NOTE *Immunology*

Isolation and Chemiluminescent Properties of Ferret (*Mustela putorius furo*) Polymorphonuclear Cells

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ABSTRACT. Ferret polymorphonuclear cells (PMNs) and peripheral blood mononuclear cells (PBMCs) were separated from whole blood by density gradient centrifugation. Using a 50% Percoll solution (density=1.066), PMNs and PBMCs were successfully isolated after centrifugation; the purities of the PMNs and PBMCs were 94.2% and 95.6%, respectively. To evaluate the function of isolated ferret PMNs, we measured the superoxide generation with a MCLA-dependent chemiluminescence assay. The isolated ferret PMNs responded to phorbol 12-myristate 13-acetate (PMA) with kinetics similar to that of human PMNs. The ferret PMNs did not respond to N-formyl-Met-Leu-Phe (fMLF), unlike human PMNs, which rapidly responded. Thus, authors established a method for the rapid separation of highly purified populations of functional PMNs from the whole blood of ferrets.

KEY WORDS: chemiluminescence assay, ferret (*Mustela putorius furo*), polymorphonuclear cell.

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Ferrets (*Mustela putorius furo*) are a useful animal model for toxicology and human respiratory infectious diseases, such as influenza and severe acute respiratory syndrome [14, 21–23]. Recently, the reports of tumors in domestic ferrets have increased as the number of domestic ferrets used as companion animals has increased. However, few reports have investigated the fundamental inflammation response of ferrets, in which neutrophils play an important role.

Polymorphonuclear cells (PMNs) adhere to endothelial cells in the blood vessels at sites of infection and inflammation, then, the PMNs infiltrate into tissues showing active chemotaxis. PMNs are responsible for the phagocytosis and killing of invading pathogenic microorganisms. Once stimulated, PMNs generate superoxide due to the activation of NADPH oxidase, and powerful oxidants derived from superoxide sterilize foreign microorganisms. Moreover, PMNs are also activated with chemotactic factors, immunocomplexes, and cytokines and have strong relevance to the first line of defense against infections [7].

In the present study, authors isolated highly purified ferret PMNs by using density gradient centrifugation and then studied the functional responses of the isolated PMNs with a MCLA-dependent chemiluminescence assay.

Four male, clinically healthy ferrets ranging in age from 10 months to 3 years were used in this study. They were obtained from Marshall Pet Products (NewYork, U.S.A.) as an experimental animal, and maintained at Nihon University Veterinary Research Center (NUVERC). The studies were approved by Ethical Committee for Animal Experiments, NUVERC. Peripheral blood samples (7~8 m*l*) were drawn from the anterior vena cava into a heparin- anticoagulation tube. White blood cells were counted by Celltac α (Nihon Coden, Japan), and the differential white blood count was analyzed by blood smear plate.

The whole blood was mixed with 2% dextran solution (dextran T500: Pharmacia, in saline), and then the mixture sat for 20 min at room temperature. The upper layer containing the leukocyte-rich fraction was recovered and then layered onto 3 m*l* of Percoll solution of various densities (Amersham Biosciences, England) in a 15-m*l* tube. Percoll solution was diluted to the following concentrations: 25%, 35%, 45%, 50%, and 55% (density: 1.036, 1.048, 1.060, 1.066 and 1.072 g/m*l*, respectively). The tube was centrifuged at 400 × g for 20 min. The leukocyte populations could be separated into 4 parts: PMNs and the red blood cell pellet (called the lower layer), the Percoll phase, PBMCs and platelets (called the upper layer), and the plasma phase containing PBS (–). After hypotonic lysis of remaining erythrocytes and centrifugation (100 × g for 10 min) in the lower layer pellet, the cells of upper and lower layer pellet were washed twice with PBS (–) and resuspended with Hank's balanced salt solution (HBSS (+)). Simultaneously, human PMNs were obtained by using previously reported methods [1] for the measurement of superoxide generation. The differential white blood cell counts of the upper layer and lower layer cell populations were determined. The upper layer and lower layer cell suspensions (100 μl) isolated from each Percoll solution were cytocentrifuged (Shandon cytospin 3, Thermo Electron Corp, UK) and then used to prepare slides that were stained with May Grunwald-Giemsa. Slides were microscopically examined to determine the differential white blood cell counts.

