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ORIGINAL PAPER

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Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice

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Abstract Saliva and salivary gland extract (SGE) of *Ixodes ricinus* ticks have suppressive effects on the innate immune response of BALB/c mice. Tick saliva prevents hemolysis of sheep red blood cells (SRBC) by the human alternative pathway of complement. The adaptive immune response is also modulated by tick antigens (saliva or SGE). When stimulated in vitro with increasing doses of tick antigens, the proliferation and IL-4 production of draining lymph node T cells of mice infested with nymphal ticks increase, peak and then decrease. These results indicate that immunostimulative and immunosuppressive molecules have competing effects in tick saliva or in SGE. *I. ricinus* saliva inhibits, in a dose-dependent manner, splenic T cell proliferation in response to concanavalin A (ConA). Tick SGE or saliva injected intraperitoneally to BALB/c mice simultaneously with SRBC systemically immunosuppress the anti-SRBC response as shown in vitro by the reduced responsiveness of sensitized splenic T cells to restimulation with SRBC. In brief some components of SGE or tick saliva reduce the responsiveness of draining lymph node T cells and of sensitized splenic T cells in vitro. The responsiveness of naive splenic T cells to ConA stimulation in vitro is also decreased by tick saliva. Modulation of host responses by tick antigens may facilitate tick feeding, transmission and the propagation of pathogens.

Introduction

Ixodid ticks attach to their hosts and feed for several days during which time they may transmit various pathogens. Infested animals are immunologically tolerant or acquire resistance against ticks. One would expect that tick saliva is produced to aid feeding, as well as for the transmission and propagation of tick-borne pathogens. Few pharmacological properties of saliva molecules have been described. They are related to evasive mechanisms which facilitate feeding and pathogen transmission (Ribeiro et al. 1985; Titus and Ribeiro 1990). Depending on the tick-host association, innate and adaptive immune responses have different influences on tick feeding and pathogen transmission. Female BALB/c mice repeatedly infested with pathogen-free *Ixodes scapularis* are tolerant to tick feeding but resist the subsequent transmission of *Borrelia burgdorferi* (Wikel et al. 1997). The partial resistance of wild white-footed mice (*Peromyscus leucopus*) to repeatedly feeding ticks did not prevent the transmission of *B. burgdorferi* (Richter et al. 1998). In our model, *I. ricinus* nymphs modulate the anti-tick immune response of BALB/c mice which failed to acquire resistance against ticks (Mbow et al. 1994a). We have recently shown that some chromatographic fractions of salivary gland extract (SGE) have either stimulative or suppressive activities on the responsiveness of draining lymph node cells of BALB/c mice infested with nymphal *I. ricinus* (Mejri et al. 2001). Saliva molecules would have competing activities during infestation, acting on several levels of the immune response. Ticks attach to their hosts in varying densities in natural conditions. The amount of salivary secretion injected into the skin could influence tick feeding as well as pathogen transmission. The balance between the immunostimulative and immunosuppressive effects of tick saliva and of SGE molecules has never been addressed.

The purpose of this work is to make an in vitro analysis of the effect of different concentrations of *I. ricinus* saliva and of SGE (tick antigens) on the non-specific innate or specific acquired immunological responses of BALB/c mice. The non-specific effects of tick antigens were determined using the human alternative complement pathway and the proliferation of naive spleen cells stimulated with concanavalin

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A (ConA). The specific effects of tick antigens were studied on lymphocyte proliferation and the secretion of IL-4 by lymph node cells. Low concentrations were generally immunostimulative whereas high concentrations were immunosuppressive. The influence of tick antigens was also estimated with the systemic immune response against SRBC.

Materials and methods

Animals

Eight to 12-week-old BALB/c female mice and male rabbits (New Zealand) weighing an average of 3 kg were purchased from IFFA-CREDO (Arbresle, France) and from Elevage des Dombes (Romans, France) respectively. Ticks were reared in our laboratory as previously described (Graf 1978).

