

Protective immunity against *Ixodes ricinus* induced by a salivary serpin

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Abstract

Iris is a specific elastase inhibitor expressed in the salivary glands of the hard tick *Ixodes ricinus*. It belongs to the superfamily of serpins and interferes with both haemostasis and the immune response of the host. In this study, we first show that Iris is expressed in nymphs but not in the female midgut nor in males. We also show that Iris is present in the saliva. To examine its potency as anti-tick vaccine candidate, we set up three models of *I. ricinus* infestation on immunized animals: nymphs on mice, and adults and nymphs on rabbits. We report the rise of neutralizing antibodies following immunization of rabbits and mice. This comes with a significant protective immunity against ticks in rabbits only, resulting in a 30% mortality rate and a diminution of weight gain in both nymphs and adults and a prolongation of blood feeding time in adults. This is the first report on an anti-tick vaccine trial on *I. ricinus* using a protein able to interact with both host immunity and haemostasis, as a vaccinating antigen.

Keywords: Tick; Serpin; *Ixodes ricinus*

1. Introduction

Ticks are the most frequent ectoparasites that infest mammals, birds, reptiles, and amphibians [1]. More than 800 tick species have been reported world-wide but only about 30 of which feed on humans. In Europe, *Ixodes ricinus* is the most common tick, which bites humans and domestic animals, including pets and cattle [2]. Because of its role as vector of an encephalitis virus and *Borrelia burgdorferi*, the causative agent of Lyme disease, *I. ricinus* is of prime medical importance. At present, the control of tick infestations relies mostly on acaricides. However, the use of chemical compounds carries several draw-backs such as pollution of environment and the food chain, but also the emergence of acaricide resistance

in ticks [3]. Nevertheless, alternative control methods exist [4–6]. They include the breeding and engineering of genetically resistant animals, biological control using pheromones and vaccination. Among these, vaccination seems to be the most promising alternative due to its practicality and sustainability. The feasibility of tick vaccination is supported by the emergence of immunity often detected in animals repeatedly bitten by *Ixodid* ticks. This has been partially reproduced by artificial immunization with pooled tick antigens. In this case, immunity affects tick feeding, moulting, reproduction, and survival [7,8].

Development of new vaccines relies on the identification and characterization of tick vaccine candidates [8–12]. There are clearly a large number of antigens worthy of further investigation. The biochemical categories into which these antigens fall include structural proteins, proteases and their inhibitors, as well as modulators of the immune response. About 10 years ago, Willadsen et al. [13,14] proposed the

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use of two intestinal “concealed” antigens (Bm86 and Bm91) from *Boophilus microplus* to protect cattle against tick infestation in Australia. However, on grassland, immunization with these antigens is better improved by using acaricides. Vaccination against other tick species such as *Rhipicephalus*, *Amblyomma* and *Haemaphysalis*, which have a high economic impact on agriculture, is under trial but still far from being a reality [15–17]. To date, there is no vaccine against *I. ricinus* ticks.

Amongst possible candidates, saliva proteolytic enzyme inhibitors, such as serine protease inhibitors (serpins), are of particular interest. An efficient immune response would indeed associate both a reaction against this tick antigen and neutralization of its enzymatic activity essential to the blood meal and tick survival. Human plasmatic serpins are important regulators of serine proteases involved in inflammation, blood coagulation, fibrinolysis, and complement activation [18–20]. Exogenous serpins could therefore interfere at the bite site with the regular immune, inflammatory and coagulation reactions from the host. Serpins have been recently isolated from the hard ticks *Amblyomma hebraeum*, *Amblyomma variegatum*, *B. microplus*, *Haemaphysalis longicornis*, *Ixodes scapularis*, and *Rhipicephalus appendiculatus* [16,21–23]. An example of the action of tick serpins on the host defence mechanisms is the protein HLS2 from *H. longicornis* which delays coagulation time and inhibits thrombin activity [16]. Although the mechanism of action of other tick serpins has not yet been investigated, data from other arthropods support an essential role of serpins in the control of proteolytic cascades [24].

Accordingly, Mulenga et al. have recently proposed the use of tick serpins as vaccine antigens [15]. Andreotti et al. [25] have observed a significant reduction of both tick number and egg weight after vaccination of cattle against a serine protease inhibitor from *B. microplus*. In addition, Imamura et al. [16,17] have demonstrated an increased mortality of *H. longicornis* and *R. appendiculatus* during their blood meal on cattle immunized with serpins.

We have recently characterized a new serpin from the hard tick *I. ricinus*. This protein modulates both the innate and acquired immunity of the host [26]. Accordingly, we have named the protein Iris for “*Ixodes ricinus immunosuppressor*”. We have also shown that Iris is a specific elastase inhibitor that interferes with the contact phase coagulation pathway and fibrinolysis, and disrupts platelet adhesion [27]. To our knowledge, this is the first ectoparasite serpin that interferes with both haemostasis and the immune response.

In this report, we have evaluated the potential interest of recombinant Iris (rIris) as a candidate vaccine antigen on three models of *I. ricinus* infestation: nymphs on mice, adults on rabbits and nymphs on rabbits. We have also studied the expression pattern of Iris throughout tick development stages and shown that Iris was expressed in female adult salivary glands and nymphs but not in the female midgut nor in males. Moreover, we have shown that the protein is present in the saliva. In addition, we have demonstrated that immunized

rabbits and mice developed neutralizing antibodies specific to Iris. We have also found a partial protection of immunized rabbits against infestation with *I. ricinus* adult females and nymphs, with, in particular, effects on the tick mortality, weight gain and blood feeding time.

2. Materials and methods

2.1. Ticks and animals

Specimens of *I. ricinus* were raised in the tick breeding facility at the Laboratoire de Parasitologie, Université de Neuchâtel (Switzerland). Founders of the colony were initially collected in a wood land near Neuchâtel and were maintained on rabbits (adults and nymphs) and SWISS mice (larvae).

