence of hemosiderin. Bovine hepatic macrophages had neither black pigments nor marked yellow pigments. Electron microscopy revealed that extremely large amounts of electron-dense homogenous or granular materials were present presumably within lysosomes of Kupffer cells; these appeared to be black pigments by light microscopy (Fig. 9). Most of dark pigments in Kupffer cells of Risso's dolphins showed autofluorescence, but some pigments did not (Fig. 10).

Distribution of Kupffer cells in the hepatic lobules: Statistically, the number of SRA-E5-positive Kupffer cells in three zones showed significant differences among cetacean species by the one-factor ANOVA (Table 2). The total mean of the SRA-E5-positive Kupffer cells in three zones was greatest in pantropical spotted dolphins (Fig. 1b), and smallest in Baird's beaked whales (Fig. 1c). Pearson's correlation coefficient test revealed that there was a significant negative correlation between the total mean of Kupffer cell number and the mean body-length of each species (Fig. 8).

In comparison among three zones, the one-factor ANOVA showed the significant difference only in pantropical spotted dolphins which were smallest species of cetaceans examined in this study; the Kupffer cell number was largest in zone 3 (Table 2). The Kupffer cell number of other two dolphins also tended to be greater in zone 3 than in zone 1, although there was no statistical significance. In comparison with cattle whose Kupffer cells did not show a significant difference among three zones, the total mean of Kupffer cell number in each of dolphins was significantly greater than that in cattle (Table 2). The Kupffer cell number in zone 3 of Risso's dolphins and in three zones of bottlenose dolphins and pantropical spotted dolphins were significantly greater than that of corresponding zones in cattle (Table 2).

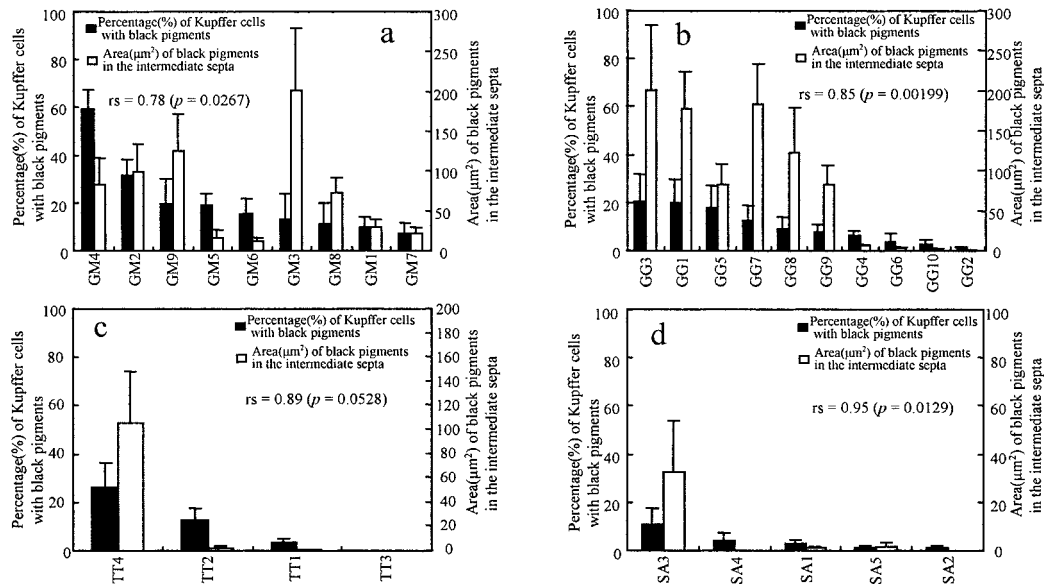


Fig. 7. The relationship between the percentage (%) of Kupffer cells containing black pigments and area (μm^2) of black pigments in the intermediate septa in short-finned pilot whales (a), Risso's dolphins (b), bottlenose dolphins (c) and pantropical spotted dolphins (d). Correlation coefficients (r_s) were calculated by the Pearson's correlation coefficient test or the Spearman's correlation coefficient by rank test. Significant positive correlations ($p < 0.05$) are recognized in three species (short-finned pilot whales ($r_s = 0.78$), Risso's dolphins ($r_s = 0.85$) and pantropical spotted dolphins ($r_s = 0.95$)). Values are mean \pm S.E. BB, Baird's beaked whales. GM, short-finned pilot whales. GG, Risso's dolphins. TT, bottlenose dolphins. SA, pantropical spotted dolphins.