Superoxide generation was measured by using a M-*Cypridina* luciferin analog (MCLA)-dependent chemiluminescent assay as described by Nishida *et al.* [16]. MCLA is a novel compound synthesized from CLA [15, 19] and is highly specific for and sensitive to superoxide. Briefly, 2×10^5 PMNs were added to a 96-well white plate containing 1 μM MCLA (Tokyo Kasei, Japan) in HBSS (+) and incu-

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bated for 5 min at 37°C. After determining the chemiluminescence baseline, each stimulant (phorbol 12-myristate 13-acetate: PMA or N-formyl-Met-leu-Phe: fMLF) was added to the wells for activation of the NADPH oxidase in PMNs. The chemiluminescence intensity was measured with a Centro LB960 luminometer (Berthold, Wildbad, Germany) as previously described [3]. Human PMNs that react with both stimulants were used as a control in this study. The stimulants were replaced with HBSS (+) buffer as a negative control. The stimulants used to measure the superoxide generation were PMA (Sigma Chemical Corp, St. Louis, Missouri) and fMLF (WAKO Pure Chemical, Osaka, Japan). PMA and fMLF were dissolved in dimethylsulfoxide (DMSO: WAKO Pure Chemical) and stored at –80°C. The two stimulants were diluted with HBSS (+) before use. The final concentration of PMA was 100 *n*g/m*l*, and the two final concentrations of fMLF were 1×10^{-5} and 1×10^{-7} M.

The mean number of white blood cells was 5,500 cells/μl and the mean percentages of neutrophils, eosionophils, basophils, lymphocytes and monocytes cell count in whole blood are 50.1 ± 4.4, 8.2 ± 5.5, 2.5 ± 0.9, 38.8 ± 8.4, 0.4 ± 1.1%, respectively. These results are consistent with previous reports [12, 17].

The purities of PMNs in the lower layer and PBMCs in the upper layer are shown in Table 1. Since there were few PBMCs in the upper layers of 25% and 35% Percoll, the purity of these cells could not be evaluated. The purity of PBMCs in the upper layer of 45% Percoll was 92.0%, but the PMN population in the corresponding lower layer was contaminated with about 10% PBMCs. In 50% Percoll, PMNs in the lower layer were obtained in good yield and high purity (94.2%), and the PBMC purity in the upper layer was also the highest (95.6%) (Fig. 1). In contrast, few PMNs were visible in the lower layer of 55% Percoll, and the corresponding upper layer was mixed with PMNs. Therefore, the most efficient isolation of ferret PMNs and PBMCs can be achieved by using 50% Percoll (density: 1.066). In 50% Percoll, approximately 1.0×10^7 PMNs and 7.0×10^6 PBMCs can be obtained from 7 m*l* of peripheral blood. There are many reports describing the isolation of PMNs or PBMCs from whole blood cells in sheep [4], humans, canines [1, 8], and bovines [5]. However, since the size and density of leukocytes in each species are different, efficient centrifugal isolation requires different densities for different species. We studied the effectiveness of different densities of Percoll, and found that the highest purities of PMNs and PBMCs were isolated when using a density of 1.066 (a 50% concentration of Percoll). Moreover, it was carried out to separate the ferret PMNs from the RBC-enriched layer using hypotonic lysis of the erythrocytes [4]. This procedure was able to do as same as human PMNs isolation. This method can avoid any uptake of chemical reagents that could influence the PMNs function [24]. Viability, which was checked by trypan blue exclusion, was greater than 98% in every case.

The kinetics of superoxide generation by stimulated ferret PMNs are shown in Fig. 2. The chemiluminescence of PMA-stimulated ferret and human PMNs increased gradually, and the peaks appeared at 6 to 8 min. Ferret PMNs stimulated with fMLF did not produce a detectable chemiluminescent response; in contrast, human PMNs stimulated with fMLF had a significant peak within 2 min of the stimulation. The PMA-induced superoxide generation by ferret PMNs was as strong as that of human PMNs. This result suggests that the isolated ferret PMNs were capable of producing a respiratory burst. PMA is the most popular poten-

Table 1. Purity of isolated cells using Percoll

Percoll (%)	Density	PMN(%)[a)]	PBMC(%)[b)]
25	1.036	64.5 ± 1.5	NE
35	1.048	85.4 ± 6.8	NE
45	1.060	90.7 ± 2.5	92.0 ± 2.4
50	1.066	94.2 ± 4.1	95.6 ± 4.3
55	1.072	96.5[c)] ± 3.1	89.0 ± 2.4

a) The data are shown as the means ± SD (n=4) of percentage of PMN in the lower layer.
b) The data are shown as the means ± SD (n=4) of percentage of PBMC in the upper layer.
c) Very low yield of cells.
NE: Not evaluated.

