Effects of Salivary Gland Extract from *Rhipicephalus sanguineus* on Immunoglobulin Class Productivity of Canine Peripheral Blood Lymphocytes

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**ABSTRACT.** Effects of salivary gland extract (SGE) from *Rhipicephalus sanguineus* on immunoglobulin class productivity of canine peripheral blood lymphocytes (PBL) *in vitro* were studied. The detectable limit of the ELISA for canine total immunoglobulin, IgM and A was at least 1, 1 and 15 ng/ml, respectively, and it seems to be useful for the evaluation of non-specific immunoglobulin class productivity *in vitro*. SGE from *R. sanguineus* suppressed pokeweed mitogen- or lipopolysaccharide-induced total immunoglobulin and IgA productivity of canine PBL although IgM productivity was not suppressed. These results suggested that the suppression was caused partly by the direct effect of SGE on B lymphocytes.

**KEY WORDS:** canine, immunoglobulin class productivity, *Rhipicephalus sanguineus.*

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It is known that tick infestation itself has immune suppressive effects on host animals, which aids the transmission of tick-borne diseases [9, 10]. *Rhipicephalus sanguineus* is the three-host dog tick, and is known to be a vector for various diseases of dogs. Suppressive effects of the infestation of *R. sanguineus* on dogs, such as antibody production, neutrophil function and lymphocyte blastogenic response have been found in our previous works [2–4]. The effects of tick saliva on the inhibition of neutrophil function and lymphocyte blastogenic response have been proven by *in vitro* experiments; however, immunoglobulin productivity has not been proven yet. In this study, we examined the effects of salivary gland extract (SGE) of adult *R. sanguineus* on *in vitro* immunoglobulin class productivity of canine peripheral blood lymphocytes (PBL) induced by pokeweed mitogen (PWM) or lipopolysaccharide (LPS) stimulation.

SGE was obtained from females of partially-engorged *R. sanguineus* kept in our laboratory [3]. The ticks were allowed to feed for 4 days on the ears of rabbits (Japanese white, Kyudo, Japan) and collected. Salivary glands were dissected from each tick and washed with phosphate buffered saline (PBS, pH 7.2) and homogenized with a ground glass homogenizer in 1.0 ml of PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Nakalai Tesque, Japan) over an ice bath. This crude extract was centrifuged at 7740 × g for 5 min. Supernatant was filtered through a 0.22 µm filter and the protein concentration was determined with a DC Protein Assay Kit (Bio-Rad, U.S.A.). The obtained SGE was stored at −20°C until use.

Peripheral blood was collected in heparinized tubes (20 IU/ml) from six tick-naïve, adult male Beagles bred at Yamaguchi University. A density gradient method with Lymphoprep (1.077 g/ml, Nycomed Pharma AS, Oslo, Norway) was used to separate mononuclear cells containing lymphocytes from peripheral blood [5]. Contaminated red blood cells were lysed with ammonium chloride Tris buffer (0.017 M Tris, pH 7.2 containing 0.75% NH₄Cl). The mononuclear cells were washed twice with sterile PBS, and finally resuspended at 1 × 10⁷ cells/ml in Dulbecco’s Modified Eagle Medium (GIBCO BRL, U.S.A.) containing 10% heat-inactivated fetal bovine serum (FBS) (ICN Biomedical Japan), Antibiotic-Antimycotic [penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml) and amphotericin B (0.25 µg/ml), GIBCO BRL, U.S.A.], and PWM (50 µg/ml, EY Laboratories Inc., U.S.A.) or LPS (100 µg/ml, Sigma Chemical Co., U.S.A.). Aliquots of 100 µl of PBL suspension were added to wells of flat-bottom 96-well microculture plates (Flow Laboratories Inc., U.S.A.). Then, aliquots of 100 µl of SGE solution were added to those wells at the final concentrations of 100, 25, 6.25, 1.56 and 0 µg/ml. The microculture plates were centrifuged, and the supernatant was collected to determine total immunoglobulin, IgM and A productivity of canine peripheral blood lymphocytes *in vitro*. The plates were incubated for 6 days at 37°C in a humidified chamber of 5% CO₂. After six-day-culture, the viability of PBMC was low, but no difference was seen between control and SGE addition sample. Then the microculture plates were centrifuged, and the supernatant was collected to determine total immunoglobulin, IgM and A concentrations by the following method.

