Effects of Serum on the Shape Change Response of Polymorphonuclear Leukocytes in the Dog

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ABSTRACT. The effects of canine serum on the cellular configuration *in vitro* were studied in polymorphonuclear leukocytes (PMN) from the peripheral blood of clinically healthy adult beagle dogs. PMN rapidly changed their shape from spherical to ruffled, uropod, or polarized within 1 to 5 min after incubation with freshly isolated autologous or allogeneic canine serum at 37°C, as observed in PMN incubated with zymosan-activated serum and casein. The maximal shape change response (approximately 68%) was observed at 5 min after PMN were exposed to 10% serum. The neutrophils changed their shape faster and at a higher percentage than did eosinophils. Furthermore, the heat treatment (56°C for 30 min) of serum did not affect the PMN shape change response. These results indicate that serum has a potent effect on the induction of the PMN shape change response.—**KEY WORDS:** chemoattractant, polymorphonuclear leukocyte, shape change, zymosan.

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The leukocyte behaviors are demonstrated to be influenced by chemoattractants [2, 8, 13, 14] such as a complement-derived component activated with bacterial substance (lipopolysaccharide; LPS, and zymosan), referred to as C5a, LPS- or zymosan-activated serum (ZAS), the arachidonic acid metabolite leukotriene (LTB₄), the platelet-activating factor (PAF), bacterial chemotactic factors (BCF), casein, and synthetic peptides (e.g., N-formyl-L-methionyl-L-phenylalanine; FMP, and N-formyl-L-methionyl-L-leucyl-L-phenylalanine; FMLP).

The characteristic morphological changes from a spherical shape to a bipolar shape have been reported to occur rapidly after bovine polymorphonuclear leukocytes (PMN) were exposed to chemotactic factors (ZAS and alpha casein) [14]. The morphological features of PMN were not altered by agents with less chemotactic activity. These findings on the interaction of PMN with chemoattractants show that these chemotactic factors are substantially effective subst-

ances for the evaluation of the *in vitro* responsiveness (shape change response and chemotactic response) of PMN to chemotactic agents. However, little information concerning the effect of serum on the leukocyte shape change has been provided. Considering that serum itself is less chemotactic [17] and that leukocyte behaviors are influenced by the PMN-serum interaction [9, 14, 17], it is of importance to define whether serum itself has a potent effect on the induction of the morphological change of PMN.

In the present study, the effect of canine serum on the canine PMN shape change response *in vitro* was evaluated by the development of morphologically deformed cells.

MATERIALS AND METHODS

Animals: Five clinically healthy beagle dogs (2 females and 3 males ranging from 2 to 3 years old) were used in the present study. They were kept at the Department of Veterinary Internal Medicine, Faculty of

Agriculture, University of Tokyo. No abnormalities were detected by hematological and biochemical examinations, urinalysis, and fecal examination. They were also negative for microfilariae of *Dirofilaria immitis*.

Separation of PMN: Heparinized blood samples were collected from the cervical vein of the beagle dogs. For the preparation of PMN suspensions, the blood samples supplemented with dextran 70 (1/5 parts of whole blood volume) (6 w/v%, Green Cross Co., Japan) were allowed to stand for 1 hr at 37°C to prepare leukocyte-rich plasma following the sedimentation of erythrocytes. The resulting leukocyte-rich plasma was centrifuged at 200g for 10 min, and the pelleted cells were washed one time in Ca^{+2} - Mg^{+2} -free phosphate-buffered saline (0.01 M PBS, pH7.2) to remove the plasma. Two parts of the leukocytes were layered on the top of one part of Ficoll-Hypaque (Lymphoprep; specific gravity 1.077, Nyegarrd Co., Norway) [6], and then centrifuged at 1200g for 40 min. The pellet, which contained granulocytes and erythrocytes, was mixed with a 0.83% ammonium chloride solution which lysed the erythrocytes. The PMN were washed twice with PBS and suspended in PBS to give a final concentration of 1×10^7 viable cells/ml. The viability of the PMN was greater than 95%, as determined by the trypan blue (0.25%) dye exclusion test. The PMN consisted of more than 98% granulocytes, of which, more than 95% were neutrophils, and the remaining cells were eosinophils. A significant number of mononuclear cells were not seen in any cell preparations.

The PMN suspension was immediately placed at 4°C. No change due to 5-hr incubation at 4°C was detected in the cellular configuration evaluated by the technique described below.

