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Co-inoculation of *Borrelia afzelii* with Tick Salivary Gland Extract Influences Distribution of Immunocompetent Cells in the Skin and Lymph Nodes of Mice

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ABSTRACT. The impact of *Ixodes ricinus* salivary gland extract (SGE) on inflammatory changes in the skin and draining lymph nodes of mice, elicited by the infection with the important human pathogen, *B. afzelii*, was determined using flow cytometry. SGE injected together with spirochetes reduced the numbers of leukocytes and -T lymphocytes in infected epidermis at early time-points post infection. In draining lymph nodes, the anti-inflammatory effect of SGE was manifested by the decrease of total cell count compared with that in mice treated with inactivated SGE. Changes in subpopulations of immunocompetent cells apparently reflected the effect of SGE on the proliferation of spirochetes in the host. The significance of tick saliva anti-inflammatory effect for saliva activated transmission of *B. afzelii* is shown.

Abbreviations

BP	band pass	PMNs	polymorphonuclear cells
FACS	fluorescein-activated cell sorter	RPE	rhodamine-phycoerythrin
FCS	fetal calf serum	SAT	saliva-activated transmission
FITC	fluorescein isothiocyanate	SGE	salivary gland extract
PBS	phosphate-buffered saline	TCR	T-cell receptor
p.i.	post infection		

Hard ticks feed on their hosts for several days, providing an opportunity for the host immune system to affect the tick. The feeding success depends on an array of pharmacologically active substances in tick saliva operating against the host's response. Immunomodulatory molecules, which suppress effective mechanisms of anti-tick immunity, represent a part of these substances.

The immunomodulatory activities of tick saliva affect both innate (nonspecific) and adaptive (specific) immunity of the host. Tick saliva or SGE have been shown to inhibit activation of the alternative pathway of complement (Ribeiro 1987), to inactivate anaphylatoxins (Ribeiro and Spielman 1986) and to prevent phagocytosis and the production of nitric oxide by neutrophils (Ribeiro *et al*. 1990). Inhibition of NK cell activity (Kopecký and Kuthejlová 1998), antiviral action of interferon (Hajnická *et al*. 2000) and histamine-binding capacity (Paesen *et al*. 1999) have been reported.

Tick feeding *in vivo* or SGE *in vitro* impaired the ability of lymphocytes to proliferate after stimulation with T mitogens (Wikel 1982; Kovář *et al*. 2001). Polarization of the host immune response to a T_H2 cytokine profile has been repeatedly demonstrated after feeding of various tick species (Ferreira and Silva 1999; Schoeler *et al*. 1999) or in cultures of immunocompetent cells treated with tick SGE (Kovář *et al*. 2002). Among T_H2 cytokines, those with strong anti-inflammatory activity (IL-4, IL-10, TGF-) are upregulated (Ferreira and Silva 1999; Kopecký *et al*. 1999).

Increasing evidence strongly suggests that a key factor in successful transmission of tick-borne pathogens is the ability of these pathogens to exploit the immunomodulatory properties of tick saliva (Nuttall 1999). Promotion of vector-borne pathogen transmission, *via* the action of vector saliva on the host, has been termed saliva-activated transmission (SAT; Nuttall and Jones 1991). The SAT phenomenon has been demonstrated for several tick-borne pathogens including Thogoto virus (Jones *et al*. 1989), tick-borne encephalitis virus (Alekseev *et al*. 1991; Labuda *et al*. 1993), *Borrelia afzelii* (Pechová *et al*. 2002), *B. burgdorferi* sensu stricto and *B. luisitaniae* (Zeidner *et al*. 2002) and *Francisella tularensis* (Kročová *et al*. 2003).

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In naïve hosts, mainly nonspecific mechanisms of innate immunity operate at the tick feeding site. The important component of these mechanisms is inflammation including the influx of inflammatory cells to the damaged site. As the effect of tick saliva on several components of inflammatory reaction (complement, histamine, cytokines) has been demonstrated, we focused on the effect of *Ixodes ricinus* SGE on recruitment of inflammatory cells in the skin and draining lymph nodes after inoculation of *B. afzelii* spirochetes.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 mice 6–8-week-old were purchased from *Charles River*, Germany.

