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Modulation of human lymphocyte proliferation by salivary gland extracts of ixodid ticks (Acari: Ixodidae): effect of feeding stage and sex

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Abstract. Ixodid ticks remain attached to their hosts for several days to weeks. During this extended feeding process new proteins involved in the modulation of host immune responses are expressed in tick salivary glands. In our study a stimulatory or inhibitory effect of salivary gland extracts (SGE) of unfed and partially fed female *Ixodes ricinus* (Linnaeus, 1758), female and male *Amblyomma variegatum* (Fabricius, 1794) and *Rhipicephalus appendiculatus* Neumann, 1901 ticks on human lymphocyte proliferation induced by Concanavalin A (ConA) and phytohaemagglutinin (PHA), respectively, was investigated. SGE of all female ticks examined suppressed proliferation of ConA-induced lymphocytes; highly significant suppression was observed in the presence of unfed *I. ricinus* and 9-day fed *A. variegatum* SGE. SGE of partially fed *A. variegatum* and *I. ricinus* females suppressed PHA responses of lymphocytes. Lymphocytes showed reduced PHA and ConA responses in the presence of SGE of unfed and 2-day fed *R. appendiculatus* females, while SGE of 6-day fed females enhanced PHA responses, but reduced their ConA responses; generally SGE of 2-day fed females displayed the strongest inhibition. *Amblyomma variegatum* male SGE slightly enhanced PHA, but significantly reduced ConA responses of lymphocytes and their inhibitory effect increased during feeding. SGE of unfed and 2-day fed *R. appendiculatus* males enhanced PHA and ConA responses and those of 6-day fed males suppressed lymphocyte proliferation. The results suggest that (i) species- and sex-specific differences exist in the effects of tick salivary gland antigens on human lymphocyte proliferation and (ii) effect of SGE on human lymphocyte responses to mitogens varies depending on the tick feeding status.

Ticks are obligate blood-feeding ectoparasites, with many species remaining attached to their hosts and acquiring a bloodmeal over a period ranging from days to weeks (Sonenshine 1991). The long contact between a tick and its host provides a host time to develop innate and adaptive host immune responses against the parasites. Not surprisingly, ticks have developed a number of countermeasures to evade the host immune response. Several genes are induced in tick salivary glands during the feeding process, leading to the expression of new proteins. These proteins are secreted into the tick saliva and are potentially involved in modulation of host immune and haemostatic responses. Tick saliva contains a large number of pharmacologically active molecules that possess antihaemostatic, vasoactive and immunosuppressive properties (Ribeiro 1995a, b, Wikel and Bergman 1997, Ribeiro and Francischetti 2003). Consequently, modulation of the host immune response facilitates both tick feeding and transmission of various tick-borne pathogens (Wikel and Alarcon-Chaidez 2001).

The effect of salivary gland extracts (SGE) or saliva of different tick species has been mainly demonstrated in laboratory models (e.g., mouse, rabbit, guinea pig), however, that on human immune cells has been limited to *in vitro* studies. The inhibitory effect of tick SGE on NK cells (Kubeš et al. 1994, 2002, Kopecký and Kuthejlová 1998) and on the production of nitric oxide by macrophages (Kuthejlová et al. 2001) were reported. An IL-2 binding protein was identified in the saliva of the Lyme disease vector, *Ixodes scapularis* (Gillespie et al. 2001). The anti-chemokine activity of SGE from the ixodid ticks *Amblyomma variegatum*, *Dermacentor reticulatus* and *Rhipicephalus appendiculatus* was demonstrated by Hajnická et al. (2001) and Kocáková et al. (2002). It was also reported that ticks suppressed production of macrophage proinflammatory cytokines as well as secretion of T_H1 cytokines, however, the synthesis of T_H2 cytokines was up-regulated, indicating T_H2 polarisation of the immune response (Fuchsberger et al. 1995, Ferreira and Silva 1999, Kovář et al. 2001, 2002, Leboulle et al. 2002). The tick-induced suppression of the host immune defences is also characterized by reduced ability of murine and human lymphocytes to proliferate *in vitro* in the presence of T-lymphocyte mitogens (Kovář et al. 2001, Leboulle et al. 2002).

There are only a few reports on changes of the immunomodulatory effects of tick saliva during their

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feeding (e.g., Kubeš et al. 2000, 2002, Bior et al. 2002, Rolníková et al. 2002). In the present study, the effects of SGE derived from male and female *Amblyomma variegatum* (Fabricius, 1794), *Rhipicephalus appendiculatus* Neumann, 1901 and female *Ixodes ricinus* (Linnaeus, 1758) ticks on *in vitro* proliferation of human peripheral T-lymphocytes were compared and changes in lymphocyte responses to SGE of unfed and feeding ticks were investigated.

