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ORIGINAL PAPER

Identification and characterization of *Rhipicephalus (Boophilus) microplus* candidate protective antigens for the control of cattle tick infestations

Consuelo Almazán · Rodolfo Lagunes · Margarita Villar · Mario Canales · Rodrigo Rosario-Cruz · Frans Jongejan · José de la Fuente

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Abstract The cattle ticks, *Rhipicephalus (Boophilus)* spp., affect cattle production in tropical and subtropical regions of the world. Tick vaccines constitute a cost-effective and environmentally friendly alternative to tick control. The recombinant *Rhipicephalus microplus* Bm86 antigen has been shown to protect cattle against tick infestations. However, variable efficacy of Bm86-based vaccines against geographic tick strains has encouraged the research for additional tick-protective antigens. Herein, we describe the analysis of *R. microplus* glutathione-S transferase, ubiquitin (UBQ), selenoprotein W, elongation factor-1 alpha, and subolesin (SUB) complementary DNAs (cDNAs) by RNA interference (RNAi) in *R. microplus* and *Rhipicephalus annulatus*. Candidate protective antigens were selected for vaccination experiments based on the effect of gene knockdown on tick mortality, feeding, and fertility. Two cDNA clones encoding for UBQ and SUB were used for cattle vaccination and infestation with *R. microplus* and *R. annulatus*. Control groups were immunized with recombinant Bm86 or adjuvant/saline. The highest vaccine efficacy for the control of tick infestations was obtained for Bm86. Although with low immunogenic response, the results with the SUB vaccine encourage further investigations on the use of recombinant subolesin alone or in combination with other antigens for the control of cattle tick infestations. The UBQ peptide showed low immunogenicity, and the results of the vaccination trial were inconclusive to assess the protective efficacy of this

C. Almazán
Facultad de Medicina Veterinaria y Zootecnia,
Universidad Autónoma de Tamaulipas,
Km. 5 carretera Victoria-Mante,
CP 87000 Victoria, Tamaulipas, Mexico

R. Lagunes
Facultad de Medicina Veterinaria y Zootecnia,
Universidad Nacional Autónoma de México,
Avenida Universidad 3000,
CP 04510 Mexico, DF, Mexico

M. Villar · M. Canales · J. de la Fuente (✉)
Instituto de Investigación en Recursos Cinegéticos
IREC (CSIC-UCLM-JCCM),
Ronda de Toledo s/n,
13005 Ciudad Real, Spain
e-mail: jose_delafuente@yahoo.com

R. Rosario-Cruz
Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria,
Carretera Federal Cuernavaca-Cuautla 8534, Col. Progreso,
Jiutepec, Morelos CP 62550, Mexico

F. Jongejan
Utrecht Centre for Tick-borne Diseases (UCTD),
Department of Infectious Diseases and Immunology,
Faculty of Veterinary Medicine, Utrecht University,
Yalelaan 1,
3584CL Utrecht, The Netherlands

F. Jongejan
Department of Veterinary Tropical Diseases,
Faculty of Veterinary Science,
University of Pretoria,
Private Bag X04,
0110 Onderstepoort, South Africa

J. de la Fuente
Department of Veterinary Pathobiology,
Center for Veterinary Health Sciences,
Oklahoma State University,
Stillwater, OK 74078, USA

antigen. These experiments showed that RNAi could be used for the selection of candidate tick-protective antigens. However, vaccination trials are necessary to evaluate the effect of recombinant antigens in the control of tick infestations, a process that requires efficient recombinant protein production and formulation systems.

Introduction

Rhipicephalus (Boophilus) spp. ticks are distributed in tropical and subtropical regions of the world with range expansion for some species due to changes in climatic conditions (Barker and Murrell 2004; Estrada-Peña et al. 2006; Olwoch et al. 2007). Infestations with the cattle tick, *Rhipicephalus microplus*, economically impact cattle production by reducing weight gain and milk production, and by transmitting pathogens that cause babesiosis (*Babesia bovis* and *Babesia bigemina*) and anaplasmosis (*Anaplasma marginale*; Peter et al. 2005). *Rhipicephalus annulatus* is present in regions of Asia, Latin America, and Africa (Estrada-Peña et al. 2006) where it may also affect cattle production and transmit pathogens.

