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RESEARCH NOTE

EFFECT OF THE SALIVARY GLAND AND MIDGUT EXTRACTS FROM *IXODES RICINUS* AND *DERMACENTOR RETICULATUS* (ACARI: IXODIDAE) ON THE GROWTH OF *BORRELIA GARINII* IN VITRO

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The interaction between pathogen and tick vector seems to be crucial for vector-borne pathogens. For instance, *Borrelia burgdorferi* s.l., the Lyme disease agent, must overcome at least two main barriers in the tick vector body to be effectively transmitted: the midgut and the salivary glands. It has been found that salivary gland extract (SGE) of ixodid ticks affects the immune system of vertebrate hosts (Ribeiro J.M.C. 1989: Exp. Appl. Acarol. 7: 15–20; Kuthejlová M., Kopecký J., Štěpánová G., Macela A. 2001: Infect. Immun. 69: 575–578) and also contributes to the transmission of *B. afzelii* to the host (Pechová J., Štěpánová G., Kovář L., Kopecký J. 2002: Folia Parasitol. 49: 153–159).

In earlier studies it was observed that borreliae did not occur in host-seeking *Dermacentor reticulatus* (Fabricius, 1794) in contrast to *Ixodes ricinus* (L., 1758) (Hubálek Z., Halouzka J., Juřicová Z. 1998: Folia Parasitol. 45: 67–72), and that *B. garinii* survived in *I. ricinus* after experimental inoculation while it rapidly disappeared from *D. reticulatus* (Mátlová L., Halouzka J., Juřicová Z., Hubálek Z. 1996: Folia Parasitol. 43: 159–160).

The aim of this study was to examine the effect of SGE and midgut extract (MGE) from both *I. ricinus* and *D. reticulatus* on the growth, motility and morphology of the *B. garinii* spirochaete *in vitro*. This might address the question of vector competence of these two tick species for *B. burgdorferi* s.l. at the level of their compartments (salivary glands and midgut).

Salivary glands and midgut were removed from 40 unfed female *I. ricinus* and 20 unfed female *D. reticulatus*. This study attempted to simulate the effect of SGE and MGE on borrelial growth under conditions of host-seeking ticks, and therefore unfed individuals only were used. Dissected organs were homogenized with a small glass blender in phosphate-buffered saline pH 7.0 (PBS; Oxoid), placed in microtubes, and centrifuged at 9,000 *g* for 10 min. Clarified extracts were sterilized by filtration through the 0.2 µm Nanosep MF centrifugal device (Pall Corporation) and stored at –20°C. Final protein content (µg/ml) of the extracts was estimated (Bradford J. 1976: Anal. Biochem. 72: 248–254) as 32.8 (SGE) and 30.9 (MGE) in *I. ricinus*, and 29.3 (SGE) and 39.2 (MGE) in *D. reticulatus*. In the experiments, 100 µl of each extract (or PBS in the control) were mixed with 100 µl of a 3-day culture of *B. garinii* strain BR 14 (about 10^7 spirochaetes per ml) in BSK-H medium with 6% of rabbit serum (Sigma) in 96-well U-bottomed sterile microplates (Sarstedt), and covered with a sterile sealing film (Denville Scientific). The microplates were placed in a 33°C incubator for 11–12 days. Concentration of motile spirochaetes (the number of motile cells/ml of medium) was determined at intervals (0, 2, 4, 7, 9 and 11 days in *I. ricinus*, and 0, 2, 5, 7, 9 and 12 days in *D. reticulatus*), using darkfield microscopy. (1) Estimation of per cent motility was determined in 3 wells per variant, when 100 randomly selected spirochaetes were screened for motility per well. (2) Concentration of all spirochaetes (motile plus non-motile) was estimated in 10-µl volumes of appropriately diluted cultures on a microscope slide with a 20 × 20 mm coverslip (Hubálek Z., Halouzka J., Heroldová M. 1998: J. Med. Microbiol. 47: 929–932); for each variant, 3 wells with 5 counts (total, 15 repetitions) were used. The data were analysed with the two sample *t*-test using SOLO (BMDP Statistical Software). Significant differences in the concentration of motile spirochaetes were considered at $P < 0.01$.

The results are summarised in Tables 1 to 3. The proportion of motile spirochaetes decreased more slowly with the extracts of *Ixodes ricinus* than in corresponding control (C) since day 9 post inoculation (p.i.). On the other hand, the percentage of spirochaetal motility decreased more rapidly with SGE and MGE from *D. reticulatus* than in C since day 9 p.i. With *I. ricinus*, the concentration of motile spirochaetes increased significantly from days 2 to 11 (p.i.) with both SGE and MGE compared to C. In addition, the growth of spirochaetes was enhanced to a greater degree with SGE than with MGE on days 4, 7 and 9 p.i. With *D. reticulatus*, a significant increase in concentration of motile spirochaetes was only detected with SGE (compared to C) on day 5 p.i., while a marked decrease in concentration of motile spirochaetes was observed on day 9 p.i. with MGE, and on day 12 p.i. with both extracts compared to C. Moreover, many spirochaetes grown in the presence of *D. reticulatus* MGE were morphologically changed (compared to C and SGE) by 9 days p.i.; the cells were damaged (e.g., less discernible walls), shorter, and with a lower number of coils.