MATERIALS AND METHODS

Ticks and tick salivary gland extracts (SGE)

Adult *I. ricinus* were collected by flagging the vegetation in selected localities of south-western Slovakia known to be free of tick-borne encephalitis virus. Their salivary gland homogenates were screened for *Borrelia burgdorferi* by PCR and only negative samples were used in subsequent assays. *Rhipicephalus appendiculatus* and *A. variegatum* were obtained from laboratory colonies maintained at the Institute of Zoology of the Slovak Academy of Sciences. Salivary glands were dissected from unfed adult ticks or ticks that were allowed to feed in groups within retaining cells attached to the backs of laboratory animals. Females of *I. ricinus* were fed on laboratory mice for 2 or 5 days, females and males of *R. appendiculatus* were fed on guinea pigs for 2 or 6 days. Adult *A. variegatum* were fed on California rabbits for 2 or 9 days; hosts were always exposed to males for 4 days before females were added.

Ticks were gently removed from laboratory animals at different stages of feeding. Both removed and unfed ticks were washed in water, rinsed quickly in 70% ethanol and subsequently in distilled water. Their salivary glands were dissected under chilled 0.15 M NaCl, washed twice with 0.15 M NaCl and pooled in eppendorf tubes (10–50 SG pairs/50 µl 0.15 M NaCl, depending on tick species, sex and feeding stage) and immediately frozen at –70°C until required. Prior to the assays, batches of salivary glands were quickly thawed, homogenized using a pestle and centrifuged at 12,000 *g* for 10 min. Supernatants were removed, pellets resuspended in 0.15 M NaCl and re-centrifuged. Supernatants obtained (SGE) were pooled and the protein concentration of the SGE was determined using the Bradford assay (Bradford 1976). SGE were diluted in 10% RPMI 1640 medium at concentrations 0.1 µg, 0.5 µg and 1.0 µg soluble protein/50 µl medium.

Cell culture

Blood was obtained from healthy human donors. Lymphocytes were isolated from heparinized venous blood by separation on a Ficoll-Verografin gradient (specific gravity 1.078, centrifugation at 440 *g* for 30 min at room temperature). Lymphocytes from the interface were removed, washed three times in balanced saline solution and resuspended (10^6 cells/ml) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, and 10% heat-inactivated human serum. Lymphocyte suspensions (100 µl/well) were distributed to flat-bottom 96-well plates (TC Microwell 96, NUNC).

Lymphocyte proliferation assay (LPA)

To each well of the 96-well plate containing lymphocyte suspension either 50 µl of RPMI medium (non-stimulated cells) or 50 µl of Concanavalin A (ConA, Sigma; 1 µg/50 µl RPMI) or phytohaemagglutinin (PHA, Sigma; 5 µg/50 µl RPMI) and 50 µl SGE (containing 0.1 µg, 0.5 µg or 1.0 µg soluble protein/50 µl RPMI, respectively) were added. Mitogens were added simultaneously with SGE. Lymphocyte cultures were incubated for 66 h at 37°C in 5% CO_2. To measure cell proliferation, 37 Bq of tritiated thymidine (Amersham) ([^{3}H]TdR)/20 µl 10% RPMI 1640) were added to each well 6 h before cell harvest. The cells were harvested on glass fibre filters (Whatman) using an automatic cell harvester (Auto-Mash, Dynex). The amount of incorporated [^{3}H]TdR into DNA of proliferating lymphocytes was determined by liquid scintillation counting on a beta counter (Spectral, LKB). All samples were tested in triplicate and each assay was repeated 6–8 times, using blood from different donors. Mean disintegrations per minute (DPM) were determined for each sample. Absolute values measured for individual donors were transformed to relative values and are expressed as percentage of inhibition/stimulation of cell proliferation as compared to control lymphocytes (without addition of SGE). The percentage of stimulation/inhibition was calculated as follows:

Percentage of stimulation/inhibition of cells without addition of mitogens (S/I):

$$S\ (I) = \frac{SGE - Control\ DPM}{Control\ DPM} \cdot 100$$

Percentage of stimulation/inhibition of cells with addition of mitogens (S_M/I_M):

$$S\ (I) = \frac{SGE - mitogens\ DPM}{mitogens\ DPM} \cdot 100$$

Statistical analysis

The effects of tick feeding stage and SGE concentrations on lymphocyte proliferation were evaluated using one-way analysis of variance (ANOVA), followed by the Bonferroni test for pair-wise comparisons. Responses of lymphocytes to SGE of male and female ticks of the same species and feeding stage were compared by two-sample analysis (*t*-test). For statistical analyses, percentages were transformed to arcsine $\sqrt{p}$ to meet the criteria of parametric statistical tests. In all statistical analyses, $P \leq 0.05$ was considered significant.