2.2. RNA extraction and RT-PCR analysis

PolyA+ RNA was extracted from (i) salivary glands harvested on dissected adult females ticks at day 0, 1, 3, and 5 of the blood meal, (ii) midguts of unfed and fed females adults, (iii) salivary glands and midguts of adult males, and (iv) whole fed nymphs. RNA extraction and cDNA synthesis were performed, respectively, with the MicroFastTrack and Genracer kits according to the manufacturer’s instructions (Invitrogen, USA). Aliquots (0.5 µl) of the RT product were used as templates in 25 µl standard PCR reaction mixtures containing gene-specific primers Iris or β-actin (Iris sense primer: 5'-ATGGAGGCAGTCTGAGCAACC-3'; Iris anti-sense primer: 5'-TTAGAGCTCACGGATGGATGG-3'; β-actin sense primer: 5'-TGTGACGACGAGGTTGCCG-3' and β-actin anti-sense primer: 5'-GAAGCACTTGAGGTGGACAATG-3'). Each primer was used at a final concentration of 0.4 µM. PCR were performed using Extaq polymerase (Roche) for 45 cycles under the following conditions: 45 s at 94 °C, 45 s at 54 °C, and 90 s at 72 °C. Ten microliters of the PCR reactions were analyzed on a 1% agarose gel containing 1 µg/ml ethidium bromide. The recombinant plasmid bearing a full-length Iris clone was used as a positive control template, while milliQ water was used as the negative control. The presence of a specific band at 1.1 kpb (the expected size of both Iris and Actin PCR products) was monitored on a U.V. transilluminator. All polyA+ samples were submitted to the whole procedure omitting the addition of reverse transcriptase to exclude amplification from contaminating genomic DNA.

2.3. Western blot analysis on saliva and glands salivary extracts

Saliva was collected from *I. ricinus* females fed for 5 days on a non-immunized rabbit. Five microliters of phosphate buffer saline (PBS) (Invitrogen) containing 0.02% dopamine, were injected into the hemocoel using a fine nee-

dle (0.1 mm). Saliva was then collected in a capillary placed around the hypostome, and immediately processed. Salivary glands were harvested from dissected ticks, and washed in PBS before processing.

For Western blot analysis, saliva (10 μ l), salivary glands extracts (5 μ g), and recombinant Iris (50–100 ng) denatured in sample buffer were subjected to electrophoresis on a 10% polyacrylamide gel (SDS-PAGE) and transferred onto a nitrocellulose Hybond-C membrane (Amersham). Recombinant Iris was produced on a baculovirus system according to Prevot et al. [27]. The membrane was revealed with the rIris rabbit antiserum or pre-immune serum from the same rabbit as negative control (1:1000 dilution on PBS-0.1% Tween 20–0.5% Instagel) at room temperature for 1 h, and washed three times with PBS-Tween. The membrane was then incubated with peroxidase-conjugated anti-rabbit IgG (Amersham, USA) (1:7500 dilution) at room temperature for 30 min, and washed three times. Positive signals were visualized by using 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indozyl phosphate (Roche, USA).

2.4. Generation of seras in repeatedly bitten rabbits

Eight rabbits were repeatedly infested (three times at 3-week intervals) with 50 pairs of adult ticks. Sera were then harvested and analyzed by Western blotting on recombinant Iris.

2.5. Immunisation and challenge infestation

Five female 6-week old BALB/c mice (Harlan Netherlands) and four female New Zealand white rabbits (3 kg, Harlan Netherlands, CEGAV France) were injected subcutaneously with purified rIris in PBS pH 7.5 emulsified in Freund's adjuvant. First injections were performed in complete Freund's adjuvant and boosters in incomplete Freund's adjuvant. Doses contained 20 μ g purified protein in 50 μ l PBS per individual mouse and 50 μ g for each rabbit. They were injected at 3-week intervals. The animals were bled at the day of injections then 2 weeks after each booster as well as before challenge infestation. Four rabbits and five mice were mock-immunized following the same protocol with PBS in Freund's adjuvant as negative controls.

Vaccinated and control animals were challenged with tick infestations following three experimental models: (i) nymphs on mice: 15 individuals per mouse, (ii) nymphs on rabbit: 50 nymphs ticks per rabbit's ear, (iii) adults on rabbits: 30 adult couples per rabbit's ear. Each experiment was performed independently.

Nymphs were held on mice backs with plastic capsules glued on the shaven back with beewax/colophane mixture [28]. Nymphs and adults were placed in soft fabric bags held on rabbit's ears with tape according to Schorderet and Brossard [29].

Animals were monitored two times per day for signs of discomfort or skin lesions, and ticks were counted and exam-

ined for attachment. Disanchored ticks were weighed and placed in individual tubes for monitoring (25 °C; 85% of humidity). Overall, the following parameters of the blood meal were measured: (i) death on host, (ii) weight at fall-off, (iii) duration of the blood meal, (iv) moulting (nymphs) and oviposition (adults).

All animals were maintained and handled according to local and national approved ethical guidelines.

2.6. ELISA

The humoral response of vaccinated mice and rabbits was measured by a home-made ELISA. Two hundred and fifty nanograms of rIris in PBS were initially coated onto 96-well plates (Nunc, Immunsorb) overnight at 4 °C. Wells were then saturated 1 h in PBS-Tween 20 0.1%–bovine serum albumin (BSA) 1%. The coated plates were incubated with various dilutions of immune or pre-immune sera for 2 h. Antibody binding was then detected by adding a biotinylated anti-IgG antibody (dilution 1:10,000) for 1 h, followed by the incubation of streptavidin (1:10,000) associated to peroxidase for 30 min. Finally, the TMB chromogen (Sigma) was added for 10 min. Absorbance at 450 and 630 nm was read with a Model 680 microplate reader (BioRad). Values were expressed as antibody titres as defined by the serum dilution at the inflection point of the curve.