Infestations

Mice were infested with 15 *I. ricinus* nymphs each. These were placed into a small plastic capsule glued to shaved skin at the site drained by brachial and axillary lymph nodes of the host shoulder using a mixture of one part beeswax and four parts colophonium (Mbow et al. 1994b). Each experiment was done using a group of five mice. To prepare tick antigens, female *I. ricinus* were applied to a rabbit's ears and allowed to feed for 5 days. They were contained in a nylon bag stuck to the ear with an adhesive band. A collar was placed around the rabbit's neck to prevent grooming.

Saliva preparation

Partially engorged female ticks were removed. Saliva was collected in a glass capillary tube fitted over the mouth-parts of the tick. From 0.3 to 0.5 µl of saliva was collected per tick after 10–30 min. Saliva from 80 partially fed ticks was pooled, sterilized through a 0.22 µm filter and stored at –20°C until used.

Salivary gland extract

Another group of partially fed *I.ricinus* females which had been attached to a rabbit's ears for 5 days were used to prepare SGE as previously described (Rutti and Brossard 1989). Eighty pairs of the salivary glands were dissected out and homogenized in 1 ml of ice-cold extraction buffer consisting of 50 mM phosphate-buffered saline (PBS) pH 7.4 supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM ethylene diaminetetraacetic acid (EDTA). The homogenate was centrifuged at 16,000 *g* for 30 min at 4°C. The supernatant was dialyzed overnight in 10 mM (PBS) pH 7.4 in a cellulose ester membrane tube with a molecular weight cut-off of 100 Da (Spectrum, Socochim, Switzerland). Dialysate was sterilized through a 0.22 µm filter and stored at –20°C until used. The protein concentration of the saliva and the SGE was determined using a BCA Protein Assay Kit (Pierce, Socochim, Switzerland).

Hemolysis test

The hemolysis test was performed according to a modified method of Ribeiro (1987) using human serum, sheep red blood cells (SRBC) (BioMérieux, Switzerland) and tick saliva. Briefly, 400 µl of SRBC at 50% (v/v) were washed twice and resuspended in 1.5 ml Veronal buffer (VBS) pH 7.35 supplemented with 0.1% BSA, 2 mM $MgCl_2$ and 5 mM EGTA (Sigma, Switzerland). SRBC at 6% were then incubated with 10 µl rabbit anti-SRBC Ab (Nordic, The Netherlands) for 30 min at 37°C. Serial dilution of the saliva protein (15, 30, and 45 µg/ml) plus 10 µl of human serum were added to the VBS-BSA-Mg^{2+} buffer followed by 30 µl erythrocytes giving a final volume of 0.1 ml. The mixture was then incubated for 1 h at 37°C. The optical density of the supernatant was measured at 405 nm with a spectrophotometer (Dynatech, Switzerland).

Proliferation of draining lymph node cells restimulated with tick antigens

Mice were killed 9 days after infestation and the axillary and brachial lymph nodes were removed. A total of 10^6 lymph node cells per well were cultivated in 200 µl culture medium containing RPMI-1640 (Gibco, Basel, Switzerland), supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids (Sigma, St Louis, Mo.), 0.05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin (Gibco) and 25µg/ml Fungizone (Gibco). Cells were stimulated for 96 h during incubation at 37°C in a 5% CO_2 saturated atmosphere, with serial dilutions of respectively dialyzed and filtered SGE (1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/ml) or saliva (1.09, 2.18, 4.37, 8.75 and 17.5 µg/ml). At 18–24 h before harvesting, they were pulsed with 1 µCi/well of methyl [^{3}H] thymidine (specific activity 25 Ci/mmol) (Amersham, UK). Methyl [^{3}H] thymidine incorporation was determined using a liquid scintillation counter (MR-300 DPM, Kontron, Switzerland).

Quantification of interleukin-4

Similarly to the cell cultures described above, culture supernatants collected 96 h after stimulation with serial dilutions of SGE or saliva were used for IL-4 quantification using an enzyme-linked immunosorbent assay (ELISA) (Ganapamo et al. 1995). Dilutions of rIL-4 ranging from 12.5 to 400 U/ml (Pharmingen, Germany) were used to construct a standard curve. The assay for IL-4 has a sensitivity of 10 U/ml.

