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SHORT COMMUNICATION

Immunomodulatory arsenal of nymphal ticks

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Abstract. Ticks have developed their own immunomodulatory mechanisms to inhibit the host inflammatory response. One of them involves the ability to subvert the cytokine network at the site of tick feeding by secreting cytokine binding molecules. Most studies have focused on the immunomodulatory prowess of adult female ticks. Here we describe anti-cytokine activity in salivary gland extracts (SGEs) prepared from 2-day-fed nymphs of *Dermacentor reticulatus* Fabricius, *Ixodes ricinus* L., *Rhipicephalus appendiculatus* Neumann and *Amblyomma variegatum* Fabricius. Anti-CXCL8 activity was detected in nymphs of all species. Relatively high activity against CCL2, CCL3 and CCL11 was observed in SGEs of *R. appendiculatus* and *A. variegatum* nymphs, whereas SGEs of *I. ricinus* nymphs showed comparatively high anti-interleukin-2 (-IL-2) and anti-IL-4 activities. These data show that nymphs, which epidemiologically are usually more important than adults as disease vectors, possess a range of anti-cytokine activities that may facilitate pathogen transmission.

Key words. Cytokine inhibitors, Ixodid ticks, nymph salivary glands.

Feeding ticks stay attached to their hosts for several days or weeks, depending on the species and developmental stage. The prolonged feeding period provides ample time for inflammation to promote haemostasis at the feeding site. Host immune mechanisms may reduce the feeding success of ticks by enhancing inflammatory reactions. Ticks have developed mechanisms to subvert the host response, presumably as an adaptation to obtain larger bloodmeals that would result in increased tick fitness. In particular, the saliva of ticks has anti-inflammatory and immunosuppressive properties (Brossard & Wikel, 2004).

The host response to foreign antigens requires the co-ordinated action of innate and acquired components of the immune system, which is regulated by small secreted proteins known as cytokines (Borish & Steinke, 2003). Cytokines are a diverse group of soluble messenger proteins involved in the activation, growth, control and repair of cells, and regulation of immune events. Chemokines, a sub-set of cytokines, play an important role in controlling leucocyte migration. In previous studies, saliva and/or salivary gland extract (SGE) of ixodid (hard) adult tick species was shown to bind numerous cytokines (interleukin-2, IL-4 and some important chemokines) and suppress the activity of immune cells that are responsive to their stimulation. Results varied between species, and also between adult males and females of the same species (Gillespie *et al.*, 2001; Hajnická *et al.*, 2001, 2005). Manipulation of the host cytokine network by ticks provides a mechanism to help ticks feed and may also facilitate tick-borne pathogen transmission (Nuttall & Labuda, 2004).

The nymphal stage is often the most important in tick-borne pathogen transmission. Several studies have shown that nymphal feeding induces changes to host haemostatic and immune responses, with some evidence of differences between nymphs and adults (Brossard & Wikel, 2004; Narasimhan *et al.*, 2007; Pedra *et al.*, 2007). To determine whether nymphs have immunomodulatory mechanisms similar to adults, we compared anti-cytokine activity in SGEs prepared from nymphs of four ixodid tick species, *Dermacentor reticulatus*, *Ixodes ricinus*, *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, all of which are important vectors of tick-borne pathogens (De Vos, 1981; Camus & Barre, 1992; Nambota *et al.*, 1994; Hubalek *et al.*, 1997; Labuda & Nuttall, 2004; Foldvari *et al.*, 2005; Kelly, 2006; Sreter-Lancz *et al.*, 2006; Skarphedinsson *et al.*, 2007). The materials and methods used followed those described in our previous studies with adult ticks (Hajnická *et al.*, 2005; Vančová *et al.*, 2006).

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Nymphs were allowed to feed on rabbits, on which complete engorgement takes approximately 5–8 days for *D. reticulatus* nymphs, 3–6 days for *I. ricinus*, 5–9 days for *R. appendiculatus*, and 5–9 days for *A. variegatum* (Honzáková, 1971; Jones *et al.*, 1988). As anti-cytokine activity of ixodid species is most consistently detected after feeding commences but prior to engorgement, nymphs were collected when they had completed approximately 2 days of feeding (Vančová *et al.*, 2006). Approximately 300 partially fed nymphs of each species were collected and SGEs prepared as described previously (Slovák *et al.*, 2000). The total amounts of protein from 10 nymphs obtained from two independent feeding sessions were 2.8 μg and 3.9 μg in *D. reticulatus*, 5.1 μg and 3.5 μg in *I. ricinus*, 5.5 μg and 6.8 μg in *R. appendiculatus*, and 11.6 μg and 9.7 μg in *A. variegatum*. Pooled SGE was prepared as 10, 5, 2.5, 1 or 0.5 nymphal equivalents per 5 μl. Salivary gland extracts were screened by ELISA for activity against human CXCL8, CCL2, CCL3, CCL5, CCL11, IL-2 and IL-4 using commercial ELISA kits obtained from R&D Systems (Xxx) and/or Bender MedSystems Diagnostics (Xxx), as described previously, with duplicate assays of each sample (Hajnická *et al.*, 2005). The results represent the means obtained with the two batches of SGEs derived from independent feeding sessions. A reduction in the detectable level of a particular cytokine, compared with the control, was interpreted as evidence of putative cytokine binding activity.