2.7. Neutralisation assays

We tested whether antisera neutralized the antiprotease activity of rIris on porcine pancreatic elastase (PPE) as described previously [27]. Briefly, rIris was pre-incubated with dilutions of immune and control sera for 5 min at 37 °C in Tris buffer. Porcine pancreatic elastase was added and the incubation proceeded for 1 h. The substrate for PPE was added and absorbance at 405 nm was read in a Model 680 microplate reader (BioRad). OD₄₀₅ values were recorded as estimates of residual PPE activity. Therefore, neutralizing activity of antisera translates into a recovery of PPE activity as measured by increased OD₄₀₅. Controls included ovalbumine for rIris and sera from mock-immunized animals for rIris immunized mice or rabbits.

2.8. Statistical analyses

Data are represented as mean \pm S.D. Significance was then assessed using one-way ANOVA or Student *t*-test implemented in the MedCalc for Windows, version 8.2.0.1 (MedCalc Software, Mariakerke, Belgium).

3. Results

3.1. Detection of Iris specific messengers

To assess the potential of Iris as a putative natural immunogen which is in contact with the host during the tick blood

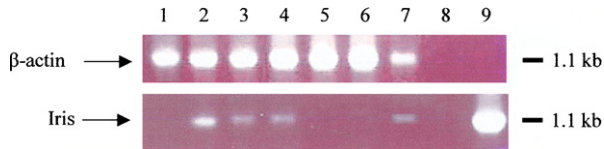


Fig. 1. Analysis of Iris transcripts during a blood meal. RNA was extracted from tick tissue samples and subjected to RT-PCR using pairs of primers specific for Iris and β -actin. 1, unfed female salivary glands; 2, 1-day fed female salivary glands; 3, 3-days fed female salivary glands; 4, 5-days fed female salivary glands; 5, 5-days fed female midgut; 6, male salivary glands; 7, total nymphs; 8, milliQ water, negative control; 9, recombinant plasmid pBlueBac 4.5/Iris, Iris positive control.

meal, we first monitored its expression in the salivary glands. Messenger RNA was extracted from various organs of unfed and fed adults and from whole fed nymphs. These RNAs were then analyzed by RT-PCR using Iris and β -actin specific pairs of primers. Results are shown in Fig. 1. The presence of a 1.1 kb PCR product generated by β -actin primers demonstrated the good quality of the RNA preparations from all tissues (lanes 1–7) and the specificity of the reaction as no product was generated on the plasmid template or in absence of template (lanes 8 and 9). A similar specificity was observed using the Iris primers as a PCR product of the expected size was generated from the cloned gene used as a template but not in its absence (lane 8). The specific 1.1 kb PCR product was generated on RNAs from fed nymphs (lane 7) as well as from salivary glands of feeding adult females at day 1, day 3 and day 5 of the blood meal (lanes 2–4). On the contrary no positive PCR signal was seen on unfed females (day 0 of the blood meal) (lane 1), on males (lane 6) or on fed female midgut (lane 5). These results indicated that an Iris specific RNA is present in feeding nymphs and is induced very early in salivary glands of female feeding ticks.

3.2. Detection of native Iris in tick saliva by Western blotting

In order to confirm that the observed Iris RNAs are translated into protein, salivary glands and saliva were analyzed with an anti-rIris antiserum from an immunized rabbit (see Section 2.5) by Western blotting analysis. Fig. 2 indicates that rabbit anti-rIris serum (lane 3) – but not pre-immune serum from the same rabbit (lane 2) – detected a discrete band at 42 kDa in salivary glands of fed ticks as well as in saliva (lane 8). The specificity of these signals was indicated by the detection of a discrete band at the expected molecular weight of 48 kDa when recombinant Iris was loaded onto the gel (lanes 5 and 6). The difference between the natural and recombinant proteins could be explained by the presence of V5 epitope and His tag on the C-terminus of rIris [27]. An additional band was also detected at 54 kDa in salivary gland extracts (lane 3). These results demonstrated that the expression of the Iris protein is induced in salivary glands during the blood meal and is secreted in the saliva.

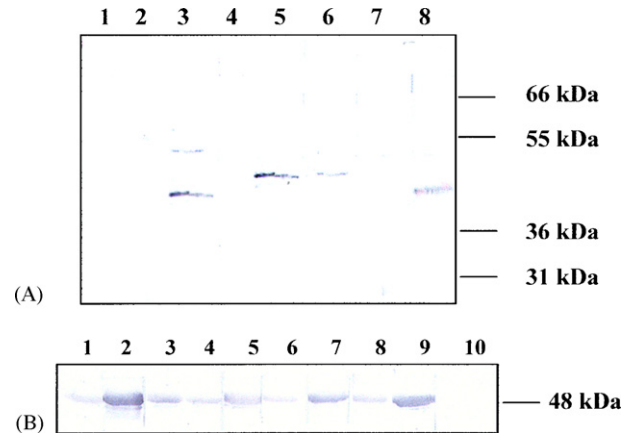


Fig. 2. Western blot analysis of Iris expression during a blood meal. (A) Protein extracts were subjected to electrophoresis, transferred onto a nitrocellulose Hybond-C membrane, and analyzed using anti-Iris specific or pre-immune rabbit antibody (1:1000); lane 1. 5 μ g of unfed female salivary gland extracts; lanes 2 and 3. 5 μ g of 5-days fed female salivary gland extracts; lanes 4 and 5. 20 ng of recombinant Iris; lane 6. 5 ng of recombinant Iris; lanes 7 and 8. 10 μ l of 5-days fed female saliva. Lanes 1, 3, 5, 6 and 8 were analyzed using a rabbit polyclonal anti-Iris while lanes 2, 4 and 7 using a preimmune serum from the same rabbit. (B) One hundred nanograms of recombinant Iris were subjected to electrophoresis, transferred onto a nitrocellulose Hybond-C membrane, and analyzed using preimmune serum (lane 10) or serum from repeatedly bitten rabbits (lanes 1–8). Lane 9 was analyzed using a rabbit polyclonal anti-Iris, as positive control.

