Published in International Journal for Parasitology 38, issue 5, 549-560, 2008
which should be used for any reference to this work

The impact of gene knock-down and vaccination against salivary metalloproteases on blood feeding and egg laying by *Ixodes ricinus*

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Abstract

Two cDNAs coding homologous putative metalloproteases (Metis 1 and Metis 2, expected molecular weights of 55.6 and 56.0 kDa, respectively) were identified from the hard tick *Ixodes ricinus*. The expression of Metis genes was induced in salivary glands during tick blood meal. RNA interference was used to assess the role of both Metis 1 and Metis 2 in tick feeding. It was found that salivary gland extracts lacking Metis 1–2 had a restricted ability to interfere with fibrinolysis. RNAi against Metis 1–2 also induced a high mortality rate. An immune reaction was raised in repeatedly bitten animals against Metis 1 and 2. Vaccination of hosts with the recombinant Metis 1 protein produced in a eukaryotic system partially interfered with completion of the blood meal. Although vaccination did not alter the survival rate or feeding time of ticks, their weight gain and oviposition rate were reduced. This will affect their reproductive fitness in the field. We believe this is the first report of an anti-tick vaccine trial using a metalloprotease derived from *I. ricinus*.

Keywords: Tick; RNA interference; Metalloprotease; *Ixodes ricinus*

1. Introduction

Ticks are blood-feeding arthropods. They infest a wide variety of reptiles, birds and mammals worldwide (Sonenshine, 1993). Their hosts include humans and domestic animals. They harm the infested host through several means. They take up blood, an action which can provoke anaemia. They can trigger poisoning or paralysis through saliva injection. They are also vectors of many pathogens which they transmit while feeding. In humans they are second only to mosquitoes as disease vectors and they transmit the agents of ehrlichiosis, encephalitis and Lyme disease (Sonenshine, 1991; Anderson, 2002). Ten thousand new cases of the latter are reported annually in North America and Europe where it is provoked by spirochetes of the genus *Borrelia* transmitted by ticks of the genus *Ixodes* (Jongejan and Uilenberg, 2004). In cattle, ticks also transmit protozoa of the genera *Theileria* and *Babesia*. Of the 1.2 billion cattle in the world, the majority are exposed to babesiosis. Its various consequences (abortions, loss of milk and meat production, decreased strength, mortality) and control measures together account for estimated losses of tens of millions of $US annually in Africa and Asia

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(Bock et al., 2004). Several hundred million cattle are also at risk from East coast fever, a variant of theileriosis, on its own causing an annual $US 200 million economic loss in Africa (Bishop et al., 2004).

A variety of tick control measures, including biological and chemical strategies, have been attempted. However, the success of tick control has so far been very limited. Vaccination is a method offering advantages such as efficiency, sustainability, low environmental impact and low risk of resistance. The proof of concept has already been obtained and a vaccine has been developed and used in Australia to protect cattle and sheep against *Boophilus microplus* (Rand et al., 1989; De Rose et al., 1999). This vaccine (commercialised as TickGUARD™ in Australia and GAVAC™ in South America) is based on a midgut membrane protein termed Bm86. It reduced the weight gain and survival of the ticks as well as their fecundity, thereby reducing the number of larvae in subsequent generations. Although the vaccine was not sufficient to eradicate ticks, it allowed a reduction in acaricide use, which lowered overall environmental impact and risks of resistance.

To date, there have been very few reports of putative vaccinating antigen candidates and even fewer vaccine attempts against *Ixodes ricinus* (Brossard et al., 1991; Prevot et al., 2007). The validity of a vaccine strategy is, however, supported by the observation of a protective immune reaction in repeatedly bitten animals (Bowessidjaou et al., 1977; Brossard, 1982). This points to the identification of vaccine candidates through screening of the antigens eliciting a reaction in repeatedly bitten animals and study of the tick's biology. Nevertheless the only commercial anti-tick vaccine is based on a concealed antigen (Willadsen, 2004; de la Fuente and Kocan, 2006; Nuttall et al., 2006). Good vaccine candidates may also be identified through high throughput screening independent of a prior knowledge of their biological role or immunogenicity (de la Fuente and Kocan, 2003).

In order to feed, ticks anchor on their host, take a blood meal and then detach. As opposed to many other blood-feeding arthropods, they remain attached to their host for long periods (sometimes up to 14 days). Therefore, they have to disrupt tissues and counteract various defence mechanisms of the host such as haemostasis, immune reaction or pain (Ribeiro, 1987, 1989; Ribeiro and Francischetti, 2003). Various proteases are putative bioactive factors of these tasks as they are involved in tissue disruption and the coagulation or complement activation cascades. Among others, an aspartic protease able to hydrolyse haemoglobin has been characterised in *Haemaphysalis longicornis* (Boldbaatar et al., 2006). Various metalloproteases are involved in tissue remodelling or disruption through digestion of structural components (Markland, 1991, 1998; Gomis-Ruth et al., 1993). Inoculation of metalloproteases found in the saliva of various biting animals could also participate in disrupting the balance of haemostasis. Therefore, the toxicity of snake venoms is partly due to several metalloproteases which interfere with haemostasis, disrupt basal laminae or extracellular matrices and trigger haemorrhages. In the case of a tick blood meal, metalloproteases could perform essential anti-haemostatic enzymatic activities. They are therefore adequate vaccine candidates on a dual basis: an efficient immune reaction would both reject a tick component and inhibit an enzymatic activity essential to the blood meal. According to this rationale, vaccine attempts have been reported based on peptidases or protease inhibitors in different tick species (Imamura et al., 2005, 2006; Leal et al., 2006) and in other invading parasite species such as *Brugia* (Pokharel et al., 2006). It is worth mentioning that these are also the target of some snake venom antisera.

Here, we show that expression of two homologous putative metalloproteases is induced in the saliva during the blood meal of *I. ricinus*. Based on their putative properties, we have called these tick proteins "Metis" 1 and 2 for "Metalloprotease from *Ixodes ricinus*" 1 and 2. The use of RNA interference (RNAi) ex vivo demonstrated the role of these proteins which interfered with the ability of tick salivary gland extracts to modulate fibrinolysis. This likely accounted, in part, for the observation that knock-down of these genes by RNAi in vivo prevented completion of the tick's blood meal. We showed that these proteins are recognised by the immune system of repeatedly bitten dogs and rabbits. We expressed and purified the corresponding recombinant proteins in mammalian cells and used them to vaccinate rabbits. We showed that Metis 1 elicited an antibody response which cross-reacted with Metis 2 and reduced the feeding ability of ticks as well as their egg laying efficiency.

2. Materials and methods

2.1. Ticks, salivary gland extracts and saliva

Ixodes ricinus ticks were bred and maintained at the Institute of Biology, University of Neuchâtel (Switzerland). Founders of the colony were initially collected in the field near Neuchâtel and have been maintained on rabbits and mice for over 20 years. Pairs of adult (female and male) ticks were allowed to anchor and feed on rabbits. For preparation of salivary gland extracts (SGE), unfed and 5 days engorged female ticks were dissected under the microscope. Salivary glands and midguts were harvested in ice-cold PBS. Tissues were then disrupted and homogenised using a dounce homogeniser. Samples were then centrifuged for 5 min at 10,000*g* and supernatants were recovered and stored at −20 °C. For saliva harvesting, ticks were stuck to glass slides using double-sided tape and a sterile glass micropipette was placed around the hypostome. Salivation was then induced by a single injection of 5 μl PBS containing 0.02% dopamine. Saliva was collected in the capillary as long as salivation occurred (usually 30–45 min). Samples were then expelled into ice-cold microcentrifuge tubes.