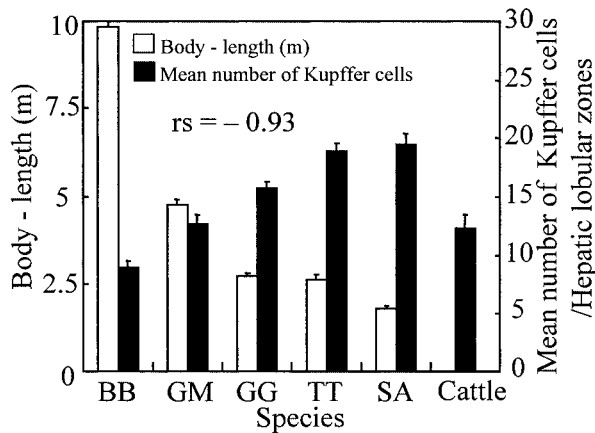


Fig. 8. The relationship between the mean body-length and the total mean of SRA-E5-positive Kupffer cell number. Pearson's correlation coefficient test showed a significant negative correlation between the total mean of Kupffer cell number and the mean body-length of each species. ($r_s = -0.93$, $p < 0.05$). Values are means \pm S.E. BB, Baird's beaked whales. GM, short-finned pilot whales. GG, Risso's dolphins. TT, bottlenose dolphins. SA, pantropical spotted dolphins.

DISCUSSION

Histopathological findings in livers: Histopathological changes observed in the livers have been reported as common findings of small cetaceans [2, 16, 24], but such find-

ings in the present cases were not considered to affect clinical conditions. The features of parasites in larger bile ducts were consistent with those of *Campylobacter* spp. [31].

Immunohistochemical reactivity for SRA-E5 in the cetacean livers: Since the MSR is implicated to play important roles in scavenging foreign bodies or waste products, the SRA-E5 is a useful marker to detect Kupffer cells and to evaluate their endocytosis [4, 27]. We have previously reported that macrophages in short-finned pilot whales and Risso's dolphins could be detected with SRA-E5 [11]. In this study, the immunohistochemistry with SRA-E5 showed the cross-reactivity to other three species of cetaceans in Delphinidae (bottlenose dolphin and pantropical spotted dolphin) and Ziphiidae (Baird's beaked whale). The MSRs are membrane-associated receptors, and they are internalized through coated pits and small vesicles and transferred to endosomes [17]. The positive reactions to SRA-E5 were seen diffusely all over in the cytoplasm of Kupffer cells in cetaceans (Fig. 2a), whereas those of bovine Kupffer cells was punctuated (Fig. 2b). These findings may indicate that cetacean Kupffer cells show higher expression of MSRs on and in the cellular membrane and have a greater phagocytic activity than bovine Kupffer cells.

Nature of black and yellow pigments in hepatic macrophages: In the present study, the most prominent materials engulfed by hepatic macrophages of cetaceans were black and yellow pigments. Such black pigments have not been observed in the terrestrial mammals such as cattle, monkeys, dogs and rodents. Our investigations showed that the major

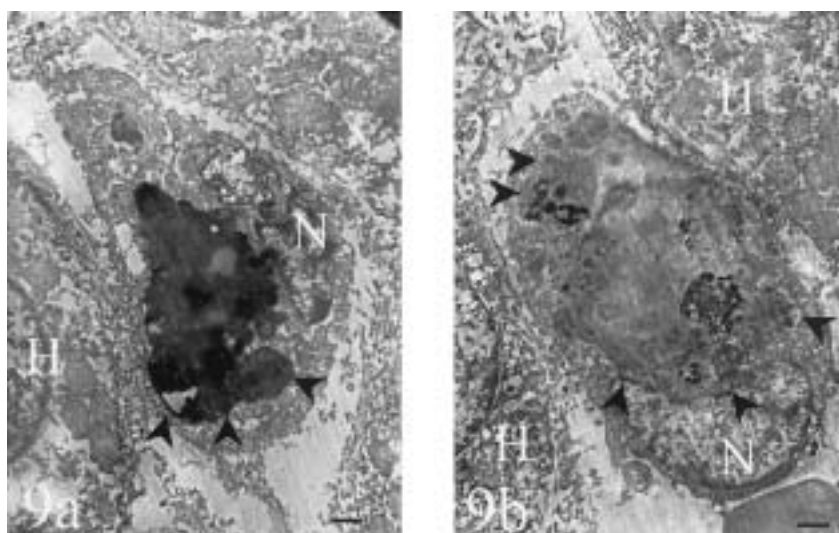


Fig. 9. The electron microscopic observation of Kupffer cells in zone 3 of short-finned pilot whale. Large amounts of electron dense homogeneous (a) or granular (b) materials (arrow-heads) within lysosomes are observed in their cytoplasm. H, the hepatocyte. Bar=1 μ m.