Acaricide application constitutes a major component of integrated tick control strategies (Graf et al. 2004). However, use of acaricides has had limited efficacy in reducing tick infestations and is often accompanied by serious drawbacks, including the selection of acaricide-resistant ticks, environmental contamination, and contamination of milk and meat products with drug residues (Graf et al. 2004). All of these issues reinforce the need for alternative approaches to control tick infestations such as the use of hosts with natural resistance to ticks, pheromone-impregnated decoys for attracting and killing ticks, biological control agents, and vaccines (de la Fuente and Kocan 2006; Sonenshine et al. 2006; Willadsen 2006).

In the early 1990s, vaccines inducing immunological protection on vertebrate hosts against tick infestations were developed and commercialized. The commercial vaccines, Gavac and TickGARD, contained the recombinant *R. microplus* Bm86 gut antigen (Willadsen et al. 1989; Rand et al. 1989; Rodríguez et al. 1994; de la Fuente and Kocan 2003; de la Fuente et al. 2007a). These vaccines reduce the number of engorging female ticks, their weight, and reproductive capacity. Thus, the greatest vaccine effect was the reduction of larval infestations in subsequent generations. Vaccine-controlled field trials in combination with acaricide treatments demonstrated that an integrated approach resulted in control of tick infestations while reducing the use of acaricides (de la Fuente et al. 1998, 2007a; de la Fuente and Kocan 2003). These trials demonstrated that control of ticks by vaccination has the advantages of being cost-effective, reducing environmental contamination, and preventing the selection of drug resistant ticks that result from repeated acaricide application. In addition, these vaccines may also prevent or reduce transmission of pathogens by reducing tick populations and/or affecting tick vector capacity (de la Fuente et al. 1998, 2007a; Rodríguez Valle et al. 2004).

Controlled immunization trials have shown that *R. microplus* Bm86-containing vaccines also protect against related tick species, *R. annulatus* and *Rhipicephalus decoloratus* (Fragoso et al. 1998; de la Fuente et al. 2000a; de Vos et al. 2001). However, *R. microplus* strain-to-strain variations in the susceptibility to Bm86 vaccination have been reported, and the efficacy of the Bm86 vaccine is higher against *R. annulatus* than against *R. microplus* strains, suggesting that tick genetic and/or physiological differences may affect the efficacy of tick vaccines in different regions (Fragoso et al. 1998; García-García et al. 1999; 2000; de la Fuente et al. 1999, 2000a, b; de Vos et al. 2001; de la Fuente and Kocan 2006; Sossai et al. 2005; Canales et al. 2009a). These results together with the need to improve vaccine efficacy have encouraged research for additional tick protective antigens for the control of cattle tick infestations (de la Fuente and Kocan 2003, 2006; Sonenshine et al. 2006; Willadsen 2006).

In the experiments reported herein, selected *R. microplus* complementary DNA (cDNA) sequences were characterized by RNA interference (RNAi) in unfed and replete adult ticks. Tick subolesin and ubiquitin were used for vaccination trials with recombinant proteins in cattle to evaluate their capacity to protect against cattle tick infestations.

Materials and methods

Tick strains The *R. microplus* (susceptible Media Joya strain, CENAPA, Mexico) and *R. annulatus* (Mercedes strain, Texas, USA) ticks were obtained from laboratory colonies maintained at the University of Tamaulipas, Mexico. Originally, these tick strains were collected from infested cattle in Tapalpa, Jalisco, Mexico and Mercedes County, Texas, USA for *R. microplus* and *R. annulatus*, respectively. Tick larvae were fed on cattle and collected after repletion to allow for oviposition and hatching in humidity chambers at 12 h light:12 h dark photoperiod, 22–25°C, and 95% relative humidity (RH). Larvae were 15 days old at the time of infestations.