Serum samples and chemotactic agents: Autologous and allogeneic serum samples

were collected from the beagle dogs. An aliquot of serum samples was subjected to heat-treatment (56°C for 30 min). ZAS was prepared by incubating 10 mg zymosan (Sigma Chemical Co., St. Louis, MO) with 1ml of fresh canine serum for 45 min at 37°C, and removing the zymosan particles by centrifugation at 900g for 20 min as previously described by Forsell *et al.* [3]. Concentration was expressed as dilutions of the original volume of serum and ZAS samples. Since the preliminary experiment showed that ZAS was maximally effective in the PMN shape change response at the concentration of 10%, it was mainly used at that concentration. Purified alphacasein from bovine milk (Sigma) was dissolved in PBS to a concentration of 25 mg/ml as previously described [3], and diluted further with PBS to the concentration of 5 mg/ml, which gave the maximal PMN shape change response.

Assessment of changes in PMN shape: A modification of the method of Forsell *et al.* [3] was employed for the assessment of morphological changes in PMN. PMN (1×10^7 cells/ml) suspended in PBS were exposed to the various conditions and reagents described above. An aliquot (0.1ml) of the PMN suspension (1×10^7 cells/ml) was added to a sterile polyethylene test tube (Eiken Kizai Co., Japan) containing 0.9ml of PBS alone (controls) or PBS supplemented with serum samples and chemotactic agents, and incubated in a water bath at 37°C. At the appropriate time (1 to 15 min), the PMN undergoing shape changes were fixed by the dropwise addition of 3 ml of cold (4°C) 1% glutaraldehyde in PBS. The PMN suspensions were mixed constantly, while the glutaraldehyde solution was added. Glutaraldehyde-fixed PMN were held at 4°C overnight and then examined for cellular shape changes with a phase-contrast microscope (Nikon Optical Co., Japan). The PMN were classified as round (spherical

shape) or unspherical (ruffled, uropod or bipolar shape) (Fig. 1). Cytospin smears were also prepared from each cell suspension, and stained with Giemsa-stain for more detailed evaluation of shape changes in neutrophils and eosinophils. At least 100 cells on each sample were scored for the percentage of deformed cells in cell suspensions. The shape change response was expressed as the mean percentage and error of duplicate or triplicate experiments. Student's *t* test was employed to assess statistical significance. The level of significance was set at $p < 0.05$.

RESULTS

The appearance of PMN fixed with glutaraldehyde is shown in Fig. 1. When kept at 4°C or incubated in PBS without fresh canine serum, the PMN generally appeared round (Fig. 1a; neutrophils, and b; eosinophils). The addition of fresh serum to the cell suspension resulted in the shape changes in cells from spherical to unspherical. The PMN had a slightly ruffled membrane on a small portion of the surface and an overall form that was oval to elongated (uropod/bipolar) (Fig. 1c, d and e). These PMN shape changes were detected within 1 to 5 min after the addition of canine serum. These morphological changes in PMN exposed to serum were observed in eosinophils as well as neutrophils (Fig. 1d and e).

Fig. 2 shows the morphological change of PMN incubated with freshly isolated autologous or allogeneic sera at the concentration ranging from 0.1% to 20% for 5 min at 37°C. PMN maintained in PBS at 4°C during the test period did not show any significant morphological change. The proportion of deformed cells in the PMN suspension was less than 8%. Although the percentage of deformed cells varied with the different concentration of serum, the shape change

response of PMN incubated in fresh allogeneic serum was similar to that of PMN incubated in fresh autologous serum. The maximal response was observed in the incubation of either 10% autologous or allogeneic serum. The mean percent of deformed cells in PMN was 64% and 68%, respectively.

Subsequently, the time course for the PMN shape change response was studied by using the PMN suspension incubated with fresh allogeneic serum at the concentration of 2% and 10%. As seen in Fig. 3, PMN in PBS maintained for 1 to 15 min at 37°C showed a slightly higher percentage of deformed cells (approximately 8%) as compared with those kept at 4°C. PMN in the cell suspension with an unspherical form rapidly appeared within 1 min after the addition of serum. The percentage of deformed cells in PMN exposed to 10% serum remained high (approximately 70%) by 5 min after the exposure to serum, and then gradually fell to low levels by 15 min after the exposure. The elimination of the PMN shape change response was more distinct in 2% serum incubation than in 10% serum incubation.

