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Characterization of three *Ixodes scapularis* cDNAs protective against tick infestations☆

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Abstract

cDNA expression library immunization (ELI) and analysis of expressed sequenced tags (EST) in a mouse model of tick infestations was used to identified cDNA clones that affected *I. scapularis*. Three protective antigens against larval tick infestations, 4F8, with homology to a nucleotidase, and 4D8 and 4E6 of unknown function, were selected for further characterization. All three antigens were expressed in all *I. scapularis* stages and localized in adult tick tissues. 4D8 was shown to be conserved in six other tick species. Based on immunization trials with synthetic polypeptides against larvae and nymphs and on artificial feeding experiments of adults, these antigens, especially 4D8, appear to be good candidates for continued development of a vaccine for control of tick infestations and may be useful in a formulation to target multiple species of ticks.

Keywords: Tick; Vaccine; Expression library immunization; EST; Recombinant protein

1. Introduction

Ticks are ectoparasites of wild and domestic animals and humans, and are considered to be the most important vector of pathogens in North America [1]. *Ixodes* spp. (Acari: Ixodidae) are distributed worldwide and are vectors of pathogens affecting humans and wild and domestic animals [1,2]. Members of the *I. ricinus* complex are the primary tick vectors of *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, the causative agents of Lyme disease and human granulocytic anaplasmosis, respectively [1,2]. Throughout eastern and north-central United States, southern Canada and northern Mexico, *I. scapularis* (the black-legged tick) is the main vector of these pathogens [1,2], while the closely related species, *I. pacificus* and *I. ricinus*, vector these pathogens in western United States and Europe, respectively [2].

Some tick species such as *Boophilus microplus* complete the life cycle while feeding on a single host. In other tick species such as *I. scapularis*, larvae, nymphs and adults feed on different hosts. Therefore, the control of tick infestations is especially difficult for multi-host ticks because several host species may need to be considered when implementing tick control strategies. Presently, tick control is effected by integrated pest management in which different control methods are adapted to a geographic area against one tick species with consideration to their environmental effects (reviewed by [3]). Recently, development of vaccines against one-host *Boophilus* spp. has provided new possibilities for identification of protective antigens for use in vaccines for control of tick infestations (reviewed by [4,5]). Control of

☆ The Genbank accession numbers for sequences of *I. scapularis* cDNA clones 4F8, 4D8 and 4E6 are AY652654–AY652656.

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ticks by vaccination would avoid environmental contamination and the selection of drug resistant ticks that result from repeated acaricide applications. Tick vaccines could also be designed to include multiple tick and pathogen antigens that may target a broad range of both tick species and associated pathogens (reviewed by [4]).

Development of high throughput screening and sequencing technologies and bioinformatic tools facilitate the study of biological systems and provide information for the identification of potential vaccine candidates [6–10]. Recently, we reported the use of cDNA expression library immunization (ELI) and analysis of expressed sequenced tags (EST) in a mouse model of tick infestations for identification of cDNAs protective against *I. scapularis*[11,12]. The combination of cDNA ELI and EST analysis resulted in the selection of 351 cDNA clones that affected tick larval development [11,12]. These clones were grouped according to their putative function, and some cDNAs resulted in inhibition of tick infestation while others promoted tick feeding [11,12].

Herein, we describe the characterization of three *I. scapularis* cDNAs, 4F8, 4D8 and 4E6, which reduced tick infestations in cDNA-vaccinated mice [11,12].

2. Materials and methods

2.1. Sequence analysis of protective tick cDNAs and deduced proteins

A strategy using primer walking was developed at the Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University, for sequencing both strands of cDNA inserts contained in protective clones 4F8, 4D8 and 4E6, using ABI Prism dye terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, CA, USA). Nucleotide sequences were analyzed using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA). Open reading frames (ORFs) were found and the deduced amino acid sequence of encoded proteins determined. BLAST [13] was used to search the NCBI databases and the TIGR *Amblyomma variegatum* Gene Index (AvGI; [14]) to identify previously reported sequences with identity to those obtained by ELI. Conserved domains in proteins were analyzed by searching CDD, a curated Entrez database of conserved domain alignments [15] at NCBI web site. Protein topology was analyzed using TMpred and TMHMM v.2.0 algorithms for the prediction of transmembrane helices in proteins [16,17], and the TargetP v1.01 algorithm was used to predict the localization of proteins in cells [18].

Phylogenetic analyses were implemented with 4D8 protein sequences using MEGA version 2.1 [19]. Protein sequences similar to *I. scapularis* 4D8 were included in the analysis from *Drosophila melanogaster* (Genbank accession number AAF50569), *A. variegatum* (T10865), *Danio rerio* (AAQ94594), mouse (XP_131324), human (NP_060534), *Xenopus laevis* (AAH43949), *Anopheles gambiae* (EAA04195) and *Caenorhabditis elegans* (NP_491304). Maximum parsimony (MP) tree searches were heuristic, using tree-bisection-and-reconnection (TBR) branch swapping for 10 random addition sequence replicates. Minimum evolution (ME) and neighbor joining (NJ) trees were constructed based on p-distances and pairwise deletion of gaps. Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis [20] of 1000 replications.

