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Dissociating effect of salivary gland extract from *Ixodes ricinus* on human fibroblasts: Potential impact on *Borrelia* transmission

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ABSTRACT

Understanding the mechanism of pathogen transmission is essential for the development of strategies to reduce arthropod-borne diseases. The pharmaco- and immunomodulatory properties of insect and acarine saliva play an essential role in the efficiency of pathogen transmission. The skin as the site where arthropod saliva and pathogens are inoculated - represents the key interface in vector-borne diseases. We identified tick molecules potentially involved in pathogen transmission, using micro-HPLC and mass spectrometry, followed by *in vitro* assays on human skin cells. Histone H4 isolated from *Ixodes ricinus* salivary gland extract was identified as a molecule with a dissociating effect on human primary fibroblasts. This histone might be involved in the formation of the feeding pool formed around the tick mouthparts and responsible of tissue necrosis in the vertebrate host. Thanks to its selective antimicrobial activity, it may also sterilize the feeding pool and facilitate transmission of pathogens such as *Borrelia burgdorferi* sensu lato.

1. Introduction

Hard ticks of the Ixodidae family are involved in numerous tick-borne diseases caused by diverse pathogens including bacteria, viruses or parasites. Certain *Ixodes* species are obligate hematophagous vectors for bacteria including *Borrelia burgdorferi* sensu lato, *Rickettsia*, *Anaplasma*, the parasite *Babesia* or the tick-borne encephalitis virus (de la Fuente et al., 2008). These pathogens are transmitted when a tick attaches to a host through its sophisticated mouthparts, the hypostome and the chelicerae. The skin injury eventually develops to a feeding pool (Sonenshine and Anderson, 2014). During a tick blood meal which typically lasts for several days, the hard tick concurrently inoculates saliva to secure its blood meal by forming a cement layer around the mouthparts and by the secretion of pharmacologically and immunologically active molecules (Kazimírová and Stibrániová, 2013). The salivary glands modify their structure during the blood meal and degenerate after the blood meal in some tick species (Alarcon-Chaidez, 2014; Kahl et al., 1990) and the tick detaches from the host to either molt (nymphs) or to die after laying eggs (female adults). The tissue damage very often develops to a skin necrosis after the tick blood meal (Krause et al., 2009).

Saliva plays a crucial role during the feeding process: it allows the tick to neutralize host hemostasis (vasoconstriction, platelet aggregation, blood clotting), innate immunity (inflammatory response, complement activation) and acquired immunity. For that purpose, tick saliva contains a wide range of pharmacologically active molecules (Francischetti et al., 2009; Hovius et al., 2008; Ribeiro et al., 1985) with anti-clotting (Chmelar et al., 2012; Francischetti et al., 2004; Narasimhan et al., 2004; Prevot et al., 2006), vasodilatory (Bowman et al., 1996; Dickinson et al., 1976) and anti-inflammatory properties (Beaufays et al., 2008; Paesen et al., 1999; Ribeiro and Mather, 1998; Sangamnatdej et al., 2002). In addition, tick saliva is able to target vertebrate host immune responses such as the complement cascade (Couvreur et al., 2008; Daix et al., 2007; Schroeder et al., 2007; Valenzuela et al., 2000), antimicrobial peptides (Kern et al., 2011; Marchal et al., 2011), B-cells (Hannier et al., 2004) and the T-cells (Anguita et al., 2002; Garg et al., 2006; Leboulle et al., 2002). In the context of tick-borne diseases, this cocktail of active molecules renders

Abbreviations: SGE, salivary gland extract; FB, fibroblast; PTM, post translational modification; AMP, antimicrobial peptides

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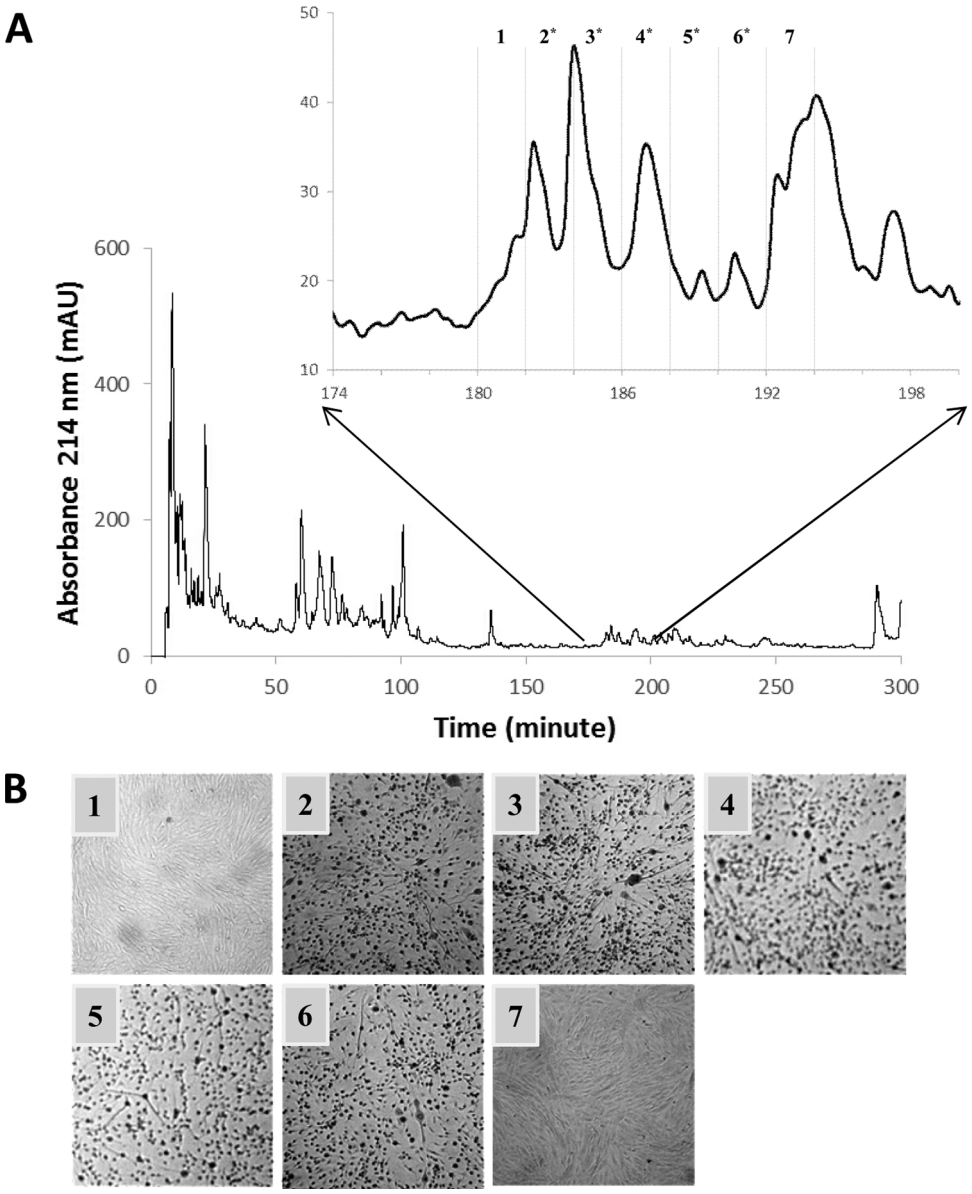