RESULTS

The *in-vitro* proliferative responses of human lymphocytes were significantly affected by exposure to tick salivary gland extracts. Different patterns of inhibition or enhancement of proliferation were observed for non-stimulated and mitogen-stimulated lymphocytes treated with SGE of the three tick species. For each tick species, lymphocyte responses also varied when cultured with SGE of females and males as well as with SGE of unfed and partially engorged individuals.

Responses of lymphocytes to tick SGE were generally dose-dependent, however, for most of the treatments the differences in percentage of stimulation/ inhibition of proliferation of cells exposed to 0.1, 0.5 and 1.0 µg SGE soluble proteins were not statistically significant (Tables 1–3). Consequently, responses of

Table 1. Stimulation/inhibition of human lymphocyte proliferation (% change compared with controls) in cultures treated with various concentrations of female *Ixodes ricinus* salivary gland extracts (SGE), without mitogens and induced by phytohaemagglutinin (PHA) and Concanavalin A (ConA).

Tick feeding stage	Treatment SGE proteins (µg/well)	No mitogens (% change) n	No mitogens (% change) Mean (S.E.)	PHA (% change) n	PHA (% change) Mean (S.E.)	ConA (% change) n	ConA (% change) Mean (S.E.)
Unfed	0.1	5	–44.4 (6.0)^{a}	8	–24.1 (3.2)^{a}	8	–33.8 (4.2)^{a}
	0.5	5	–38.7 (8.4)^{a}	8	–47.1 (3.6)^{b}	8	–45.4 (6.9)^{a}
	1.0	4	–31.3 (8.3)^{a}	8	–57.9 (2.8)^{b}	8	–63.0 (3.0)^{a}
Day 2	0.1	5	56.9 (22.4)^{a}	6	–4.9 (7.3)^{a}	5	–6.8 (4.5)^{a}
	0.5	5	58.8 (9.6)^{a}	6	–16.6 (7.9)^{a}	5	–35.4 (6.6)^{b}
	1.0	5	35.6 (13.8)^{a}	6	–13.8 (6.3)^{a}	5	–25.2 (7.3)^{ab}
Day 5	0.1	8	58.4 (12.2)^{a}	8	–31.0 (4.2)^{a}	8	–10.5 (3.8)^{a}
	0.5	8	26.8 (6.6)^{a}	7	–33.4 (7.3)^{a}	8	–9.0 (4.4)^{a}
	1.0	7	30.9 (12.6)^{a}	8	–44.3 (5.5)^{a}	8	–17.3 (3.9)^{a}

n – numbers of replicates in treatments; means indicated by the same superscripts between SGE concentrations for each treatment are not significantly different (one-way ANOVA followed by Bonferroni test, $P \leq 0.05$).

Table 2. Stimulation/inhibition of human lymphocyte proliferation (% change compared with controls) in cultures treated with various concentrations of female and male *Rhipicephalus appendiculatus* salivary gland extracts (SGE), without mitogens and induced by phytohaemagglutinin (PHA) and Concanavalin A (ConA).