2.2. cDNA library screening and rapid amplification of cDNA ends (RACE)

Analysis of a cDNA subtractive library from 5 days fed and unfed *I. ricinus* salivary glands originally identified 27 distinct sequences specific for the fed state (Leboulle et al., 2002). Specific oligonucleotides (described in Table 1) were designed from these sequences, pooled and used to screen a new cDNA library by conventional hybridisation procedures. This screening identified 44 clones. These clones were sequenced using primers M13 Forward and Reverse. Sequences were analysed using BLAST software against the original 27 clones, and two sequences were found to show 100% and 85% identity, respectively, to Seq16 (sequence homologue to a snake venom metalloprotease). Seq16 was renamed Metis 1 and the new sequence was named Metis 2.

For Metis 2, the full-length cDNA clone was not recovered. Therefore, the 3′ missing end was generated by rapid amplification of cDNA ends (RACE) using the GeneRacer Kit (Invitrogen) and the primer 5′-GCTAATCTTAGG TATGCCAGCTTCGTAT-3′. The amplification was made using ThermoZyme DNA Polymerase according to the manufacturer's instructions. The PCR product was cloned into the pCR4-TOPO vector (Invitrogen) and sequenced.

2.3. RNA extraction and RT-PCR analysis

mRNAs were isolated by oligo-dT chromatography (MicroFastTrack 2.0 mRNA Isolation Kit, Invitrogen). Sense primer: 5′-GTCAAAACATTTTTATCGTACT GCC-3′ and anti-sense primer: 5′- GATTGTTTTTTT CGCTTTTCGTTCAG-3′, designed to amplify a 423 bp-specific DNA fragment of Metis 1 (nucleotides 801–1,224 of the open reading frame (ORF)), and sense primer 5′-ATCAAAAAAATTCTATAATACCTCCGAT-3′ and anti-sense primer 5′-ATTGTTTTTGTCGTGTATCGT TCTC-3′, designed to amplify a 423 bp-specific DNA fragment of Metis 2 (nucleotides 801–1,224 of the ORF), were used to perform RT-PCR analysis of the steady-state transcripts. Reverse transcription (RT) was routinely performed in a 20 μl standard RT reaction mixture according to the instructions of the manufacturer (First-Strand cDNA Synthesis System, Invitrogen) using the oligo-dT primer. PCR was routinely performed in a 50 μl vol. of standard Takara buffer containing 2.5 U of *Taq* polymerase (Takara Ex Taq, Takara, Japan), 10 pmol of each primer and 2.5 nmol of each deoxyribonucleotide triphosphate (dNTP) (Takara). PCR conditions were 30 cycles of 30 s at 95 °C, 30 s at 58 °C and 1 min at 72 °C, preceded by an initial 4 min denaturation at 95 °C and followed by a final 10 min extension at 72 °C. A pair of primers designed to amplify a 1,131 bp fragment from the actin ORF (sense primer 5′-ATGTGTGACGACGAGG TTGCC-3′ and anti-sense primer 5′-TTAGAAGCACTT GCGGT GGATG-3′) were used as controls. Ten microlitres of the PCR reactions were analysed on 2% agarose gels. PCR products were then cloned into the pCRII-TOPO vector (Invitrogen) and sequenced.

Table 1
Primer sequences used for screening the full-length cDNA library

Clone	Probe (5′–3′)
seq1	GTCCTCTTCTAAATAAGACCCATCC
seq2	AAGTCACTTGCACTTATCAAGCTCC
seq3	TTATGCTGCCGCCTACTTTTCCTTC
seq4	AGTACCCCTGTGAACTCTGGCTTTG
seq5	ATTGCCCTTGACGTACTCTCTCAAC
seq6	GAAGGAACAGGCACAAATATACTAC
seq8	GACCGATTCCACATTGTAGTACACC
seq9	TGTGACCATATCTTTGTTTCCCCTG
seq10	ACATATCATTTGGAGGAAGGCGTAG
seq11	GTGATAACCATATCCATTCCTCACC
seq12	TGGTTTACCGTAACAAGTACACCAG
seq13	CTGCCTCTACAAAGTCAATGCCAAG
seq14	CTCACCAAACACATCAAATACCCCC
seq15	CATGCCTTCGTCGTACATATACACC
seq17	TCGAATTGCACTTCGGAACTTCCAC
seq18	TCCCCGCCCCTTGACAATCGTCCGA
seq19	ATCCGAATGAGTTGTCAAATGACAT
seq20	AGAAGAGTAAGGTTTTCACCGACAG
seq21	TGTTGCTACAGACTCGACGTTTCGA
seq22	TGAAACTTGAAATACTCCCACAGTC
seq23	GACCACCCCGTCCGAACTTGCTAAA
seq25	TCCAATCTACAATCTTTCCTCGCAC
seq26	AGAAGACTGGGAAGATAAGAAGCAC
seq27	TCACCTGCTATTCCAGAAGTACACC

2.4. Expression of recombinant proteins in mammalian cells

Pairs of primers were designed to RT-PCR amplify the two Metis ORFs from 5-days fed tick salivary gland mRNA used as a template (sense primers 5′-CGGGGT ACCCCGACCATGTCGGGACTCAGCCTGAAA-3′, 5′-CGGGGTACCCCGACCATGTCGGAACTCAGCCT GAAAT-3′ and anti-sense primers 5′-TGCTCTAGAGC AGTCCTTCTTGCTTATTTTGATTTTG-3′, 5′-TGCT CTAGAGCAGTCCGTTCTGCTTATTTTGATCG-3′ for Metis 1 and 2, respectively). Unique restriction sites were added in 5′ and 3′ of the Metis ORF by PCR in order to subclone those into the mammalian expression vector pCDNA3.1/V5-His coding for the His and V5 epitope tags. The PCRs were performed in a 50 μl reaction volume containing 2.5 U of *Taq* polymerase (Takara Ex Taq, Takara, Japan), 10 pmol of specific primers for each target region amplified and 2.5 nmol of each dNTP (Takara) in a standard buffer supplied by the manufacturer (Takara). The PCR conditions were: one cycle at 95 °C for 4 min followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. The PCR products were then purified using polyacrylamide gel electrophoresis followed by electroelution. The PCR products were cloned into the pCDNA3.1/V5-His KpnI and XbaI (for Metis 2) or BamHI and SfuI (for Metis 1) restriction sites. The MetispCDNA3.1/V5-His constructs were then transfected into COS-1 cells by means of Fugene 6 (Roche) according to the manufacturer's

recommendations. The expression of proteins recovered in the culture supernatant was confirmed by Western blot analysis.

2.5. Design of small interference RNA (siRNA)

Small interference RNAs (siRNAs) were designed to specifically target each of the Metis mRNAs and were synthesised by Eurogentec. These were 5′-ACACUC AGAUGAAGUCAAA-3′ and 5′-GCACGACAGAG GGAAAUGA-3′, respectively.

2.6. siRNA silencing in COS-1 cells

COS-1 cells were co-transfected with combinations of 500 ng of each of the two Metis/pCDNA3.1/V5-His constructs and 100 ng of each of the two specific siRNAs using 4 μl of X-tremeGENE siRNA transfection Reagent (Roche) according to the manufacturer's recommendations. Forty-eight hours post-transfection, culture supernatant was harvested and protein expression was analysed by Western blot.