PMN exposed to 10% fresh allogeneic serum for 1 or 5 min at 37°C were examined for the proportion of deformed cells in neutrophils and eosinophils (Table 1). The deformed neutrophils and eosinophils were also confirmed by microscopy of Giemsa-stained PMN smear preparations. Neutrophils changed shape faster and at a higher percentage as compared with eosinophils ($p < 0.01$ in a 1 min response, $p < 0.05$ in a 5 min response).

Furthermore, the percentage of deformed cells was not affected in PMN incubated with heat-treated (56°C for 30 min) serum for 1 or 5 min at 37°C, giving the same degree of response as did PMN exposed to fresh serum (Table 2).

The morphological change of PMN was

Fig. 1. Change in shape of PMN exposed to fresh canine serum. Two neutrophils (arrow) and two mononuclear cells (a) and two eosinophils (b) with round (spherical) feature are seen 15 min after the incubation of the PMN in PBS. Several PMN with uropod/bipolar forms (c; $\times 400$, phase-contrast), two deformed neutrophils with bipolarized forms (d) and a deformed eosinophil with uropods (e) are seen 5 min after the addition of canine serum to the PMN suspension. Each bar represents $5\mu\text{m}$.

Fig. 2. Shape changes in canine PMN exposed to various concentrations of fresh autologous or allogeneic serum (0.1–20%) for 5 min at 37°C . PBS was employed as controls instead of the serum. PMN were fixed in a 1% glutaraldehyde solution and examined with a phase-contrast microscope. Each value represents the mean and standard error of triplicate experiments.

Fig. 3. Shape changes in canine PMN exposed to fresh serum (2% or 10%) or PBS (as controls) for different incubation periods at 37°C. Each value represents the mean and standard error of triplicate experiments.

Table 1. Serum-induced shape change response of PMN, neutrophils, and eosinophils

Cells	% Deformed cells ^{a)}	
	1 min	5 min
PMN	68.5±9.6	66.8±8.5
Neutrophils	67.4±6.8	72.6±7.4
Eosinophils	15.6±4.8 ^{b)}	38.5±6.0 ^{c)}

a) PMN (1×10^6 cells/ml) were incubated with fresh sera (10%) from 5 healthy dogs for 1 min or 5 min at 37°C. Each value represents the mean and standard error of duplicate experiments in 5 dogs. The shape change response of PMN incubated in PBS (as controls) for 1 min or 5 min was $5.1 \pm 1.4\%$ and $7.2 \pm 1.5\%$, respectively.

b) $P < 0.01$, as compared with neutrophils.

c) $P < 0.05$, as compared with neutrophils.

Table 2. Effect of heat-treated serum on PMN shape change response

Stimulant	% Deformed cells ^{a)}	
	1 min	5 min
Fresh serum (10%)	68.5±9.6	66.8±8.5
Heat-treated serum (10%)	61.8±5.0	59.6±6.8
PBS	5.1±1.4	7.2±1.5

a) PMN (1×10^6 cells/ml) were exposed to each stimulant, freshly isolated serum from a healthy dog at the final concentration of 10% at 37°C. PBS was used as controls instead of the stimulant. Each value represents the mean and standard error of triplicate experiments.

Fig. 4. Shape changes in canine PMN exposed to ZAS (2% or 10%), casein (5 mg/ml) and PBS (as controls) for different incubation periods at 37°C. Each value represents the mean and standard error of triplicate experiments.

also observed within 1 min after the addition of ZAS (at the concentration of 2% and 10%) and casein (5 mg/ml) to the cells (Fig. 4). The maximal response was noted by 5 min after the addition of each chemoattractant. Then after 5 min incubation, the responses slightly decreased. The degree of the reduction was less distinct comparing with the PMN shape change response induced by serum.

DISCUSSION

In the present study, canine PMN were demonstrated to change their shape from spherical to ruffled, uropod, or bipolar forms within 1 min after the addition of autologous or allogeneic serum. This shape change response closely resembled that of PMN exposed to ZAS or casein. The maximal shape change response of PMN was found in 10% canine serum. ZAS and casein were maximally effective at 10% and 5 mg/ml, respectively. The PMN shape change response was also induced by heat-treated serum, as evaluated in deformed cell percentages. Therefore, these results suggest that canine serum contains PMN-deforming substances, which are heat (56°C

for 30 min)-resistant and apparently different from complement-related constituents.