Tick feeding stage	Treatment SGE proteins (µg/well)	No mitogens (% change) n	No mitogens (% change) Mean (S.E.)	PHA (% change) n	PHA (% change) Mean (S.E.)	ConA (% change) n	ConA (% change) Mean (S.E.)
Females Unfed	0.1	5	–10.9 (4.7)^{a}	8	–19.5 (3.5)^{a}	8	–16.0 (5.9)^{a}
	0.5	7	–17.4 (7.5)^{a}	7	–18.2 (8.3)^{a}	8	–31.3 (5.9)^{ab}
	1	6	–26.2 (7.8)^{a}	6	–22.9 (7.3)^{a}	8	–41.4 (5.7)^{b}
Females Day 2	0.1	6	–29.2 (9.2)^{a}	8	–45.9 (7.7)^{a}	8	–51.2 (4.0)^{a}
	0.5	7	–29.2 (9.2)^{a}	7	–45.9 (7.7)^{a}	7	–51.2 (4.0)^{a}
	1	8	–36.6 (6.6)^{a}	6	–48.7 (7.0)^{a}	8	–41.9 (8.0)^{a}
Females Day 6	0.1	6	–13.6 (4.7)^{a}	7	–15.6 (6.0)^{a}	8	–17.5 (2.0)^{a}
	0.5	6	–20.4 (6.4)^{ab}	8	19.4 (4.8)^{b}	7	–10.4 (4.3)^{a}
	1	8	–42.3 (7.3)^{b}	7	32.3 (10.3)^{b}	8	–15.6 (5.5)^{a}
Males Unfed	0.1	6	6.8 (7.0)^{a}	6	29.1 (12.7)^{a}	4	23.4 (3.1)^{a}
	0.5	6	–6.2 (4.5)^{a}	5	41.5 (16.1)^{a}	5	26.9 (19.1)^{a}
	1	6	–14.5 (9.4)^{a}	5	46.1 (19.4)^{a}	5	25.2 (11.3)^{a}
Males Day 2	0.1	8	33.7 (7.1)^{a}	6	36.0 (8.0)^{a}	7	28.9 (6.7)^{a}
	0.5	8	103.0 (21.1)^{b}	8	14.6 (4.8)^{a}	6	30.8 (4.8)^{a}
	1	7	94.5 (31.8)^{b}	5	40.7 (6.1)^{a}	8	36.7 (11.4)^{a}
Males Day 6	0.1	7	–11.8 (7.4)^{a}	8	–26.5 (6.0)^{a}	8	–28.3 (6.1)^{a}
	0.5	6	–12.7 (6.4)^{a}	8	–21.9 (5.6)^{a}	8	–20.9 (6.4)^{a}
	1	8	6.8 (7.0)^{a}	7	–13.9 (4.2)^{a}	7	–27.7 (7.6)^{a}

n – numbers of replicates in treatments; means indicated by the same superscripts between SGE concentrations for each treatment are not significantly different (one-way ANOVA followed by Bonferroni test, $P \leq 0.05$).

lymphocytes to SGE of the three tick species and sexes and to SGE from various feeding stages were further evaluated only for the dose 1.0 µg.

Responses of non-stimulated lymphocytes to SGE of unfed *I. ricinus* females were suppressed, but they were enhanced by SGE of partially engorged ticks (Fig. 1). In contrast, no significant differences between responses of non-stimulated cells were found when treated with SGE of unfed and partially fed *R. appendiculatus* females (Fig. 2) and *A. variegatum* males (Fig. 3). SGE derived from unfed and 6-day fed *R. appendiculatus* males suppressed cell proliferation, however, SGE of 2-day fed males had a 94.5% stimulatory effect. Consequently, differences between the effect of SGE of 2-day fed male and female *R. appendiculatus* were highly significant (Fig. 2). SGE of female *A. variegatum* suppressed cell proliferation and this effect significantly increased with feeding, but, except unfed ticks, the differences between sexes were not significant (Fig. 3).

Responses of lymphocytes induced by mitogens were suppressed by all feeding stages of *I. ricinus*, but SGE of unfed ticks had the strongest effect (Fig. 1). In majority of treatments, responses of mitogen-stimulated lymphocytes to male and female SGE of the same

Table 3. Stimulation/inhibition of human lymphocyte proliferation (% change compared with controls) in cultures treated with various concentrations of female and male *Amblyomma variegatum* salivary gland extracts (SGE), without mitogens and induced by phytohaemagglutinin (PHA) and Concanavalin A (ConA).