2.7. siRNA interference in live ticks

One microlitre of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA buffer only or containing 650 ng of specific Metis siRNA or control siRNA duplexes (Eurogentec, Belgium) was injected into the ventral torso of the idiosoma, away from the anal opening of adult *I. ricinus* females. The injections were carried out using Hamilton Microliter syringes with 33-gauge needles. At least 30 ticks were used per group. The ticks were allowed to recover for 1 day before infestation (together with non-injected male ticks) of the ears of New Zealand white rabbits. Ticks were monitored daily. Female ticks that detached upon repletion were collected and weighed on a digital balance.

2.8. siRNA ex vivo incubation with salivary gland extracts

The salivary glands from 10 partially (5 days) fed female ticks were incubated for 6 h at 37 °C in the presence of 5 μg of the control or Metis siRNA (individually or in combination) or buffer alone in a total volume of 200 μl of incubation buffer TC-199 (Sigma) containing 20 mM 3-[*N*-Morpholino]propanesulphonic acid (MOPS), pH 7.0.

2.9. Fibrinolysis assay: euglobulin clot lysis time (ECLT)

The euglobulin fraction was prepared as described by Zouaoui Boudjeltia et al. (2002). Briefly, 75 μl of acetic acid (0.025%) and 900 μl of deionised water were added to 100 μl of human plasma. The sample was centrifuged at 4,000*g* for 10 min at 4 °C. The pellet was resuspended in 100 μl of Owren buffer. A euglobulin clot was started by adding 25 μl (1.5 U/ml) of thrombin. After 10 min, 10 μl of SGE in Owren buffer were added to the reaction and the lysis time was measured by a semi-automatic method using a "Lysis Timer" device.

2.10. In vitro expression and purification of recombinant Metis 1

The ORF of Metis 1 was amplified by PCR using primers designed to add the unique BglII and BamHI restriction sites in 5′ and 3′, respectively. PCR was performed as described above except that the elongation step was carried out at 72 °C for 2 min. The restricted PCR product was cloned between the BglII and BamHI sites of the transfer vector p119L (Marchal et al., 2001) and sequenced. Sf9 cells were co-transfected with p119L/Metis 1 and purified viral DNA extracted from AcSLP10 virus as described previously (Chaabihi et al., 1993) and recombinant viruses isolated by plaque assay (Summers and Smith, 1987). Viral genome integrity was controlled by Southern blot analysis. The expressed protein was desalted by dialysis against the binding buffer (20 mM Tris, pH 8.5) and loaded on a XK 16 Q Sepharose column (GE Healthcare) equilibrated in the same buffer. The Metis 1 protein was then eluted in 20 mM Tris, 50 mM NaCl, pH 8.5.

2.11. SDS–PAGE and Western blot analysis

After protein dosage, recombinant proteins or tissue samples were mixed with one volume of 2× sample buffer, loaded on a 12.5% polyacrylamide gel and electrophoresed. Proteins were then blotted onto Hybond-C Extra nitrocellulose membranes (Amersham Biosciences). The membrane was incubated with the anti-Metis primary antibody at 25 °C for 1 h, and washed three times with TBS-tween (0.15 M NaCl, 0.05 M Tris, 80 0.1% Tween). The membrane was then incubated with alkaline phosphatase coupled anti-rabbit secondary antibodies (1:7,500 dilution, Promega) at 25 °C for 30 min and washed three times with 0.1% TBS-tween. Finally, proteins on blotted membranes were detected using 4-nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

2.12. Serum preparation

Serum from resistant rabbits was prepared by infesting two New Zealand white rabbits, three times at 3-week intervals with *I. ricinus* female ticks. Blood was harvested by ear puncture, allowed to clot for 1 h at 37 °C and then left at 4 °C overnight for clot retraction. It was then centrifuged for 10 min at 4,000*g* and the supernatant was harvested. The dog sera were derived from animals that had been infested once or twice (30 days apart) with adult females of *I. ricinus.*

2.13. Immunisation and challenge infestation

Female New Zealand white rabbits (3 kg, Harlan Netherlands, CEGAV France) were injected s.c. three times at 2

weekly intervals with 50 μg of purified Metis 1 in PBS with 1 mg of Alum adjuvant (final volume 1 ml). Control rabbits were mock-immunised with phosphate buffer in Alum adjuvant only. Animals were routinely bled by ear puncture 2 weeks after each booster. For challenge infestations, 10 days after the third booster, four vaccinated and four control animals were challenged with 30 pairs of adult (female and male) ticks or 50 nymphs. A daily survey was made after tick infestation. Ticks were harvested upon detachment, counted and maintained individually in humidified tubes.

2.14. Statistical analysis

The significance of the data was evaluated using one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test, the Student *t*-test implemented in MedCalc for Windows, version 8.2.0.1 (MedCalc Software, Mariakerke, Belgium), or when data were non-parametric, a Mann–Whitney rank sum test in the SigmaStat 3.5 SPSS software was used. Data are presented as medians (25–75%).

3. Results

3.1. A multigene family encoding putative metalloproteases

In the course of a differential screening between salivary gland cDNA libraries from fed and unfed ticks, our laboratory previously cloned 27 partial cDNAs (called seq 1–seq 27) corresponding to transcripts induced during a blood meal of female *I. ricinus* (Leboulle et al., 2002). Oligonucleotides were then designed from these partial sequences and pooled to screen an expression library. This allowed the isolation of 44 larger sequences. Among these, two showed a high level of identity (100% and 85%, respectively) to seq 16, a sequence homologous to snake venom metalloproteases of the reprolysin family. Primers were designed to clone the corresponding full-length cDNAs using 3′ RACE–PCR. Seq16 was renamed Metis 1 and the new sequence was named Metis 2. As illustrated in Fig. 1, bioinformatics analyses demonstrated the presence in both sequences of a signal peptide (as determined by SignalP3.0), a propeptide (by comparison with an already described *Ixodes scapularis* sequence (gi 22164294)) with a cleavage site tentatively located between positions 169 and 170, respectively, a Zn binding domain, two (Metis 1) or three (Metis 2) consensus glycosylation sites and a Met turn encoded 3′ of the putative catalytic site.