Many kinds of endogenous or exogenous chemotactic biologicals and chemicals such as C5a, ZAS, casein, FMP and FMLP have been used to investigate the PMN-chemoattractant interaction. The agents with chemotactic activity are effective in the induction of the morphological change in PMN, and agents with less chemotactic activity fail to induce such an effect [3, 14]. These chemotactic factors seem to be substantially most beneficial agents to evaluate the activity of PMN, as assessed in terms of the PMN shape change response *in vitro*. Multiple endogenous factors influencing leukocyte behaviors *in vitro* include plasma, serum albumin, C5a, LTB₄ and PAF [5, 9, 12], the concentration of Ca²⁺ and Mg²⁺ in the medium [1, 4], PMN-lysosomal enzymes (e.g., beta-glucuronidase) and PMN-chemotactic factors which are regarded to be lymphokines from activated T lymphocytes [10, 16]. However, it is uncertain whether the *in vitro* responsiveness of PMN to these chemotactic factors directly reflects the PMN behaviors under the physiological conditions.

The immediate shape change response of canine PMN and the persistence of a high response in 10% canine serum suggest that serum directly induces morphological changes in PMN. The percentages of deformed cells in PMN gradually decreased in the prolonged incubation. The similar effects have been shown in human neutrophils exposed to ZAS, BCF and C5a [14]. ZAS employed in the present study induced a high percentage of deformed cells in PMN even at a lower concentration (0.2%), while fresh serum had a negligible effect on the PMN shape change response at the same concentration. This shape change phenomenon of PMN may be induced as a result of a synergic effect of endogenous serum components and complement cleav-

ages in ZAS. when PMN were incubated with 10% fresh serum, neutrophils changed shape faster and at high percentages than did eosinophils. This result suggests that the differences of their responsiveness may be associated with the receptors or receptor density on cell surfaces of neutrophils and eosinophils, as described earlier [3]. In addition, heat-treated serum was also found to be effective in the induction of the morphological alteration of PMN. This suggests that serum components as well as complement components are involved in the PMN shape change at least in part (Table 2). However, it remains to be defined whether canine serum induces the PMN shape alteration through the physical property [11] or biochemical property [3, 11]. Some investigators reported the active substance in the serum, which stimulates the cell surface receptors and induces the morphological change [7, 15].

It is of interest that the percentage of deformed cells in the PMN suspension falls to low levels by 15 min after the exposure to serum, especially at 2% (Fig. 3). This result was not consistent with the findings reported by others for human [14] and bovine [3] PMN. In these reports, the PMN shape change response was maintained at a high level by 40 min after the exposure of PMN to chemotactic factors such as ZAS and casein. The markedly reduced response of PMN was more apparent in fresh serum than in ZAS and casein. Therefore, the PMN shape change response may be dependent on the difference and concentrations of chemoattractants, experimental conditions (including exposure time) and PMN used for the assay. In this respect, freshly isolated serum failed to induce a significant chemotactic response in the horse [17]. The percentage of bipolarized cells in human PMN exposed to chemoattractants such as C5a, ZAS, casein, FMP and BCF gradually reduced by 40 min. Forty minutes later,

when PMN were washed with medium and restimulated with chemoattractants, deformed PMN reappeared to the same extent as on the initial stimulation. This indicated that the shape change in PMN is reversible upon removal of the chemotactic factor [14]. Furthermore, continued stimulation by active agents may be necessary for the maintenance of the bipolarized cells in PMN. Canine serum used in the present study did not maintain the persistent PMN shape change response, but was active enough to induce the response. Further studies are required for defining the significance of the deformation of PMN shape in the migration, recruit and infiltration of PMN to the affected tissue.

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要 約

イヌの多形核白血球の形状に及ぼす血清の影響：廣田好和，吉川康一，長谷川篤彦，友田 勇（東京大学農学部家畜内科学教室）——健常ビーグル成犬の末梢血多形核白血球（PMN）の形状に及ぼす血清の影響について検索した。自己，または同種新鮮血清に浮遊した PMN を 37℃ に静置すると，1 ～ 5 分以内に PMN は球形から卵円形，有棘形，あるいは偏心状の細胞に変形した。このような変形は，サイモザンで活性化した血清およびカゼインに浮遊した PMN において観察される形状変化と同じであった。変形細胞の出現比率は，PMN を 10% 血清に 5 分間浮遊した場合に最大であった（約 68%）。好中球の変形は，好酸球のそれに比して迅速で，変形細胞出現率も高かった。さらに，血清を 56℃ で 30 分間加熱処理しても，PMN 中の変形細胞発現率は変らなかった。