NOTE *Immunology*

The Respiratory Burst Activity of Bottlenose Dolphin Neutrophils Elicited by Several Stimulants

Rikiya SHIRAISHI[1)], Takuya ITOU[1)]*, Hitoshi SUGISAWA[1)], Youko SHOJI[1)], Tomoko ENDO[2)] and Takeo SAKAI[1)]

[1)]Department of Preventive Veterinary Medicine and Animal Health, Nihon University School of Veterinary Medicine, 1866 Kameino, Fujisawa, Kanagawa 252–8510 and [2)]Shinagawa Aquarium, 3–2–1 Katsushima, Shinagawa-ku, Tokyo 140–0012, Japan

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ABSTRACT. To investigate the early host defense function in aquatic animals, the respiratory burst activity of bottlenose dolphin neutroph ils against soluble and particulate stimulants was measured by luminol-dependent chemiluminescence assays and compared with those of bovine and human. Dolphin neutrophils generated the respiratory burst in response to phorbol 12-myristate 13-acetate (PMA), concanavalinA (ConA), heated-plasma (HP), and homologous-plasma opsonized zymosan except *N*-formyl-Met-Leu-Phe (fMLP). However, the respiratory burst of dolphin neutrophils stimulated by lipopolysaccharide and *Staphylococcus aureus* was inferior to those of bovine and human. Furthermore, DP-OZ also induced the respiratory burst of bovine and human neutrophils. In conclusion, dolphin neutrophils responded to several soluble and particulate stimulants as well as human neutrophils, but were refractory or slightly responded to bacterial agents.

KEY WORDS: dolphin, neutrophils, respiratory burst.

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Neutrophils play an important role in host defense and are responsible for the phagocytosis and killing of invading pathogenic microorganisms by releasing superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) as well as granule-derived factors like elastase, lactoferrin, lysozyme, and myeloperoxidase [1, 3, 17]. To generate the microbicidal oxidants, neutrophils demonstrate a burst of oxidative activity, which is known as the respiratory burst. Although the study of neutrophils respiratory burst has been revealed in many terrestrial animal species, there have been a few reports in aquatic animals [8]. We have previously demonstrated that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase acts as a superoxide anion generating enzyme in bottlenose dolphin neutrophils [14], and the defined dolphin NADPH oxidase genes possessed the high homologies with those of bovine [12, 13]. In this study, we examined the early host defense mechanism in aquatic animal by measuring the respiratory burst of dolphin neutrophils against several stimulants, compared with those of bovine and human.

Neutrophils were isolated from peripheral blood of clinically healthy bottlenose dolphins, Holstein cows, and humans by heparinized vacutainer. Two female and a male bottlenose dolphins (*Tursiops truncatus*, body weight of 240–350 kg; over 5 years old) were examined. They were maintained for the purpose of exhibition and education at the Shinagawa Aquarium, and were periodically checked for health by aquarium stuffs. Blood samples were properly diluted with phosphate buffered saline (PBS) (dolphin:4-fold, bovine:1.6-fold, human:2-fold) and laid onto LYMPHOPREP (sp gr 1.077, AXIS SHIELD PoC As, Oslo, Norway). After the centrifugation at 750 × g for 20 min, the fraction of neutrophils and erythrocytes were isolated. Erythrocytes were lysed by adding 10 m*l* of cold lysis buffer (8.26 mg NH_4Cl, 1.19 mg $NaHCO_3$, 0.0378 mg EDTA-2Na/m*l*), and neutrophils were pelleted by centrifugation at 150 × g. The pellet was washed twice with PBS and suspended in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS (-) ;GIBCO, Grand Island, NY) adjusted to the concentration of 1×10^7 cells/m*l*.

Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide derived from *Escherichia coli*, EC serotype 026:B6 (LPS), and zymosan were purchased from Sigma chemical (St. Louis, Missouri). N-formyl-Met-Leu-Phe (fMLP) and concanavalin A (ConA) were provided by WAKO pure chemical (Osaka, Japan). Rabbit polyclonal IgG antibody (rabbit-IgG) specific for zymosan was from Molecular probes (Eugene, Oregon). PMA and fMLP were dissolved into dimethyl sulfoxide (DMSO; WAKO pure chemical), and diluted with HBSS before use. Heated-plasma (HP) was prepared at 60°C for 30 min. *Staphylococcus aureus* ATCC25923 (SA) was inactivated at 70°C for 60 min. Zymosan, dolphin, bovine or human plasma-opsonized zymosan (DP-OZ, BP-OZ and HP-OZ), and rabbit IgG-opsonized zymosan (IgG-OZ) were prepared according to the method described by Brown and Roth [4]. The final concentration of each stimulant was PMA: 1, 10, or 100 ng/m*l*, ConA: 250, 1250, or 2,500 μg/m*l*, fMLP: 2, 10, or 20 μg/m*l*, LPS: 50, 250, or 500 μg/m*l*, HP: 5, 10, or 20%, SA: 10^7, 10^8, or 10^9 CFUs/m*l*, zymosan, P-OZ (DP-OZ, BP-OZ, HP-OZ) and IgG-OZ : 1 mg/m*l*.