RNA interference in ticks Genes for RNAi were selected based on previous results in *R. microplus* or *Dermacentor variabilis* demonstrating the effect of gene knockdown on tick attachment, mortality, and/or fertility (de la Fuente et al. 2007b; 2008). These genes included those encoding for putative glutathione-S transferase (GST; GenBank acces-

sion number AF077609), ubiquitin (UBQ; AA257892), selenoprotein W (SEL; tentative consensus sequence TC19044, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_report.pl?gudb=b_microplus&tc=TC19044), elongation factor-1 alpha (EF-1a; EU436163), and subolesin (SUB; DQ159966).

Oligonucleotide primers containing T7 promoter sequences at the 5′-end (GST: GSTBMT75: 5′- *TAATACGACTCACTATAGGGTACT*CCATGGCTCCTGTGCTCGGCT-3′ and GSTBMT73: 5′- *TAATACGACTCACTATAGGGTACT*CATTGAGAGGCCACTTAATGC-3′; UBQ: UB2BMT75: 5′-*TAATACGACTCACTATAGGGTACT*TTGGTCCTGCGGCTCCGCGGAG-3′ and UB2BMT73: 5′-*TAATACGACTCACTATAGGGTACT*GCCACCACGGAGCCGCAGGACA-3′; SEL: SEBMT75: 5’-*TAATACGACTCACTATAGGGTACT*CCACCGTGACTGGCACAGTAG-3′ and SEBMT73: 5′-*TAATACGACTCACTATAGGGTACT*CCATACATGAATGGCTTTCCA-3′; EF-1a: GII5: 5′-*TAATACGACTCACTATAGGGTACT*GGCCCACGGACAAACCCCTC-3′ and GII3: 5′-*TAATACGACTCACTATAGGGTACT*CGACCGTTTGCCTCATGTC-3′; SUB: D8BMT75: 5′-*TAATACGACTCACTATAGGGTACT*GACTGGGACCCCTTGCACAGT-3′ and D8BMT73 5′-*TAATACGACTCACTATAGGGTACT*CGAGTTTGGTAGATAGCACA-3) were synthesized and used for in vitro transcription of double-stranded RNA (dsRNA) using the Access reverse transcription–polymerase chain reaction (RT–PCR) system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA) following manufacturer's instructions. The dsRNA was purified and quantified by spectrophotometry.

Newly molted *R. microplus* and *R. annulatus* females were injected with approximately 0.3 μl of dsRNA (5×10^{10} molecules per microliter) in the lower right quadrant of the ventral surface of the exoskeleton of the tick (Nijhof et al. 2007). Thirty ticks per group were injected using a Hamilton syringe with a 1-inch, 33-gauge needle. Control ticks were injected with the unrelated GIII dsRNA or were left uninjected. The GIII sequence was identified in *R. microplus* and did not affect tick feeding, mortality, and oviposition after RNAi (unpublished results). Ticks were held in a humidity chamber for 3–5 h before surviving ticks were allowed to feed in separate patches, each one for a different group, glued on the back of a calf. Ten males were placed in each patch simultaneously with injected females. Unattached ticks were removed 2 days after infestation, and attached ticks were allowed to feed until repletion. Engorged female ticks started to drop off the host after 8 days and were collected during 4 days. All the females completing feeding were counted and weighted. The average ± standard deviation (SD) weights of engorged females were compared between test and control groups by Student's *t* test (P=0.05). Tick attachment was recorded with respect to surviving ticks after injection, and tick mortality was recorded with respect to the total number of attached ticks feeding on the animal and were compared between test and control groups by χ^2 test (α=0.01). For oviposition, individual ticks were held in a humidity chamber at 22–25°C and 95% RH, and the egg mass oviposited by each tick was weighted and compared between test and control groups by Student's *t* test (P=0.05).

Engorged *R. microplus* and *R. annulatus* females were weighted and injected with 5 μl of dsRNA (5×10^{10} molecules per microliter) in the right spiracular plate within 6 h after dropping off the host using the same methods as described above or left uninjected. They were stored individually in an incubator at 22–25°C and 80% RH. Tick weight and egg masses removed after oviposition and weighted were analyzed as described above. Egg batches were stored separately under the same conditions to estimate percent hatching by counting the number unhatched eggs that was compared between test and control groups by χ^2 test (α=0.01).