Bacteria. The CB-43 strain of *B. afzelii* isolated from an *I. ricinus* female (Štěpánová-Tresová *et al*. 1999) was grown in Barbour–Stoenner–Kelly-H (BSK-H) medium (*Sigma*) supplemented with 6 % rabbit serum at 34 °C. The number of spirochetes was calculated by dark-field microscopy. The low-passage spirochetes were used.

Salivary gland extract. Adult *I. ricinus* ticks from the colony of the *Institute of Parasitology, Academy of Sciences of the Czech Republic* in České Budějovice were screened for *B. burgdorferi* sensu lato by PCR with negative results. Ticks were fed in groups of 20 mating pairs within retaining cells attached to the backs of guinea pigs. After 5 d, engorged female ticks were removed and the salivary glands were dissected from the live ticks and pooled. The salivary glands were washed in PBS, homogenized in 1 mL PBS by sonication and clarified by centrifugation (10 000 ***g***, 10 min). The protein concentration of clarified SGE was determined using a protein estimation kit (*BioRad*, USA). Aliquots of the SGE preparation were stored at –70 °C.

Experimental infection of mice. Mice were inoculated intradermally in the lower thorax with 5 injections close to each other in a total dose of 10^7 *B. afzelii* spirochetes in a mixture of 100 L PBS and 100 L of BSK-H medium per mouse (this group of mice was labeled B). The second group (BS) was inoculated with the same dose of spirochetes in 100 L BSK-H medium mixed with 40 g of native SGE protein in 100 L of PBS. The third group (BiS) was inoculated with the same dose of spirochetes mixed with 40 g of SGE protein, inactivated by heating for 15 min at 100 °C. Control groups consisted of mice inoculated with a mixture of 100 L BSK-H medium and 100 L PBS (group C), mice, that received BSK-H medium and 40 g of SGE protein (group CS), and mice injected with BSK-H medium and 40 g of inactivated SGE (group CiS). Each group contained 9 mice. At indicated time-points (3, 6 and 10 d post infection), 3 mice from each group were sacrified by cervical dislocation. The skin was shaved, the pieces containing the sites of injection were cut off and regional lymph nodes (lymphonodus axillaris proprius, ln. axillaris accesorius, ln. subiliacus and ln. popliteus) were aseptically removed.

Preparation of cells for immunophenotyping. The pieces of skin were placed epidermal side down in a Petri dish with sterile PBS. Hypodermis fat and tissue were ablated with a scalpel. Small pieces of cleared skin were moved dermal side down into 1 % trypsin in PBS and incubated for 30 min at 37 °C. The epidermis was then peeled in RPMI 1640 medium supplemented with 10 % FCS and containing 0.025 % DNAase I (*Sigma*) and collagenase (*Sigma*, 20 U/mL). The cell suspension was poured through a cell strainer and incubated for 45 min at 37 °C. The cell suspension was washed twice in PBS, counted and resuspended in FACS buffer containing 1 % FCS in PBS. Lymph nodes were mashed through a sterile strainer, cells were washed twice in PBS, counted and resuspended in FACS buffer.