The SGEs of all the nymphal species reduced the level of CXCL8 (Fig. 1). The highest levels of inhibition were shown by *R. appendiculatus* and *D. reticulatus*. Thus there was no correlation between the total protein content of SGE from each species and the levels of inhibitory activity (*D. reticulatus* had the lowest protein content and *R. appendiculatus* the second highest). Relatively high activity against CCL2, CCL3 and CCL11 was observed in *R. appendiculatus* and *A. variegatum* nymphs, whereas activity was barely detectable in *D. reticulatus* and undetectable in *I. ricinus*. Only *A. variegatum* showed significant levels of activity with CCL5. Anti-IL-2 activity was detected in SGE of *I. ricinus* nymphs and low levels of activity in SGE of *D. reticulatus* nymphs, whereas anti-IL-4 was demonstrated in SGE of *I. ricinus* and *R. appendiculatus*. Thus nymphs of four ixodid tick species showed contrasting patterns of anti-cytokine activity after 2 days of feeding on rabbits. Similar results were obtained using murine (rather than human) cytokines (data not shown), reflecting the high degree of amino acid identity between mammalian cytokines and the likelihood that anti-cytokine activity is effective irrespective of (mammalian) host species.

For *I. ricinus* and *A. variegatum*, nymphal anti-cytokine profiles were similar to those recorded for adults, whereas adult *D. reticulatus* showed a much greater repertoire of anti-cytokine activity compared with conspecific nymphs (Table 1). The differences between species and between stages may reflect differences in host preference. However, *I. ricinus* has probably the most catholic 'taste', but appears to have the poorest anti-cytokine repertoire. An alternative explanation may be that anti-cytokine activity reflects the size of the mouthparts and/or the duration of feeding. For example, *A. variegatum* has large mouthparts that penetrate deep into the dermis, and takes a comparatively long time to reach engorgement (Stewart *et al.*, 1998). The mechanics and physiology of *A. variegatum* feeding may antagonize different cytokines to those provoked by a species such as *I. ricinus*, which has much smaller mouthparts and engorges faster.

Soon after tissue damage, specific leucocyte subsets emigrate from the circulation into the affected area. These leucocytes function as the primary line of host defence in the destruction of micro-organisms and initiation of tissue repair. The histopathology of tick-bite lesions shows that, depending on the tick species and host species, the predominant cells infiltrating attachment sites are neutrophils (in mammals) or heterophils (in non-mammals), eosinophils and basophils (Latif *et al.*, 1990; Szabo & Bechara, 1999; Van der Heijden *et al.*, 2005). Neutrophils are the first infiltrating cell type in the dermis; their migration to inflammatory sites is directed by the chemokine CXCL8. The molecular structure of CXCL8 has been determined for various vertebrate species and shown to be similar. Indeed, the most ancient chemokine, found in a primitive group of vertebrates, resembles mammalian CXCL8, indicating high conservation of this chemokine since the evolution of early vertebrates (Najakshin *et al.*, 1999). The chemotactic ability of human CXCL8 is not species-specific; granulocytes from many vertebrate species migrate to this chemokine *in vitro* (Rot, 1991). Polymorphonuclear neutrophils inform and shape immune responses. Thus it is perhaps not surprising that all the ixodid species and stages showed anti-CXCL8 activity, presumably indicating the importance to ixodid ticks of controlling neutrophil activity. Even adult female *A. variegatum*, which has low anti-CXCL8 activity at 5 days of feeding, shows comparatively higher activity earlier in feeding (Vančová *et al.*, 2006).

The CC chemokines have pleiotropic activities; they are potent attractants for monocytes, eosinophils, basophils, natural killer cells and memory T cells (Laing & Secombes, 2004). The importance of cells of the host immune system infiltrating the tick feeding site resides in their ability to produce cytokines that modulate the downstream response (Falcone *et al.*, 2001). Subversion of the activity of the four CC chemokines examined appears important for the two larger nymphal species, *R. appendiculatus* and *A. variegatum.*

Because of the relatively long duration of tick blood-feeding, ticks must suppress host immune reactions at all levels. The main function of IL-2 is to stimulate the growth and cytotoxic response of activated T lymphocytes. In addition, IL-2 is implicated in the development, homeostasis and function of natural killer cells. For nymphal *I. ricinus* in particular, the results suggest the importance of suppressing one or more of these functions.