Fig. 1. SGE fractionation and detection of dissociating effect on human FBs. (A) UV chromatogram of SGE of *Ixodes ricinus* females, fed on rabbit for three days. Fractions were collected every two min. Fractions whose dissociating effect on human fibroblasts was measured are indicated (fractions 1–7). Fractions exhibiting an effect on human fibroblasts are indicated by a star (*).
(B) *In vitro* cultures of human FBs co-incubated with *I. ricinus* SGE obtained after microHPLC separation. Dissociating effect tested for fractions (1), (2), (3), (4), (5), (6) and (7). Activity was observed for (2) to (6). Scale bar corresponds to 100 µm.

the infection of a host more efficient.

The skin of the vertebrate host is the key interface where the vector bites and inoculates its saliva containing the pathogens (Bernard et al., 2015, 2014). The epidermis with its keratinocytes represents the first barrier encountered by ticks. They overcome this barrier using their biting pieces to penetrate deeply into the skin and reach the dermis, where the saliva interacts with resident cells like dermal dendritic cells, mast cells or fibroblasts. Some pathogens already multiply in the dermis before disseminating to the target organs. Investigating these initial steps is essential to understand the further development of most of the vector-borne diseases and to develop new strategies to control these diseases (de la Fuente et al., 2017).

We have selected the causative agent of Lyme disease, *Borrelia burgdorferi* sensu stricto (ss), which is transmitted by certain *Ixodes* species in Europe to study these transmission mechanisms. In a previous study, we have shown the antialarmin effect of tick saliva on human keratinocytes (Marchal et al., 2011). We observed also that crude salivary gland extract induced a dissociating effect on fibroblast monolayer adherent to tissue culture plate (Schramm et al., 2012). Identification of the tick molecules responsible of this specific effect could help to understand the formation of the feeding pool, which is essential for the tick blood meal process and thus for the transmission of pathogens. Therefore, we performed a fractionation of tick salivary gland extract by micro RP-HPLC and tested the different chromatographic fractions for a potential dissociating effect on human dermal fibroblasts. A tick-borne histone H4 was identified in the active fractions by enzymatic digestion and nanoLC-MS/MS. This protein was further characterized by mass spectrometry. The biological significance of this protein is discussed in the context of *B. burgdorferi* sl transmission.

Table 1
Proteins identified in each active fraction of *Ixodes ricinus* SGE.

Fraction[a]	Retention time (min)	Identified proteins[b]	Accession number	Dissociating activity
1	180-182	cyclophilin A	gi|67084095	No
		heat shock protein, putative	gi|240972667	
		histone H2B, putative	gi|241568982	
		calreticulin	gi|39725993	
2	182-184	small heat shock protein, putative	gi|241860225	Yes
		histone H2B, putative	gi|241568982	
3	184-186	histone H4, putative	gi|241568987	Yes
		histone H2B, putative	gi|241568982	
		ornithine decarboxylase, putative	gi|24200134	
4	186-188	histone H4, putative	gi|241568987	Yes
		histone H2B, putative	gi|241568982	
		hypothetical protein IscW_ISCW008323	gi|241652216	
5	188-190	histone H4, putative	gi|241568987	Yes
6	190-192	cyclophilin A	gi|67084095	Yes
		histone H4, putative	gi|241568987	
		histone 2A	gi|241251590	
		fructose 1,6-bisphosphate aldolase, putative	gi|241690479	
		ixoderin B4	gi|126360866	
		hypothetical protein IscW_ISCW008323	gi|241652216	
7	192-194	histone H4, putative	gi|241568987	No

[a] Fractions are described by their number indicated in Fig. 1.
[b] NanoLC-MS/MS data obtained after reduction of disulfide bridge, alkylation of cysteines and enzymatic digestion by trypsin were analyzed using MASCOT and OMSSA search engines against an in-house generated target-decoy protein database composed of protein sequences of *Ixodes*.

MSGRGKGGKGLGKGGAKRHRKVLR**DNIQGITKPAIR**RLARRGGV
KR**ISGLIYEETRGVLKVFLENVIRDAVTYTEHAK**RK**TVTAMDVV**
YALKRQGRTLYGFGG

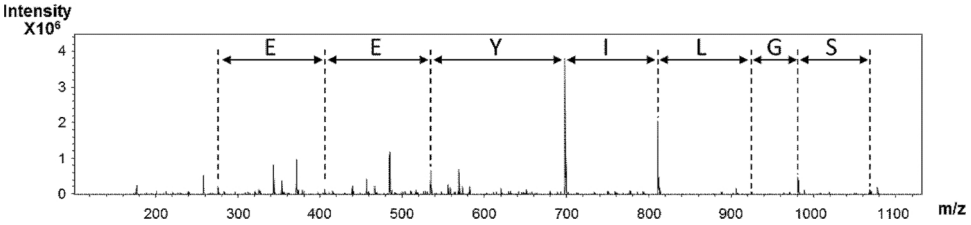

Fig. 2. Histone H4 identification. The sequence of histone H4 protein and the MS/MS spectra of the peptide **ISGLIYEETR** are presented. The protein was identified with a maximum of 11 peptides (highlighted in bold) representing a sequence coverage of 66%.