ConA stimulation of spleen cells

Spleen cell suspension was obtained by teasing the spleen from naive BALB/c mice with the large striated end of a forceps and red blood cells were removed by incubation in hypotonic lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA). Spleen cells were plated into 96-well culture plates (Falcon) at 10^6 cells per well in RPMI-1640 supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 0.05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin and 25 µg/ml Fungizone. The cells were then incubated with or without different protein concentrations of saliva (0.6, 1.2, 6 and 30 µg/ml) for 2 h at 37°C and 5% CO_2 before being stimulated with ConA (1 µg/ml) in a total volume of 200 µl according to the modified procedure of Urioste et al (1994). Cells were pulsed 24 h later for 18–24 h with 1 µCi/well of methyl [^{3}H] thymidine. The degree of proliferation was determined by scintillation counting.

In vitro restimulation of SRBC-sensitized spleen T cells

SRBC were washed twice in 50 mM PBS containing 0.1% BSA before intraperitoneal injection into three BALB/c mice. The mice were injected with 10^7 SRBC, a mixture of 10^7 SRBC and 25 µg of SGE or 5 µg of saliva respectively in 2 ml total volume of PBS pH 7.3. One week later, the spleens of the three treated mice were removed and 7×10^5 spleen cells/well were plated onto 96 well plates. Sensitized splenic T cells from each mouse were then restimulated in vitro with the following numbers of SRBC (5×10^4, 10^5, 5×10^5 and 10^6) according to Titus (1998). Cells were incubated for 96 h at 37°C in a 5% CO_2 saturated atmosphere. One µCi/well of methyl [^{3}H] thymidine was added 18–24 h before harvesting the cells.

Methyl [^{3}H] thymidine incorporation was determined by liquid scintillation counting.

Results

Anti-complement activity of tick saliva

To examine whether the human alternative pathway of complement was affected by tick saliva, we performed a hemolysis test using different protein concentrations of saliva. The full activity of the human alternative complement pathway, corresponding to 0% of the SRBC hemolysis inhibition, was obtained in the absence of tick saliva in the culture medium. The presence of tick saliva inhibited the SRBC hemolysis in a dose-dependent manner (Fig. 1). Anti-complement activity increased with increasing concentration of saliva. The optimum of anti-complement activity was reached with a saliva protein concentration of 30 μg/ml. This inhibited about 70% of the SRBC lysis.

In vitro proliferation of tick-sensitized lymph node cells

To study the influence of tick saliva and SGE on the adaptive immune response, we stimulated tick-specific lymph node cells in vitro with increasing doses of tick antigens. The proliferation of primed lymph node cells was influenced by the concentration of tick saliva and SGE applied in the cell cultures. This proliferation increased with the protein concentration of the saliva and SGE which ranged between 1.09–2.18 μg/ml and 1.56–12.5 μg/ml respectively (Figs. 2a, 3). With higher protein concentrations of saliva and of SGE, ranging between 2.18–17.5 μg/ml and 12.5–100 μg/ml respectively, the proliferation of primed T cells decreased. Low doses of tick antigens were immunostimulative whereas higher doses were immunosuppressive.

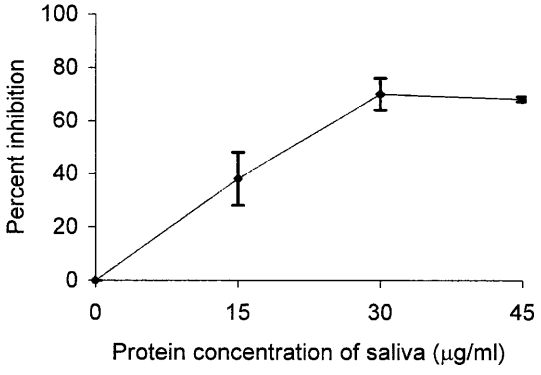

Fig. 1 *Ixodes ricinus* salivary inhibition of SRBC hemolysis by the human alternative complement pathway. Results are presented as a percentage of inhibition of complement activity. No inhibition of complement activity was measured when tick saliva was absent. Results are representative of three different experiments. Each value represents the mean of quadruplicate wells ± SD

