NOTE *Clinical Pathology*

Evaluation of the Polymorphonuclear Cell Functions of Bottlenose Dolphins

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ABSTRACT. The functions of polymorphonuclear cells (PMN) are the important non-specific defense mechanisms in the immune system. Especially marine mammals are protected by these mechanisms from the aquatic environment with a large variety of microorganisms. Therefore, we examined the PMN functions of bottlenose dolphins in order to obtain the normal ranges and to standardize the techniques. PMNs were isolated by using lymphocyte isolate solution whose density was 1.077; superoxide production was assessed by nitroblue tetrazolium reduction test (NBT) and phagocytosis was tested by using polystyrene latex beads. We showed that the optimal incubation time was 30 min in NBT assay and 12 hr in phagocytosis assay for dolphin PMNs.

KEY WORDS: dolphin, function, PMN.

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Marine mammals share the aquatic environment with a large variety of microorganisms. It is presumed that they are protected from those organisms by a nonspecific biogenic defense system. Because polymorphonuclear cells (PMN) contribute to the nonspecific immune system, they play an important role in protecting the animal from microbial infection. These PMN functions depend on their capacity to ingest particles (phagocytosis) and to destroy them with products of their oxidative metabolism (intracellular killing). These systems have been well investigated in terrestrial mammals,but there is little information available on oxygen-dependent microbicidal activity or phagocytosis in marine mammals [3, 4].

The aim of the present study was to set up quantitative assays in dolphins to measure two key functions of PMN, namely phagocytosis and intracellular killing. The present paper reports on the evaluation of these functions in bottlenose dolphins (*Tursiops truncatus*).

Four bottlenose dolphins (*Tursiops truncatus*, body weight of 200–250 kg; over 5 years old; 1 male and 3 females) were examined. They were maintained for the purpose of exhibition and education and were periodically checked for health by the staff. None of the dolphins exhibited clinical or hematological signs indicative of a pathological condition.

Peripheral blood samples were drawn from a superficial vessel on the ventral aspect of the tail fluke and kept cool until analysis. All samples were examined within 8 hr after blood collection. The white blood cell count was performed in duplicate with a Celltac-α automated hematology analyzer (Nihonkoden, Tokyo). In pilot experiment, the white blood cell counts by an automatic and a direct method were $8100 \pm 100/\mu l$ and $7356 \pm 765.8/\mu l$ (mean ± standard deviation; n=4), respectively. There was no significant difference between these methods in the white blood cell count ($p<0.05$). The leukocyte differential count was done on Giemsa stained smears. The blood was diluted 1:1 with Hanks's balanced salt solution without Ca^{2+} (Nissui Pharmaceutical, Co., Ltd., Tokyo) (HBSS), and after mixing by gentle inversion, was layered on lymphocyte separation solution (d=1.077 g/m*l*) (Nakarai Tesque, Co., Ltd., Kyoto). The solutions were layered in conical plastic tube (Falcon Plastics, Los Angeles, CA) and centrifuged at 400 × g for 20 min at 4°C. After centrifugation, the supernatant containing mononuclear cells was removed by aspiration and the packed erythrocyte layer was lysed by the addition of NH_4Cl-Tris solution with gentle mixing at 37°C. This was done until most of the erythrocytes were lysed. The cell pellet obtained after centrifugation at 400 × g for 20 min at 4°C was washed twice with HBSS and suspended in HBSS. Viable cells were determined by trypan blue exclusion and counted in a Burker-Türk counting chamber. The viability of PMN was over 95%. The final cell concentrations were adjusted to 5.0×10^6/viable cells/m*l* of HBSS.

NBT reduction due to PMN was evaluated by a modification of the procedure of Murata *et al.* [10]. Tests were conducted in duplicate in 15 × 105 mm silicon-coated glass tubes. The NBT (Sigma Chemical Co., MO) solution was prepared by suspending in HBSS at a concentration of 1 mg/m*l*. Insoluble NBT was removed by filtration. After Zymosan A (Sigma Chemical Co., MO) suspended in distilled water was dipped into boiling water for 30 min, the suspension was centrifuged at 250 × g for 10 min, and the supernatant was discarded. Opsonized zymosan A in fresh serum of bottlenose dolphin at a concentration of 25 mg/m*l* was incubated at 37°C with shaking for 30 min. The mixture was centrifuged at 250 × g for 10 min, and the supernatant was discarded. The pellet was resuspended in HBSS and frozen at –80°C. The reaction mixture consisted of 0.5 m*l* of cell suspension (5.0×10^6/m*l*), 0.4 m*l* of the NBT solution and 0.1 m*l* of opsonized zymosan A suspension (10 mg/m*l*). The reduction of NBT by resting cells was determined in a similar set of tubes from which opsonized zymosan A was omitted. After incubation at 37°C for 0.5, 1, 1.5 and 2 hr, the reaction was terminated by adding 1.0 m*l* of 0.5 N HCl. The mixture was then centrifuged at 1,000 × g for 10 min,

