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Chemotactic properties and absence of the formyl peptide receptor in ferret (*Mustela putorius furo*) neutrophils

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ABSTRACT

This study describes a chemotaxis assay of ferret polymorphonuclear cells (PMNs). The optimal conditions for this chemotaxis assay were investigated for three chemoattractants: zymosan activated serum (ZAS), recombinant human interleukin-8 (rhIL-8) and N-formyl-Met-Leu- Phe (fMLF). In this study, ferret polymorphonuclear cells (PMNs) reacted to ZAS and rhIL-8, but not fMLF. The optimal concentration of ZAS and rhIL-8 were 5% and 100 ng/ml, respectively. The optimal incubation time of each reagent was 60 min. Due to the lack of response shown from fMLF, the existence of formyl peptide receptors (FPR) on ferret PMNs was investigated by evaluating FPR binding using flow cytometry. The receptor was not detected, implying that ferret neutrophils may lack FPR. This study confirms the fundamental experimental conditions for ferret PMNs chemotaxis and elucidates new findings concerning FPR in ferret neutrophils.

Neutrophil chemotaxis is an important first line of defense against infection. When infection or inflammation occurs, adhesion molecules appear on the surface of activated endothelial cells and bind with adhesion molecules on the leukocyte surface. Polymorphonuclear cells (PMNs) then migrate out of blood vessels and infiltrate tissues, a process termed active chemotaxis which is lead by several chemoattractants (Edwards, 1994; Tizard, 2004). The ferret (*Mustela putorius furo*) is a useful animal model for studying human infectious disease, especially those involving microbes such as *Helicobacter pylori*. The naturally occurring infectious agent in ferrets, *Helicobacter mustelae*, may be closely related to *H. pylori* as it shares many virulence factors (O'Rourke and Lee, 2003; Nedrud and Blanchard, 2001). The ferret also serves as an animal model for influenza and severe acute respiratory syndrome (ter Meulen et al., 2006; van Riel et al., 2006; Maher and DeStefano, 2004). Furthermore, the number of domestic ferrets kept as companion animals has recently increased. Despite the important role the ferret plays in the field of veterinary and human medicine, there are few reports on ferret neutrophil chemotaxis. However, *in vitro* chemotaxis assays have been applied to several other animal species (Sugawara et al., 1995).

The initial aim of this study was to determine optimal conditions for the chemotaxis assay of ferret PMNs and responses to each ferret PMNs chemoattractant. Upon finding that the ability of chemotaxis on ferret PMNs was not responsive to fMLF, the presence of the formyl peptide receptor (FPR) on ferret neutrophils was also investigated.

The animals used in this study were clinically healthy male ferrets (2 years old; $n = 3$). They were obtained from Marshall Pet Products (New York City, NY) and maintained in individual cages at Nihon University Veterinary Research Center (NUVERC), Japan. The ferrets were fed a commercial dry-food diet. This study was approved by the Ethical Committee for Animal Experimentation, NUVERC. Isolation of PMNs was performed as previously described (Nakata et al., 2007). In brief, the whole blood was mixed with 2% dextran solution (dextran T500: Pharmacia, in saline), and then left for 20 min at room temperature. The upper layer containing the leucocyte-rich fraction was recovered and layered onto 50% Percoll solution, and then centrifuged at 400*g* for 20 min. The lower layer containing PMNs was subjected to hypotonic lysis and washed twice with PBS (−). Finally, the cells were resuspended in Hank's balanced salt solution (HBSS)(+) containing 0.3% bovine serum albumin (BSA). The number of cells was adjusted to 1.0×10^6 cells/ml. Simultaneously, human PMNs were obtained as a control using previously reported methods (Boyum, 1968). The chemotactic factors used were zymosan-activated serum (ZAS), recombinant human interleukin-8 (rhIL-8) and N-formyl-Met-Leu-Phe (fMLF). ZAS is a complementary chemoattractant and stimulates the alternative complement pathway. The major active component of ZAS is presumed to be complement fragment C5a (Edwards, 1994). rhIL-8 is a potent chemotactic cytokine for human neutrophils with only minor effects on eosinophils, monocytes, basophils (White et al., 1989), and subsets of human peripheral T lymphocytes (Larsen et al., 1989). fMLF is a potent chemotactic factor for human neutrophils of the pro-inflammatory bacterial peptide,