In vitro IL-4 production

IL-4 secretion also depended on the protein concentration of the tick saliva as well as of the SGE used to restimulate primed lymph node cells in vitro. Increasing protein concentrations of saliva (1.09–4.37 μg/ml) and SGE (1.56–25 μg/ml) induced increased production of IL-4 which reached a maximum at a concentration of 4.37 μg/ml for saliva and 25 μg/ml for SGE (Figs. 2b, 3b). Thereafter increasing protein concentrations of saliva (4.37–17.5 μg/ml) and of SGE (25–100 μg/ml) triggered the decrease of IL-4 production by primed T cells.

I. ricinus saliva inhibited splenic T cell proliferation to ConA

To show the non-specific immunosuppressive activity of tick saliva, naive spleen cells were preincubated in vitro for 2 h with different protein concentrations of *I. ricinus* saliva ranging between 0.6 and 30 μg/ml. Thereafter they were stimulated in vitro with ConA. A high reduction (>90%) of cell proliferation in response to ConA was observed with 30 μg/ml of saliva proteins (Fig. 4). The inhibitory effect of tick saliva was dose-dependent. It diminished with decreasing saliva

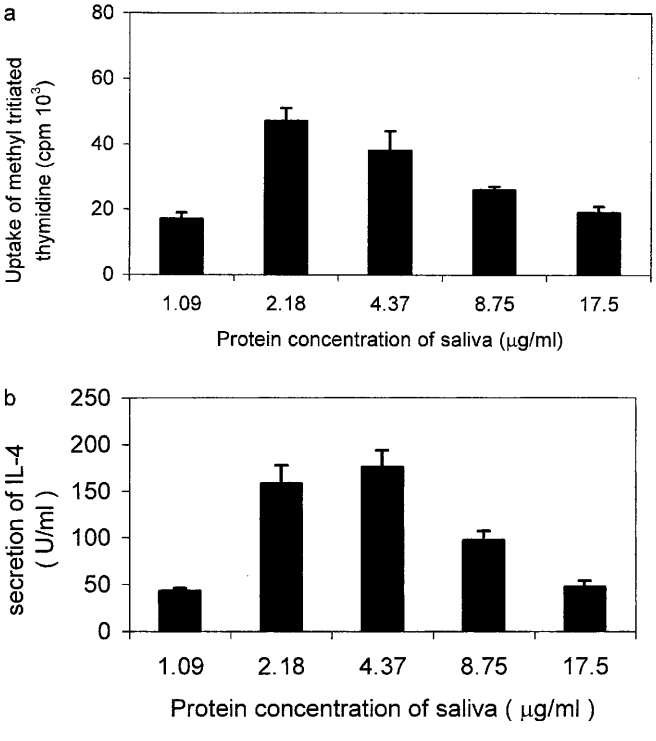

Fig. 2a, b Effect of the protein concentration of tick saliva on the activation of tick-sensitized lymph node T cells. The activation of primed lymph node T cells incubated with increasing protein concentrations of tick saliva was assessed by the determination of the degree of proliferation (**a**) and the measurement of the production of IL-4 (**b**). Each column represents an average of triplicate wells ± SD