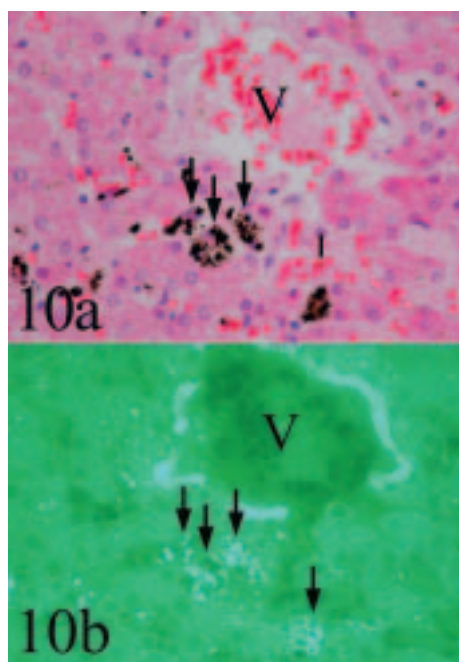


Fig. 10. The fluorescent microscopic observation of black pigments in Kupffer cells of Risso's dolphin. Most of black pigments observed in Kupffer cells of a section stained by hematoxylin-eosin stain (a: arrows) show autofluorescence (b: arrows). V, the central vein. a and b, $\times 300$.

component of these pigments were lipofuscin but not ceroid, because they showed weak acid-fast nature. The fine structures of dark pigments by electron microscopy were also consistent with those of lipofuscin [28, 33].

Accumulation of black pigments in hepatic macrophages:

The excessive pigment accumulation in the cetacean liver has been already reported. Rawson *et al.* [20,21] reported that Atlantic bottlenose dolphins stranded along the coast of Florida revealed abundant dark lipofuscin pigments in the hepatic intermediate septa, which were associated with the accumulation of mercuric selenide. Because these dolphins with rich hepatic pigments exhibited active hepatic disease, they suggested that excessive pigment accumulation might be related to toxic effects of mercury. But, Woshner *et al.* [32] reported that because mercury and lipofuscin, both which increased with age, were not colocalized in light microscopic liver sections, mercury was not a crucial factor for hepatic lipofuscin deposition in beluga (*Delphinapterus leucas*). They also reported that hepatic lipofuscin was rich in the liver of bowhead whale (*Balaena mysticetus*) and young beluga with low hepatic mercury concentrations. In our study, excessive dark pigments were also seen in hepatic macrophages of four species, but significant pathological changes could not be recognized, hence it was considered that the accumulation of lipofuscin pigments in hepatic macrophages of cetaceans was not abnormal change but physiological finding.

In our study, because hepatocytes with rich yellow lipofuscin were seen in association with the appearance of pigment-containing hepatic macrophages, the development of these lipofuscin pigments might be related to the metabolism of hepatocytes. Lipofuscin is commonly formed as a result of oxidative stress and intracellular damage; this may be related to aging and some causes such as anti-oxidant deficiencies, leading to the accumulation of lipofuscin in hepatocytes, mainly of zone 3 [32]. Such conditions might be responsible for the increased number of Kupffer cells with black pigments. Because antecedent materials such as

Table 2. The distribution of Kupffer cells detected by the immunohistochemistry with SRA-E5

Species	Zone 1*	Zone 2*	Zone 3*	Total mean
BB	8.31 \pm 0.53 ^{a)}	9.26 \pm 0.78	8.99 \pm 0.82	8.31 \pm 0.52
GM	13.58 \pm 1.08	12.85 \pm 0.94	11.57 \pm 0.76	12.67 \pm 0.84
GG	14.29 \pm 0.87	16.02 \pm 0.67	16.77 \pm 1.00***	15.69 \pm 0.65***
TT	18.15 \pm 0.80***	18.58 \pm 0.81***	19.89 \pm 1.08***	18.87 \pm 0.65***
SA**	15.19 \pm 0.89***	19.99 \pm 1.22***	23.19 \pm 1.30***	19.45 \pm 0.90***
Cattle	12.83 \pm 1.71	12.97 \pm 1.51	11.23 \pm 0.87	12.34 \pm 1.28

a) Mean \pm S.E.* Significantly different among species by one factor ANOVA ($p < 0.05$).** Significantly different among zones by one factor ANOVA ($p < 0.05$).*** Significantly higher than cattle by student *t*-test ($p < 0.05$).