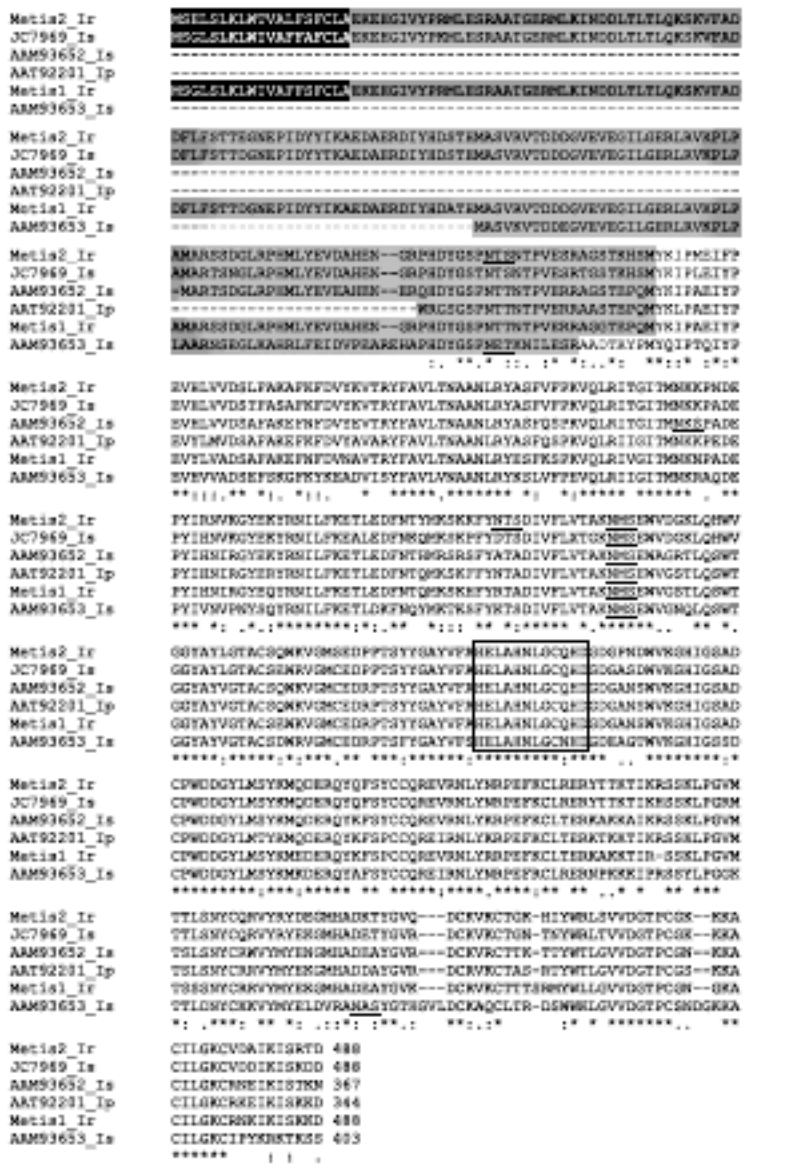

Fig. 1. Alignment of metalloprotease amino acid sequences from *Ixodes pacificus, Ixodes scapularis* and *Ixodes ricinus*. Amino acid sequences were predicted by in silico translation of the open reading frames of the sequenced cDNAs. The predicted signal peptides are shown in reverse background. The predicted prosequences are grey-shaded. The zinc-binding motifs HEXXHXXGXXH/D are boxed. The putative methionine turn found after the zinc-binding domain is in bold. The predicted glycosylation site is underlined. Asterisks indicate identities. Dots indicate similarities.

3.2. Expression of Metis 1 and 2 transcripts in salivary glands

We verified that the expression of these two genes is induced during the blood meal as previously suggested for seq16. mRNAs were extracted from various organs of dissected fed or unfed adults as well as from whole fed or unfed larvae and nymphs. These RNAs were analysed by RT-PCR using Metis 1-, Metis 2- and β-actin-(in order to verify the integrity and correct loading of the samples) specific primers. As shown in Fig. 2, Metis 1 and 2 mRNAs were only detected in fed nymphs (lane 2) and salivary glands of fed adult females (lane 7), but not in unfed nymphs (lane 1) or any other adult tissues (unfed female salivary gland or midgut, and male salivary gland or midgut; lanes 3–6). These results suggest that Metis 1 and 2 mRNAs are only expressed in fed *I. ricinus* tick salivary glands and that the transcription of these genes is induced during blood feeding.

3.3. Metis proteins contributed to the completion of the blood meal

In order to investigate the role of Metis 1 and 2, we observed ticks under in vivo Metis knock-down by RNAi.

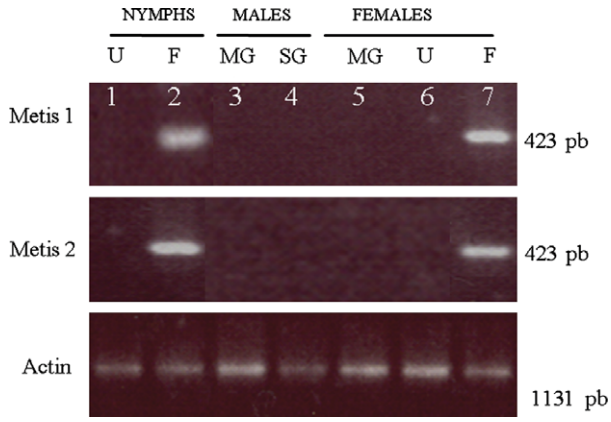

Fig. 2. RT-PCR analysis of Metis expression. The steady-state Metis 1 and Metis 2 mRNA levels were analysed by RT-PCR. PolyA + RNA was extracted from whole unfed (U) or fed (F) nymphs, midguts (MG) and salivary glands (SG) of males as well as midguts and salivary glands from unfed (U) and 5-day fed females (F). PolyA + RNA was reverse transcribed using Metis 1, 2 or actin-specific primers and the RT product was amplified by PCR using Metis 1, 2 or actin-specific pairs of primers generating a 423 bp (Metis 1 and 2) and a 1,131 bp (actin) product, respectively.

Gene-specific siRNA was designed and its specificity assessed by co-transfection with each of the two pcDNA3.1 expression vectors in COS-1 cells. Supernatants of each co-transfection were harvested and submitted to Western blot analysis using anti-his antibodies. Fig. 3a shows that an siRNA was able to specifically suppress expression of the matching expression vector amongst several co-transfected expression vectors. These results showed that each of the designed siRNA was functional as it knocked down the expression of the corresponding protein and it was highly specific as it did not affect the expression of the other family member.

We then proceeded to analyse the in vivo effect of these siRNAs. For that purpose, groups of 30 adult female ticks were microinjected with the two siRNAs, alone or pooled, in the ventral torso of the idiosoma. As shown in Fig. 3b, injection of buffer alone or irrelevant siRNA as controls caused a $30.0 \pm 0.0\%$ and $29.2 \pm 1.7\%$ mortality rate, respectively. Injection of the pooled Metis 1–2 siRNAs raised the mortality rate to $70.0 \pm 9.4\%$. This effect was likely due to the action of the Metis 2 siRNA as it showed a similar effect on its own while the Metis 1 siRNA on its own did not increase the mortality rate at all. These results suggested that the two Metis proteins are differentially required for the survival of ticks during a blood meal.

3.4. Metis proteins interfered with fibrinolysis

We then investigated the mechanism affected by the action of Metis. Despite repeated attempts, we failed to express an active form of the Metis proteins (see Section4). In order to deal with this, we set up an ex vivo siRNA interference assay. Tick salivary gland extracts or purified proteins have been shown to interfere with several physiological processes in vitro. In particular, metalloproteases with fibrino(geno)lytic activity in *I. scapularis* saliva, where they may function as anti-haemostatic or anti-angiogenic factors, have been described previously (Francischetti et al., 2003; Packila and Guilfoile, 2002; Valenzuela et al., 2002). In order to verify these data, the action of tick salivary gland extracts was monitored in a fibrinolysis assay. In the assay, 5 μg of salivary gland extracts of 5 days fed female ticks were mixed with a preformed fibrin clot and the fibrinolysis was monitored by euglobulin clot lysis time. Under these conditions, the extracts accelerated fibrinolysis as the fibrinolysis time was decreased (in a dose-dependent manner – results not shown) by $41.1 \pm 2.2\%$ compared with only $2.3 \pm 2.4\%$ upon addition of ungorged salivary extracts (Fig. 3c). As shown in Fig. 3d, Metis 1–2 siRNA added to salivary gland extracts interfered with their ability to reduce fibrinolysis time as they reduced it by half (a reduction of $22.4 \pm 3.2\%$ in fibrinolysis time compared with $40.1 \pm 2.8\%$ and $41.5 \pm 1.2\%$ reduction upon addition of salivary gland extracts supplemented with buffer or a control siRNA, respectively). Therefore, the putative enzymatic activity coded by the *metis* 1 and 2 genes, their induction during the blood meal and their need for the blood meal to be correctly completed pointed to those being ideal vaccine candidates.