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and probably the most commonly-used activator of human neutrophils *in vitro*. It is used as a model agonist to study receptor-mediated processes, generating intracellular signaling molecules that then activate cell functions (Edwards, 1994). ZAS was generated by incubating 10 mg zymosan (Sigma Chemical Corp., St. Louis, MI) with 1 ml fresh ferret serum at 37 °C for 60 min. The zymosan was removed by centrifugation with 600*g* for 15 min, and the resultant ZAS was then heat-inactivated at 56 °C for 30 min and stored at −80 °C until use. As ferret recombinant IL-8 was not commercially available, recombinant human IL-8 (rhIL-8) (R&D Systems, Inc., Minneapolis, MN) was used instead. rhIL-8 was dissolved in PBS with 0.1% bovine serum albumin, while fMLF (WAKO Pure Chemical, Osaka, Japan) was dissolved in dimethylsulfoxide (DMSO, Wako Pure Chemical) and stored at −80 °C. The final concentrations for each of the chemoattractants were adjusted to 5, 10, and 20% for ZAS, 1, 10, 100 ng/ml, and 1 μg/ml for rhIL-8, and 10^{-10} to 10^{-6} M for fMLF.

The chemotaxis assay was performed using a 96-well modified Boyden chemotaxis chamber (Chemotx96 type: 106, Neuro Probe Inc., Gaithersburg, MD). Bottom wells were filled with 30 μl buffer solution containing chemoattractant or no chemoattractant serving as a negative control (NC), and put on 3-μm pore size polycarbonate filters. The chamber was pre-incubated for 30 min at 37 °C in a 5% CO_2-humidified atmosphere. After pre-incubation, 65 μl PMN suspension in HBSS(+) was placed in the upper side of the filter and then incubated at 37 °C under 5% CO_2-humidified atmospheric conditions for 15, 30, 60 and 120 min. After the incubation the upper suspensions were removed and centrifuged at 77*g* for 5 min, and then the filters were removed. Then 5 μl of CellTiter-Glo™ luminescent cell viability solution (Promega, Madison, WI) was added to the bottom wells and incubated for 2 min at room temperature. The number of cells migrating to the bottom well of the chamber was counted as chemiluminescence intensity using a luminometer (Centro LB960 luminometer, Berthold, Wildbad, Germany), similar to a previously reported method (Frevert et al., 1998). In brief, the number of cells was adjusted to 15,000 per well of the 96-well white micro plate and then the two fold serial dilutions of cells were prepared. CellTiter-Glo™ was added to each well and the number of cells is counted with the degree of chemiluminescence. The standard curve was then produced. After checking if the value between the number of cells and the degree of chemiluminescence could be correctly evaluated based on the standard curve, the cell number in each bottom well sample was evaluated. Results are shown as mean ± SEM of three to five independent experiments obtained from three ferrets. The data is presented as the cell migration ratio of the number of cells migrating to the bottom wells divided by the number of PMNs in the upper suspensions (15,000 cells/well) and multiplied by 100. To estimate the significance of the differences between the migration ratio of each concentration of ZAS, rhIL-8, or fMLF and NC, the two-directional paired Student's *t*-test was performed. The cells collected from the bottom wells were cytocentrifuged and stained with May Grunwald–Giemsa. Slides were microscopically examined to determine the differential of the migratory cells.

The FPR binding assay was performed on the basis of a porcine report (Fletcher et al., 1990). Since it was reported that human neutrophils have the expression of FPR and bind to formyl peptide (Sklar et al., 1984), they were used as positive control. For the FPR binding assay, the fluorescein isothiocyanate-labeled formyl peptide analog (formyl-Nle–Leu–Phe–Tyr–Lys) was added to both ferret and human PMN solutions in HBSS(+) at a final concentration of 10^{-10} to 10^{-8} M. After the incubation for 30 min at 4 °C, the FPR binding assay was evaluated using FACSCanto flow cytometer (BD Biosciences, Franklin Lake, NJ) at room temperature.

The majority of cells obtained from the bottom of each well were neutrophils (>99%). Therefore, it is suggested that this experiment could estimate neutrophil chemotaxis.