Flow cytometry. To 45 L of cell suspension containing 5×10^5 cells, 5 L of normal rat serum and 5 L of fluorochrome-conjugated monoclonal antibody was added and the mixture was incubated for 45 min at 4 °C. Stained cells were washed in FACS buffer, resuspended in 0.5 mL of this buffer and analyzed. The following antibodies were used for a three-color analysis: Cy-Chrome-conjugated rat anti-mouse CD45 (IgG2b, clone 30-F11; *Pharmingen*, USA), rat anti-mouse CD3 conjugated to FITC (IgG2a, clone KT3; *Serotec*, UK), rat anti-mouse CD19 conjugated to RPE (IgG2a, clone 6D5; *Serotec*), rat anti-mouse CD4 conjugated to FITC (IgG2a, clone YTS 177.9; *Serotec*), rat anti-mouse CD8 conjugated to RPE (IgG2a, clone KT15; *Serotec*), mouse anti-mouse MHC II: I-A^d, I-A^b conjugated to FITC (IgM, clone 28-16-8S; *Serotec*), rat anti-mouse F4/80 conjugated to RPE (IgG2b, clone CI: A3-1 (F4/80); *Serotec*) and hamster anti-mouse TCR conjugated to FITC (IgG, clone GL3; *Serotec*). Appropriate isotype controls were included into the analysis.

Cell analysis was performed on an EPICS XL-MCL flow cytometer (*Coulter Corporation*, USA), equipped with a 15-mW 488 nm argon-ion laser. Emission peaks were collected using a 530 nm BP filter for FITC, a 585 nm BP filter for RPE and 620 nm BP filter for CyChrome. Labeled cell populations were analyzed using SYSTEM II software (*Coulter Corporation*). Triggering was set on the forward scatter channel and the threshold was adjusted to exclude debris. Cells positive for CD45 were gated for separation of leuko-

cytes. Ten thousand events of viable leukocytes were analyzed in each sample. All other markers were also measured from the gate of CD45+ cells but the results for marker-positive cells were recalculated to express the percentage from all analyzed cells present.

Statistical analysis. The significance of any differences between experimental groups was evaluated by Student's *t*-test.

RESULTS

In the skin, the average proportion of CD45+ cells was ≈3 %. *Borrelia* infection significantly increased the percentage of these cells on 6 d p.i.; 10 d p.i. this trend continued but the difference from the noninfected group was not significant (Fig. 1). Three d p.i. a significant decrease in CD45+ cells was recorded in infected, SGE-treated mice (group BS) compared to the control (C) group; 6 d p.i. a significant reduction in CD45+ leukocytes was observed in the BS group compared with group B. Native and inactivated SGE alone did not exert any significant effect on the number of leukocytes in the skin (*data not shown*).

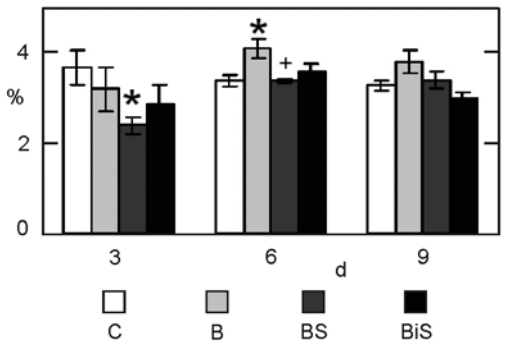

Fig. 1. Proportion (%) of CD45+ cells in the epidermis of mice intradermally injected with 10^7 *B. afzelii* spirochetes with or without *I. ricinus* SGE (40 g per mouse) as analyzed by flow cytometry; C denotes the group of mice injected with a diluent, B with spirochetes, BS with spirochetes + SGE and BiS with spirochetes + inactivated SGE; mean of three animals ± SD; statistically significant difference ($p < 0.05$) is indicated: * – from the group C, + – from the group B.

Among the subsets of CD45+ cells, SGE showed the strongest effect on CD3+ and -T-receptor-positive cells (Fig. 2A,B). On days 3 and 6 p.i. SGE significantly reduced the percentage of all T lymphocytes (CD3+) and also their subset with the receptor, which dropped to about one-half of the values in the control group. In the *Borrelia*-infected group an increase in CD3+ cells was recorded since day 6 p.i., and 10 d p.i. the difference from the control group was significant (Fig. 2C). In this group, also + cells significantly outnumbered those in the control and BiS groups. In groups B and BS, more MHC II+/F4/80+ cells (mostly Langerhans cells) were observed 10 d p.i. compared with the other groups (Fig. 2C). CD19+ B lymphocytes represented a minority subpopulation (<0.3 % of all epidermal cells) and no effect of SGE or infection on this subset could be demonstrated. Among the noninfected control groups (C, S, iS), no significant differences in monitored leukocyte subpopulations were registered (*data not shown*).