3.3. Analysis of the antibody response against Iris

The question then asked was whether Iris is fully exposed as a salivary antigen to the immune system of a bitten host. To that end, we assessed the anti-rIris reactivity of sera collected from rabbits that had been repeatedly bitten. Fig. 2B shows that the sera from all eight multi-infested rabbits recognized a recombinant rIris as determined by Western blot analysis. We also investigated whether recombinant Iris is able to trigger an efficient immune response. To do this, we immunized mice and rabbits with the recombinant protein produced in a baculovirus system, using a three boost protocol and Freund's adjuvant. The specific antibody response was analyzed by ELISA after each injection. All mice (4/4) and rabbits (4/4) developed a response to immunization by Iris, and the humoral response was detected after only one injection. Three injections were necessary to reach a maximal antibody titre (data not shown). Sera titre values were around 10^6 and 2.5×10^6 , for mice and rabbits, respectively. IgG₁ and total IgG levels were identical (10^6) whilst a lower level (8.0×10^3) of IgG_{2a} and no IgE were detected. Pre-immune sera were consistently negative (titres < 250). These data indicate that recombinant Iris is able to elicit a high and specific antibody response in mice as well as in rabbits.

3.4. Antibodies inhibited rIris antiprotease activity

In order to evaluate the quality of the antibodies, we tested whether anti-rIris rabbit and mice anti-sera neutral-

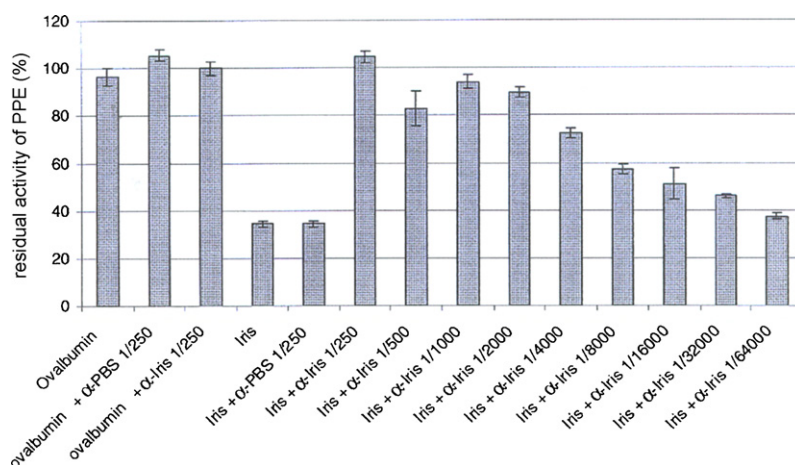


Fig. 3. Neutralization of Iris antiprotease activity by mouse rIris (α -Iris) or PBS (α -PBS) anti-sera. One microgram of rIris was incubated with various sera dilutions for 5 min at 37 °C, and for an additional hour with equimolar ratio (to Iris) of pancreatic porcine elastase (PPE). Residual activity in the presence of 1 μ g ovalbumin, a serpin with no inhibitory activity, is used as the 100% reference.

ized the antiprotease activity of the protein. This activity was monitored by testing its ability to inhibit the pancreatic porcine elastase (PPE) activity [27]. As reported previously, rIris inhibits elastase activity by around 70%, while the same amount of ovalbumin has no effect. Addition of increasing concentrations of mice anti-rIris sera progressively alleviated this inhibition indicating that the Iris antiserum is able to inhibit rIris activity in a dose dependent manner. Fig. 3 shows that mice sera could be diluted around 1000 times, but nevertheless keep their blocking activity on 1 μ g of rIris (as the residual activity of PPE remains around 100%). Addition of sera from mice injected by PBS had no effect on rIris activity. Identical experiments were performed with rabbit antisera with similar results (data not shown).

3.5. Nymph infestation of vaccinated mice

The above results indicate that immunization with a recombinant Iris elicited a good titre of antibodies able to neutralize the enzymatic activity of the protein. We therefore assessed their ability to counteract laboratory infestation in immunized animals. For that purpose, a set of mice were infested by *I. ricinus* nymphs. These nymphs were then followed to obtain data such as the feeding time, the weight gain, the mortality and moulting. As detailed in Table 1, there is no significant difference between nymphs fed on immunized or control mice.

3.6. Nymph infestation of vaccinated rabbits

We then performed the same monitoring on immunized rabbits, which revealed a different response. Sets of 50 nymphs were allowed to infest immunized as well as control rabbits. They were monitored according to several parameters as detailed in Table 1. The attachment rates were first examined during the initial 24 h of tick contact with the rabbit's ears. No difference could be detected between the nymphs

infesting the immunized or control rabbits. Ticks completed their blood meal within 3–6 days on all rabbits (immunized or not). However, a higher mortality was observed as 30% of the nymphs died feeding on immunized rabbits as opposed to normal rates. Calculation of the exact mortality rates gave figures of 33.6% on immunized rabbits. These calculations took into account total number of ticks which either died on rabbits or fell off and died without moulting. These dead ticks collected from rIris-vaccinated rabbits appeared dry. Several surviving nymphs fed on vaccinated rabbits also presented a parched appearance, an observation never before made on ticks collected from PBS-injected rabbits. All surviving ticks were weighed. An analysis of the plotted weights showed the expected bimodal distribution values (Fig. 4). As male-to-be nymphs are known to feed less, the lower weight peak corresponded to future males whereas the higher one corresponded to future females [30]. Furthermore, recorded weight values of nymphs fed on control rabbits were in accordance