Tick feeding stage	Treatment SGE proteins (µg/well)	No mitogens (% change) n	No mitogens (% change) Mean (S.E.)	PHA (% change) n	PHA (% change) Mean (S.E.)	ConA (% change) n	ConA (% change) Mean (S.E.)
Females Unfed	0.1	6	17.9 (6.9)^{a}	8	69.2 (5.5)^{a}	8	40.1 (9.8)^{a}
	0.5	5	8.0 (10.3)^{a}	8	109.1 (11.3)^{b}	8	23.5 (10.5)^{a}
	1	5	4.1 (17.6)^{a}	8	79.0 (7.9)^{ab}	6	20.6 (6.7)^{a}
Females Day 2	0.1	6	21.2 (10.7)^{a}	8	19.5 (5.7)^{a}	7	–10.8 (4.4)^{a}
	0.5	7	–14.5 (4.3)^{b}	7	26.1 (5.3)^{a}	7	–21.0 (5.8)^{a}
	1	8	–17.2 (5.5)^{b}	8	34.6 (4.8)^{a}	8	–17.3 (6.3)^{a}
Females Day 9	0.1	7	–11.1 (4.8)^{a}	6	–22.6 (6.9)^{a}	6	–24.7 (4.8)^{a}
	0.5	7	–3.9 (1.9)^{a}	8	–53.7 (7.3)^{b}	7	–46.5 (7.9)^{a}
	1	6	–35.5 (8.2)^{b}	8	–57.5 (7.5)^{b}	7	–48.9 (8.4)^{a}
Males Unfed	0.1	8	–35.3 (5.4)^{a}	7	19.1 (5.0)^{a}	8	–14.9 (5.3)^{a}
	0.5	8	–37.4 (5.1)^{a}	7	15.4 (2.9)^{a}	8	–22.1 (6.1)^{a}
	1	8	–47.0 (8.4)^{a}	8	23.1 (5.2)^{a}	7	–26.7 (3.9)^{a}
Males Day 2	0.1	8	6.5 (6.6)^{a}	7	–31.7 (5.6)^{a}	8	–26.6 (4.8)^{a}
	0.5	8	8.5 (6.4)^{a}	7	–13.4 (6.2)^{ab}	8	–40.4 (4.1)^{ab}
	1	4	–16.9 (7.4)^{b}	6	–0.03 (8.5)^{b}	8	–43.4 (3.9)^{b}
Males Day 9	0.1	5	3.5 (2.1)^{a}	8	4.5 (2.3)^{a}	8	–34.2 (4.1)^{a}
	0.5	7	–7.4 (3.1)^{b}	8	7.7 (3.0)^{a}	8	–59.8 (2.9)^{b}
	1	7	–26.9 (7.8)^{b}	8	3.6 (7.5)^{a}	8	–67.9 (3.5)^{b}

n – numbers of replicates in treatments; means indicated by the same superscripts between SGE concentrations for each treatment are not significantly different (one-way ANOVA followed by Bonferroni test, $P \leq 0.05$).

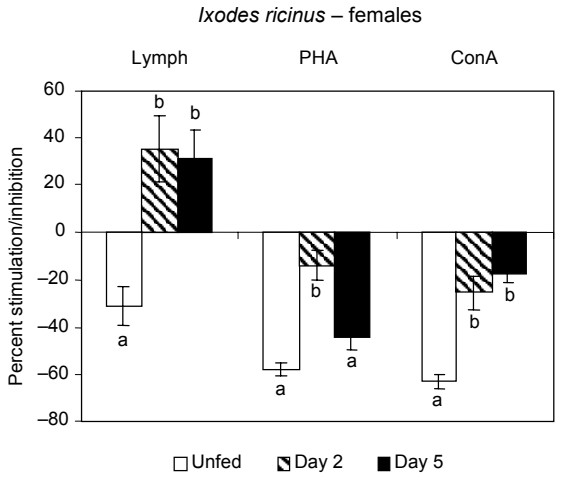

Fig. 1. Proliferation of human lymphocytes (% change compared with control lymphocytes) after treatment with 1 µg of SGE proteins of unfed and partially fed *Ixodes ricinus* females. Lymphocytes were non-stimulated with mitogens (Lymph) or stimulated with PHA and ConA. Values represent means ± SEM. Means within a treatment indicated by the same letters are not significantly different (one-way ANOVA followed by Bonferroni test, $P \leq 0.05$). Results of ANOVA: non-stimulated lymphocytes ($F = 12.79$, $P < 0.001$); lymphocytes stimulated with PHA ($F = 30.50$, $P < 0.001$); lymphocytes stimulated with ConA ($F = 20.62$, $P < 0.001$).

species significantly differed within a feeding status (Figs. 2, 3). Proliferative responses of mitogen-induced lymphocytes to SGE of *R. appendiculatus* also differed between the two mitogens applied (Fig. 2). While proliferation of Con-A-induced lymphocytes was suppressed by *R. appendiculatus* female SGE, proliferation of PHA-induced lymphocytes was suppressed only by 2-day fed females. SGE of unfed and 2-day fed males had a stimulatory effect on mitogen-induced proliferation, and only 6-day fed males suppressed proliferation.

Responses of mitogen-induced lymphocytes to *A. variegatum* SGE also varied with feeding stage and sex (Fig. 3). Proliferation of lymphocytes was enhanced when treated with SGE of unfed and 2-day fed females, but it was suppressed by SGE of 9-day fed females. The suppressive effect of male *A. variegatum* SGE on Con-A-stimulated proliferation significantly increased with duration of feeding, however, this effect was not observed in PHA-stimulated lymphocytes.