BB, Baird's beaked whale. GM, Short-finned pilot whale. GG, Risso's dolphin. TT, Bottlenose dolphin. SA, Pantropical spotted dolphin. Zone 1, The periportal zone. Zone 2, The mid-zonal zone. Zone 3, The perivenous zone.

lipid, protein and nucleic acid englobed by macrophages were synthesized into ceroid/lipofuscin in lysosomes by oxidative reaction [25, 28, 33], the diet might be also associated with the formation of abundant lipofuscin. Studies on feeding habits of cetaceans will be needed to analyze the relationship between lipofuscin production and diets.

Dark pigments in hepatic macrophages tended to be smaller in younger animals (Table 1), hence it was considered that bottlenose dolphins and pantropical spotted dolphins, most of which were young cases, might show lower amount of pigments than short-finned pilot whales and Risso's dolphins, most of which were adult. Along with the reasons why dark pigment-containing hepatic macrophages were not seen in Baird's beaked whales, the mechanisms of the accumulation of pigments in cetacean livers remain to be investigated.

Zonal heterogeneity of Kupffer cell distribution and phagocytosis: It has been reported in rats that Kupffer cells in zone 1 were larger in size to contain more heterogeneous large lysosomes and to be more active in phagocytes [5, 30], and the distribution of Kupffer cells in zones 1, 2 and 3 was 4:3:2 or 4:3:3 in ratio [1, 14, 22], indicating the highest number in zone 1. Generally, it is considered that Kupffer cells are predominantly distributed in zone 1 because they must monitor the blood entering into the liver [14]. In the present study, three species of dolphins showed the greatest number of SRA-E5-positive Kupffer cells in zone 3 and the smallest number in zone 1 (Table 2) as opposed to those reported in rats [1, 14, 22]. Kupffer cells are regarded as fixed tissue macrophages, but they have the ability of migration along sinusoid walls with or against the blood flow [3, 15]. Thus, also in cetaceans, Kupffer cells with dark pigments might migrate around the central vein and even into the hepatic intermediate septa where macrophages are usually believed not to show an active englobement [25]. Since Kupffer cells with few microspheres could migrate whereas those with many microspheres were never seen to migrate [15], Kupffer cells with abundant lipofuscin might have remained; it may be explained by the findings in Risso's dolphins, that aggregations of swollen macrophages with

abundant pigments were scattered not only in the intermediate septa but also around central veins (Fig. 4b). As described above, the mechanisms for the appearance of black pigment-containing hepatic macrophages, that was a characteristic finding of the cetacean livers, should be investigated.

Difference of Kupffer cell number among species: It is interesting to note that there was difference in the total mean of Kupffer cell number among species; especially, the total mean of the number was significantly greater in dolphins than in whales (Table 2). As shown in Fig. 8, the Kupffer cell number increased as the body-length was small. The reasons could not be explained. However, the small number of Kupffer cells may be enough in cetacean species with the long body-length, because they had the large-sized liver.

In conclusion, the present study has reported for the first time the distributive and phagocytic characteristics of hepatic macrophages in five different species of cetaceans belonging to two families. Kupffer cells in the liver play important roles in the pathophysiology or the maintenance of the homeostasis [18]. Our present research indicates the unique characteristics of Kupffer cells in the cetaceans living in the aquatic environment. It is interesting to pursue the more detailed functions of cetacean Kupffer cells under the physiological or pathological conditions.

ACKNOWLEDGEMENTS. We are grateful to T. Kishiro in the Cetacean Population Biology Section, National Research Institute of Far Seas Fisheries, and T. Hara and H. Sato of cetacean researchers for providing liver samples. This work was supported in part by a Grant-in-Aid (no.15380217 to J. Yamate) for Scientific Research B, the Ministry of Education, Science, Sports and Culture, Japan.

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