The results of the cell migration ratio for each chemoattractant are shown in Fig. 1. Ferret PMNs appear to migrate to ZAS at all concentrations tested ($p < 0.05$) (Fig. 1A). In comparison, the migration responses at each concentration showed no significant difference. The optimal ZAS concentration was then determined to be 5%, serving as a minimum concentration in order to lessen the influence of other factors in the chemotaxis assay. Therefore, it was suggested that the satisfactory chemotactic factor was contained at 5% ZAS. Incubation times were also compared. Although the cells of the upper suspensions fell to the bottom of the wells by gravity, the optimal incubation time was determined based on the maximal migratory rate of cells compared with a background (in a buffer without chemoattractants) well. The greatest number of migratory cells was calculated after 120 min incubation; how-

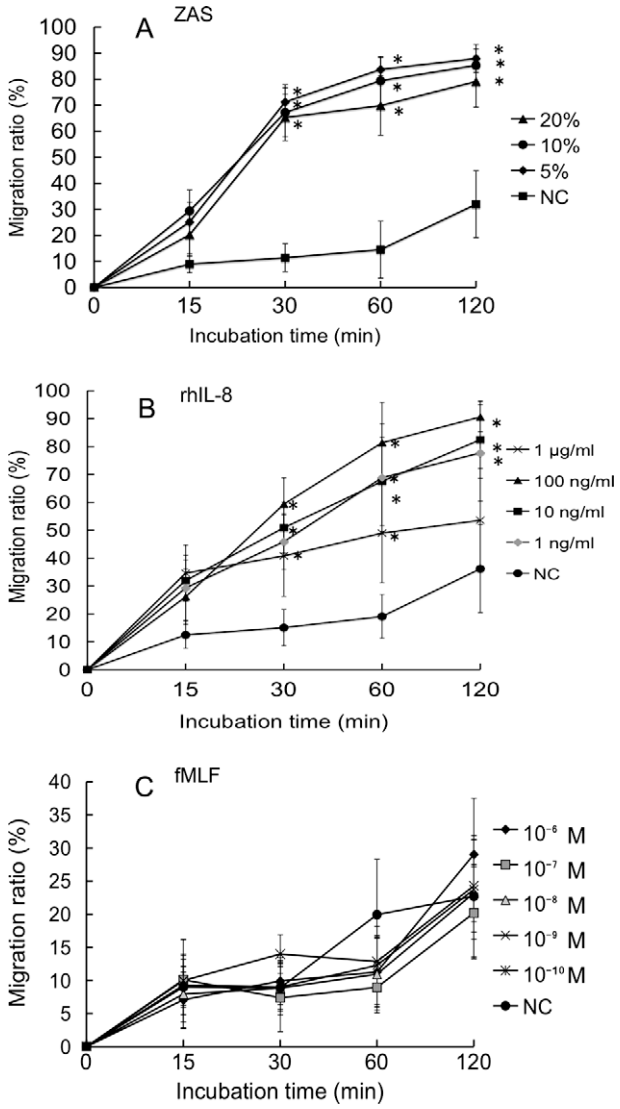

Fig. 1. The chemotactic response of ferret neutrophils. Concentration or incubation time chemotactic response of ferret neutrophils to ZAS (A), rhIL-8 (B) and fMLF (C) represented as a migration ratio. (A) ZAS concentrations were adjusted to 5, 10 and 20%. (B) rhIL-8 concentration was adjusted to 1, 10, 100 ng/ml and 1 /ml. (C) fMLF concentration was adjusted to 10^{-10} to 10^{-6} M. These results are presented as the mean ± SEM ($n = 3$–5). Asterisks indicate significant increases compared with the data from NC ($^{*}p < 0.05$, Student's *t*-test). Representative responses from at least three independent experiments are shown.