2. Materials and methods

2.1. Tick salivary gland extract preparation and HPLC fractionation

This research has been reviewed and approved by the Institutional Animal Care, CREMEAS (Comite Régional d'Ethique en Matière d'Expérimentation Animale). Protocols for all animal experiments were prepared according to their guidelines. Adult *I. ricinus* females, either originating from our in-house tick colony or collected in the field, were either fed on rabbit or on mouse for three days before being dissected. Tick salivary gland extract (SGE) was prepared from blood-fed females on day 3 as described previously (Marchal et al., 2011). SGE was tested for the presence of endotoxin by the Limulus assay.

Tick SGEs were then fractionated on a micro-HPLC system U3000 (Thermo Fischer Scientific, San Jose, USA). The solvent system consisted of 0.05% TFA in water (solvent A) and 0.045% TFA in ACN (solvent B). Detection was performed at 214 nm. The system was equipped with a Dionex Acclaim PepMap C18 enrichment column (500 µm x 15 mm, 5 µm) and an Agilent Zorbax 300SB-C18 analytical column (300 µm x 15 cm, 3.5 µm). SGE corresponding to the equivalent of 3 ticks was loaded during 3 min on the enrichment column at a flow rate of 15 µL/min with 0.1% TFA in water. Elution was performed at a flow rate of 4 µL/min with the following step gradient: a 5 min stage at 2% B followed by a 2–9.5% B linear gradient over 15 min, then a 9.5–29.5% B linear gradient over 80 min, then a 29.5–48% B linear gradient over 185 min and a final 5 min stage at 80% B.

2.2. Measurement of the effect of tick SGE on human fibroblasts (FBs)

Primary human dermal fibroblasts (FBs) (NHDF, Promocell, Germany) were maintained in FGM2 medium. Cells were used at passage 3–5 and plated into 48-well plates. At confluence and one day before the assays, FGM2 medium was replaced by FGM medium without fetal calf serum. SGE micro-HPLC fractions were transferred onto FBs and incubated for 24 h. Controls were performed with equivalent H_2O/0.05% TFA (v/v) and ACN/0.045% TFA (v/v) mixtures. The SGE effect was measured by observation of the cells under inverted microscope.

To evaluate more precisely the effect of SGE and histone, recombinant human histone H4 with his-tag (Sigma) (data not shown) or without his-tag (Biolabs) was incubated for 24 h at different concentrations (0, 5, 10 and 20 µg/mL) with FBs in 24-well plates. Positive control was performed with crude *I. ricinus* SGE at 20 µg/ml. An irrelevant recombinant protein (Human HMGN1: High mobility group nucleosome-binding domain-containing protein 1, R&D) was also tested on fibroblasts as negative control.