In draining lymph nodes, both infection and SGE exerted a profound effect on the absolute numbers of cells (Fig. 3). Infection with spirochetes increased the number of cells in all three time-points roughly twice compared with noninfected controls. The effect of SGE differed, depending on the time post infection. Whereas 3 d p.i. the highest number of cells was counted in the group injected with inactivated SGE (BiS), native SGE (group BS) reduced the cell number almost to that in the group injected only with the spirochetes (B); on day 6 p.i. the effect of SGE was reversed. Among the infected groups, the highest number of cells was in group BS, whereas the lowest in group BiS. Ten d p.i. the lowest cell number was counted again in group BiS.

For monitoring the effect of spirochete infection and SGE on leukocyte subpopulations, the proportional expression (in %) instead of absolute cell numbers was chosen, because the latter only reflected the effect on total cell counts, bringing no additional message. The analysis revealed the main changes in the ratio of CD19+ and CD3+ cells (Fig. 2D–F). An increase in the percentage of CD19+ cells was observed in all groups infected with *B. afzelii*. This increase was at the expense of the CD3+ subset. In most of the infected groups, an increase in the percentage of MHC II+/F4/80– cells was recorded. CD4+ cells significantly decreased 3 and 10 d p.i. in the infected groups compared with the noninfected control. By contrast, the CD8+ subpopulation dropped markedly 6 d p.i. Infection of mice led also to changes in the ratio of CD4+ : CD8+ cells. Whereas in the control group this ratio was approximately 2 : 1, it was almost 3 : 1 on day 6 p.i. and 1 : 1 on day 10 p.i. in the infected groups. Both native and inactivated SGE (groups BS and

BiS) increased the percentage of CD19+ and MHC II+/F4/80– cells (mostly B lymphocytes) on day 3 p.i. compared with the group injected only with spirochetes (group B). At the same time, a significant decrease in CD4+ cells was recorded in both SGE-treated groups (BS, BiS) compared with group B (Fig. 2D). On day 6 p.i., an increase in the CD19+ subset and a drop in the CD8+ subset were observed in group BS in comparison with group B (Fig. 2E). In the last time-point, the only significant difference among infected groups was the reduction of CD19+ cells and MHC II+/F4/80- cells in the group treated with inactivated SGE (BiS) (Fig. 2F).