DISCUSSION

Ticks have evolved various strategies to evade the immune response of hosts they feed on. Biologically active compounds in their salivary glands play the main role in immunosuppression and immunomodulation. Tick-induced immunosuppression of the host is characterized by decreased primary antibody responses to T-cell-dependent antigens, decreased production of T_H1 cytokines (IL-2, IFN-γ) and enhanced production of T_H2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) (Willadsen and Jongejan 1999, Gillespie et al. 2000, Schoeler and Wikel 2001, Wikel and Alarcon-Chaidez 2001, Ribeiro and Francischetti 2003).

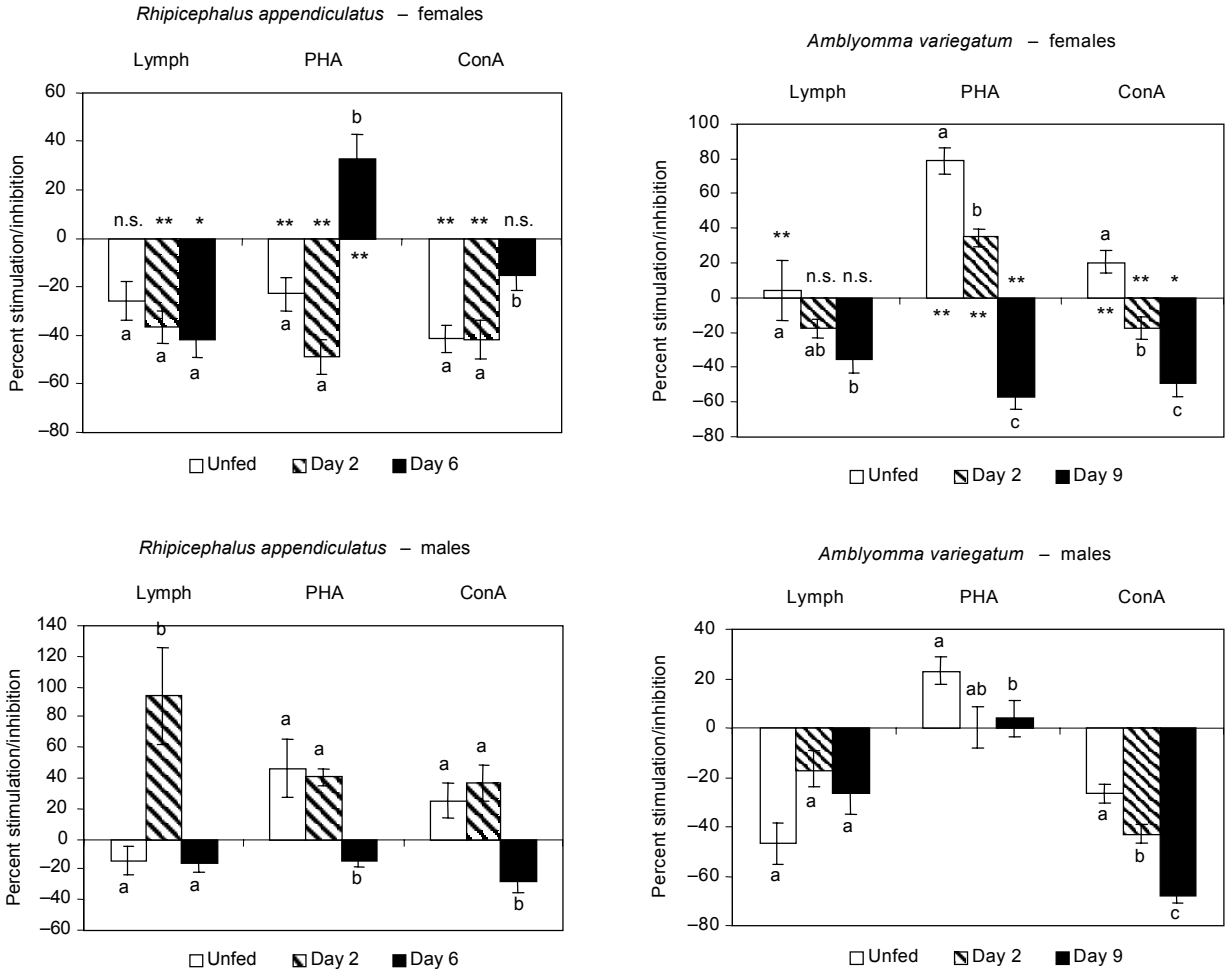

Fig. 2. Proliferation of human lymphocytes (% change compared with control lymphocytes) after treatment with 1 µg of SGE proteins of unfed and partially fed *R. appendiculatus* females and males. Lymphocytes were non-stimulated with mitogens (Lymph) or stimulated with PHA and ConA. Values represent means ± SEM. Means within a treatment indicated by the same letters are not significantly different (one-way ANOVA followed by Bonferroni test, $P \leq 0.05$). Results of ANOVA for female ticks: non-stimulated lymphocytes ($F = 1.31$, n.s.); lymphocytes stimulated with PHA ($F = 26.00$, $P < 0.001$); lymphocytes stimulated with ConA ($F = 5.67$, $P < 0.05$). Results of ANOVA for male ticks: non-stimulated lymphocytes ($F = 12.05$, $P < 0.001$); lymphocytes stimulated with PHA ($F = 19.13$, $P < 0.001$); lymphocytes stimulated with ConA ($F = 21.65$, $P < 0.001$). Differences between responses to male and female SGE are indicated above or below the values for females as n.s. (not significant), * $P < 0.05$ or ** $P < 0.01$ (*t*-test).

Fig. 3. Proliferation of human lymphocytes (% change compared with control lymphocytes) after treatment with 1 µg of SGE proteins of unfed and partially fed *A. variegatum* females and males. Lymphocytes were non-stimulated with mitogens (Lymph) or stimulated with PHA and ConA. Values represent means ± SEM. Means within a treatment indicated by the same letters are not significantly different (one-way ANOVA followed by Bonferroni test, $P \leq 0.05$). Results of ANOVA for female ticks: non-stimulated lymphocytes ($F = 78.04$, $P < 0.001$); lymphocytes stimulated with PHA ($F = 125.32$, $P < 0.001$); lymphocytes stimulated with ConA ($F = 39.26$, $P < 0.001$). Results of ANOVA for male ticks: non-stimulated lymphocytes ($F = 3.08$, n.s.); lymphocytes stimulated with PHA ($F = 4.04$, $P < 0.05$); lymphocytes stimulated with ConA ($F = 33.48$, $P < 0.001$). Differences between responses to male and female SGE are indicated above or below the values for females as n.s. (not significant), * $P < 0.05$ or ** $P < 0.01$ (*t*-test).