3.5. Metis proteins shared antigenic epitopes

The two Metis ORFs were cloned in the pcDNA3.1/V5-His mammalian expression vector. Constructs were transfected in COS-1 cells for transient expression. The ability of these vectors to drive the expression of whole V5-His fusion proteins was checked by Western blot analysis using an anti-V5 antibody (Fig. 4). These two constructs were then used for genetic immunisation of mice. The resulting sera were tested by Western blot analysis of the corresponding Metis proteins expressed in COS cells, which showed that antibodies were only raised in mice immunised with pcDNA3.1/V5-His Metis 1 (Fig. 4). Each of these sera was then submitted to Western blot analysis of each of the recombinant proteins. As shown in Fig. 4, the polyclonal antibody serum specific to Metis 1 reacted with Metis 1 and cross-reacted with Metis 2. These results suggest that some antigenic properties are shared between Metis 1 and 2.

3.6. Immunogenicity of Metis 1 and 2 in repeatedly bitten animals

To be good vaccination candidates, Metis 1 and 2 proteins should be accessible to the immune system in vivo. In order to verify that, dogs and rabbits were submitted to repeated tick infestations. Sera from such animals were used to probe the recombinant antigens in Western blot analysis. Such an analysis is shown in Fig. 5 and indicates that Metis 1 and 2 were indeed detected by the serum of repeatedly bitten dogs and rabbits (lanes 3 and 5, respec-

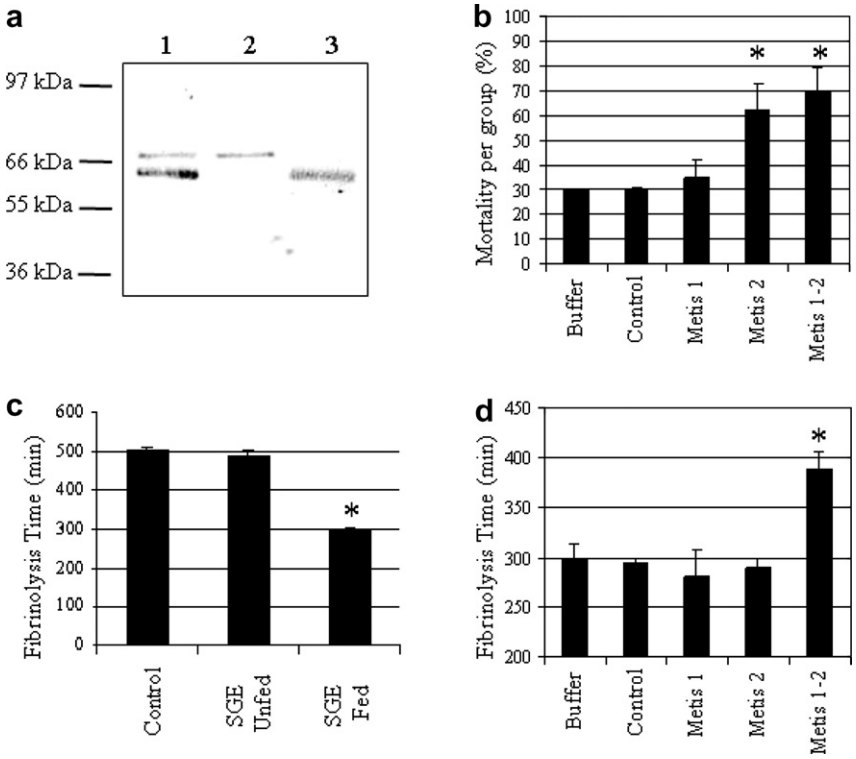

Fig. 3. The action of Metis small interference RNAs (siRNAs) on tick viability and fibrinolysis. (a) Metis siRNAs specifically silence expression from their targeted Metis genes. Combinations of the pCDNA3.1/V5-His vectors expressing Metis 1 and Metis 2 were co-transfected in COS-1 cells with each of the siRNAs; control siRNA duplexes (lane 1), Metis 1 (lane 2) and Metis 2 (lane 3). The Metis protein expression was analysed by Western blot using anti-V5 antibodies. (b) Metis knock-down by RNA interference affects tick viability. Buffer alone or siRNA targeting an irrelevant gene (control), Metis 1, Metis 2 or Metis 1 and Metis 2 together (Metis 1–2) were injected into the idiosoma of *Ixodes ricinus* adults (30 ticks per experimental group). Ticks were observed daily until day 5 post-infestation. The percentage of dead ticks and the SD from three independent experiments are shown. $^{*}P < 0.001$ compared with control (one-way ANOVA and Student–Newman–Keuls test). (c) Salivary glands extracts (SGEs) accelerate fibrinolysis. SGEs were incubated with a preformed clot and the time required for clot reduction was measured using a euglobulin clot lysis time assay. Five micrograms of SGE from unfed and 5-day fed adult females or buffer alone were added to the fibrin clot. $^{*}P < 0.001$ compared with control (one-way ANOVA and Student–Newman–Keuls test). (d) Metis knock-down interferes with the stimulation of fibrinolysis by SGEs. SGEs were incubated with the indicated siRNA (Metis 1, Metis 2, Metis 1–2) or buffer alone for 6 h prior to incubation with a preformed fibrin clot. The time necessary for clot reduction was measured using the euglobulin clot lysis time assay. $^{*}P < 0.005$ compared with control (one-way ANOVA and Student–Newman–Keuls test).

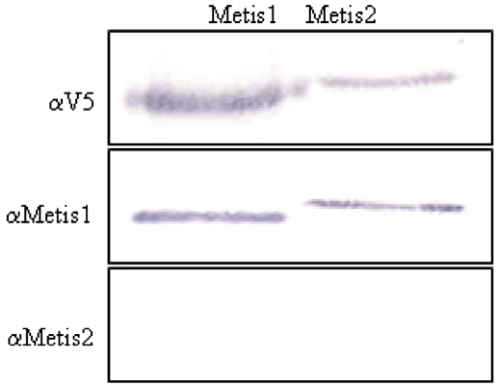

Fig. 4. The Metis 1 and 2 proteins share antigenic epitopes. The pCDNA3.1/V5-His expressing Metis 1 and 2 were used for protein expression in COS-1 cells and genetic immunisation of mice. Standardised amounts of the Metis proteins from culture supernatants were submitted to Western blot analysis using anti-V5 antibodies or sera from immunised mice. Analysis with anti-V5 antibodies demonstrated equal loading of the two Metis (αV5). The anti-Metis1 serum recognised both recombinant Metis 1 and Metis 2 (αMetis 1). Mice immunised with Metis 2 did not raise detectable antibodies (αMetis 2).

tively). These results showed that the immune system of bitten hosts was stimulated and reacted against Metis 1 and 2 further supporting Metis 1 and 2 as vaccinating antigens.