Table 1
The influence of rIris vaccination on fed nymphs

	Immunized animals	Control animals
Nymphs fed on mice		
Mortality (%)	4.9 (\pm 4.5)	0.0 (\pm 0.0)
Feeding period (days)	4.25 (\pm 0.54)	4.13 (\pm 0.44)
Weight—first peak (mg)	2.93 (\pm 0.51)	3.05 (\pm 0.56)
Weight—second peak (mg)	4.85 (\pm 0.32)	4.93 (\pm 0.46)
Moulting (%)	97.6 (\pm 4.3)	99.1 (\pm 1.5)
Nymphs fed on rabbits		
Mortality (%)	33.6 (\pm 8.3)*	7.2 (\pm 2.9)
Feeding period (days)	3.73 (\pm 0.23)	3.93 (\pm 0.48)
Weight—first peak (mg)	2.73 (\pm 0.37)**	3.08 (\pm 0.35)
Weight—second peak (mg)	4.60 (\pm 0.44)**	5.37 (\pm 0.41)
Moulting (%)	92.2 (\pm 8.1)	94.9 (\pm 7.4)

Results are expressed as average (\pm S.D.) of each group. The control group was immunized with PBS buffer. Mortality rate was calculated from the ticks that died either during feeding or without moulting.

* $P < 0.001$ compared to control (Anova one-way of variance).

** $P < 0.001$ compared to control (Student's test).

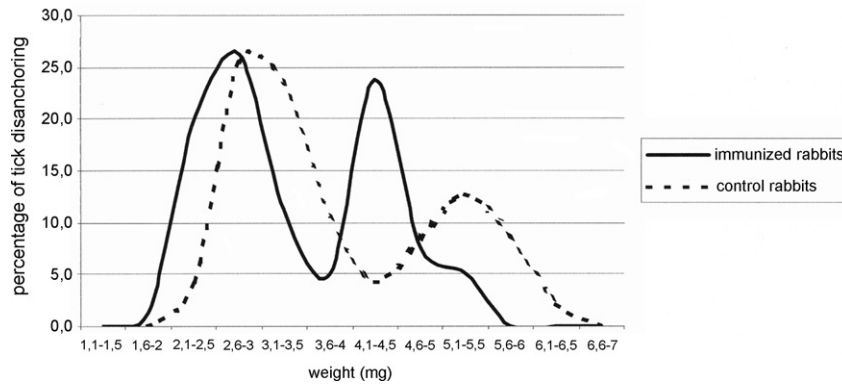


Fig. 4. rIris vaccination influences the weight of engorged nymphal ticks at disanchoring. Nymphal stages were fed on vaccinated and control rabbits. The disanchoring was observed to start on day 3 and finish on day 5 post-infestation. Living disanchored engorged nymphs were harvested. The percentage of the total number of disanchored living nymphs is plotted against the weight by 0.5 mg sections. The significance of the result was assessed using the Student's *t*-test ($P < 0.001$).

with values previously published [30]. However, an obvious shift of peaks of both future males and females to lower weights was observed in nymphs fed on immunized rabbits when compared to the normal values obtained on control rabbits (Fig. 4). This corresponded to mean decreases of 0.3 and 0.8 mg, respectively, as indicated in Table 1. The Student's *t*-test indicated that these differences are significant ($P < 0.001$). All these observations were fully reproduced on a rabbit immunized following an identical protocol but using alum as adjuvant, with values of 25.0% for mortality rate and decreases of 0.3 and 0.8 mg for weight peaks, while no difference in moulting rate was observed. Moreover, in this case, nymphs needed a longer feeding time (+ 0.7 day). Finally, all engorged tick nymphs were observed to moult whether fed on control or immunized rabbits. These results indicated that vaccination provokes a 30% mortality rate in feeding nymphs and that all surviving nymphs were able to moult despite a decreased weight gain.

3.7. Adult ticks infestation of vaccinated rabbits

The effect of vaccination was also monitored on the blood meal of adult female ticks. Results are shown in Table 2. The values are from two independent experiments each comprising two rabbits per condition tested. The attachment rates were first examined during the initial 24 h of tick contact with the rabbit's ears. No difference could be detected between the females infesting the immunized or control rabbits. On control rabbits, ticks accomplished their blood meal within 6–10 days. However, on immunized rabbits, 21.6% of ticks needed between 11 and 23 days to complete feeding and detach. As shown in Table 2, this phenomenon translated into an increase of the average blood meal time by more than 1 day on immunized rabbits (9.0 ± 0.6 days) as compared to controls (7.6 ± 0.4 days) ($P < 0.02$). Within addition, a high mortality rate of $33.6 (\pm 3.9)\%$ for ticks fed on rIris-immunized rabbits versus $4.2 (\pm 4.7)\%$ on control group was observed. This mortality rate figure takes into account the total number of ticks which died either on rabbits, or detached and died later with-

out laying eggs. On visual examination, dead ticks were dry although they were fully gorged or nearly-fully gorged, suggesting that they had completed their blood meal. Most live ticks taken from vaccinated rabbits or from controls appeared identical. Nevertheless, several living adult ticks fed on vaccinated rabbits had a parched appearance which had never been observed on live ticks collected from PBS-injected rabbits. All ticks were weighed after detachment and as shown in Table 2, found to insert into a similar weight window whether they were fed on immunized or control rabbits. There was no difference either in the oviposition rate between ticks surviving on vaccinated or control rabbits (Table 2). Finally, no obvious skin alteration at the feeding spot or behaviour difference could be observed between vaccinated and control rabbits during the whole course and after the infestation period. All these observations were fully reproduced on a rabbit immunized following an identical protocol but using alum as adjuvant, with values of 23.1% for mortality rate and