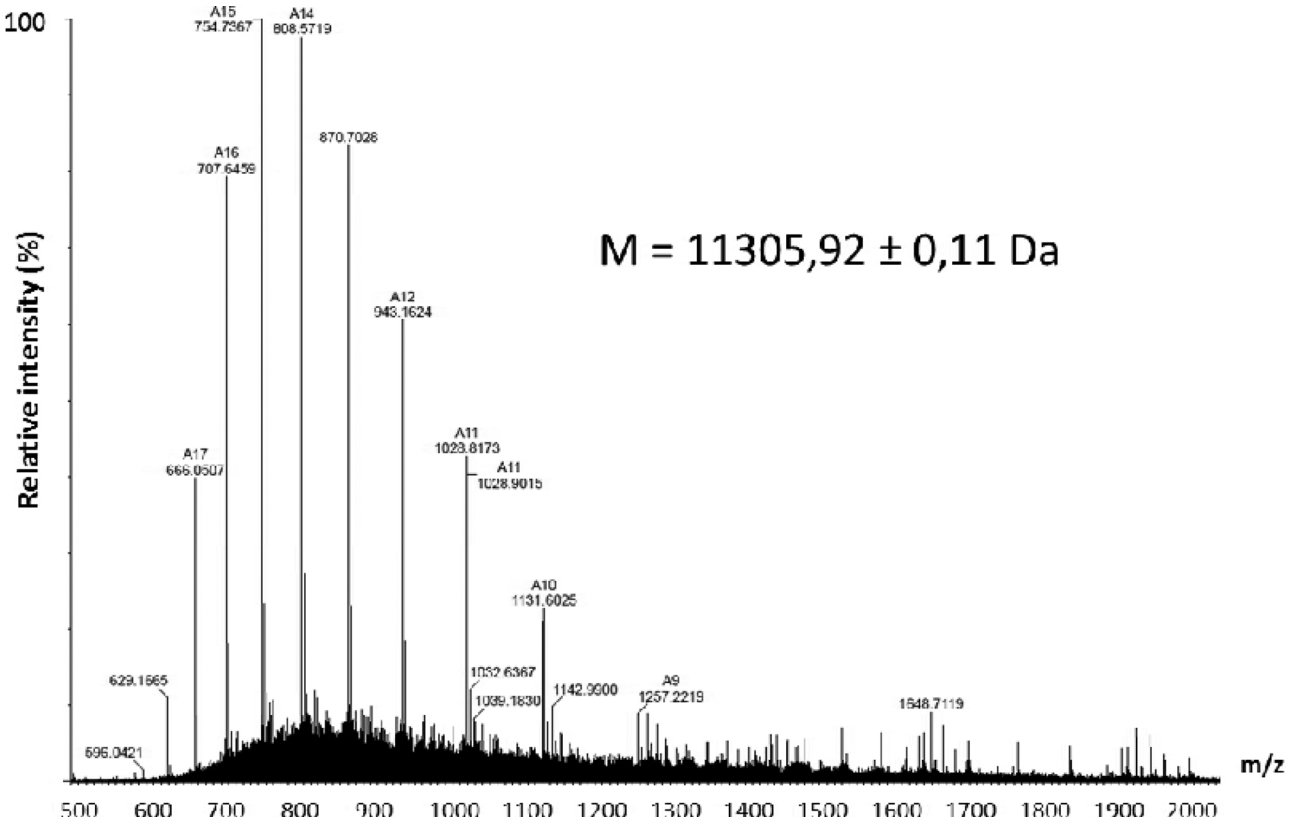

Fig. 3. Intact mass measurement of histone H4 isolated from SGE. The MS spectrum showing the different charge states was obtained by infusion on a Triversa NanoMate/Synapt G2 instrument. A protein with an average mass of 11,305.92 ± 0.11 Da was mostly observed.

The viability of FBs was assessed by Trypan blue. Supernatants of control FBs, FBs treated with SGE or with histone (20 µg/ml) were collected and cells counted with Trypan blue. Then, the remaining adherent FBs were counted in a Neubauer cell after trypsination, and FBs viability was determined by Trypan blue staining.

2.3. Mass spectrometry measurements

Intact mass measurements of proteins present in fractions of interest were performed on a hybrid electrospray quadrupole time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Manchester, U.K.) coupled to an automated chip-based nanoelectrospray device (Triversa Nanomate, Advion Biosciences, Ithaca, U.S.) operating in the positive ion mode.

For nanoLC-MS/MS, proteins present in fractions of interest were digested by using a liquid trypsin digestion protocol as described in supplementary data. Digested peptides were analyzed on an Agilent 1100 series nanoHPLC-Chip system (Agilent Technologies, Palo Alto, USA) hyphenated to an amaZon ion trap (Bruker Daltonics, Bremen, Germany) as described (Schnell et al., 2014) with an ACN gradient in 0.1% HCO_2H. Detailed chromatographic and MS conditions are provided in supplementary data. For the characterization of histone H4, proteins present in fractions of interest (or recombinant histone H4), were digested by using a liquid AspN digestion protocol as described in supplementary data. Digested peptides were analyzed on a nanoACQUITY UPLC system coupled to a Q-Exactive Plus (Thermo Fisher Scientific, MA) equipped with a nanospray Flex™ ion source (supplementary data). The peptides were trapped on a nanoAcquity UPLC precolumn (Symmetry C18 Trap, 5 µm, 180 µm × 20 mm, Waters), then separated on a nanoAcquity UPLC column (BEH C18, 1.7 µm, 75 µm × 150 mm, Waters) with an ACN gradient in 0.1% HCO_2H. Detailed chromatographic and MS conditions are given in supplementary data.

2.4. MS/MS data interpretation

Collected mass data were converted into mgf files using DataAnalysis 4.0 (AmaZon-Bruker Daltonics) or MSConvert 3.0 (Q-Exactive Plus) softwares. The MS/MS data obtained from AmaZon were analyzed using two search engines: the MASCOT 2.3.2 (Matrix Science, London, UK) and OMSSA 2.1.9 algorithms as described (Schnell et al., 2014) against an in-house generated target-decoy protein database composed of protein sequences of *Ixodes* downloaded from National Center for Biotechnology Information non redundant database (NCBInr) and of common contaminant proteins such as porcine trypsin and human keratins (2 × 21,571 entries on December 16, 2012). Spectra were searched with a mass tolerance of 0.5 Da for MS and MS/MS data. For the data obtained from AspN digestion and Q-Exactive Plus analyses, spectra were searched with a mass tolerance of 5 ppm for MS and 0.07 Da for MS/MS data, allowing a maximum of one missed cleavage with AspN enzyme and with acetylation, mono-, di- and trimethylation of both lysine and arginine and oxidation of methionine specified as variable modifications.

2.5. Biological activity of histone H4 on pathogenic and commensal bacteria

B. burgdorferi sensu lato (sl) were used at low passage. *B. burgdorferi* ss 297 (isolated from cerebrospinal fluid in the United States- Dr. A. Steere) were grown at 33 °C in Barbour-Stoenner-Kelly (BSK) medium supplemented with 6% rabbit serum for 4 days. *B. afzelii* strain NE4049 was cultured in modified BSK-H complete medium (Sigma) at 33 °C in anaerobic conditions. We used a starting concentration of 10^5 *Borrelia*/well in 100 µl. *Staphylococcus epidermidis* ATCC12228, *Micrococcus luteus* and *Escherichia coli* ATCC 25922 were grown in Mueller-Hinton (MH) medium overnight. A secondary culture in fresh MH medium was incubated for 3 to 4 h. Then, *S. epidermidis*, *M. luteus* or *E. coli* cultures were centrifuged and incubated with different concentrations (from 0