3.7. Expression of recombinant Metis 1 and antibody production

In order to evaluate the vaccination potential of Metis proteins, we checked the immunogenicity of a recombinant version of Metis 1. The ORF was cloned in a baculovirus vector. The protein was produced in insect cells and purified to homogeneity. The recombinant protein was then used to immunise a rabbit. Sera collected after each injection were analysed by Western blotting on two different sources of recombinant immunogens produced in either baculovirus or mammalian cells. As illustrated in Fig. 6 anti-Metis 1 antisera detected both Metis 1 and 2 proteins around 60 kDa. The expected molecular weight of Metis 1 and 2, 55.6 and 56.0 kDa, respectively, as well as the fuzzy aspect of the detected bands, suggested a post-translational

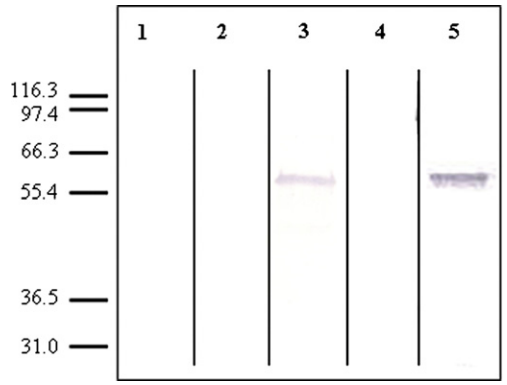

Fig. 5. Western blot analysis of sera from repeatedly bitten dogs and rabbits. Two hundred nanograms of recombinant Metis 1 was submitted to SDS–PAGE and analysed by Western blot using pooled serum from two naïve (pooled) dogs (lane 1) and dogs infested once (lane 2) or twice (lane 3) with 30 *Ixodes ricinus* female ticks as well as serum from naïve rabbits (lane 4) and rabbits infested three times (lane 5).

modification, likely to be a glycosylation. The antibody response was analysed by Western blot. This allowed us to estimate the antibody titre at 1: 256,000 after the final booster injection. These results indicate that recombinant Metis 1 produced in a baculovirus system was able to elicit a humoral immune response in rabbits.

3.8. Detection of the native metis protein(s) in the tick saliva

We also evaluated whether antibodies raised in immunised rabbits were able to recognise endogenous tick protein(s). Salivary glands and saliva extracts were analysed by Western blot using rabbit immune sera. Fig. 7 shows that these sera detected a specific band at 40 kDa in salivary gland extracts and saliva of fed females (lanes 5 and 6, arrow) but not in salivary glands of unfed females (lane 4). The observed molecular weight of Metis 1 and/or 2 matched the expected weights of the peptides (identical for both Metis 1 and 2) devoid of their preprosequence. It was similar to the size of metalloproteases previously detected in the saliva of *I. scapularis* (Valenzuela et al., 2002). Extra bands (lanes 3 and 6, asterisks) were likely to be non-specific since they were also detected by the pre-immune sera (lane 3). This result showed that the protein(s) was induced in the salivary glands upon a blood meal of adult females and that its expression pattern paralleled the RNA expression pattern. Taken together, the results described above showed that the Metis 1 and/or 2 RNAs was translated into a protein(s) present in the salivary glands and saliva of fed females. They showed that the native protein(s) was recognised by the serum of immunised rabbits in vitro. They further suggested that the endogenous protein(s) was accessible to the immune system of bitten animals in vivo. These data justified vaccination trials.

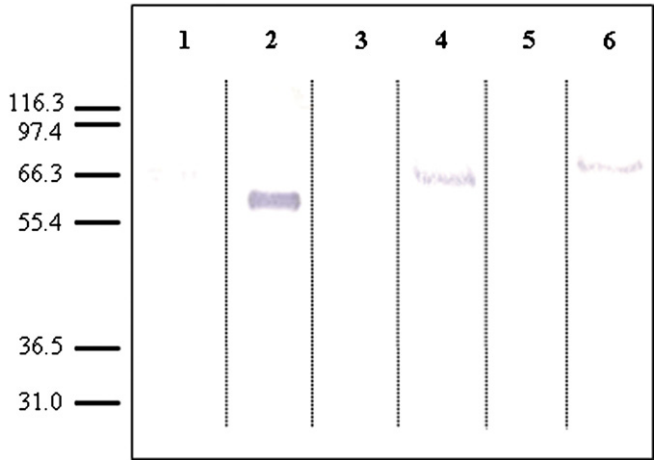

Fig. 6. Western blot analysis of rabbit immune serum. A female rabbit was immunised by repeated injections of 50 μg of recombinant Metis 1 in alum adjuvant. Serum collected 10 days after the final booster immunisation was checked for antibody titre by Western blot analysis of 200 ng of Metis 1 expressed in baculovirus (lane 1 and 2) or Metis 1 and Metis 2 expressed in COS-1 (lanes 3, 4 and 5, 6, respectively) submitted to SDS–PAGE. After blotting, the membranes were probed with 1:4,000 dilutions of the serum. Lanes 1, 3 and 5: pre-immune serum; lanes 2, 4 and 6: immune serum.

3.9. Vaccination of rabbits against Metis 1 and infestation of vaccinated rabbits

Rabbits were immunised with Metis 1 recombinant protein (or PBS for control animals) in alum adjuvant. After assessing the immune reaction using Western blots, the effect of vaccination on the blood meal of female adult ticks was evaluated. Ticks were monitored according to several parameters. Firstly, ticks were found to attach comparably on vaccinated or control rabbits. The overall mortality rates were evaluated to be $3.33 \pm 4.21\%$ and $6.65 \pm 11.21\%$ for the four control and four vaccinated rabbits, respectively. This difference is not statistically significant. Next, no significant difference could be observed in the duration of the blood meal between the control and immunised rabbits, as more than 93% of the ticks fed for 6–11 days, irrespective of the

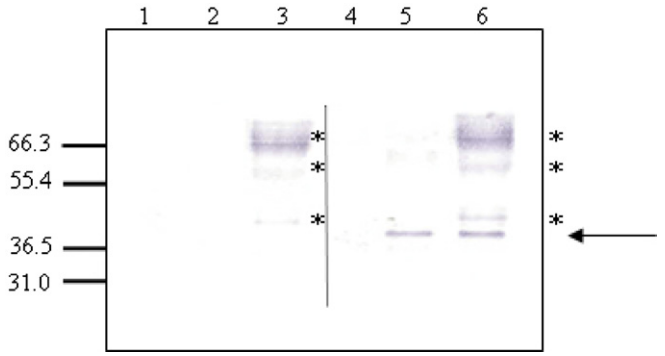

Fig. 7. Western blot analysis of tick salivary glands and saliva. Five micrograms of salivary gland extracts from unfed (lanes 1 and 4) and 5-day engorged (lanes 2 and 5) female ticks as well as 1.5 μl of saliva from 5-day engorged (lanes 3 and 6) female ticks were submitted to SDS–PAGE and analysed by Western blot using the pre-immune rabbit serum (lanes 1–3) or an anti-Metis 1 immune serum (lanes 4–5). A specific band is detected in salivary glands and saliva of fed ticks (arrow). Asterisks indicate non-specific signals also detected with a pre-immune serum.

rabbit they infested (see Fig. 8a). Host behaviour (feeding, drinking and scratching) was similar during the duration of infestation. The skin at the feeding site did not reveal any alteration. However, the weight of ticks was drastically different depending on the rabbits on which they fed. The average weight per tick was 126.7 ± 47.3 mg on vaccinated rabbits compared with 269.6 ± 39.8 mg on control rabbits. As shown in Fig. 8b, while the weight distribution of ticks fed on control rabbits was in accordance with the values usually recorded in the laboratory, a strong shift to lower weights was observed for ticks fed on vaccinated rabbits [Mann–Whitney rank sum test, $P < 0.001$, 120 mg (41–188) compared with 267.6 mg (228–326)]. Thus, the peak of the distribution accounting for 30% of the tick population shifted from weights of 251 to 300 mg to weights of less than 50 mg. Furthermore, as shown in Fig. 8c, the ability to lay eggs was impaired in ticks fed on vaccinated rabbits as a laying was observed in only $48 \pm 12\%$ of ticks fed on vaccinated rabbits compared with $86.6 \pm 11.7\%$ for ticks fed on controls. These results suggested that vaccination with Metis 1 had no effect on the attachment or mortality of ticks nor the length of the adult tick blood meal. However, it impaired weight gain in adult females and their egg laying rate.