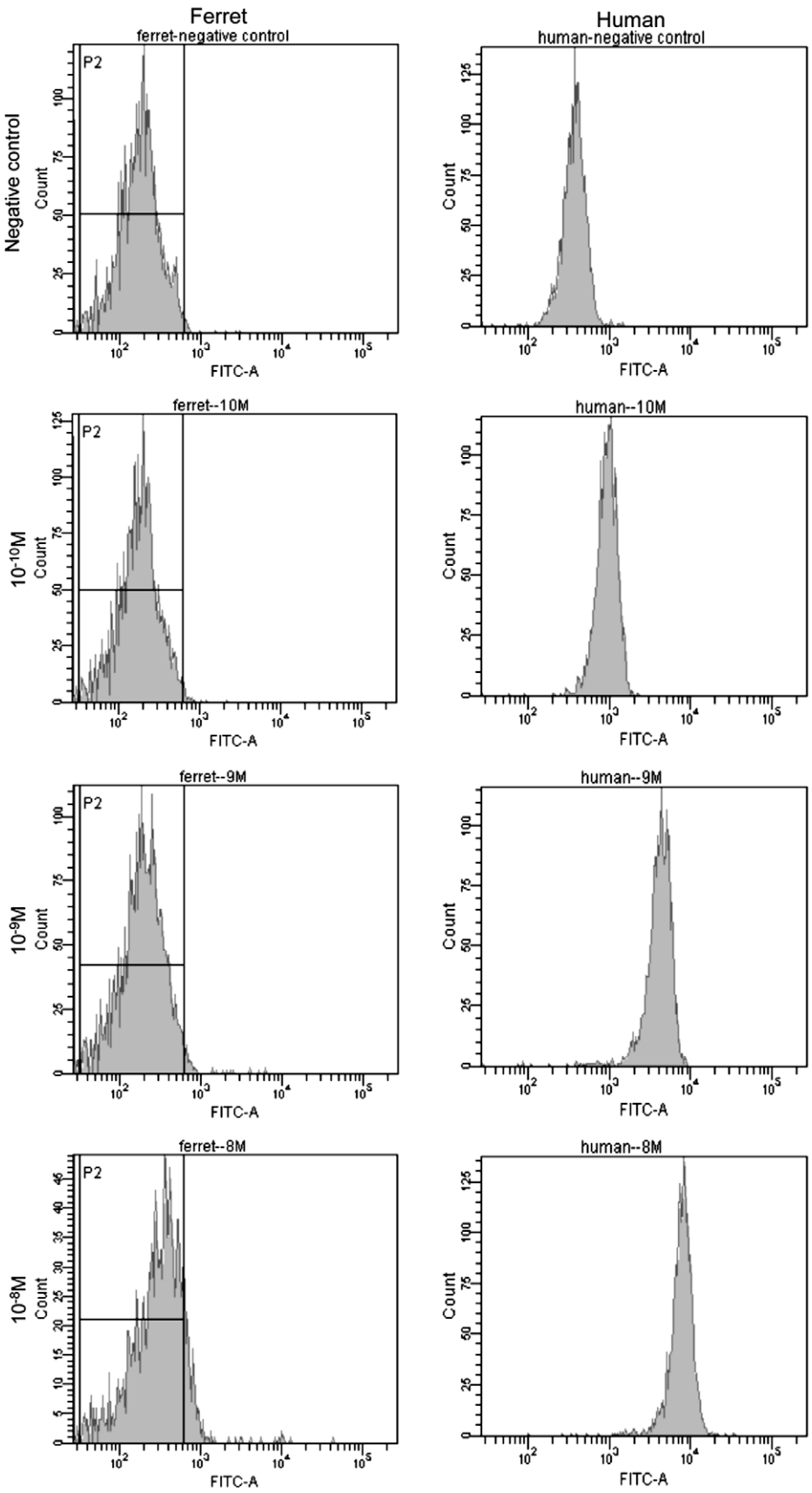

Fig. 2. Fluorescein-labeled formyl peptide receptor binding assay using flow cytometry. The fluorescein isothiocyanate-labeled formyl peptide analog was adjusted to 10^{-10} to 10^{-8} M. The fluorescent intensities of all concentrations are presented as compared with a control (absence of fluorescein-labeled formyl peptide analog).