Table 2
The influence of rIris vaccination on females adults fed on rabbits

	Females fed on immunized rabbits	Females fed on control rabbits
Mortality (%)	32.5 (± 3.9)*	4.2 (± 4.7)
Feeding period (days)	8.99 (± 0.64)**	7.63 (± 0.41)
Tick disanchoring after 10 days (%)	21.62 (± 4.08)***	1.02 (± 0.11)
Weight (mg)	227.2 (± 57.9)	268.8 (± 38.5)
Oviposition (%)	95.2 (± 5.1)	91.7 (± 7.3)

Results are expressed as average (\pm S.D.) of each group. The control group was immunized with PBS buffer in association with AFC/AFI. Mortality rate was calculated from the ticks that died either during feeding or without oviposition.

* $P < 0.001$ compared to control (Anova one-way of variance).

** $P < 0.02$ compared to control (Student's test).

*** $P < 0.02$ compared to control (Anova one-way of variance).

a significant increase of blood feeding period to 12.4 days. The latter is explained by a high proportion (42%) of ticks, which accomplished their blood feeding after the day 10.

4. Discussion

Ticks are the most frequent blood feeding ectoparasites that infest various animal species including humans, pets and cattle [2]. Because of their role as vector of various pathogens, they impose a double burden to the host populations: one relating to the blood intake, and a more serious one being associated to tick-borne diseases (e.g. Lyme disease, encephalitis, rickettsiosis, theileriosis, and babesiosis). Anti tick strategies are therefore of medical and veterinary importance although none has proven to be sufficiently efficacious so far. Vaccination would be the best solution. Some proofs of concept have already been obtained [13,14]. One anti-tick vaccine has even been commercialized. However, its efficiency is limited and it is generally used in combination with acaricides [31].

Serine protease inhibitors (Serpins) are involved in the control of many biological processes. Therefore, it is particularly interesting to use serpins as anti-tick vaccine antigens because different members of this family of proteins are key regulators of either arthropod metabolism and/or host haemostasis. In addition, promising results have been reported using as vaccinating proteins serpins from other hard ticks *A. hebraeum*, *A. variegatum*, *B. microplus*, *H. longicornis*, and *R. appendiculatus* [16,21–23] in their respective infestation models.

We have recently isolated and characterized Iris, a serpin expressed in salivary glands of the hard tick *I. ricinus*. This tick species is the most common one in Europe. It carries *Borrelia burgdorferi*, the causal agent of Lyme disease. Iris modulates both the innate and acquired immunity of the host [26]. It is a specific elastase inhibitor that interferes with the coagulation, fibrinolysis, and platelet adhesion [27], but also with the host immune system [26].

In this report, we tested its potential interest as a candidate vaccine antigen in three models of *I. ricinus* infestation: nymphs on mice, and adults and nymphs on rabbits. Firstly, we evaluated whether Iris could be accessible to the host immune response. Using RT-PCR, we confirmed that Iris mRNA is expressed in female salivary glands [26] (Fig. 1), where it is induced by feeding. Western blot analysis confirmed that the protein is found in salivary glands and saliva, and that it is recognized by the serum of repeatedly bitten animals. Therefore, Iris is naturally exposed to the immune response of the bitten host, and is expected to be a good vaccine candidate. Moreover, it could display a booster effect when ticks are in contact with vaccinated hosts.

Iris is not expressed in female midguts nor in males. A possible explanation could be that Iris is not involved in the physiological processes associated to the midgut nor in males as they bite but do not ingest blood [2]. The *Iris* gene is also

expressed in the feeding nymphs. Therefore, Iris expression is induced in female saliva and it can be reasonably assumed it is also the case in the nymph saliva. Provided it is induced in larvae, it would be injected in the host during the blood meal of the three development stages. Furthermore, an additional band at 54 kDa is detected by anti-Iris antibodies in salivary gland extracts. This indicates that Iris could be a member of a multigene family expressed in tick salivary glands [32]. This would be reminiscent of a family of four genes described by Mulenga et al. [22] in *R. appendiculatus*, coding for four serpins (RAS-1 to RAS-4) with predicted molecular weights of 41.9, 42.7, 43.2 and 53.9 kDa, respectively.

We next assessed the immunogenicity of the recombinant protein. IgG titres, as determined by ELISA on serum from all immunized mice and rabbits reached $\sim 10^6$ and 2.5×10^6 , respectively. An humoral response could be detected as soon as after the first injection of rIris, but a maximal titre required three injections. Western blotting analysis indicated that the antibodies elicited upon vaccination strongly recognized the protein in salivary gland extracts. In mice, detection of IgG2a (8×10^3) and absence of IgE suggested that immunization by Iris elicited a Th1 response. This is indeed expected while using Freund's adjuvant known to polarise the response to Th1 components as compared to other adjuvants such as alum [33]. On the contrary, in BALB/c mice, nymph infestations have been typically reported to polarize the immune system towards a Th2 response, an effect related to immunosuppression [34]. Accordingly, we assumed that Th1 polarization should facilitate protection of the host.

We then tested whether anti-rIris rabbit and mice sera were able to neutralize the activity of the protein. These tests showed that both mice and rabbit sera inhibited rIris antiprotease activity (Fig. 4). Taken together, these results indicated that Iris is injected in the host as a part of the saliva and is highly immunogenic to rabbits and mice. Antibodies not only recognized the natural protein but also blocked its antiprotease activity.