Ten ticks per group were dissected after oviposition and pooled for RNA extraction to evaluate gene expression by semiquantitative RT–PCR using the same oligonucleotide primers used for dsRNA synthesis. The messenger RNA (mRNA) levels (nanograms per amplicon) were estimated in ethidium bromide-stained agarose gels by comparison to a standard curve of the target gene and compared between target gene dsRNA and GIII dsRNA-injected control ticks to calculate the percent of gene silencing after RNAi.

Vaccine formulations R. microplus UBQ and SUB were produced for vaccination trials. Recombinant SUB was expressed in *Escherichia coli* from a synthetic gene (GenBank accession number GQ456170) optimized for codon usage in *E. coli* and purified by Ni affinity chromatography (Genscript Corporation, Piscatway, NJ, USA; www.genscript.com). The UBQ peptide NH2-YNIQKESTLHLVLRLRGGMQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIF AGKQLED was synthesized and purified at the Bioanalytics, Pharmacology, and Proteomics facility (Barcelona Biomedical Research Park, Spain). The recombinant Bm86 (susceptible strain) used as positive control was secreted in *Pichia pastoris* and purified as reported previously (Canales et al. 2008). Protein adjuvation was made by mixing a solution of anhydromannitoletheroctodecenoate (Montanide ISA 50 V; Seppic, Paris, France) with the recombinant protein solution in batch-by-batch processes using a high-speed mixer Heidolph DIAX 900 (Heidolph Elektro, Kelheim, Germany) at 8,000 rpm, and the vaccine was filled manually under sterile conditions in glass bottles of 20 ml (Wheaton, Millville, NJ, USA) at a concentration of 100 μg/2 ml dose. Quality controls were made by testing

mechanical and thermal stability of vaccine emulsions as described previously (Canales et al. 1997).

Cattle immunization with recombinant proteins and tick infestations Four 1-year-old European crossbred calves per group were each immunized with three doses (weeks 1, 4, and 6) containing 100 μg per dose of purified recombinant proteins formulated as described above. Negative controls were injected with adjuvant/saline alone. Cattle were injected intramuscularly with 2 ml/dose using a 5-ml syringe and an 18-gauge needle. Two weeks after the last immunization, cattle in vaccinated and control groups were infested with 10,000 *R. annulatus* (Mercedes, Texas, USA strain) and *R. microplus* (susceptible, Mexico strain) larvae/animal applied individually to each animal in separate cotton cells attached to the back of the animals. Cattle were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals.

Data collection and evaluation Adult engorged female ticks dropping from cattle were daily collected, counted, and weighted. All the collected adult female ticks were assessed for oviposition and egg fertility (de la Fuente et al. 1999). The personnel collecting the ticks were "blinded" as to which group animals belonged. The efficacy of vaccine formulations was evaluated employing the following formulae (de la Fuente et al. 1999; Canales et al. 2009a).

Effect on the number of adult female ticks $(DT) = 100[1-(NTV/NTC)]$, where NTV is the number of adult female ticks in the vaccinated group, and NTC is the number of adult female ticks in the control group.

Effect on tick weight $(DW) = 100[1-(WTV/WTC)]$, where WTV is the average adult female tick weight in the vaccinated group, and WTC is the average adult female tick weight in the control group.

Effect on oviposition $(DO) = 100[1-(PATV/PATC)]$, where PATV is the average weight of the eggs per survived tick in the vaccinated group, and PATC is the average weight of the eggs per survived tick in the control group.

Effect on egg fertility $(DF) = 100[1-(PPLOV/PPLOC)]$, where PPLOV is the average weight of the larvae per gram of eggs in the vaccinated group, and PPLOC is the average weight of the larvae per gram of eggs in the control group.

Vaccine efficacy (E) was calculated as $100[1-(CRT \times CR0 \times CRF)]$, where CRT=NTV/NTC, CR0=PATV/PATC, and CRF=PPLOV/PPLOC that represent the reduction in the number of adult female ticks, oviposition, and egg fertility as compared to the control group, respectively.