To quantify the total immunoglobulin concentration, U-bottom 96-well microplates (Greiner, Germany) were incubated overnight at 4°C with rabbit anti-dog IgG (1 µg/ml, H&L-chain, Rockland, U.S.A.) in 0.05 M carbonate buffer (CB, pH 9.6). Plates were rinsed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 1% gelatin in CB for 2 hr at 37°C. The plates were then rinsed three times with PBS-T. Supernatant of the lymphocyte culture was added to each well (100 µl) and incubated for 1 hr at 37°C. The plates were again rinsed three times with PBS-T.
T, and 100 µl of horseradish peroxidase conjugated rabbit anti-dog IgG (1:5000 in PBS-T, H&L-chain, Rockland, U.S.A.) was added to each well. The plates were incubated for 1 hr at 37°C and rinsed three times with PBS-T. After the addition of 100 µl of ABTS peroxidase substrate (Kirkegaard & Perry Laboratories Inc., U.S.A.) to each well, the plates were incubated for 1 hr at 37°C. The optical density (OD) in each well was read on an ELISA reader (Multi-scan Bichromatic, Labsystem, U.S.A.) at a wavelength of 405 nm, and the total immunoglobulin concentration was determined by comparison with control canine immunoglobulin. To determine IgM and A, goat anti-dog IgM (1 µg/ml, µ-chain specific, Bethyl, U.S.A.) and horseradish peroxidase conjugated goat anti-dog IgM (1:10,000 in PBS-T, µ-chain specific, ICN, U.S.A.), and goat anti-dog IgA (0.5 µg/ml, α-chain specific, Bethyl, U.S.A.) and horseradish peroxidase conjugated goat anti-dog IgA (1:10,000 in PBS-T, α-chain specific, Bethyl, U.S.A.) were used, respectively. The standard curves were obtained by measuring OD of control immunoglobulin, IgM and A. The control immunoglobulin and IgM were two time serial dilutions from 500 ng/ml to 1 ng/ml, and the control IgA was from 15,000 ng/ml to 15 ng/ml. To analyze standard curves, the paired t test was used.

For analyzing the effects of SGE from R. sanguineus on immunoglobulin class productivity, the paired t test was also performed. The standard curves of total immunoglobulin, IgM and A are shown in Fig. 1 a, b and c, respectively. Each concentration of total immunoglobulin, IgM or A had significant differences from other concentrations, suggesting the detectable limit of the ELISA for canine total immunoglobulin, IgM and A was at least 1, 1 and 15 ng/ml, respectively.

The effects of SGE on the total immunoglobulin, IgM and A productivity of PBL to PWM are shown in Fig. 2 a, b and c, respectively. Significant suppression was observed for total immunoglobulin productivity at the SGE protein concentrations of 1.56 and 25 µg/ml or more and IgA at 25 µg/ml or more, but IgM was not suppressed by any concentration of SGE used. The suppressive effects of SGE on PWM induced total immunoglobulin and IgA productivity seemed to be dose-dependent. The effects of SGE on the total immunoglobulin, IgM and A productivity of PBL to LPS are shown in Fig. 3 a, b and c, respectively. Dose-dependent-like suppressive effects of SGE on LPS induced IgA productivity were observed; however, significant suppression only occurred for total immunoglobulin productivity at the SGE protein concentrations of 6.25 and 25 µg/ml and IgA at 25.25 and 100 µg/ml. No suppressive effect was observed in IgM productivity.

In the present study, we developed an ELISA to quantify canine immunoglobulin class productivity in vitro. The detectable limit of the ELISA for canine total immunoglobulin, IgM and A was 1, 1 and 15 ng/ml, respectively (Fig. 1 a, b and c). It seemed to be useful for the evaluation of non-specific immunoglobulin class productivity of B lymphocytes in vitro. Using this method, we observed the effects of SGE from R. sanguineus on PWM- or LPS-induced immunoglobulin class productivity of canine PBL. PWM is known to be T and B lymphocyte mitogen, and LPS is effective on B lymphocyte proliferation. Both mitogens induced non-specific immunoglobulin production of lymphocytes [6]. In this experiment, SGE from R. sanguineus suppressed
immunoglobulin productivity induced by both PWM and LPS. These results suggested that the suppression of immunoglobulin productivity was caused partly by the direct effect of SGE on B lymphocytes, because LPS has the effect on B lymphocyte proliferation [6]. Interestingly, suppressive effects were found on total immunoglobulin and IgA, but not IgM. This difference might be caused by suppression of cytokines produced by helper T lymphocytes (Th). Th cytokines are known to influence a class switch to immunoglobulin classes of B lymphocytes [6], and our previous study revealed that mitogenic responses of T lymphocytes from dogs infested with *R. sanguineus* were suppressed [2].

The cytokine-mediated mechanisms of immunosuppression caused by ticks were demonstrated in mice [1, 7–9]. The production of Th1 cytokines such as interleukin-2 and interferon-γ was suppressed by SGE of ticks in mice [9], but there are no reports in dogs.

In this study, total immunoglobulin and IgA productivity of canine PBL was suppressed by SGE from *R. sanguineus* in vitro [3], which may be caused by tick saliva. The suppression of immunoglobulin productivity, neutrophil function [4] and lymphocyte blastogenic response [2] in dogs by *R. sanguineus* in vivo [3], which may be caused by tick saliva.
sanguineus may play an important role in transmitting tick-borne diseases such as Babesia canis, B. gibsoni, Hepatozoon canis and Ehrlichia canis. Further studies are required to explain the detailed mechanism of the suppressive effects of SGE from R. sanguineus on the canine immune system.

REFERENCES