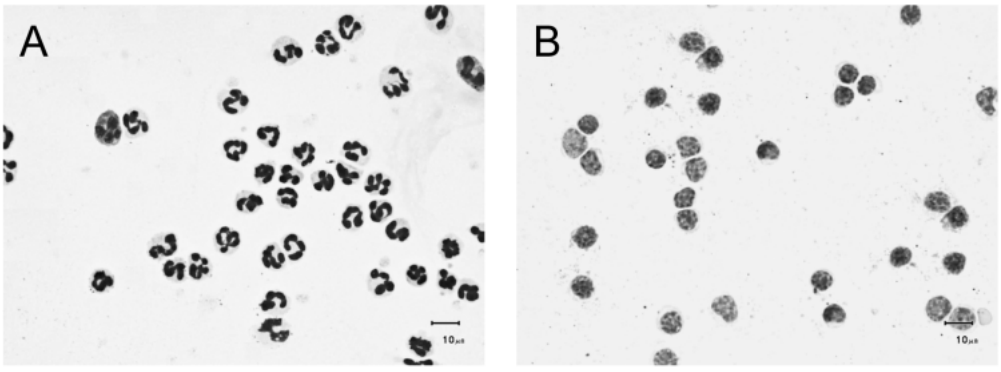

Fig. 1. Morphological evaluation of ferret PMNs and PBMCs. After density gradient centrifugation using 50% Percoll solution, the lower (A) and upper (B) layer-derived cell populations were cytocentrifuged, and stained with May-Grunwald Giemsa (× 400).

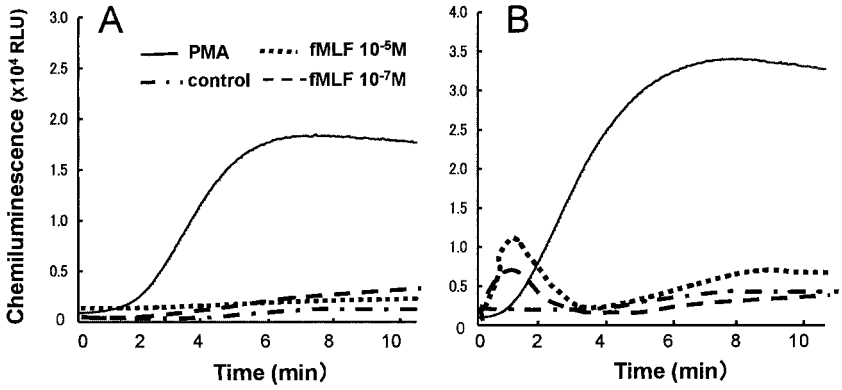

Fig. 2. MCLA-dependent chemiluminescence in ferret or human PMNs after the addition of a stimulant (PMA, 10^{-5} M fMLF, or 10^{-7} M fMLF) or HBSS (+) buffer (as the negative control). A and B show the responses of ferret and human PMNs, respectively. The horizontal axis indicates the time (minutes), and the vertical axis indicates the intensity of chemiluminescence (RLU). Data of representative experiments are shown (n=4).

tiator used to evaluate neutrophilic bactericidal activity such as the respiratory burst, degranulation, and chemotaxis. PMA is a stimulant that activates the protein kinase C (PKC)-mediated signaling pathway [25]. PKC is an important factor for the activation of NADPH oxidase in humans, rats, and mice [6]. Additionally, the PKC-mediated signaling pathway was recently discovered in bovine neutrophils [26]. Thus, we suspect that ferret PMNs containing neutrophils also have a PKC-mediated mechanism for superoxide generation that is the same as the previously reported mechanism found in other animals.

A bacterial chemotactic peptide, fMLF, did not induce superoxide generation in ferret PMNs; however, human PMNs strongly responded to both concentrations of fMLF that were tested. PMNs from other mammals such as bison [20], cats [11], sheep [2], and pigs [9] do not react to fMLF. In contrast, PMNs from humans, rodents, and dogs do respond to fMLF [13,18]. Gray *et al.* [10] reported that bovine neutrophils lack the receptor for fMLF. Therefore, we speculate that ferret neutrophils may also lack the fMLF receptor.

In the present study, authors isolated highly purified PMNs by using Percoll density gradient centrifugation, and evaluated the respiratory burst activity of the isolated PMNs. We also simultaneously isolated highly purified PBMCs.

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