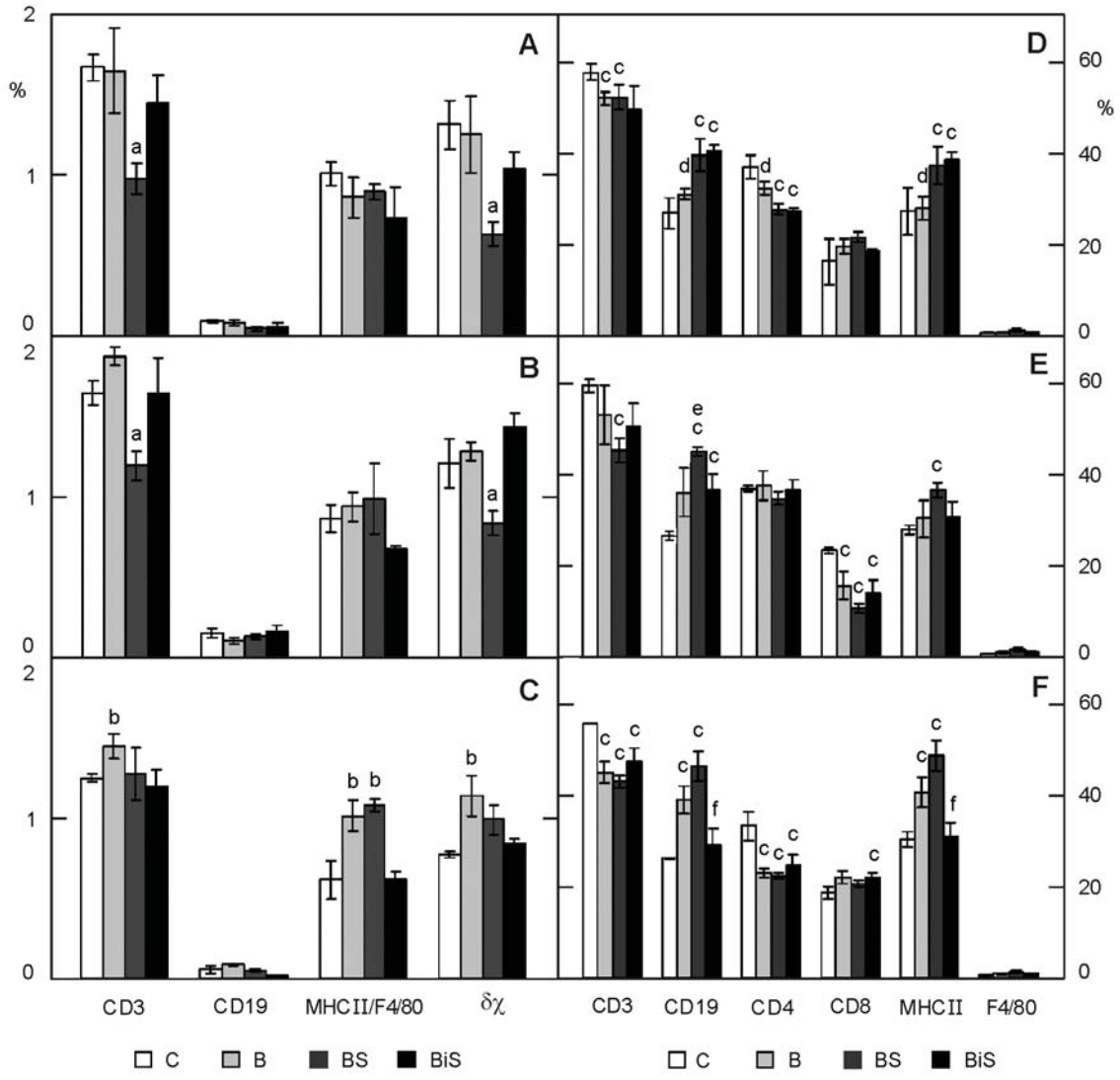

Fig. 2. Proportion (%) of immunocompetent cell subpopulations in epidermis (**A**, **B**, **C**) and draining lymph nodes (**D**, **E**, **F**) of mice after intradermal injection of 10^7 *B. afzelii* spirochetes with or without *I. ricinus* SGE (40 g per mouse) as analyzed by flow cytometry; the analysis was performed 3 (A, D), 6 (B, E) and 10 d (C, F) p. i.; all markers were measured from the gate of CD45+ cells, but the results for marker-positive cells were recalculated to express the percentage from all analyzed cells present; for individual experimental groups *see* Fig. 1; mean of three animals ± SD; statistically significant difference ($p < 0.05$) is indicated: a – from groups C, B and BiS, b – from group C and BiS, c – from group C, d – from groups BS and BiS, e – from groups B and BiS, f – from group BS.

In the noninfected control groups both native and inactivated SGE induced an increase in absolute numbers of cells in draining lymph nodes, particularly 3 d p.i. The changes in leukocyte subsets, induced with both SGE preparations, followed similar patterns as in SGE-treated infected groups, but the differences between individual groups were much smaller, mostly not significant (*data not shown*).

DISCUSSION

There is increasing evidence that SAT is mediated *via* immunomodulatory molecules in tick saliva. In the mosaic of these immunomodulatory effects, information is lacking about the impact on the development of inflammation, characterized by changes in the distribution of immunocompetent cells.