and the supernatant was discarded. The precipitate was dissolved with 3 m*l* of dimethyl sulfoxide (DMSO), heated in boiling water for 5 min and cooled. After the mixture was clarified by centrifugation at 500 × g for 5 min, the optical density at 565 nm was determined immediately with a DMSO blank in a spectrophotometer (Shimadzu Co., Kyoto).

For the evaluation of phagocytosis, we used polystyrene latex beads (diameter=1.0 μm) (Polysciences, Inc., PA). First, 2 m*l* of the cell suspension (5.0×10^6/m*l*) and 2 m*l* HBSS including 25% autologous serum were preincubated at 37°C with shaking for 15 min. After the preincubation, 15 μl of the 0.1 % nonopsonised polystyrene latex beads were added to the mixture, and then the mixtures were incubated at 37°C and 0°C for 1, 3, 12 and 18 hr, respectively. After the incubation, the reaction was terminated by adding 6 m*l* washing solution which was HBSS containing 0.2% Bovine Serum Albumin (JRH Biosciences. Co., Tokyo). The mixture then was centrifuged at 400 × g for 7.5 min at 4°C, and the supernatant was discarded. This was done repeatedly. The precipitate was dissolved in 1 m*l* of Phosphate Buffer Solution (PBS). The solution were added 10 m*l* of 1M $MgSO_4$ and 10 μl of Trypsin (Sigma Chemical Co., MO) solution (10 mg/m*l*). Then the mixture was incubated at 37°C for 30 min without shaking. After the incubation, the glass tube was put into cold water to terminate the reaction. Ten μl of the Soy Bean Trypsin Inhibitor (SBTI) (Sigma Chemical Co., MO) solution (10 mg/m*l*) and 20 μl of the Deoxyrybonuclease solution (20 mg/m*l*) (Sigma Chemical Co., MO) were added the mixture. After 60 sec when adding them, the mixture was washed with washing solution and centrifuged at 400 × g for 7.5 min at 4°C, repeatedly. The pellets were smeared on 3 slide glasses with Cytospin (Shandon Co., PA). The smear was stained with Giemsa solution, and the number of cells ingesting beads per 600 PMN was counted microscopically. Phagocytic activity was expressed as the percentage of PMN phagocytizing 3 or more particles.

Data are shown as the means ± standard deviation (SD) and the coefficient of variation (CV) has calculated to evaluate the variation in data in the incubation period.

The PMN isolation procedure performed on 4 separate blood samples from apparently healthy animals yielded the results shown in Table 1. The average total WBC count was 6875 ± 2187/μl, and the average PMN percentage in whole white blood was 70.8 ± 5.0%. An average of 2.8×10^6 PMNs were recovered per m*l* of blood, which represented an average recovery of 71.0% of the PMNs present in whole blood. The final PMN preparation contained an average of 85.6% neutrophils, 12.4% eosinophils and 2.0% mononuclear cells.

The average (± SD) NBT reduction for four bottlenose dolphins with regard to the time course from 0.5 to 2 hr is shown in Fig. 1. The NBT reduction after 0.5 hr ranged from 0.05 to 0.28, with an average (± SD) of 0.17 ± 0.12, and a CV of 69.5%. It had a smaller CV than those after 1, 1.5 and 2 hr.

Table 1. Yield and purity of PMNs isolated from 4 samples of blood from healthy adult dolphins

	mean	± SD
Percent PMNs in the final preparation		
Neutrophils	85.6	9
Eosinophils	12.4	10.4
Mononuclear cells	2	2.6
PMN yield per m*l* of whole blood ($\times 10^6$)	2.8	1.2
Percent recovery of PMNs	71	8.3

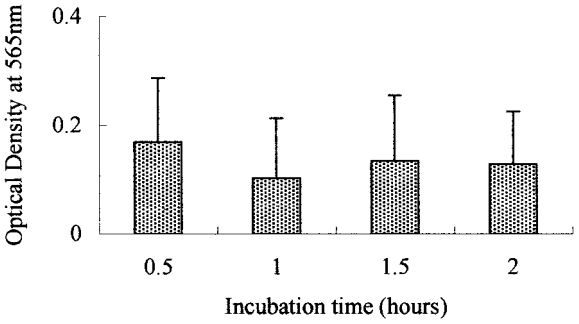


Fig. 1. Time kinetics for NBT reduction test of healthy bottlenose dolphins.