Inhibition of T-cell responsiveness to ConA could result from the direct effect of salivary gland proteins on lymphocytes or from their production of IL-10 (Wikel 1999, Schoeler and Wikel 2001, Wikel and Alarcon-Chaidez 2001). The suppression of T-lymphocyte proliferation can also be due to prostaglandin E_2 which inhibits IL-2 and IFN-γ production and T-lymphocyte proliferation and is present in high concentrations in tick saliva (Bowman et al. 1996). PGE_2 inhibits *in vitro* cytokine production by T_H1 cells; however, it has no effect on T_H2 cells. Up-regulation of IL-4 and IL-10 probably leads to the development of a T_H2 response,

resulting in a reduction of the T_H1 response (Ramachandra and Wikel 1992, Wikel 1999, Kovář et al. 2001, Schoeler and Wikel 2001, Wikel and Alarcon-Chaidez 2001).

Tick-mediated suppression of the T_H1-lymphocyte reactivity may inhibit expansion of antigen specific T-lymphocyte clones (IL-2), differentiation of B-lymphocytes (IL-4, IL-6, IL-13), activation of macrophages (IFN-γ), and natural killer cell activity (IL-12, IL-15, IFN-γ). The tick-induced T_H2 cytokine profile seems to be advantageous for the survival of the tick because of the anti-inflammatory effect of T_H2 cytokines. These anti-inflammatory mechanisms may also enhance the transmission of tick borne pathogens (Schoeler and Wikel 2001, Wikel and Alarcon-Chaidez 2001).

In our experiments, *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Ixodes ricinus* SGE modulated proliferation of human peripheral blood T-lymphocytes *in vitro* in various ways, depending on feeding stage and sex. In majority of treatments, responses of lymphocytes to tick SGE when stimulated with mitogens simultaneously or 2 h after exposure to SGE showed no significant differences (Rolníková et al., unpublished results).

The suppressive effect of the *R. appendiculatus* SGE on the production of a number of cytokines (IFN-α, IFN-γ, IL-1, IL-5, IL-6, IL-7 and IL-8) in human leucocytes has already been demonstrated (Fuchsberger et al. 1995). Kubeš et al. (2000) reported that salivary gland extracts derived from *A. variegatum* females decreased *in vitro* natural killer (NK) activity of healthy persons. Hajnická et al. (2001) showed that the *A. variegatum* SGE reduced the level of IL-8, resulting in inhibition of chemotaxis of neutrophils. As the SGE-induced recruitment of leucocytes provides a defence against a parasitic attack, the anti-IL-8 activity may facilitate tick blood-feeding. Additionally, Kocáková et al. (2002) reported that *R. appendiculatus* and *A. variegatum* SGE reduced levels of both, IL-8 and MIP-1α, a chemokine responsible for chemotaxis of human monocytes.