A Student's *t* test with unequal variance (P=0.05) was used to compare the results of adult female tick number, tick weight, oviposition, and egg fertility between vaccinated and control groups.

Determination of serum antibody levels by enzyme-linked immunosorbent assay Before each immunization and 21 days after the last immunization, blood samples were collected from each calf into sterile tubes and maintained at 4°C until arrival at the laboratory. Serum was then separated after centrifugation and stored at −20°C. Serum antibody titers were determined using an antigen-specific indirect enzyme-linked immunosorbent assay (ELISA). Purified UBQ, SUB, and Bm86 (susceptible strain) antigens (0.1 μg per well) were used to coat ELISA plates overnight at 4°C. Sera were serially diluted to 1:10, 1:100, and 1:1,000 in phosphate-buffered saline with Tween 20 (PBST; PBS/0.5% Tween 20, pH 7.2) and 10% fetal bovine serum (Sigma). The plates were incubated with the diluted sera for 1 h at 37°C and then incubated with 1:10,000 rabbit antibovine immunoglubolinG (IgG)–horseradish peroxidase conjugates (Sigma) for 1 h at 37°C. The color reaction was developed with 3,3′,5,5′- tetramethylbenzidine (Sigma), and the optical density$_{450\ nm}$ (OD) was determined. After incubation, the plates were washed with PBST. Antibody titers were considered positive when they yielded an $OD_{450\ nm}$ value at least twice as high as the preimmune serum. Antibody titers in immunized cattle were expressed as the $OD_{450\ nm}$ value for the highest serum dilution (1:1,000) and compared between vaccinated and control cattle using an analysis of variance (ANOVA) test ($P<0.05$). Correlation analyses were conducted in Microsoft Excel to compare antibody titers at the time of tick infestation and vaccine efficacy calculated in individual cattle.

Results and discussion

The discovery and characterization of new tick-protective antigens is important to improve existing vaccines for the control of cattle tick infestations (de la Fuente et al. 2007a). However, the expenses and difficulties associated with the screening of tick-protective antigens and cattle vaccination trials constitute an obstacle toward this goal. Recently, RNAi has been proposed as a method for the rapid screening of tick-protective antigens (de la Fuente et al. 2005, 2007c). However, vaccination trials are ultimately needed to characterize new candidate tick-protective antigens.

In these experiments, RNAi was used to select the antigens for vaccination trials based on the effect of gene knockdown on cattle tick mortality, feeding, and fertility. The GST, UBQ, SEL, EF-1a, and SUB genes were selected for RNAi experiments in *R. annulatus* and/or *R. microplus* based on previous results in *R. microplus* or *D. variabilis* demonstrating an effect of gene knockdown on tick attachment, mortality, and/or fertility (de la Fuente et al. 2007b, 2008). The results showed that GST and SEL gene

Table 1 Summary of RNAi experiments in unfed and engorged *R. microplus* female ticks

Parameter	*R. microplus* cDNA group						
	GST	UBQ	SEL	SUB	EF-1a	GIII	UNI
Unfed female *R. microplus*							
Tick mortality (%)	43	77*	50	73*	77*	53	27
	ND	70*	53	67*	73*	53	ND
Tick weight (mg)	214±115**	85±129***	211±124**	8±2***	55±140***	307±125	485±113
	ND	227±95	191±130**	7±3***	4±5***	292±125	ND
Eggs/tick (mg)	80±60	0±0***	59±64	8±2**	22±58	26±39	103±64
	ND	0±0***	121±43	0±0***	0±0***	61±51	ND
Engorged female *R. microplus*							
Tick weight (mg)	301±67	314±18	302±47	284±45	324±39	364±22	288±72
Eggs/tick (mg)	151±34	89±30***	109±14**	101±83	123±89	191±10	158±46
Egg hatching (%)	70	30*	30*	10*	5*	70	80