Finally, nymphs were used for infestations and, according to the same parameters as for adult infestations, were found to behave similarly on vaccinated and control rabbits. Thus, in vaccinated and control rabbits, respectively, the future males weighed 3.3 ± 0.2 and 3.2 ± 0.2 mg and the future females 5.3 ± 0.2 and 5.4 ± 0.1 mg upon detachment; the feeding time was 3.9 ± 0.5 and 3.9 ± 0.2 days; the mortality rate 9.0 ± 1.4 and $7.2 \pm 2.9\%$; and the moulting rate 90.7 ± 5.4 and $92.9 \pm 7.4\%$, respectively. This suggested that vaccination with Metis 1 interfered in a different manner with adult and nymph blood meal.

4. Discussion

The proof of concept for vaccination against ticks has already been obtained. On one hand, immune protection has been reported in some animal species repeatedly bitten by *I. ricinus* ticks, the vectors of Lyme disease in Europe (Bowessidjaou et al., 1977; Brossard, 1982). On the other hand, two commercialised vaccines have been shown to confer partial protection to cattle and sheep against

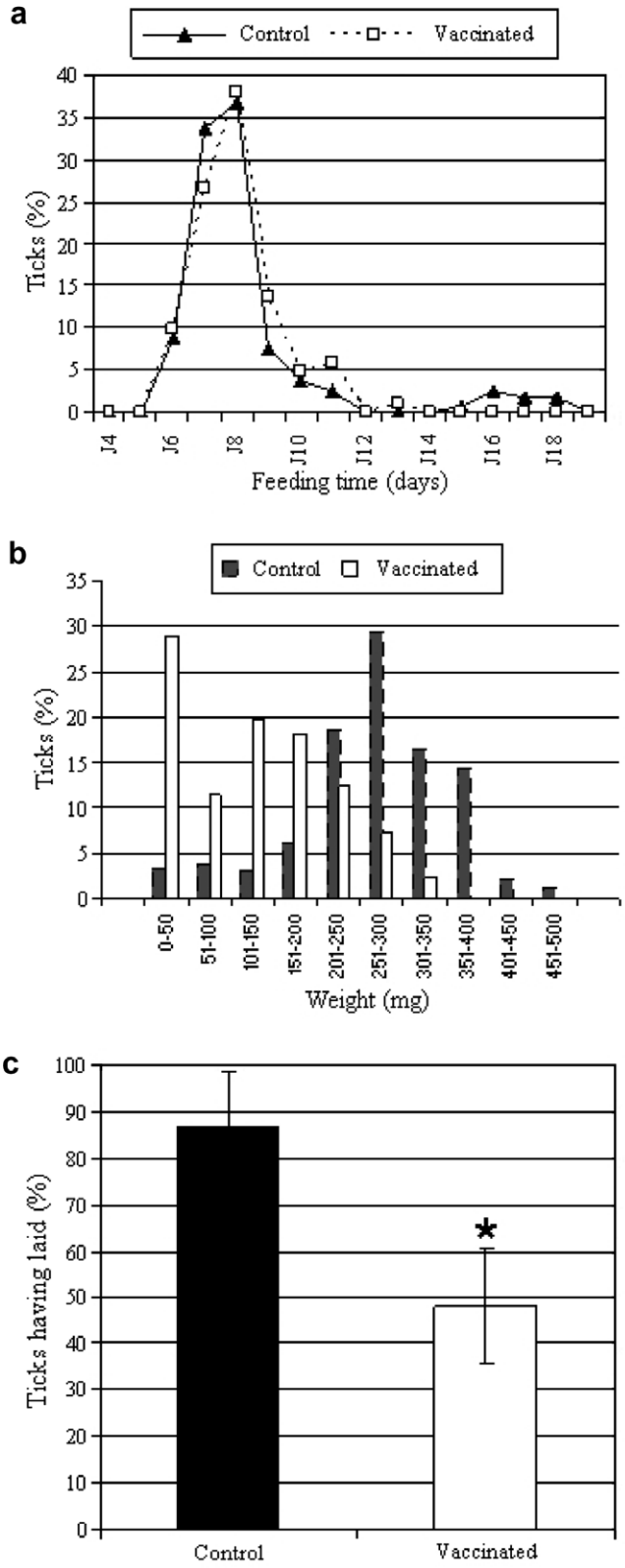

Fig. 8. Biology of ticks fed on control and vaccinated rabbits. (a) Feeding time (in days) of female ticks fed on control or vaccinated rabbits. Infested rabbits were checked daily. Anchored and detached ticks were counted. The number of detached ticks (expressed as a percentage of the whole population) was plotted daily. (b) Weight gain in female ticks fed on control or Metis 1-vaccinated rabbits. Infested rabbits were checked daily and detached ticks were harvested and weighed. Ticks were sorted into 50 mg categories then plotted against the percentage of the whole population found in each of those. Comparison of the curves indicates a shift towards lower weights in ticks fed on vaccinated rabbits. $^{*}P < 0.001$ compared with control (Mann–Whitney rank sum test). (c) Oviposition rate in female ticks fed on control or vaccinated rabbits. Rabbit ears were infested with 30 pairs of ticks and checked daily. Detached female ticks were harvested, kept individually in humidified test tubes and surveyed for 2 months. The percentage of ticks having laid eggs is indicated. $^{*}P < 0.005$ compared with control (one-way ANOVA and Student–Newman–Keuls test).

B. microplus in Australia (Rand et al., 1989; De Rose et al., 1999). A report indicated that one of the vaccines promoted a reduction in tick numbers and reproductive capability, combined with weight gain in treated cattle herds (Willadsen, 2006). According to another report, TickGuard™ and GAVAC™ vaccines resulted in a 10-fold reduction in pasture contamination after one season of vaccination (Willadsen, 2004). This resulted in a reduced acaricide treatment programme (Willadsen, 2004). Therefore these vaccines, although not sufficient on their own to eradicate ticks, allowed reduced use of acaricides and, on a long term basis, should also reduce the occurrence of acaricide resistance.

Antigens considered for vaccination fall into two categories: concealed and exposed antigens. The latter are accessible to the host immune system during natural tick infestations and could be involved in observed cases of naturally acquired resistance to tick infestation. The best examples are proteins of the saliva injected at the bite site. The immune reaction against these antigens is repeatedly boosted by renewed exposure upon repeated bites. Concealed antigens are not exposed to the host, do not stimulate the immune response in the field and their use requires repeated immunisations. An example is gut membrane proteins targeted by immunoglobulins found in the ingested blood and able to disrupt the digestive tract. The use of concealed antigens prevents the possible appearance during host–parasite co-evolution of a host defence mechanism against tick-exposed antigens.