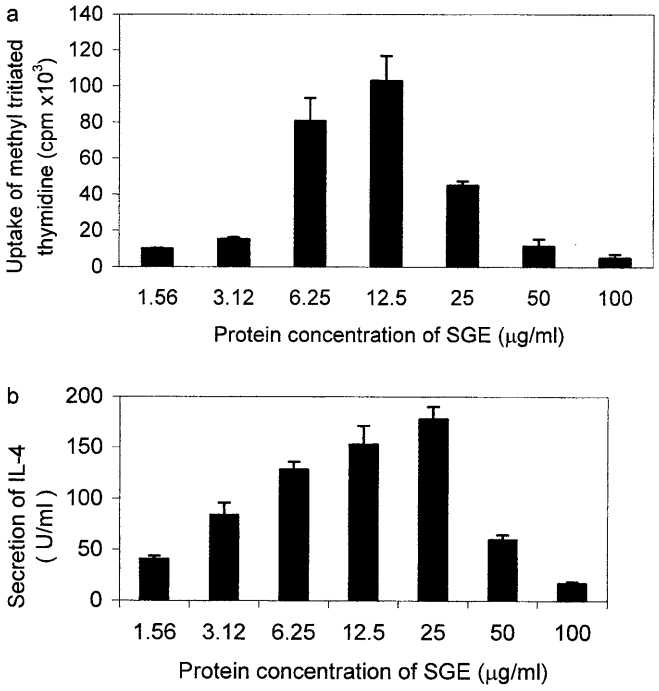

Fig. 3a, b Effect of the protein concentrations of SGE on the activation of tick-sensitized lymph node T cells. The activation of primed lymph node T cells incubated with increasing doses of SGE was assessed by the determination of the degree of proliferation (**a**) and the measurement of the production of IL-4 (**b**). Each value represents the mean of triplicate wells ± SD

concentrations and remained evident at 0.6 μg/ml of saliva proteins. Spleen cells not treated with saliva and stimulated with ConA acted as controls.

Systemic immunosuppressive effect of tick antigens

To investigate the systemic immunosuppressive activity of tick antigens we injected a mixture of saliva or SGE and 10^7 SRBC intraperitoneally. At 1 week post-injection, mice elicited an anti-SRBC response as shown by the proliferation of splenic T cells stimulated with SRBC in vitro. Spleen cells from the control mouse, which was only injected with 10^7 SRBC, showed a high proliferative response of T cells when restimulated in vitro with 0.5×10^5–10×10^5 SRBC, whereas splenic T cells from the mouse injected with a mixture of saliva or SGE and 10^7 SRBC showed a reduction in proliferation when they were restimulated in vitro with SRBC (Fig. 5). The impairment of splenic T cells to proliferate after stimulation with SRBC was more pronounced when mice were treated with SGE than with saliva.

Discussion

Ixodid ticks need several days to feed. During this time, hosts acquire resistance or develop tolerance to the ectoparasites. In contrast to resistant hosts, for example

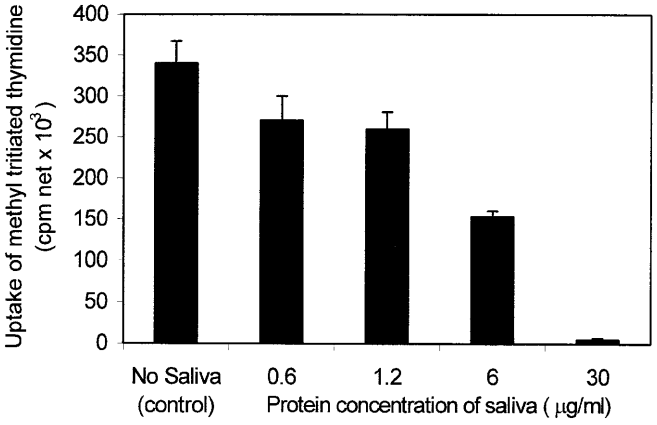

Fig. 4 *I. ricinus* tick saliva inhibition of naive splenic T cell proliferation in response to ConA. 10^6 splenic cells/well were preincubated in medium containing increasing protein concentrations of tick saliva and stimulated in vitro with ConA. Results are presented as the mean of triplicate cultures ± SD