Respiratory burst of 1×10^5 neutrophils induced by each stimulant was measured by LB953 luminometer (Berthold, Wildbad, Germany) as previously described [6], and the integral value of chemiluminescence (ICL) for 30 min after stimulation was evaluated as the ability of neutrophils respi-

* CORRESPONDENCE TO: ITOU, T., Department of Preventive Veterinary Medicine and Animal Health, Nihon University School of Veterinary Medicine, 1866 Kameino, Fujisawa, Kanagawa 252–8510, Japan.

Table 1. Respiratory burst by dolphin, bovine and human neutrophils in the presence of several stimulants

Stimulants		Integral of chemiluminescence (ICL; $\times 10^5$ counts per minute) of neutrophils obtained from		
		Dolphin	Bovine	Human
PMA	(ng/m*l*)			
	1	3397.5 ± 479.9*	150.2 ± 33.3	4629.3 ± 1557.3
	10	7830.4 ± 1755.1	261.0 ± 49.0	12308.8 ± 1856.4
	100	6620.1 ± 1144.3	281.7 ± 67.1	10911.9 ± 1135.6
Con A	(μg/m*l*)			
	250	90.3 ± 55.0	59.4 ± 26.8	148.0 ± 100.3
	1250	54.1 ± 20.2	56.9 ± 23.3	460.4 ± 264.7
	2500	45.7 ± 19.2	66.9 ± 28.0	219.3 ± 179.7
FMLP	(μg/m*l*)			
	2	0 ± 0	0.1 ± 0.8	19.5 ± 7.7
	10	0.3 ± 1.2	0.1 ± 1.2	28.8 ± 10.1
	20	1.2 ± 0	0 ± 1.6	32.9 ± 14.3
HP	(%)			
	5	465.0 ± 153.6	23.3 ± 11.4	2182.1 ± 447.9
	10	612.3 ± 202.3	60.3 ± 21.0	3180.3 ± 543.4
	20	766.7 ± 166.4	82.0 ± 35.6	3318.9 ± 547.0
LPS	(μg/m*l*)			
	50	1.2 ± 5.2	2.3 ± 2.4	15.5 ± 7.0
	250	0.9 ± 3.3	20.4 ± 10.2	18.4 ± 7.3
	500	3.4 ± 9.5	45.3 ± 20.9	30.7 ± 12.5
SA	(CFU/m*l*)			
	10^7	6.4 ± 1.9	45.9 ± 19.4	162.5 ± 82.0
	10^8	17.3 ± 5.2	190.1 ± 64.3	753.4 ± 358.2
	10^9	34.3 ± 4.0	234.1 ± 63.6	1417.9 ± 799.5

* The data are shown as the mean ± SEM of ICL for 30 min after stimulation from 3 samples.

ratory burst.

Respiratory burst of dolphin, bovine and human neutrophils induced by several stimulants is shown in Table 1. Dolphin neutrophils generated the oxygen radicals in response to PMA, ConA and HP, as well as to LPS and SA, though weakly, but didn't respond to fMLP. Respiratory burst of bovine neutrophils was induced by all stimulants except fMLP, although bovine neutrophils showed the less oxygen radical generation than other two animals in the stimulation with PMA and HP. Human neutrophils responded strongly to all stimulants.

Respiratory burst by dolphin, bovine and human neutrophils in the presence of non-opsonized and opsonized zymosan is shown in Table 2. Non-opsonized zymosan weakly induced the respiratory burst of neutrophils in all species. The largest activity of respiratory burst in dolphin neutrophils was obtained by the zymosan opsonized with homogeneous plasma as well as other two species, and dolphin plasma had the opsonic activity to bovine and human neutrophils. In response to rabbit IgG-OZ, dolphin and bovine neutrophils generated considerably less oxygen radicals than did the neutrophils of human.

In this study, the respiratory burst of dolphin neutrophils induced by PMA was as strong as that of human. This suggests that dolphin neutrophils fundamentally have the capability of the respiratory burst enough to resist pathogenic microorganisms and play an important role in anti-infection mechanism as in any terrestrial animals. PMA is the most popular potentiator used for the evaluation of neutrophils bactericidal activity such as respiratory burst, degranulation, and chemotaxis, and has been known as a stimulant which activates protein kinase C (PKC)-mediated signaling pathway [25]. In human and experimental animals such as mouse and rat, PKC is known as one of the important factor for the activation of NADPH oxidase which is necessary for the generation of respiratory burst [7]. In domesticated animal, PKC-concerned signaling pathway has been found in bovine neutrophils, too [26]. It is suspected that dolphin neutrophils also have the same mechanism in the generation of respiratory burst.