Adult ticks (N=30 for unfed and N=3 for engorged females) were injected with dsRNA or left uninjected (UNI). Two experiments were conducted in unfed ticks while a single experiment was done with engorged female ticks. Tick mortality and egg hatching are shown in percent and were compared to the GIII dsRNA-injected control ticks by $\chi 2$ test (*α<0.01). Tick weight and oviposition (eggs per tick) are shown as average ± SD and were compared to the GIII dsRNA-injected control ticks by Student's t test (**P<0.05; ***P<0.01)

ND not done, UNI *uninjected*

knockdown affected tick attachment (60% and 65% tick attachment, respectively, when compared to 91% attachment in the controls; α<0.01) as in previous experiments with *D. variabilis* (de la Fuente et al. 2007b) but had no effect on *R. microplus* mortality and oviposition (Table 1) and were not characterized further. As in previous experiments (de la Fuente et al. 2007b, 2008), RNAi with UBQ, EF-1a, and SUB increased *R. microplus* mortality and reduced oviposition when compared to controls (Table 1). Despite the fact that gene expression was silenced between 75% and 100% in target gene dsRNA-injected *R. microplus* and *R. annulatus* ticks when compared to controls, in *R. annulatus*, only EF-1a knockdown had an effect on both tick mortality and oviposition (Table 2). However, tick-to-tick variations in RNAi results were higher in *R. annulatus* than in *R. microplus* probably because RNAi conditions are better established for *R. microplus* (Nijhof et al. 2007).

R. microplus UBQ, SUB, and EF-1a were selected for vaccination trials based on their effect on tick mortality and/or oviposition after RNAi (Tables 1 and 2). However, recombinant EF-1a could not be produced in sufficient quantities for protein purification and vaccination trials were conducted with recombinant UBQ and SUB only in comparison with Bm86 (positive control) and adjuvant/saline (negative control).

Except for cattle immunized with recombinant SUB, antibody titers in vaccinated cattle increased after successive immunizations (Fig. 1). However, anti-UBQ antibody titers in immunized cattle were not significantly different from control animals (Fig. 1). The poor immunogenicity of the UBQ vaccine could reflect self-tolerance to a highly conserved molecule in eukaryotes and the fact that short peptides are often not immunogenic and require conjugation to a carrier protein or the use of potent adjuvants to

Table 2 Summary of RNAi experiments in unfed *R. annulatus* female ticks

Parameter	*R. microplus* cDNA group				
	UBQ	SUB	EF-1a	GIII	UNI
Tick mortality (%)	63*	47	90*	47	53
Tick weigh (mg)	235±113	69±86***	25±22***	206±108	199±128
Eggs/tick (mg)	97±70	70±83	0±0***	114±56	116±71
Hatching (%)	80	20*	0*	100	90

Adult unfed female ticks (N=30) were injected with dsRNA or left uninjected (UNI). Tick mortality and egg hatching are shown in percent and were compared to the GIII dsRNA-injected control ticks by χ^2 test (*α<0.01). Tick weight and oviposition (eggs per tick) are shown as average ± SD and were compared to the GIII dsRNA-injected control ticks by Student's t test (**P<0.05; ***P<0.01)