ever, the cell number in background wells was also high. Therefore, although the cell migration ratio was significant at 30, 60 and 120 min of the incubation ($p < 0.05$), the optimal incubation time was determined to be 60 min. The chemotactic responses of human, dog, and rat neutrophils for ZAS depend on the origin of ZAS, and the majority of neutrophil migrate to homologous ZAS (Sugawara et al., 1995). In our preliminary experiments on this chemotactic assay, human and bovine ZAS were also used, but the migration ratio for ferret ZAS was slightly higher than human and bovine ZAS (data not shown). These results suggested that zymosan-induced C5a activity differs depending on species, and neutrophil response is more potent in response to homologous ZAS than heterologous ZAS, as previously reported (Edwards, 1994). In this study, homologous ZAS was used as the zymosan-induced ferret C5a, which showed strong chemotactic responses.

rhIL-8 response data are shown in Fig. 1B. A significant response of rhIL-8 to ferret neutrophil was seen at all concentrations except 1 μg/ml. The migration ratio of 1 μg/ml was lower than others. The change of this concentration dependency was thought to be based on the typical 'bell-shaped dose response', where the migration ratio of neutrophils decreased after exceeding a certain concentration (Itou et al., 2006). Furthermore, a 30-min incubation time was required for a significant response to be observed. Although there were no significant differences between concentrations 1, 10 and 100 ng/ml, the latter concentration was the most stable in all experiments. Therefore, the optimal concentration of rhIL-8 was determined to be 100 ng/ml. Additionally, the optimal incubation time to estimate ferret neutrophil chemotaxis response to rhIL-8 was 60 min, as with the ZAS-induced response (Fig. 1B). rhIL-8 is a CXC chemokine consisting of 72 amino acids. IL-8 has been found to be an important regulator of human neutrophil recruitment and activation (Gale and McColl, 1999). Furthermore, bovine neutrophils have been reported to respond by migrating to hrIL-8 (Galligan and Coomber, 2000). The migration effect of ferret neutrophils to hrIL-8 indicates that the rhIL-8 can be triggered through ferret CXC receptors.

The results of chemotaxis for fMLF are presented in Fig. 1C. Ferret PMNs showed no significant reaction with fMLF at any concentration or incubation time. Therefore, the binding between ferret neutrophils and formyl peptide were investigated using flow cytometry to determine the possibility of ferret FPR. The ferret PMNs expressed a complete lack of binding to formyl peptide at all concentrations tested (Fig. 2), while human PMNs bound fluorescent-labeled formyl peptide at a concentration (10^{-10} M) even lower than previously reported (Allen et al., 1992). FPR, one of the chemotaxis receptors that exists on human neutrophils, belongs to the GPCR (G protein-coupled receptor) family. Reports of a FPR binding assay using flow cytometry in human and pig neutrophils (Fletcher et al., 1990; Allen et al., 1992; Graves et al., 1992) found that human, but not porcine, neutrophils bind to the formyl peptide. Thus, the binding reaction to the formyl peptide appears to differ between species.

It has been previously reported that neutrophils of human, rodents, rabbit and guinea pigs have chemotaxis or superoxide generating ability induced by fMLF (Sugawara et al., 1995; Zimmerli et al., 1986). Superoxide generation is an essential function of neutrophils, and concerned with FPR when human neutrophils respond to fMLF. Dog neutrophils were reported to show no chemotaxis in response to fMLF (Sugawara et al., 1995; Stickle et al., 1985). Conversely, the dog neutrophils have superoxide generation ability in response to fMLF (Lindena and Burkhardt, 1988) and the presence of FPR (Linnekin et al., 1990). According to several other reports, PMNs of bison (Swain et al., 2000), cattle (Gray et al., 1982), cat (Gray et al., 1986), sheep (Buchta, 1990) and pig (Fletcher et al., 1990) show no fMLF-induced superoxide generation ability. Equine neutrophils also do not demonstrate any chemotactic response to fMLF, and there is no inflammatory reaction when fMLF is injected intradermally, despite the presence of FPR (Brazil et al., 1998). Moreover, the authors previously reported that ferret PMNs do not generate superoxide in response to fMLF (Nakata et al., 2007). Taken together, ferret neutrophils appear to lack FPR and consequently do not have chemotaxis and superoxide generation activity through the FPR.

In conclusion, this study confirms the fundamental experimental conditions for ferret PMNs chemotaxis and elucidates new information concerning FPR expression in ferret neutrophils.

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