ConA and HP induced the respiratory burst of dolphin neutrophils. It has reported that bovine neutrophils recognize ConA by ConA receptor [19], and HP binds with Fc receptor for IgA, IgG or IgE-dependent immune complexes which were produced in the process of heating plasma or serum [16, 23, 28]. Thus, it is suggested that ConA and Fc receptor-mediated respiratory burst activity exists in dolphin neutrophils.

Table 2. Respiratory burst by dolphin, bovine and human neutrophils in the presence of non-opsonized, plasma-opsonized, and rabbit IgG-opsonized zymosan

Stimulants	Integral of chemiluminescence (ICL; $\times 10^5$ counter per minute) of neutrophils obtained from		
	Dolphin	Bovine	Human
Zymosan	35.2± 23.4	13.5± 8.9	11.5± 3.8
DP-OZ	158.4± 62.3*	91.0± 26.0*	253.4± 94.5**
BP-OZ	38.0± 24.8	135.1± 12.1*	23.9± 9.0
HP-OZ	33.8± 17.6	16.4 ± 0.4	1547.7± 540.4**
IgG-OZ	73.8± 22.0*	22.6± 9.3	2569.0± 721.8**

DP-OZ, BP-OZ and HP-OZ indicate the opsonized zymosan with dolphin, bovine, and human derived plasma, respectively. The data are shown as the mean ± SEM of ICL for 30 min after stimulation from 3 samples. Asterisks indicate significant increase compared with the data obtained from non-opsonized zymosan-stimulated neutrophils (*; $p<0.05$, **; $p<0.01$, Student's *t*-test).

fMLP didn't induce the respiratory burst at all in dolphin neutrophils. This result was the same as that of bovine neutrophils, but it was in contrast to that of human. It has been reported that neutrophils from other animals such as bison [24], feline [11], ovine [5], and porcine [9], have also no response to fMLP, while canine and rat neutrophils response to fMLP [15]. Since bovine neutrophils don't have the receptor for fMLP [10], dolphin would have no fMLP receptor, either.

LPS induced little respiratory burst of dolphin neutrophils in contrast to that of bovine and human. Human neutrophils recognize LPS using with Toll-like receptor [18] or CD14 [20], and bovine CD14 expresses on neutrophils [21]. Neutrophils from buffalo and goat release oxygen radicals in response to LPS [22], but that from equine didn't [2]. To investigate whether or not dolphin neutrophils have a particular mechanism for LPS recognition, analysis of dolphin LPS receptor is needed. SA also induced weakly the respiratory burst of dolphin neutrophils in contrast to those of bovine and human. Thus, dolphin neutrophils may hardly respond to bacteria-derived stimulants.

Zymosan, P-OZ, and IgG-OZ were used for the examination of phagocytic activity of neutrophils. In bovine neutrophils, zymosan and P-OZ concerned with p38 mitogen-activated protein kinase (MAPK), PKC, and phosphatidyl inositol 3 kinase (PI3-K) in the signaling pathway of NADPH oxidase [26]. Dolphin neutrophils responded to homologous plasma-OZ stronger than non-opsonized and heterogeneous plasma-opsonized zymosan as well as bovine and human neutrophils. Furthermore, DP-OZ also induced the respiratory burst of bovine and human neutrophils. This shows that dolphin plasma has the opsonic activity to heterogeneous neutrophils. Because the cross reactivity of opsonic activity has also been shown among bovine, ovine, and human [27], it is suggested that the common region exists partially in mammalian complement 3 fragment or Fc of immunoglobulin.

The respiratory burst activity of human neutrophils was higher than those of dolphin and bovine neutrophils in nearly all stimulants. Additionally the respiratory burst of human neutrophils induced by rabbit-derived IgG-OZ was incommensurable with those of dolphin and bovine neutrophils. These imply that human neutrophils may originally have a high ability in recognition and clearance of extraneous microbes. Interestingly, the maximal response of bovine neutrophils induced by PMA or HP was markedly weak, though bovine NADPH oxidase genes show high homologies with those of dolphin. It seems possible that bovine neutrophils possess the mechanism that controls a redundant production of oxidants via a regulating the activation of the oxidase.

In the present study, we indicated that dolphin neutrophils occur during the respiratory burst in response to not only PMA but also any other stimulants. However, in the stimulation by bacterial agents, the reactivity of dolphin neutrophils was inferior to those of bovine and human neutrophils. This may result from the differences in habitat between terrestrial and aquatic animals where there is another extraneous antigen. In order to reveal the neutrophil functions in marine mammals, we will investigate the susceptibilities to various microorganisms in dolphin neutrophils.

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