Finally, we tested the potential interest of Iris as a candidate vaccine antigen in models involving infestation of two species (mice and rabbits) by two developmental stages (nymphs and adults). The adult-on-mouse model was however not investigated here because we previously observed that mice die when infested with two or more adult *I. ricinus* (unpublished data). Rabbit and mice infestations were considered as complementary models. Indeed, rabbits are known to develop an immune resistance after repeated cycles of tick bites, whereas parameters of the blood meal do not change on mice after successive infestations [30]. Even if comparisons of the two animal models could be done only with nymphs infestations, it appears that the two species behave differently while reacting against infestations. There was no perturbation in several parameters of nymph blood meal in the mice vaccinated model (Table 1). This is contrary to the higher mortality rate observed in nymphs feeding on vaccinated rabbits. This difference between mice and rabbits is intriguing.

It could be related to the weaker antibody response observed in mice. It is anyway in keeping with the acquired resistance in multi-infested rabbits never observed in mice [35].

Higher mortality was reproduced in adult females feeding on the vaccinated rabbits. Our data also suggest that vaccination does not have a significant effect at the beginning of blood meal. Mortality affects only already well fed ticks which suggested that ticks begin to die at the end of the blood meal. Dead ticks collected from rIris-vaccinated rabbit appeared dry and most living ticks had a parched appearance compared to control tick. This could be related to our observation that the expression of Iris is up-regulated during the blood meal with a maximum at the end of the blood meal [26], which is also the time when the *I. ricinus* female starts to swallow the largest amount of blood [1,2], suggesting that Iris is particularly useful in this late feeding step. At this step, Iris could be ingested to the midgut along with the blood meal, and could facilitate both blood intake and protection of the midgut walls. These data also suggested that Iris has an important role in both feeding females and nymphs. Nevertheless, vaccination also had differential effects on nymphs and adults. Thus, a significant reduction of weight gain was observed only with nymphs ($P < 0.001$) although their feeding time remained the same (Table 2). On the opposite, a significant proportion of adults had at times a largely increased feeding time on vaccinated rabbits compared to control.

Haemostasis and inflammation are powerful barriers to blood feeding by haematophagous parasites. In parallel, ticks have developed countermeasures such as salivary proteins able to inhibit one or both processes [8,27,36–39]. These mechanisms are obviously good targets for vaccination. As such Iris is probably a suitable candidate as a specific elastase inhibitor. Indeed, elastase is a key player in haemostasis and inflammation and therefore is probably detrimental to different steps of the ticks' blood meal among which the activation of a pro-inflammatory response [40,41], the digestion of the extracellular matrix and the destruction of tick's gut tissue.

Though significant, protection acquired by immunization with rIris (~30% of tick mortality) is not sufficient. Similar results were obtained upon vaccination with other tick serpins [16,17]. The observed mortality rates in adult ticks would result in a reduced egg production and a subsequent reduction of tick population on grassland conditions. This is indeed an observed effect of the only current commercial veterinary vaccine. However, much higher protection levels should be aimed at. This goal could be reached through: (i) vaccinating with several members of a gene family. This would allow targeting a larger number of ticks if there are individual differences in the expression of different members of a gene family. Mulenga et al. [22] have indeed identified four members of a serpin multigenic family in *R. appendiculatus*. On the other hand, Imamura et al. [17] went on to test a vaccinating cocktail of two serpins in cattle. However, the relative protection conferred by the single-antigen or dual antigen candidate vaccine was not assessed; (ii) searching for other tick antigens offering a better protection upon vaccination; (iii) vaccination

with cocktails of antigens involved in distinct mechanisms against host defences. The efficiency of such a strategy is supported by the detection of multiple antigens by the sera of animals acquiring protection after repeated bites; and (iv) combination of the above approaches using super-cocktails of several families of distinct antigens.

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References

- [1] Sauer JR, McSwain JL, Bowman AS, Essenberg RC. Tick salivary gland physiology. *Annu Rev Entomol* 1995;40:245–67.
- [2] Sonenshine DE. *Biology of ticks*, vol. 1. Oxford: Oxford University Press; 1991.
- [3] Shapiro SZ, Voigt WP, Fujisaki K. Tick antigens recognized by serum from a guinea pig resistant to infestation with the tick *Rhipicephalus appendiculatus*. *J Parasitol* 1986;72(3):454–63.
- [4] Sonenshine DE. *Biology of ticks*. Oxford: Oxford University Press; 1993.
- [5] Brossard M. The use of vaccines and genetically resistant animals in tick control. *Rev Sci Tech* 1998;17(1):188–99.
- [6] Willadsen P. Anti-tick vaccines. *Parasitology* 2004;129(Suppl.):S367–87.
- [7] Barriga OO. Evidence and mechanisms of immunosuppression in tick infestations. *Genet Anal* 1999;15(3–5):139–42.
- [8] Wikel SK. Host immunity to ticks. *Annu Rev Entomol* 1996;41:1–22.
- [9] Kay BH, Kemp DH. Vaccines against arthropods. *Am J Trop Med Hyg* 1994;50(Suppl. 6):87–96.
- [10] McKenna RV, Riding GA, Jarmey JM, Pearson RD, Willadsen P. Vaccination of cattle against the *B. microplus* using a mucin-like membrane glycoprotein. *Parasite Immunol* 1998;20(7):325–36.
- [11] Opdebeeck JP. Vaccines against blood-sucking arthropods. *Vet Parasitol* 1994;54(1–3):205–22.
- [12] Wikel SK. Immunological control of hematophagous arthropod vectors: utilization of novel antigens. *Vet Parasitol* 1988;29(2–3):235–64.
- [13] Willadsen P, Bird P, Cobon GS, Hungerford J. Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology* 1995;110(Suppl.):S43–50.
- [14] Willadsen P, Smith D, Cobon G, McKenna RV. Comparative vaccination of cattle against *Boophilus microplus* with recombinant antigen Bm86 alone or in combination with recombinant Bm91. *Parasite Immunol* 1996;18(5):241–6.
- [15] Mulenga A, Sugimoto C, Onuma M. Issues in tick vaccine development: identification and characterization of potential candidate vaccine antigens. *Microbes Infect* 2000;2:1353–61.
- [16] Imamura S, da Silva Vaz Junior I, Sugino M, Ohashi K, Onuma M. A serine protease inhibitor (serpin) from *Haemaphysalis longicornis* as an anti-tick vaccine. *Vaccine* 2005;23:1301–11.