In general, *in-vitro* proliferative responses of mouse, human, or cattle lymphocytes to SGE of partially engorged female ticks were found to be suppressed (Bergman et al. 1995, Schoeler et al. 2000, Kovář et al. 2001, Turni et al. 2002), however, no data have been available on the effects of SGE derived from unfed female or male ixodid ticks. Sexual behaviour of some tick species evokes speculation about possible co-operation between sexes in the interaction with the host (Bior et al. 2002). The specific function of male *R. appendiculatus* ticks in female tick feeding has been reported (Wang et al. 1999). *Rhipicephalus appendiculatus* male ticks mate on the host with the feeding females, and then help their mates to complete successful engorgement by secreting immunosuppressive saliva components into the co-feeding site. Differences in male and female SGE protein profiles presented by Gašperík et al. (2000) demonstrated the existence of sexual dimorphisms in *R. appendiculatus*, *Dermacentor reticulatus* and *A. variegatum*. The support of female tick feeding by male SGE indicates different functions of saliva from both sexes and the effective cooperation between males and females during their feeding on the host. The different role of the sexes in host immunomodulation is also supported by our study, showing, in some cases, controversial responses of human lymphocytes to male and female SGE isolated from *R. appendiculatus* and *A. variegatum*, respectively. The strong stimulation of lymphocytes by 2-day fed *R. appendiculatus* males is also very interesting. The results of different, sometimes quite opposite effects of male and female SGE on lymphocyte proliferation points to the importance of "cooperation" of sexes during their feeding on the host. The mechanism how female and male ixodid ticks modulate the immune response of their hosts needs to be studied in more detail.

Our experiments with SGE of *I. ricinus* females, derived from various stages of engorgement, showed their inhibitory effect on PHA- and ConA-induced proliferation of human peripheral blood T-lymphocytes. Very interesting is our finding on the strongest inhibitory effect of SGE of unfed *I. ricinus* females. The presence of immunosuppressive substances in unfed ticks is not surprising, as they have to counteract host defences as soon as they attach to their hosts. Also the expression of novel proteins during engorgement of hard ticks is well known, leading to production of a wide array of biologically active compounds in their salivary glands (Sonenshine 1991, Sauer et al. 1995). We treated human lymphocytes with certain concentrations of soluble SGE proteins (i.e. a cocktail of immunosuppressive as well as antigenic compounds). Based on our results we suppose that the ratio of immunosuppresive compounds in *I. ricinus* SGE changes during feeding, being probably lower in partially engorged ticks. Recently, Leboulle et al. (2002) have detected a novel protein – immunosuppressor (Iris) in the SGE of 5-day fed female *I. ricinus*. They found that expression of Iris was induced in the salivary glands of *I. ricinus* during the feeding process and that Iris was secreted into the tick saliva. It suppressed T-lymphocyte proliferation and induced a T_H2 type immune response and inhibited the production of pro-inflammatory cytokines (IL-6 and TNF). Kovář et al. (2001) investigated the tick-mediated (*I. ricinus* SGE) modulation of human T-cell proliferation and cytokine synthesis using human peripheral blood mononuclear cells (PBMCs) stimulated with ConA or LPS. The SGE significantly inhibited the *in vitro* production of IL-2 and IFN-γ. In contrast, the production of IL-4, IL-6, and IL-10 was significantly increased. In a subsequent study the authors showed that the SGE of partially engorged *I. ricinus* inhibited T-lymphocyte proliferation (Kovář et al. 2002).

In general, our results confirmed the suppressive effect of the SGE from partially engorged female ixodid ticks tested on mitogen-stimulated human peripheral blood T-lymphocyte proliferation. In contrast to *I. ricinus*, stimulatory effect was observed in unfed and 2-day fed *A. variegatum* females, while 9-day fed females strongly suppressed lymphocyte proliferation. We suggest that this is not the consequence of absence of immunosuppressive compounds in the saliva of unfed ticks, but more probably results from the abundance of compounds having no immunosuppressive properties.

In *R. appendiculatus* females and *A. variegatum* males, differences in responses of PHA- and ConA-stimulated lymphocytes have been found. These differences could result, in part, from different effects of SGE on various subsets of T-lymphocytes, as PHA stimulates preferentially $CD4^+$, while ConA stimulates more $CD8^+$ T-lymphocytes (e.g. Buc 2001). However, these questions should be addressed in subsequent studies dealing with effects of tick SGE on proliferation of various subsets of T-lymphocytes.

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