UNI *uninjected*

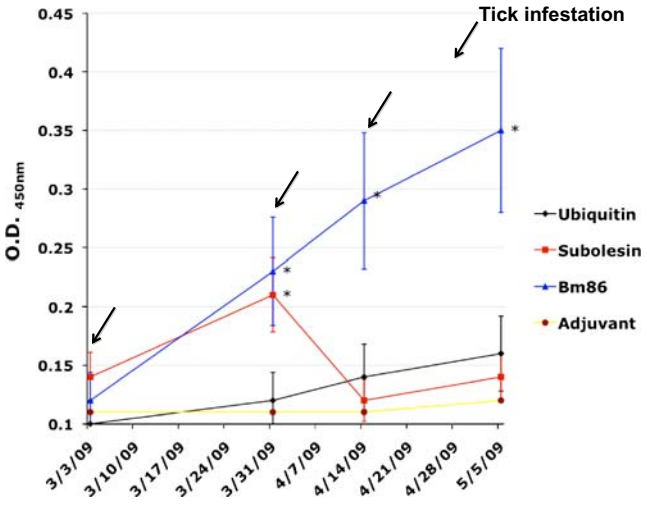

Fig. 1 Antibody response in vaccinated cattle. Bovine serum antibody titers to recombinant antigens were determined by ELISA in cattle vaccinated with ubiquitin, subolesin, Bm86, and adjuvant/saline control. Antibody titers in immunized cattle were expressed as the $OD_{450\ nm}$ value for the highest serum dilution (1:1,000) and compared between vaccinated and control cattle using an ANOVA test (*$P<0.05$). The time of vaccination shots (*arrows*) and tick infestation are indicated

increase immunogenicity (Rodríguez et al. 1994; Haro and Gómara 2004). In cattle immunized with recombinant SUB, antibody titers increased after the first immunization and then decreased after the second immunization (Fig. 1). The cause for this decrease in anti-SUB antibody titers after the second immunization is unknown but could be due to problems associated with the stability of the vaccine formulation. The vaccine containing the recombinant Bm86 antigen in Montanide ISA 50 V has been shown to be stable even at field temperature (Canales et al. 2007 and unpublished results), but vaccine stability could be different for different antigen preparations. In general, antibody titers were low in immunized cattle, even in the animals vaccinated with the control Bm86 vaccine when compared to previous studies (Canales et al. 2009a). Although cattle were apparently healthy, low antibody responses after vaccination suggested that animals were probably not in the best health condition. Nevertheless, a positive correlation (correlation coefficients R^2=0.7, 0.8, and 0.9 in UBQ-, SUB-, and Bm86-immunized cattle, respectively) between antibody titers 1 week after tick infestation and vaccine efficacy calculated in individual cattle was obtained for *R. microplus*. For *R. annulatus*, a positive correlation (R^2= 0.6) between antibody titers and vaccine efficacy was obtained in SUB-immunized cattle only. A positive correlation between antibody titers and vaccine efficacy has been previously demonstrated in Bm86-vaccinated cattle and underlines the effect of antitick vaccines on the control of cattle tick infestations (Cobon et al. 1995; de la Fuente et al. 1998).

The results of the vaccine trial showed an effect on *R. microplus* and *R. annulatus* infestations for all three antigens when compared to controls (Tables 3 and 4). The efficacy with the Bm86 vaccine were within the range reported in previous experiments (Cobon et al. 1995; Fragoso et al. 1998; de la Fuente et al. 1998, 1999, 2000a, b; García-García et al. 2000; de Vos et al. 2001; de la Fuente and Kocan 2003, 2006; Canales et al. 2009a) and was higher than that obtained with UBQ and SUB vaccines

Table 3 Control of *R. microplus* infestations in cattle vaccinated with the recombinant antigens

	R. microplus (susceptible; Mexico strain)				
	Percent reduction (vaccinated/control)[b]				E[c]
Experimental group[a]	DT	DW	DO	DF	
Ubiquitin	26%	9%	–2%	29%	55%
	(1,079±410)	(251±52)	(109±9)	(0.37±0.04)	
Subolesin	43%	0%	0%	15%	51%
	(835±179)*	(277±33)	(107±21)	(0.44±0.06)	
Bm86	51%	5%	14%	6%	60%
	(714±208)*	(261±26)	(92±16)	(0.49±0.04)	
Adjuvant/saline control	(1,454±206)	(276±14)	(107±12)	(0.52±0.29)	–

DT percentage reduction in tick infestation, *DW* percentage reduction in tick weight, *DO* percentage reduction in oviposition, *DF* percentage reduction in egg fertility

[a] Cattle were randomly assigned to experimental groups (N=4), vaccinated, and challenged with *R. microplus* and *R. annulatus* larvae

[b] The percent reduction was calculated with respect to the control group. In parenthesis are shown the average ± SD for adult female tick number, tick weight (milligram), oviposition (egg weight (milligram) per tick), and egg fertility (larvae weight per egg weight) and were compared by Student's t test with unequal variance between vaccinated and control groups (*$P<0.05$)