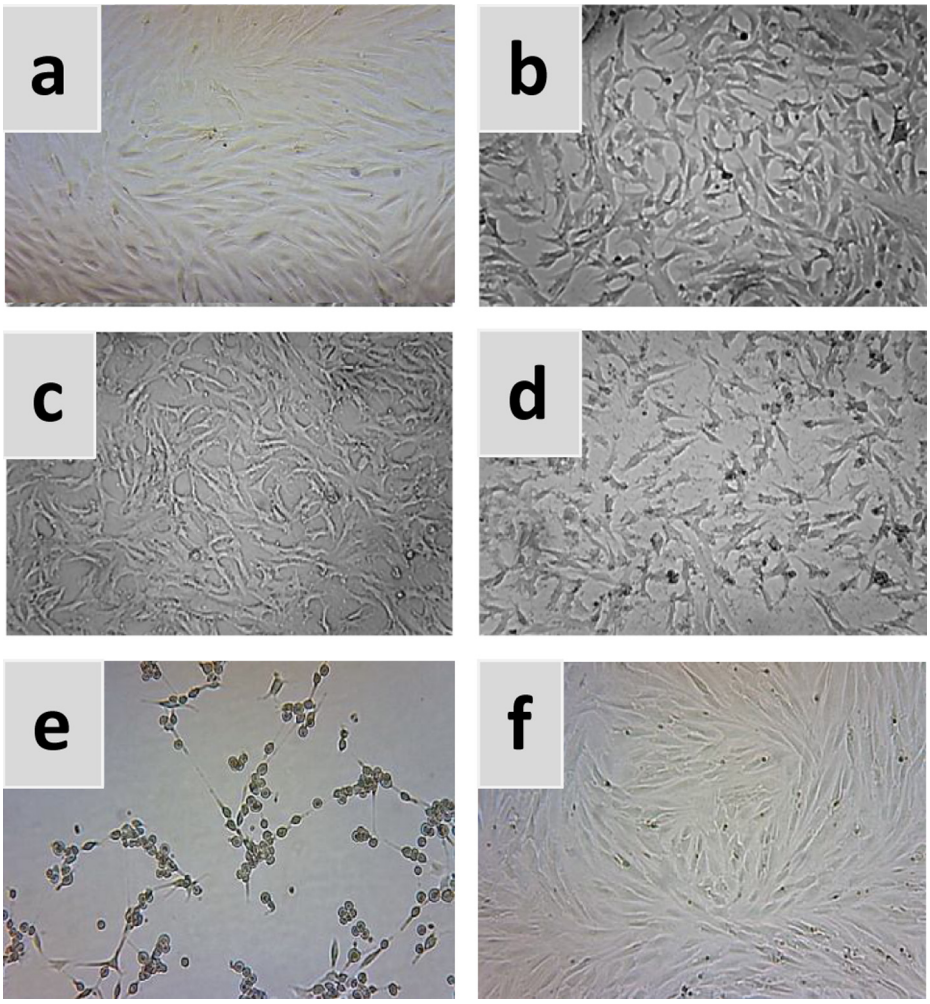

Fig. 4. Dissociating effect of Human recombinant histone H4 and crude SGE on human FBs. Images of human FBs co-incubated for 24 h with 0 (**a**), 5 (**b**), 10 (**c**) or 20 µg/mL (**d**) of recombinant histone H4, with crude SGE at 20 µg/mL of proteins (**e**) or with an irrelevant recombinant protein (**f**). Scale bar corresponds to 100 µm.

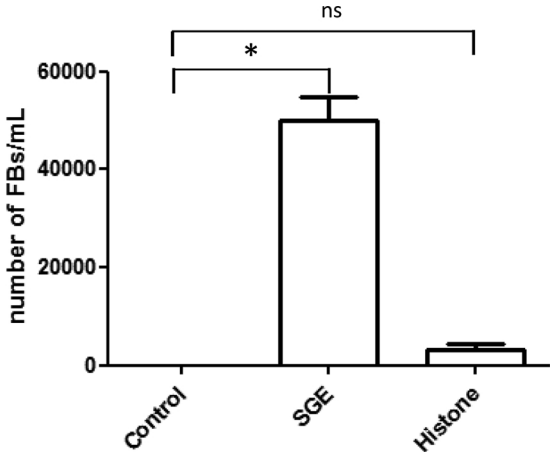

Fig. 5. Measure of cell viability and dissociating effect of salivary gland extract by Trypan blue. The effect of salivary gland extract (SGE) and human recombinant Histone H4 on human primary fibroblasts was measured by Trypan blue. Commercial purified histones (Sigma and/or Biolab) were used to compare their potential dissociating effect on SGE. Non adherent fibroblasts were first counted in the supernatant. Then, after trypsin treatment, adherent cells were counted (data not shown).

to 100 µg/mL) of gentamycin (Promocell) or human cathelicidin antimicrobial peptide (LL-37) as positive controls and recombinant human histone H4 without his-tag (Biolabs), or *I. ricinus* SGE at 37 °C for 3 h. After incubation, bacteria were plated on blood agar plates at 37 °C for 24 h. CFU were counted and percentage of survival was calculated according to the negative, *i.e.*, untreated control.

B. burgdorferi sl was incubated in microtiter plates for 24 h in serum-free FGM medium with different concentrations of gentamicin and LL-37 (positive controls), or human histone H4 and tick SGE, and then transferred in 1.5 ml-tubes of BSK for an additional 5 days. Then the *Borreliae* were counted and percentage of motility was calculated according to the negative, *i.e.*, untreated control.

3. Results

3.1. Identification of tick SGE fractions with dissociating activity

Preliminary micro-HPLC analyses were performed to assess the biological reproducibility and the amount of injected SGE needed to perform reproducible chromatograms (data not shown). An equivalent of three ticks per injection was found to be sufficient to obtain a satisfactory HPLC profile. Elution conditions were optimized with shallow gradients in the zone of SGE effect. Five active fractions between 182 and 192 min were identified with a dissociating effect on FBs (fractions 2–6 in Fig. 1A). The maximum dissociating effect was observed in the