The adaptive immune system has evolved two types of immune cells, Th1 and Th2, as the system supervisors (Kidd, 2003). Th1 cells are predominantly involved in the type-1 pathway of cellular immunity, whereas Th2 cells drive the type-2 pathway of humoural immunity. Th2 differentiation is a central process in the protection against parasites such as helminths. Tick infestation also results in a Th2 immune response, as shown by the cytokine profile induced in murine lymph node cells (Ferreira & Silva, 1999). IL-4 is a key cytokine in the induction of Th2 immunity, mediating B-cell activation. Activated

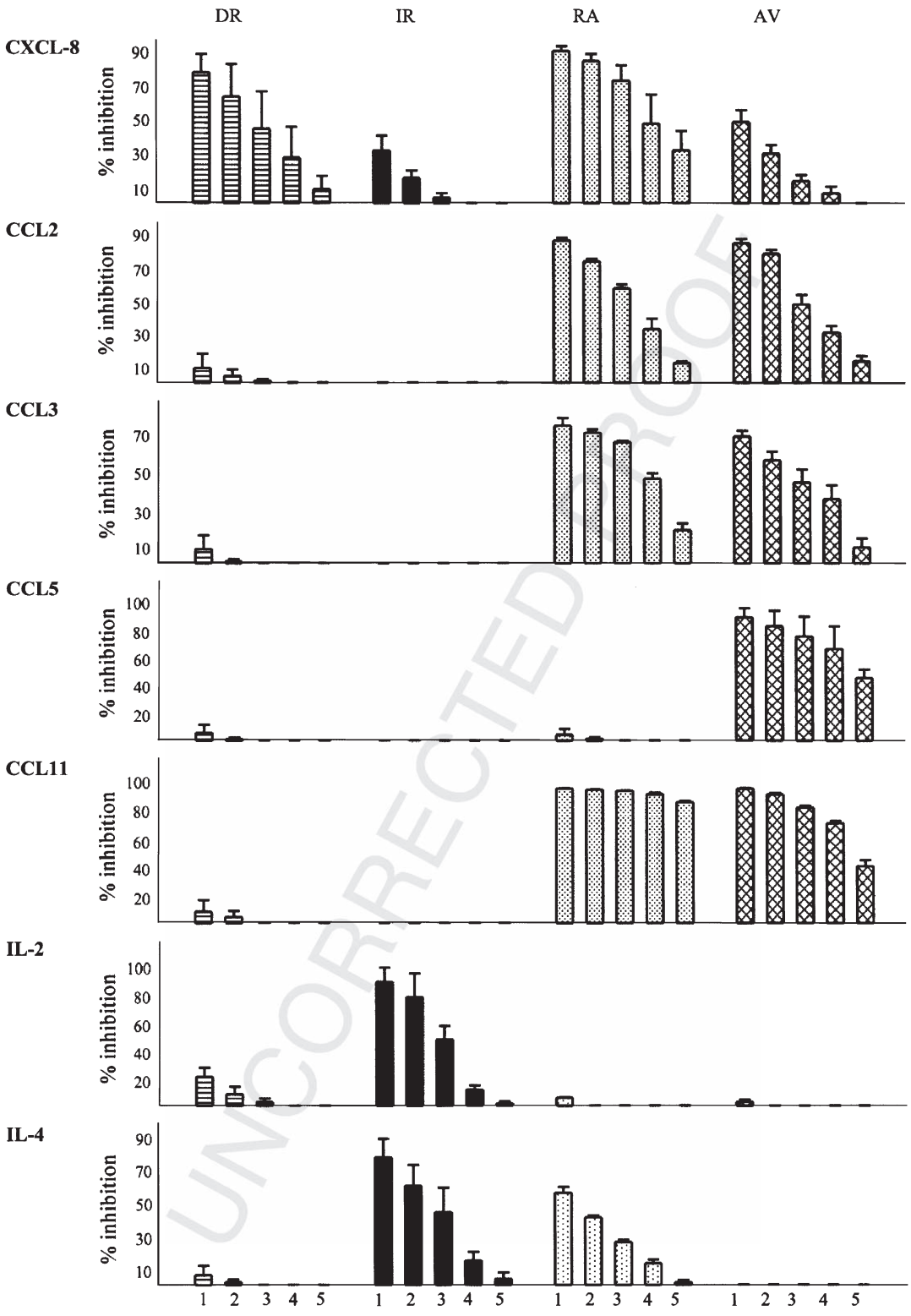

Fig. 1. Anti-cytokine activities of salivary gland extract (SGE) obtained from 2-day-fed nymphs of *Dermacentor reticulatus* (DR), *Ixodes ricinus* (IR), *Rhipicephalus appendiculatus* (RA) and *Amblyomma variegatum* (AV) ticks. Salivary gland extracts equivalent to 10.0, 5.0, 2.5, 1.0 or 0.5 nymphs (labelled 1, 2, 3, 4, 5, respectively) were pre-incubated with 50 pg of each cytokine for 90 min before ELISA analysis. Results are expressed as percentage reduction of OD reading compared with control.

Table 1. Host preferences of nymphal and adult ixodid species and their anti-cytokine activities.

	Dermacentor reticulatus			*Ixodes ricinus*			*Rhipicephalus appendiculatus*			*Amblyomma variegatum*		
	N*	F†	M†	N*	F†	M†	N*	F	M	N*	F†	M†
Preferred host	Small mammals	Medium/large mammals (e.g. deer, dog, sheep)	Medium/large mammals (e.g. deer, cattle, sheep)	Small mammals; birds, reptiles	Medium/large mammals (e.g. deer, sheep)	Negligible feeding	Small mammals	Medium/large ungulates (e.g. sheep, water buffalo)	Medium/large ungulates (e.g. sheep, water buffalo)	Mammals, birds, reptiles	Large ungulates (e.g. cattle)	Large ungulates (e.g. cattle)
*Anti-cytokine activity												
CXCL-8	++++	+++	++++	++	++	+++	++++	ND	ND	++	+	++
CCL2	+	+++	++++	–	–	+	++++	ND	ND	+++	+++	+++
CCL3	+	++	+++	–	–	+	+++	ND	ND	+++	++	+++
CCL5	+	++	+++	–	–	+	+	ND	ND	+++	++	+++
CCL11	+	+++	++++	–	–	+	+++	ND	ND	+++	+++	+++
IL-2	+	+	++	+++	+	++	+	ND	ND	+	–	++
IL-4	+	++	++	+++	++	++	+++	ND	ND	–	++	++

*Salivary gland extracts obtained from 2-day-fed nymphs: 3.5 µg *D. reticulatus*, 4.0 µg *I. ricinus*, 6.0 µg *R. appendiculatus*, 10.0 µg *A. variegatum*.