Apart from the available veterinary vaccines, several strategies have also been used to evaluate the validity of candidate vaccines (Willadsen, 2004, 2006). The strategy first considered involves molecules which are targeted by the host's natural immune response ultimately protecting it when it is bitten repeatedly. Such an immunological approach could be overestimated, however, as the observation of an immune response does not mean it is protective. Another strategy is to target factors that experiments identified to be critical for parasite physiological functions or survival. This strategy depends on knowledge which is still fragmentary. Nevertheless, RNAi recently emerged as a powerful technology to analyse the suitability of an antigen. Next, high throughput blind screening of proteins independent of a prior knowledge of their properties also lead to identification of vaccine candidates. Immunisation with a cDNA expression library allowed the identification of cDNAs protecting mice against *I. scapularis* infestations. However, this is time consuming. These strategies have already demonstrated their potential in the analyses of some tick antigens. Vaccination against subolesin (4D8), a candidate identified by high throughput screening of cDNA libraries, reduced tick infestation and oviposition (Almazan et al., 2005). This effect was mimicked by RNAi in the tick *Rhipicephalus sanguineus* which suggested a role for this protein in tick feeding and reproduction (de la Fuente et al., 2006). Vaccination against 64TRP, a cement protein, initiated an inflammatory reaction at the feeding site and damaged the tick gut through cross-reaction with midgut antigen epitopes (Trimnell et al., 2005). Both antigens vaccinated animals against several tick species. Furthermore, vaccination with 64TRP also reduced transmission of vector-borne pathogenic agents (Labuda et al., 2006). Vaccination against IRIS, a saliva elastase inhibitor, reduced weight gain and provoked a 30% mortality in both nymphs and adults (Prevot et al., 2007).

We here describe vaccination with a novel *I. ricinus* salivary protein (Metis 1) which interferes with blood meal completion. Several criteria point to this antigen as a good candidate. Firstly, its expression is induced in the salivary glands during tick feeding. Then, the corresponding recombinant protein is the target of a natural immune reaction in animals repeatedly bitten by ticks. The recombinant protein is detected by sera of these repeatedly bitten animals. Next, Metis 1 and the cross-reacting Metis 2 are putative metalloproteases. Proteases or protease inhibitors are involved in almost all described physiological mechanisms. In particular, metalloproteases have been found to be involved in (among other roles) tissue remodelling or disruption through digestion of structural components (Francischetti et al., 2003, 2005). Inoculation of metalloproteases found in the saliva of various biting animals also participates in disrupting the balance of haemostasis. For example, the toxicity of snake venom is partly due to several metalloproteases that interfere with haemostasis (for instance breaking down fibrin clots or interfering with the progression of clot formation), disrupt basal laminae or extracellular matrices and trigger haemorrhages (Ramos and Selistre-de-Araujo, 2006). Metalloproteases could perform similar functions in the saliva of blood-feeding arthropods, preparing the feeding site by tissue disruption, allowing the meal by inhibition of blood clotting or facilitating the late feeding phase by inhibition of host tissue repair. An analysis of the amino acid sequences of this Metis protein family revealed several characteristics: a signal peptide, a zinc-binding domain, a conserved critical methionine and a pattern of C terminal cysteines, indicating that these are members of the metzinin group which includes snake venom proteases. Our biochemical analysis was also in agreement with a putative protease activity, as the knock-down of Metis gene expression interfered with blood meal completion in vivo and modulated fibrinolysis by SGE ex vivo. These observations suggest that Metis stimulates fibrinolysis, an action that counteracts haemostasis, interferes with wound healing of the endothelial epithelium and therefore facilitates the blood meal. An efficient immune response would therefore not only target and reject a tick constituent but also block enzymatic activity essential to blood meal completion. However the definitive demonstration that the action of Metis requires a putative enzymatic activity will require a functional enzymatic/biochemical bioassay. Unfortunately all our attempts to perform such an assay were unsuccessful (a general feature of metalloproteases). Such an assay would also enable evaluation of the neutralising potential of our

antisera. Finally, despite the lack of effect on viability, the reduction in oviposition efficiency indicates that vaccination with this antigen would reduce reproduction efficiency and therefore the field population size. This would necessitate, however, vaccination of most host species. Our observations indicate that vaccination with Metis 1 altered blood meal parameters differently than those modified upon vaccination with another recently reported salivary antigen (IRIS) (Prevot et al., 2007). This emphasises the possibility to use antigen cocktails as multivalent vaccines likely to give higher protection rates by combining of their respective actions. The organisation in multigenic families of ticks, supported by several analyses of the sialome of *I. scapularis* (Valenzuela et al., 2002; Pedra et al., 2006), is also worth noting. In particular, Metis 1 follows this rule as it is a member of a family of at least two homologous genes. This organisation suggests that it would be interesting to test a vaccinating protein family cocktail which would also improve vaccine efficiency.

The characteristics of Metis, for example its enzymatic activity and organisation in a protein family, also come with draw-backs when considering it as a vaccine candidate. As a protease, it is a member of a very large group of enzymes with, as a likely side effect, protein redundancy. This redundancy might even be amplified because these proteins are coded by a gene family. However, this organisation also allows diversification of function. Both redundancy or functional diversification (associated, for example, with developmental stage, individual or host species specificity) might challenge the efficiency of a monovalent vaccine.

The effect of vaccination was analysed on nymphs and adult females. While it reduced weight gain and egg laying efficiency of female adults, there was no effect on nymphs, whatever the parameter analysed. This is another drawback of a Metis-based vaccine as it would not affect an important developmental stage of the tick. This is in keeping with the differences between adult and nymph meals (an average duration of 8 days and 4 days as well as weight gains of more than 250 mg compared with 3-4 mg, respectively) which could be related to functional, mechanistic and molecular variations. It is therefore possible that Metis proteins are only necessary for long lasting meals but not short ones. This hypothesis is in full agreement with the effects of Metis 1 vaccination late in the blood meal and is also supported by a lack of expression in males which take only short and occasional meals. This is reminiscent of the knock-down of salp14 which resulted in diminished engorgement weights in adult but not in nymph ticks (Ribeiro et al., 2006).

Our observations also have implications regarding the basic biology of ticks. Firstly, we observed a similar number of living ticks detached from the host after a similar blood meal duration but nevertheless with a negligible weight gain on vaccinated animals. This probably has implications regarding the nature of the signal triggering detachment. It indicates that it is not related to a threshold weight but rather to meal duration. This is, however, contradicted by the observations of delayed detachment in some situations. The simultaneous observation of reduced weight gain and oviposition rate addresses the cause-effect relationship between the two. A blood meal is mandatory for adult female ticks to lay eggs, due to an hormonal signal triggered by the blood meal. Therefore reduced weight gain could be the cause of the observed reduction in oviposition.

In conclusion, we have assessed the vaccination potential of Metis 1, a putative metalloprotease which is induced during, and necessary for the completion of, the blood meal of *I. ricinus*. The results indicate that Metis 1 elicits a strong antibody response able to recognise another homologous protein and reduces the feeding ability of ticks as well as their egg laying efficiency. To our knowledge, this is the first report of a metalloprotease as a vaccine candidate. In the near future, we will investigate the possible use of antigen cocktails (affecting different physiological mechanisms of the host) as multivalent vaccines likely to give better protection rates.

Acknowledgements

We thank Louis Delhaye and Valérie Denis for their excellent technical assistance. We thank Dr. S. Schorder-et-Weber, Novartis Centre de Recherche Santé Animale SA, CH-1566 St-Aubin, Switzerland, for the generous supply of serum samples from tick-infested dogs. We also thank Sylvie and Gyles Dickens for proofreading. Animal care and experimental procedures were carried out in accordance with local institutional guidelines (laboratory licence n° LA 1500474) and the Belgian law of 14th August 1986 as well as the royal decree of 14th of November 1993 relative to the protection of laboratory animals. This work was supported by grants from the Walloon Region (convention 415.702 and 215.055). Luc Vanhamme is a Senior Research associate at the Belgian National fund for scientific research.

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