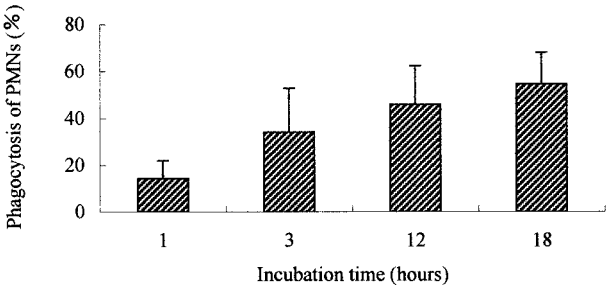


Fig. 2. Time kinetics for phagocytosis of healthy bottlenose dolphins.

The average (± SD) phagocytic activity of four bottlenose dolphins with regard to the time course from 1 to 18 hr is shown in Fig. 2. Phagocytosis in bottlenose dolphins increased for up to 18 hr in most animals. The phagocytic activity after 18 hr ranged from 36.0 to 73.6, with an average (± SD) of 54.6 ± 13.6%, and a CV of 24.9%. The CV was smaller than those after 1, 3 and 12 hr. But the morphological appearance of PMN at 18 hr was microscopically disintegrated.

The lymphocyte isolation solution whose specific gravity was 1.077 allowed us to obtain a suspention of cells of high purity that was considered satisfactory for testing PMN functions, although some variations among individuals were seen. The viability of isolated PMN cells was also enough for examination of the functions.

It has been shown that neutrophils rapidly lose generated

power via the hexose monophosphate shunt in what is termed a metabolic burst. If during the process NBT is present, it is reduced to formazan and can be detected spectrophotometrically [2, 9]. Some experiments reported the evaluation of superoxide production of cetaceans [4, 8]. de Guise *et al.* and Itoh *et al.* investigated it by chemiluminescense assay, and we obtained similar results to theirs. The means optical density for all the periods differed little, but a CV of 0.5 hr was the smallest in all periods. In this study, it took 8 hr from collection of the blood to separation the PMN. But the viability of PMN was sufficient for analysis and the optical density is in the same range as human PMN [1]. It is concluded that the time taken to separate the PMN did not effect the NBT reduction due to PMN, and the incubation time of 0.5 hr was optimal for the NBT reduction test.

Maximum phagocytosis in bottlenose dolphins was obtained after approximately 18 hr of co-incubation of peripheral blood leukocytes with latex beads. This result agrees with those reported by other authors [4]. But the morphology of the cells had changed after incubation for 18 hr. The CV of 12 hr was 36.1% and it is concluded that the incubation time of 12 hr was optimal. This period of time is relatively long compared with that in the test on cattle and pigs where the maximum was reached within approximately 1 hr [3, 6]. de Guise *et al.* considered that the use of fetal calf serum in the phagocytosis assay and non-opsonized particles might be responsible for that time difference [4]. It is considered that other factors may have an effect on this time difference, for example, blood transport time until the analysis and hemolysis by NH_4Cl-Tris. They used NH_4Cl for hemolysis, and obvious morphological and functional problems were not observed when using this method. But there was no consideration of the effect of blood transport time on the time course of phagocytosis. We also checked the morphology and viability of PMN before analysis, and there was no change in the morphology or decrease in viability. So the different results obtained in our study from those on other animals could be explained by the use of non-opsonized particles. We should investigate the relationship between these factors and the time course of phagocytosis.

Variation in both functions was rather large. This might be due to the small size of the sample, but the variation in the evaluation of phagocytosis and intracellular killing in other species was also relatively high [5, 7, 11]. Further analysis of a larger number of animals is needed to reduce the variation.

In this report, we described how the use of a lymphocyte separation solution (d=1.077) could obtain a satisfied purification of dolphins PMN. PMN cell functions in dolphins could be evaluated by means of the NBT reduction test for oxidative metabolism and polystyrene latex beads for phagocytosis. We expect further investigation of immune functions of cetaceans affected by their environment and many other kinds of situations.

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