rabbits infested with *I.ricinus* adults (Bowessidjaou et al. 1977), no difference was observed in the percentage of attachment or the weight of engorged larvae or nymphs of *I. ricinus* during reinfestations of BALB/c mice (Christe et al. 1998). In order to feed as well as to transmit pathogens successfully ticks must maintain blood flow and control the immune response of their host. They must develop countermeasures to antagonize the mechanisms of the innate as well as the adaptive immune response. Some bioactive molecules confer antihemostatic, anti-inflammatory and immunosuppressive properties to tick saliva (Ribeiro et al. 1985). The innate immunity constitutes the first line of defense against noxious or innocuous antigens. It is followed by the specific immune response (Fearon and Locksley 1996). Activation of the alternative pathway of complement is important in the innate defense and in the development of some tissue inflammation. We showed that the saliva of *I. ricinus* inhibits the hemolysis of SRBC by the human alternative pathway of complement. Accordingly, the saliva of *Ixodes dammini* (= *Ixodes scapularis)* also has an anti-complement activity on the human serum (Ribeiro 1987). Impairment of the innate immune system, either by the inhibition of the alternative pathway of complement or by downregulation of the activities of cellular components such as NK cytotoxicity and macrophage killing of pathogens (Kopecky et al. 1999), would contribute to the reduction of skin inflammation and protection against ticks and favor the transmission and propagation of tick-borne pathogens.

BALB/c mice infested with *I. ricinus* ticks develop a Th2 immune response characterized by CD4+ T cells secreting IL-4, IL-5 and IL-10 (Ganapamo et al. 1995, 1996). SDS-PAGE analysis showed some similarities in polypeptide profiles between tick saliva and SGE (Mejri et al. 2001). Therefore, both of these were used to study the dose-dependent influence of tick antigens on the responsiveness of tick-sensitized lymph node T cells. The immunosuppressive effects of saliva and SGE from

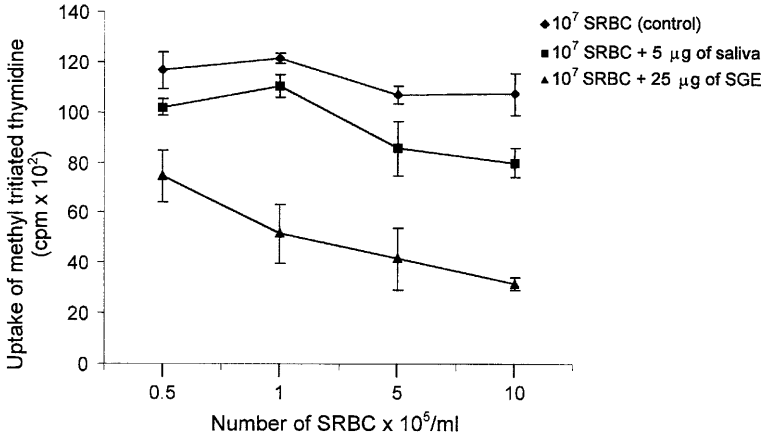

Fig. 5 Immunosuppressive effect of tick saliva and SGE on the proliferation of SRBC-sensitized splenic T cells. 7×10^5 spleen cells/well from BALB/c mice injected with SRBC (control) or SRBC and tick-antigens were stimulated in vitro with increasing numbers of SRBC. Results of proliferation are expressed as the mean of triplicate wells ± SD

I. ricinus were obtained with high concentrations of tick saliva or SGE. BALB/c mice tolerated frequent feedings by pathogen-free larval or nymphal *I. ricinus*. The two instars seem to engorge more effectively on repeatedly exposed mice (Christe et al. 1998). In nature, *I. ricinus* larvae and nymphs feed abundantly on yellow-necked mice (*Apodemus flavicollis*) and black-striped mice (*Apodemus agrarius*). These mice infect more ticks with *B. burgdorferi* than do other rodents (Matuschka et al. 1991, 1992). Both species fully tolerate repeated experimental feedings (Dizij and Kurtenbach 1995). The susceptibility of either laboratory or wild mice to larval or nymphal *I. ricinus* feeding or to pathogen transmission may be due to the prolonged and massive exposure to these ectoparasites which deposit a high amount of salivary secretions at the site of their attachment. We suggest that the immunosuppressive activities overcome the competitively immunostimulative activites in injected saliva resulting in the reduction of primed lymph node T cell responsiveness. In vivo effector lymphocytes formed in the draining lymph nodes of infested BALB/c mice circulate and infiltrate the skin at the site of tick attachment. Numerous T cells have been observed in skin infested with nymphal tick. CD4 + T cells outnumbered CD8 + T cells from a primary to a tertiary infestation (Mbow et al. 1994b). These cells could be locally activated by salivary antigens to proliferate and produce higher levels of IL-4 than IFN-γ. In situ hybridization of skin sections showed a positive signal for IFN-γ mRNA in some infiltrating mononuclear cells in the dermis near the tick hypostome and chelicerae beside a few cells positive for IL-4 mRNA (Mbow et al. 1994c). The weak activity of locally primed Th2 cells could be due to the high amounts of salivary secretion deposited at the site of attachment of nymphal ticks. This would be in agreement with our finding showing a reduced activity of primed lymph node T cells stimulated in vitro with higher protein concentrations of saliva or SGE.