- [17] Imamura S, Namangala B, Tajima T, Tembo ME, Yasuda J, Ohashi K, et al. Two serine protease inhibitors (serpins) that induce a bovine protective immune response against *Rhipicephalus appendiculatus* ticks. *Vaccine* 2006;24(13):2230–7.
- [18] Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 1991;30(43):10363–70.
- [19] Hekman CM, Loskutoff DJ. Fibrinolytic pathways and the endothelium. *Semin Thromb Hemost* 1987;13(4):514–27.
- [20] Rubin H. Serine protease inhibitors (SERPINS): where mechanism meets medicine. *Nat Med* 1996;2(6):632–3.
- [21] Kazimirova M, Jancinova V, Petrikova M, Takac P, Labuda M, Nosal R. An inhibitor of thrombin-stimulated blood platelet aggregation from the salivary glands of the hard tick *Amblyomma variegatum* (Acari: Ixodidae). *Exp Appl Acarol* 2002;28:97–105.
- [22] Mulenga A, Tsuda A, Onuma M, Sugimoto C. Four serine proteinase inhibitors (serpin) from the brown ear tick, *Rhipicephalus appendiculatus*; cDNA cloning and preliminary characterization. *Insect Biochem Mol Biol* 2003;33:267–76.
- [23] Sugino M, Imamura S, Mulenga A, Nakajima M, Tsuda A, Ohashi K, et al. A serine proteinase inhibitor (serpin) from ixodid tick *Haemaphysalis longicornis*; cloning and preliminary assessment of its suitability as a candidate for a tick vaccine. *Vaccine* 2003;21:2844–51.
- [24] Kanost MR. Serine proteinase inhibitors in arthropod immunity. *Dev Comp Immunol* 1999;23:291–301.
- [25] Andreotti R, Gomes A, Malavazi-Piza KC, Sasaki SD, Sampaio CA, Tanaka AS. BmTI antigens induce a bovine protective immune response against *Boophilus microplus* tick. *Int Immunopharmacol* 2002;2(4):557–63.
- [26] Leboulle G, Crippa M, Decrem Y, Mejri N, Brossard M, Bollen A, et al. Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks. *J Biol Chem* 2002;277:10083–9.
- [27] Prevot PP, Adam B, Boudjeltia KZ, Brossard M, Lins L, Cauchie P, et al. Anti-hemostatic effects of a serpin from the saliva of the tick *Ixodes ricinus*. *J Biol Chem* 2006;281(36):26361–9.
- [28] Mejri N, Rutti B, Brossard M. Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. *Parasitol Res* 2002;88(3):192–7.
- [29] Schorderet S, Brossard M. Changes in immunity to *Ixodes ricinus* by rabbits infested at different levels. *Med Vet Entomol* 1993;7(2):186–92.
- [30] Mbow ML, Christe M, Rutti B, Brossard M. Absence of acquired resistance to nymphal *Ixodes ricinus* ticks in BALB/c mice developing cutaneous reactions. *J Parasitol* 1994;80(1):81–7.
- [31] De la Fuente J, Rodriguez M, Montero C, Redondo M, Garcia-Garcia JC, Mendez L, et al. Vaccination against ticks (*Boophilus* spp.): the experience with the Bm86-based vaccine Gavac. *Genet Anal* 1999;15(3–5):143–8.
- [32] Ribeiro JM, Alarcon-Chaidez F, Francischetti IM, Mans BJ, Mather TN, Valenzuela JG, et al. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. *Insect Biochem Mol Biol* 2006;36:111–29.
- [33] Vermout S, Denis M, Losson B, Mignon B. Choix d'un adjuvant lors d'essais de vaccination. *Ann Méd Vét* 2003;147:393–401.
- [34] Mejri N, Francini N, Rutti B, Brossard M. Th2 polarization of the immune response of BALB/c mice to *Ixodes ricinus* instars, importance of several antigens in activation of specific Th2 subpopulations. *Parasite Immunol* 2001;23(2):61–9.
- [35] Christe M, Rutti B, Brossard M. Susceptibility of BALB/c mice to nymphs and larvae of *Ixodes ricinus* after modulation of IgE production with anti-interleukin-4 or anti-interferon-gamma monoclonal antibodies. *Parasitol Res* 1998;84(5):388–93.
- [36] Champagne DE. Antihemostatic molecules from saliva of blood-feeding arthropods. *Pathophysiol Haemost Thromb* 2005;34(4–5):221–7.
- [37] Ribeiro JM. Blood-feeding arthropods: live syringes or invertebrate pharmacologists? *Infect Agents Dis* 1995;4:143–52.
- [38] Kopecky J, Kuthejllova M. Suppressive effect of *Ixodes ricinus* salivary gland extract on mechanisms of natural immunity in vitro. *Parasite Immunol* 1998;20:169–74.
- [39] Brossard M, Wikel SK. Tick immunobiology. *Parasitology* 2004;129:161–76.
- [40] Shepherd JC, Aitken A, McManus DP. A protein secreted in vivo by *Echinococcus granulosus* inhibits elastase activity and neutrophil chemotaxis. *Mol Biochem Parasitol* 1991;44:81–90.
- [41] Maizels RM, Gomez-Escobar N, Gregory WF, Murray J, Zang X. Immune evasion genes from filarial nematodes. *Int J Parasitol* 2001;31:889–98.