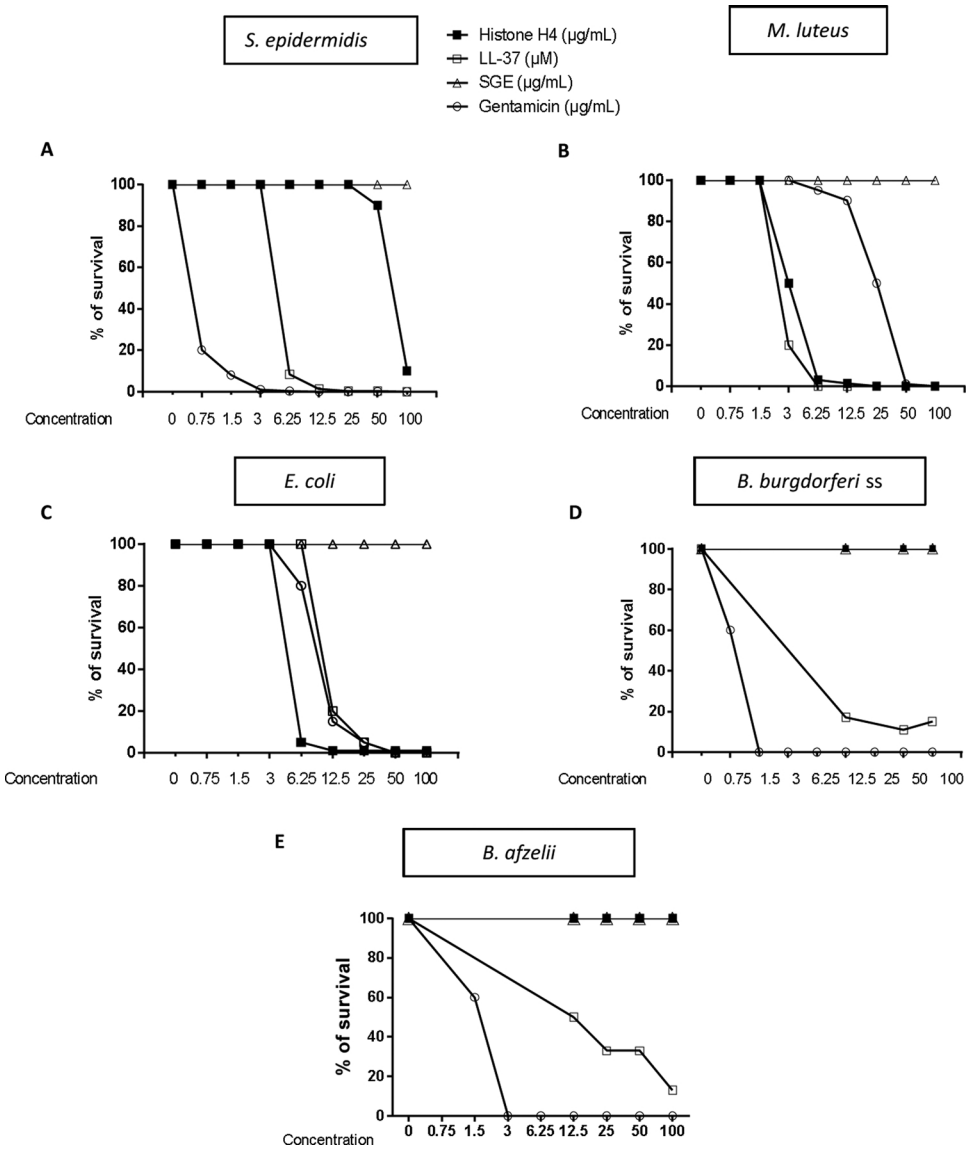

Fig. 6. Histone H4 but not whole SGE shows antimicrobial activity against pathogenic and commensal bacteria. (A) *Staphylococcus epidermidis* (*S. epidermidis*), **(B)** *Micrococcus luteus* (*M. luteus*) and **(C)** *Escherichia coli* ATCC 25922 (*E. coli*) were incubated for 3 h in PBS with different concentrations of LL-37 or gentamicin (positive controls), tick salivary gland extract (SGE) or human recombinant histone H4. Then bacteria were plated on agar for 24 h. CFU were counted and percentage of survival was calculated according to the negative control. **(D)** *Borrelia burgdorferi* ss (297) and **(E)** *B. afzelii* (NE4049) were incubated for 24 h in KGM medium with different concentrations of gentamicin (positive control), LL-37, SGE or histone H4. Then the *Borreliae* were counted and percentage of survival was calculated according to the negative control. Results (± SEM) are representative of at least three independent experiments with similar results.

fractions eluted between 184 min and 192 min (fractions 3–6 in Fig. 1B). No effect was observed before 182 min and after 192 min (fractions 1 and 7 in Fig. 1B).

3.2. Histone H4 identification

To identify tick SGE proteins responsible for the dissociating effect on FBS, fractions of interest were analyzed by a nanoLC-MS/MS strategy after a tryptic digestion. As a large number of proteins was expected, fractions neighboring active samples were analyzed as well to exclude proteins which might also be present in non-active fractions. We identified a total of 11 different proteins in active fractions (Table 1). Histone H4 was present in four (fractions 3–6) out of the five active fractions. It was present as single peptide in the active fraction 5, and with only one other peptide in fractions 3, 5 and 6, but with as many as 11 peptides (with OMSSA) in fraction 4. The sequence of the protein is presented in Fig. 2. The identified peptides correspond to 66% sequence coverage. The missing part can be explained by a high proportion of lysine and arginine, leading to short peptides not well detected during nanoLC-MS/MS. Tick SGE histone H4 was found to be 100% identical to human histone H4. We were able to show by Western blot that the tick histone present in SGE is recognized by a polyclonal rabbit serum directed to human histone, as well as to human recombinant histone H4 (Supplementary data).

ESI-TOF mass spectrometry of histone H4 isolated from SGE revealed an average mass of 11,306 Da (Fig. 3). On the other hand, the average mass calculated for the sequence without post-translational modifications (PTM) was 11,236 Da, resulting in a difference of 70 Da, which corresponds to one acetylation and two methylations on the N-terminal part. Further characterization of PTMs present on histone H4 by using AspN digestion and nanoLC-MS/MS confirmed the identification of histone H4 with 77% sequence coverage. However, identification of the N-terminal peptide carrying the PTMs was not successful.