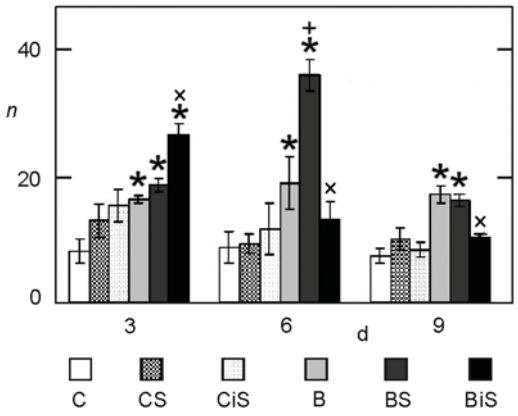

Fig. 3. Total cell counts (*n*) in draining lymph nodes after intradermal injection of 10^7 *B. afzelii* spirochetes with or without *I. ricinus* SGE (40 g per mouse); C denotes the group of mice injected with a diluent, CS with SGE, CiS with inactivated SGE, B with spirochetes, BS with spirochetes + SGE, and BiS with spirochetes + inactivated SGE; mean of three animals ± SD; statistically significant difference ($p < 0.05$) is indicated: * – from group C, × – from group BS, + – from group B.

We used *B. afzelii* for induction of inflammatory changes in the skin and draining lymph nodes of mice. The ability of *Borrelia* spirochetes to induce an erythema migrans rash and lymphadenopathy in the area of tick attachment was demonstrated by Summers *et al*. (2005). A high dose of spirochetes was applied to induce strong inflammatory response, in which the anti-inflammatory effect of tick SGE could be recorded. Using this *I. ricinus* tick-transmitted pathogen, SAT was demonstrated (Pechová *et al*. 2002), as well as the suppressive effect of SGE on the killing of spirochetes by macrophages (Kuthejlová *et al*. 2001) or on the production of pro-inflammatory cytokines induced by the spirochete *in vitro* (Kuthejlová *et al*. 2000).

In the epidermis, no remarkable increase in CD45+ cells (leukocytes) was recorded 3 d after intradermal injection of live spirochetes. Later, 6 and 10 d p.i., leukocytes in infected skin outnumbered those in the skin of noninfected controls. Chong-Cerrillo *et al*. (2001) reported immunohistochemical analysis of the skin of rabbits infected intradermally with *B. burgdorferi*. They recorded a marked infiltration of PMNs 1 d p.i. which decreased by 2 d. Three d p.i. PMNs were replaced by a few infiltrating macrophages. At the time of erythema migrans (2 weeks p.i.) PMNs and macrophages were replaced by a lymphocytic infiltrate. Because the first time-point p.i. analyzed in our work was 3 d, the first increase in PMNs was probably missed. At later time-points an increase in T lymphocytes (CD3+), especially -T cells, and macrophages and/or Langerhans cells was detected. No erythema migrans was observed on the skin of infected mice.

Tick SGE significantly reduced the percentage of CD45+ cells in the epidermis 3 d p.i. This reduction was not recorded with inactivated SGE. Subpopulation analysis revealed a reduction in the CD3+ subset, mostly in -T lymphocytes. Similar reduction was measured 6 d p.i., whereas no significant effect was observed on day 10 p.i. No decrease in the percentage of epidermal -T cells was observed in noninfected mice injected with SGE. It indicates that the anti-inflammatory action of SGE is in some way dependent on current infection, *i.e.* on the interaction of the immune system with spirochete antigens. The significant decrease of -T lymphocytes in infected, SGE-treated skin compared with noninfected untreated controls can be explained by apoptotic death of these cells or their migration out of the injected site. Recognition of *Borrelia* lipoproteins by -T lymphocytes was reported (Vincent *et al*. 1996; Glatzel *et al*. 2002) and an extensive and preferential death of these cells was shown to be connected with another pathogen containing lipoprotein components in the cell wall, *Listeria monocytogenes* (Mukasa *et al*. 2002). -T lymphocytes, often classified as innate T lymphocytes, represent an important component of innate immunity operating against infection (Jameson *et al*. 2003). Protective response at early time-points of infection with *L. monocytogenes* (Hiromatshu *et al*. 1992), *Mycobacterium tuberculosis* (Ladel *et al*. 1995) and *Toxoplasma gondii* (Hisaeda *et al*. 1995) was dependent on -T cells, the depletion of which led to a more severe course of infection.