†Salivary gland extracts obtained from 5-day-fed adults: 5 µg *D. reticulatus*, 10 µg *I. ricinus*, 5 µg *A. variegatum* (from V. Hajnická *et al.* 2005 and unpublished data).
‡Average values of the inhibition of 50 pg cytokines detection by ELISA: –, undetectable; +, 1–25%; ++, 26–50%; +++, 51–75%; ++++, 76–100%. ND, not done.
N, xxx: F, xxx, M, xxx.

basophils with enhanced IL-4 production are candidates to mediate the innate-adaptive link for Th2 responses during helminth infections (Min *et al.*, 2006). As the Th2 cells mature they also produce IL-4, which generates an autocrine loop to the naïve T cells to make more Th2 cells. As for IL-2, *I. ricinus* nymphs appear to target IL-4, suggesting they manipulate the Th2 response. When fed on laboratory mice, *I. ricinus* nymphs induce a Th2 response, but do not elicit a host rejection response (Brossard & Wikel, 2004). However, on natural hosts, *I. ricinus* nymphs induce resistance in *Apodemus flavicollis*, although not in *Clethrionomys glareolus* (Dizij & Kurtnebach, 1995). Thus the interplay between tick-induced manipulation of host innate and acquired immunity, and Th1 and Th2 responses, for different host species, is highly complex. Identification of the tick molecules active in cytokine manipulation is needed to help unravel this complexity.

The importance of disrupting the host cytokine/chemokine network is demonstrated by the many 'large' DNA viruses that produce cytokine/chemokine inhibitors (Deane *et al.*, 2000; Webb & Alcami, 2005). Arthropod-borne viruses (arboviruses) are 'small' RNA viruses (with one exception, African swine fever virus) and appear to exploit the immunomodulatory properties of their vector's saliva (Nuttall & Labuda, 2004). Our results show that even nymphal ticks, which epidemiologically are generally more important than adults as vectors of arboviruses (and other tick-borne pathogens), produce a wealth of cytokine/chemokine inhibitors. Such important immunosuppressors will need to be considered in designing novel vaccines that target ticks and block arbovirus transmission.

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