3.3. Effect of salivary gland extract and recombinant histone on human fibroblasts

As *I. ricinus* histone H4 is 100% homologous to human histone, we used the commercially available human recombinant histone H4 to confirm the dissociating effect of histone H4 on FBs. The stimulation on FBs confirmed the ability of this surrogate histone protein to induce a cell effect within 24 h. The activity was concentration-dependent with a significant effect at 20 µg/mL (Fig. 4a to d). It was, however, less strong than the effect observed with crude SGE (Fig. 4e). This could be due to the fact that native histone H4 in SGE has a more potent effect than the recombinant histone that lacks post-translational modifications (PTMs),

especially on the N-terminal part. The effect is protein-specific since an irrelevant recombinant human protein, HMGN1 had no effect on the cell monolayer (Fig. 4f). It is also cell-specific since human primary keratinocytes were not affected at confluence by crude tick SGE (data not shown). In addition, we observed a retention time of 187 min when analyzing the recombinant histone H4 protein by micro-HPLC, which is consistent with the active fractions in SGEs.

To ensure that the dissociating effect of SGE on FBs was not only present in *Ixodes* ticks maintained under laboratory conditions, we also tested SGE of adult ticks collected in the field. Indeed, the SGE from these ticks showed a similar effect on FBs (data not shown). Furthermore, two different hosts, rabbit and mouse, were tested to verify that the effect was not linked to a specific vertebrate host. We observed a dissociating effect of SGE in any host the tick used for the blood meal: tick SGE isolated after feeding on either rabbit or mouse produced a dissociating effect (data not shown).

To assess more precisely the effect of SGE on human FBs, the cell viability was measured by Trypan Blue. No cells were seen in the supernatant of the untreated FBs (Fig. 5). Recombinant Human histone had a significant effect on the shape of the cells but very few cells were found in the suspension. SGE induced a strong dissociating effect on FBs with around 50% of cells in suspension (Fig. 5). Subsequently, the remaining adherent cells in each well were treated with trypsin. Trypan blue staining and counting of these cells demonstrated that more than 90% of the cells were viable. We can therefore conclude that SGE has rather a dissociating than a lytic effect on FBs. Interestingly, FBS co-incubated with SGE extract adhered again to the wells 72 h later, confirming that they were alive (data not shown).

3.4. Antimicrobial activity assays

Histone H4 is known for its antimicrobial activity (Lee et al., 2009). To test whether the identified histone might affect *Borrelia* and the skin microflora, we co-incubated *Borrelia* or different bacteria, Gram-positive (*M. luteus* and *S. epidermidis*) and Gram-negative (*E. coli*), with histone H4 or SGE, or with gentamycin antibiotics or LL-37 (human antimicrobial peptide, cathelicidin), both as positive controls for killing effect. Histone H4 affected *S. epidermidis*, *M. luteus* and *E. coli* but not the pathogenic bacteria, *B. burgdorferi* ss and *B. afzelii* (Fig. 6). *S. epidermidis*, a commensal of the skin (Fig. 6A), was less affected by histone H4 than *E. coli* and *M. luteus* (Fig. 6B and C). In contrast, we did not notice any effect of tick SGE extract on commensal or pathogenic bacteria. On the other hand LL-37, a well-known antimicrobial peptide of the human skin, showed an antibacterial effect in all bacteria tested including *Borrelia,* as it was already described earlier (Lusitani et al., 2002). The histone H4 present in SGE did not exhibit any antimicrobial activity, whereas the pure recombinant histone H4 did.

4. Discussion

The pathogen transmission is a key event in the context of arthropod-borne diseases. In the initial mechanical process, the two chelicerae lacerate the host tissue, leading to cell injury and the formation of a feeding pool which damages the vascular endothelium and facilitates the blood meal uptake. Subsequently, the tick saliva comes into play to boost the process of the blood meal. This has been particularly well-studied in the context of anti-tick vaccines, since antibodies directed against tick proteins can block the blood meal and potentially the transmission of pathogens (Schuijt et al., 2011). At the skin interface, tick saliva exerts its pharmacological as well as its immunomodulatory properties (Kazimírová and Stibrániová, 2013; Wikel, 2013). The effect of tick saliva on different immune cells has also been well studied. However, its effect on the main resident cells of dermis, the skin FBs, has been poorly investigated. The precise mechanism of the feeding pool formation around the tick biting pieces is not elucidated. We have shown earlier the dissociating effect of *I. ricinus* tick salivary glands on primary human fibroblasts in culture plates (Schramm et al., 2012). Since this could explain the direct formation of the feeding pool during the tick blood meal, we wanted to identify and investigate the tick molecule(s) responsible for this effect on dermal fibroblasts. We identified histone H4 in tick salivary gland as the molecule inducing a partial dissociation of FBs, using micro-HPLC and mass spectrometry. The Trypan blue assay revealed that SGE in fact induces a dissociation of the FB monolayer after which most of the cells detach but remain alive in suspension after 24 h of incubation. Presumably, Histone H4 is not the only molecule responsible for this effect, since the surrogate purified recombinant molecule, a human histone H4 100% homologous to tick histone, did not produce the same strong effect as the tick SGE. Another explanation could be that the recombinant protein is not as effective as the native molecule since it misses the PTMs.

Histones (H2A, H2B, H3, and H4) and DNA form in the nucleus the nucleosomes, folded in chromatin fibers. In the genome and transcriptome of *Ixodes scapularis*, five putative histones were identified and classified as IsH1, IsH2A, IsH2B, IsH3, and IsH4 (Cabezas-Cruz et al., 2016). All these histones except IsH4 were closely related to other tick histones from *Ornithodoros coriaceus*, *Amblyomma variegatum*, and *Nuttalliella namaqua*. The IsH4 was closely related to the mammalian histone H4, but not to H4 from other arthropods (Cabezas-Cruz et al., 2016). Transcriptomic studies on salivary glands and midguts, performed during blood feeding of *I. ricinus* on the vertebrate host, revealed 34 transcripts coding for proteins associated with histone modifications (Kotsyfakis et al., 2015). PTMs (acetylation, phosphorylation, methylation or ubiquitination) are essential for gene transcription and part of the “histone code” (Gardner et al., 2011). The most common modifications observed for histone H4 are the mono-, di- or tri-methylation of lysine 20 (Jørgensen et al., 2013; Yang and Mizzen, 2009), the histidine phosphorylation (Besant and Attwood, 2012), and acetylation of lysine 16 (Shahbazian and Grunstein, 2007; Shia et al., 2006). We have not been able to identify the PTMs on the histone purified from SGE. However, the measured mass in tick salivary gland is the same as the one previously observed by Kawasaki (Kawasaki et al., 2008). These PTMs are important for the varied histone activities, including antimicrobial and lytic/dissociating activities. Histones are primarily located in the nucleus, but they can also be found in the cytoplasm or on the cell surface. They are abundantly synthesized, have a low turnover and constitute 30% of total protein synthesis (Thepparit et al., 2010). The *Ixodes* histone H4 is 100% homologous to the human histone H4. This is the reason why we used the recombinant human H4, commercially available, to run our different experiments.