To test whether tick saliva inhibits the development of an immune response non-specifically, we stimulated naive splenic T cells in vitro with ConA. This T cell mitogen mimics the action of antigens on primed T cells (Sharon 1983). We demonstrated that *I. ricinus* saliva inhibited the proliferation of splenic naive T cells to ConA in a dose-dependent manner. Accordingly *I. scapularis* saliva inhibited T cell proliferation to ConA (Urioste et al. 1994). SGE from *Dermacentor andersoni* fed for 9 days also suppressed ConA-stimulated T cell proliferation in vitro (Ramachandra and Wikel 1992). Few immunosuppressive molecules have been described in the salivary glands of ixodid ticks. We previously showed that some chromatographic fractions in the SGE of *I. ricinus* inhibit tick-sensitized lymph node T cells proliferation (Mejri et al. 2001). Prostaglandin E2, detected in the saliva of *I. ricinus* females (unpublished result), has a suppressive effect on Th1 cytokine elaboration (Betz and Fox 1991). A recombinant protein derived from a subtractive cDNA library of the salivary gland of female *I.ricinus* was also found to modulate T lymphocyte and macrophage responsiveness by inducing a T_H2 type response and by inhibiting the production of pro-inflammatory cytokines (Leboulle et al. 2001 and unpublished results). A calreticulin protein secreted in *Amblyomma americanum* saliva (Jaworski et al. 1995) as well as a protein of 36 kDa isolated from salivary glands of *D. andersoni* ticks (Bergman et al. 2000) also have immunosuppressive properties.

The hypostome and chelicerae of a nymphal tick penetrates deeply into the dermis causing skin damage (Mbow et al. 1994a). Tick saliva carried with the blood stream could stimulate leukocytes in the deep lymphoid organs such as the spleen. We therefore examined the effect of tick saliva and of SGE on the priming of splenic T cells to SRBC. The results showed that both have a systemic immunosuppressive effect on SRBC sensitized splenic T cells for at least 1 week after intraperitoneal injection. A weak effect was already evident after 4 days of culture (unpublished result). The immune response of guinea-pigs infested with *D. andersoni* was also suppressed systemically (Wikel 1982). The intraperitoneal injection of sand fly salivary gland lysate induced systemic immunosuppression in C57BL/6 and BALB/c mice (Titus 1998). As with sand flies and ticks, the salivary glands of other arthropods such as black flies (Cross et al. 1994a) and mosquitoes (Cross et al. 1994b) contain immunomodulatory molecules. The immunosuppressive

effect of *I. ricinus* saliva probably influences the transmission of tick-borne pathogens. It has been reported that the immunomodulator properties of vector saliva may be required for the successful transmission and establishment of host infection (Titus and Ribeiro 1990).

In conclusion, the impairment of the innate and acquired immune system allows BALB/c mice to tolerate tick feeding and might facilitate the transmission of tick-borne pathogens. The identification of immunomodulatory molecules in *I. ricinus* tick saliva and SGE requires further investigation. This could be useful in the conception of a vaccine against tick feeding and pathogen transmission.

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