The role of -T lymphocytes can be immunoregulatory, effector or both as demonstrated in the *M. tuberculosis* model (Bloom 1999). The immunoregulatory role of -T lymphocytes was demonstrated in Lyme borreliosis, when these cells provided B-cell help for the generation of neutralizing anti-OspC antibodies (Mbow *et al*. 2001). In this way the down-regulation of MHC II antigens on Langerhans cells, indu-

ced by *Borrelia* (Silberer *et al*. 2000), can be overcome because -T lymphocytes do not require MHC II presentation of the recognized antigen (Kabelitz *et al*. 2000). From the above data it can be assumed that reduction of -T lymphocytes in the host skin can be beneficial for the spirochete and contribute to saliva-activated transmission of this tick-borne pathogen.

A significant increase in MHCII+/F4/80+ cells in both borrelia-infected and borrelia-plus-SGE-infected groups of mice 10 d p.i. is probably connected with increased growth of spirochetes in the skin. The combined pro-inflammatory effect of inactivated SGE and spirochetes could reduce their numbers at late time-points of the experiment. In the skin, MHCII+/F4/80+ cells represent the Langerhans cells (Hume *et al*. 1983).

Intradermal injection of spirochetes with or without SGE induced substantial changes in cell numbers in draining lymph nodes. These changes reflect the inflammatory process and provide information about the course of infection and the impact of SGE on it. On day 3 p.i., an increase in lymph node cell numbers was recorded in all experimental groups compared with the control. The highest number of cells in the group injected with spirochetes plus inactivated SGE indicates a combined pro-inflammatory effect of spirochetes and SGE proteins. The infected group with native SGE also showed an increase but it was much lower compared with that in the group with inactivated SGE. As the amount of protein injected into the mice was the same for both SGE preparations, the difference in cell numbers between these two groups was obviously caused by the anti-inflammatory effect of native SGE. This transient effect was replaced by the increase in lymph node cells in the group treated with native SGE. On day 6 p.i., cells in this group outnumbered those in all remaining groups. The likely reason of this increase is the favorable effect of native SGE on the proliferation of spirochetes at early time-points of infection. The increased number of spirochetes due to the SAT effect could be responsible for this pronounced inflammatory reaction on day 6 p.i. The lower numbers of lymph node cells in the group treated with inactivated SGE at later time-points can be explained by the strongest inflammatory response at the early stage of infection and reduction of spirochete numbers due to it.

The mitogenic activity of *Borrelia* lipoproteins (Honarvar *et al*. 1994) or a lipopolysaccharide-like component (Schwarzová and Čižnár 2004) apparently induced an increase of the percentage of CD19+ B lymphocytes in draining lymph nodes. This increase was at the expense of CD3+ T lymphocytes. It is likely that also SGE proteins contributed to B cell expansion, because some increase in these cells was recorded in lymph nodes of mice injected only with any of the SGE preparations. Further changes in the B-cell subset (identical with MHC II+/F4/80– cells) apparently reflected changes in the proliferation of spirochetes due to SAT.

Changes observed in the CD4+/CD8+ ratio apparently corresponded to the course of infection. Activation and proliferation of CD4+ T lymphocytes could be influenced by the spirochete-caused down-regulation of MHC II antigens on antigen-presenting cells (Silberer *et al*. 2000).

Our study provides for the first time data on the impact of tick SGE on the inflammation induced by tick-transmitted pathogen. The demonstrated anti-inflammatory effect of SGE contributes to the list of candidate SAT factors.

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