[c] Vaccine efficacy (E) was calculated as $100[1-(CRT \times CR0 \times CRF)]$, where CRT, CRO, and CRF are the reduction in the number of adult female ticks, oviposition, and egg fertility as compared to the control group, respectively

Table 4 Control of *R. annulatus* infestations in cattle vaccinated with the recombinant antigens

	R. annulatus (Mission, TX strain)				
	Percent reduction (vaccinated/control)[b]				E[c]
Experimental group[a]	DT	DW	DO	DF	
Ubiquitin	−2%	−19%	−21%	15%	15%
	(519±96)	(275±29)	(109±15)	(0.13±0.03)	
Subolesin	18%	17%	23%	37%	60%
	(419±288)	(192±129)	(69±49)	(0.09±0.07)	
Bm86	100%	All ticks	All ticks	All ticks	100%
	(0±0)*	Died	Died	Died	
Adjuvant/saline control	(509±145)	(231±28)	(90±20)	(0.15±0.03)	–

DT percentage reduction in tick infestation, *DW* percentage reduction in tick weight, *DO* percentage reduction in oviposition, *DF* percentage reduction in egg fertility

[a] Cattle were randomly assigned to experimental groups (N=4), vaccinated, and challenged with *R. microplus* and *R. annulatus* larvae

[b] The percent reduction was calculated with respect to the control group. In parenthesis are shown the average ± SD for adult female tick number, tick weight (milligram), oviposition (egg weight (milligram) per tick) and egg fertility (larvae weight per egg weight) and were compared by Student's t test with unequal variance between vaccinated and control groups (*P<0.05)

[c] Vaccine efficacy (E) was calculated as $100[1-(\mathrm{CRT}\times\mathrm{CR0}\times\mathrm{CRF})]$, where CRT, CRO, and CRF are the reduction in the number of adult female ticks, oviposition, and egg fertility as compared to the control group, respectively

in both tick species. Particularly for *R. annulatus* control, it is difficult to improve the results obtained with Bm86 vaccines which attain >99% efficacy in all experiments (Fragoso et al. 1998; de la Fuente et al. 2000a, b; de Vos et al. 2001; Canales et al. 2009a). For SUB, this is the first vaccination trial in *R. microplus* and *R. annulatus*, and the results were similar to those reported in other tick species (Almazán et al. 2005a, b; de la Fuente et al. 2006; Canales et al. 2009b) and support further experiments with this antigen. Vaccination with the UBQ peptide had some efficacy on tick control, particularly on *R. microplus* infestations (Table 3). However, these results were affected by the low immunogenicity of the UBQ vaccine preparation and require additional trials with improved peptide formulations before a conclusion could be reached.

The genes were selected for vaccination trials based on their effect on tick mortality and fertility after RNAi experiments included those encoding for proteins involved in the regulation of mRNA and protein synthesis and metabolism with potential impact on important biological processes in eukaryotes such as cell growth and maintenance. However, these proteins are cytoplasmic and may be evolutionary highly conserved which may pose a problem for their use in vaccine formulations for the control of tick infestations. As previously discussed, the conservation of protein ortholog sequences among arthropod vectors and vertebrate hosts may raise the question of safety when using them for immunization with the potential of inducing autoimmune responses damaging to the host (Canales et al. 2009b). However, it is expected that the antibody response would be primarily directed against nonself epitopes thus reducing the possibility of detrimental effects to the host. Additionally, immunization with intracellular proteins has been effective in ticks and other invertebrate organisms and suggests a low risk to induce autoimmune responses in vertebrate hosts (Elad and Segal 1995; Almazán et al. 2005a, b; de la Fuente et al. 2006; Canales et al. 2009b).

In summary, these experiments showed that RNAi could be used for the selection of candidate tick-protective antigens. However, vaccination trials are necessary to evaluate the effect of recombinant antigens in the control of tick infestations, a process that requires efficient recombinant protein production and formulation systems. The results with the SUB vaccine encourage further investigations on the use of recombinant subolesin alone or in combination with other antigens for the control of cattle tick infestations.

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