Tick saliva activity on FBs *in vitro* has been previously described in female *I. ricinus* and in a second hard tick, female *Amblyomma variegatum*. The effect was associated with the presence of tick molecules targeting wound-healing growth factors such as the platelet-derived growth factor (PDGF). The related immunosuppressive effect on inflammation may facilitate the successful accomplishment of the tick blood meal. The tick saliva factors involved in these processes have not been identified so far (Hajnická et al., 2011). We hypothesize that it could be Histone H4 interacting with fibroblast growth factors and leading to an alteration of cytoskeletal actin filaments. Therefore, we further analyzed the morphological changes of human FBs. Trypan blue staining showed that most of the cells are viable in the supernatant but detached in high numbers. It has been shown that tick salivary glands degenerate during the blood meal, and then histone H4 might be released into saliva and the host skin (Alarcon-Chaidez, 2014). Interestingly, another histone, H2B, has also been detected in the active fractions. Histones, and in particular histone H2B, are receptors for plasminogen. Their presence would also enhance all the inflammatory process (Das et al., 2007), promoting pathogens such as *B. burgdorferi* ss to diffuse into the target organs: joints, the heart and the nervous system. Extracellular histones seem to contribute to the hypocoagulability (Johansson et al., 2013) which could also facilitate the blood

meal uptake. In addition, a histone H2B of *I. scapularis* has been shown *in vitro* to interact with *Rickettsia felis*, a bacterium transmitted by ticks, to facilitate tick cell ISE6 invasion (Thepparit et al., 2010). More recently, *Anaplasma phagocytophilum*, another bacterium transmitted by *Ixodes* spp., was shown to increase the levels of histone modifying enzymes to inhibit host cell apoptosis and facilitate pathogen infection (Cabezas-Cruz et al., 2016). Free histones have also been described to play a role in hemocytes and hemolymph of oysters involved in the process of ETosis (Extracellular Trap osis) (Bachère et al., 2015), and more generally in other invertebrates as an ancient immune defense mechanism (Robb et al., 2014). Free histones might participate in local immune response of the tick salivary glands.

Concerning the involvement of histone antimicrobial activity in innate immunity, it has been evidenced in different organisms (Kawasaki and Iwamuro, 2008; Robb et al., 2014). In invertebrates, truncated forms of histone H4 from the armyworm *Spodoptera frugiperda* Sf9 cells were reported to be antimicrobial, leading to lysis of *Bacillus megaterium* and *Escherichia coli* (Calles et al., 2005). Several studies demonstrated the histone effect in the defense of oyster (Dorrington et al., 2011; Poirier et al., 2014). In vertebrates, frog histones H2A known as buforins I and II showed potent antimicrobial activity (Park et al., 1996), whereas another work revealed the presence of histone H4 in the skin of the Japanese tree frog, *Hyla japonica,* with a lytic effect on red blood cells (Kawasaki et al., 2008). A potential anti-inflammatory function of the rat histone H4 variants was reported as well (Poirier et al., 2006). In that study, it was demonstrated that C-terminal peptides transcribed from rat histone H4 variants significantly inhibit the release of prostaglandin E2 and interleukine-8, two pro-inflammatory mediators. Finally in human, histone H4 from sebocytes has been identified to have antimicrobial activity (Lee et al., 2009).

We showed that the histone H4 produces diverse effects when tested on different bacteria. It has a lethal effect on commensal bacteria of the skin (*S. epidermidis* and *M. luteus*) and also on *E. coli*. On the other hand, it has no effect on the pathogenic *Borrelia* transmitted by the tick. This demonstrates the efficiency *B. burgdorferi* ss has developed in using its vector to assure pathogen transmission. In SGE, Histone H4 might be associated with other molecules that modulate its antimicrobial activity.

Some of the tick proteins target the pharmacology of the host (coagulation, pain, hemostasis…), whereas others target the immunity (complement cascade, innate and acquired immunity) (Kazimírová and Stibrániová, 2013; Wikel, 2013). We show here that tick histone H4 is likely to be essential in the formation of the feeding pool, thanks to its antimicrobial activity and its dissociating properties. It probably acts in synergy with other tick saliva proteins since its effect was less strong when used alone. This feeding pool could explain the inflammatory process at the bite site as well as the skin necrosis observed in certain patients, and also described in animal models (Krause et al., 2009). H4 by its antimicrobial activity could also create a sterile environment for the blood meal of the tick. The FBs, in their interaction with *Borrelia* were described as a major player in *Borrelia* pathogenicity, by secreting metalloproteases, inflammatory molecules (Lochhead et al., 2012; Marchal et al., 2009; Schramm et al., 2012; Wu et al., 2011; Zhao et al., 2007) and by internalizing *Borrelia* (Wu et al., 2011). We postulate that FBs have a pivotal role during Lyme borreliosis infection, first during the process of transmission as a target for tick saliva and by producing a sterile niche for pathogens, and second as a potential cell involved in the persistence of *Borrelia* in the skin once the direct effect of tick saliva has disappeared.

Conflict of interest

The authors state no conflict of interest.

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