The effect of SGE and MGE on the growth of *B. garinii* spirochaetes *in vitro* thus differed between the two tick species tested. While extracts derived from *I. ricinus* (a competent vector for Lyme borreliosis) stimulated growth significantly, extracts from *D. reticulatus* (a non-competent species) did not affect the growth of borreliae markedly, or even inhibited their growth on days 9 (MGE) and 12 p.i. (MGE and SGE). Our results therefore indicate that the tick compartment extracts surprisingly need not be inhibitory for pathogen survival in the body of even non-competent tick species like *D. reticulatus* in a short-term exposure. In such species, the role of a barrier in

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Table 1. Per cent motility of *Borrelia garinii* spirochaetes in BSK-H medium with salivary gland (SGE) or midgut (MGE) extracts (compared to control, C) from ticks.

Ixodes ricinus				*Dermacentor reticulatus*			
Days p.i.	C	SGE	MGE	Days p.i.	C	SGE	MGE
0	100.0	100.0	100.0	0	100.0	100.0	100.0
2	99.3	99.3	98.3	2	99.3	99.7	98.7
4	97.3	98.0	96.3	5	97.7	99.7	97.0
7	92.0	94.7	88.3	7	91.0	94.3	88.0
9	47.3	84.0	77.0	9	87.7	80.0	52.7
11	18.3	26.7	50.0	12	51.0	24.3	5.7

Table 2. Effect of SGE or MGE from *Ixodes ricinus* on the growth of *B. garinii* in BSK-H medium (compared to control, C).

Days p.i.	Concentration of motile spirochaetes [$\times 10^6$/ml of medium]						Differences (*t*-values)		
	C		SGE		MGE				
	AVG	SD	AVG	SD	AVG	SD	C vs. SGE	C vs. MGE	SGE vs. MGE
0	9.08	1.88	9.24	1.36	9.35	1.83	0.26	0.40	0.19
2	27.30	6.58	33.77	6.74	36.29	7.10	2.66*	3.60**	1.00
4	40.37	6.45	80.53	9.80	50.61	9.73	13.25**	3.39*	8.39**
7	49.49	13.48	96.24	20.37	60.48	11.25	7.41**	2.42*	5.95**
9	19.38	9.81	87.93	15.90	45.72	10.81	14.21**	6.99**	8.50**
11	2.35	2.64	15.09	5.95	12.56	4.70	7.58**	7.34**	1.29

* P < 0.01; ** P < 0.001; AVG – arithmetic average; SD – standard deviation

Table 3. Effect of SGE or MGE from *Dermacentor reticulatus* on the growth of *B. garinii* in BSK-H medium (compared to control, C).

Days p.i.	Concentration of motile spirochaetes [$\times 10^6$/ml of medium]						Differences (*t*-values)		
	C		SGE		MGE				
	AVG	SD	AVG	SD	AVG	SD	C vs. SGE	C vs. MGE	SGE vs. MGE
0	10.92	2.30	13.72	3.78	13.44	2.41	2.45	2.93*	0.24
2	47.22	11.66	44.12	9.69	41.59	7.42	0.79	1.58	0.80
5	59.03	12.39	80.30	19.58	69.56	23.29	3.27*	1.41	1.37
7	75.26	12.77	85.40	11.48	74.31	17.53	2.29	0.17	2.05
9	78.77	20.48	80.13	4.52	46.62	3.67	0.19	4.99**	5.76**
12	36.24	11.21	17.53	7.59	4.43	1.25	5.35**	10.92**	6.60*

Explanations as for Table 2.

the pathogen transmission could be another tick compartment, e.g. the haemolymph (Johns R., Ohnishi J., Broadwater A., Sonenshine D.E., De Silva A.M., Hynes W.L. 2001: J. Med. Entomol. 38: 99–107), or non-specific immune compounds like lectins (Grubhoffer L., Jindrák L. 1998: Folia Parasitol. 45: 9–13).

A stimulatory chemotactic effect of SGE from *Ixodes scapularis* on *B. burgdorferi* s.s. *in vitro* was found in a recent study (Shih C.M., Chao L.L., Yu C.P. 2002: Am. J. Trop. Med. Hyg. 66: 616–621). Our work considered the effect of SGE and MGE on spirochaetal growth, but further experiments with other genomic species of borreliae (especially *B. afzelii*, *B. burgdorferi* s.s.) are required.

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