2.2. Production and characterization of tick antigens

2.2.1. Cloning and expression in Escherichia coli of 4F8 and 4D8 recombinant proteins

For expression of 4F8 and 4D8 cDNAs in *E. coli*, coding regions were amplified from plasmid DNA by PCR using specific oligonucleotide primers introducing *Eco* RI and *Sal* I restriction sites in the 5′ and 3′ primers, respectively, to insert amplified fragments into the cloning site of pFLAG-CTC expression vector (Sigma, St. Louis, MO, USA). Recombinant plasmids were named pFNUC1 and pFEND2 for 4F8 and 4D8, respectively. In these constructs, the inserted genes were under the control of the inducible *tac* promoter and yielded full-length polypeptides with a C-terminal fusion of a FLAG marker octapeptide. The fidelity and orientation of the constructs was verified by sequencing. For expression of recombinant polypeptides, pFNUC1 and pFEND2 expression plasmids were transformed into *E. coli* K-12 (strain JM109). Transformed *E. coli* cells were inoculated in LB containing 50 μg/ml ampicillin and 0.4% glucose. Cultures were grown at 37 °C to $OD_{600\,nm}$ = 0.4. IPTG was then added to 0.5 mM final concentration, and incubation continued during 4 h for induction of recombinant protein expression. Cells were collected by centrifugation and later analyzed by SDS-PAGE and Western blot, or then used for the purification of recombinant proteins.

2.2.2. Synthesis of 4E6 peptide

A peptide corresponding to the sequence of 4E6 ORF (NH_2-MEISVKPRPTKRKRKAIIIMARMRTAFPTR-SGNSFSRT-COOH) was synthesized, analyzed by HPLC and mass spectrometry by Sigma-Genosys (The Woodlands, TX, USA), and shown to be 99% pure.

2.2.3. Protein purification

E. coli cells expressing recombinant 4F8 and 4D8 proteins were disrupted by sonication. Recombinant proteins were extracted with 0.1% Triton X-100 in Tris-buffered saline (TBS) and purified by FLAG-affinity chromatography (Sigma) following the manufacturer's instructions. The purity of recombinant proteins was estimated to be ≥90% as assayed by densitometry scanning of protein gels.

2.2.4. Protein gel electrophoresis and Western blot analysis

Expression and purification of the recombinant proteins was confirmed by SDS-PAGE [21] and immunoblotting. Protein samples were loaded on 12.5% polyacrylamide gels that were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 60 min at room temperature. Western blot analysis was performed using anti-FLAG M2 monoclonal antibodies (Sigma) for detection of recombinant fusion 4F8 and 4D8 proteins or 4F8-, 4D8-, 4E6-monospecific rabbit sera prepared in New Zealand White rabbits that were immunized subcutaneously with three doses (weeks 0, 4 and 7) each containing 50 μg purified 4F8 and 4D8 proteins or 4E6 synthetic peptide per dose in Freud's incomplete adjuvant (FIA) (Sigma). After washing with TBS, the membranes were incubated with 1:10,000 goat anti-mouse IgG or goat anti-rabbit IgG alkaline phosphatase conjugate (KPL, Inc., Gaithersburg, MD, USA). The membranes were washed again, and the color was developed using BCIP/NBT alkaline phosphatase substrate (Sigma).

2.3. Ticks

I. scapularis females, nymphs, larvae and eggs and *A. americanum*, *Dermacentor variabilis* and *Rhipicephalus sanguineus* nymphs and adults were obtained from the Oklahoma State University Tick Rearing Facility. *I. pacificus* females were field collected and kindly provided by Dr. Robert B. Kimsey (University of California, Davis, CA, USA). *I. ricinus* and *B. microplus* were kindly provided by Drs. Milan Labuda (Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia) and Robert J. Miller (Cattle Fever Tick Research Laboratory, USDA, Edinburg, TX, USA), respectively.

2.4. RNA extraction and reverse transcriptase (RT)-PCR

Total RNA was extracted from guts and salivary glands dissected from unfed adult ticks or from homogenates of eggs and whole unfed larvae and nymphs. Thirty *I. scapularis* females, approximately 100 and 1000 *I. scapularis* nymphs and larvae, respectively, the egg mass oviposited by one *I. scapularis* female, 10 *D. variabilis* males, 20 *A. americanum* adults, 20 *R. sanguineus* adults, 10 *B. microplus* adults, 10 *I. pacificus* females and 100–150 *I. ricinus* larvae were used. Total RNA was extracted from homogenized tick samples using TRI Reagent (Sigma), except for *I. ricinus* and *B. microplus* RNA which were extracted using the RNA Instapur kit (Eurogentec, Seraing, Belgium) and the RNeasy mini kit (Qiagen, Valencia, CA, USA), respectively, according to the manufacturer's instructions. The final RNA pellet was resuspended in 50–100 μl diethyl pyrocarbonate-treated distilled deionized sterile water. RT-PCR reactions were performed using the Access RT-PCR system (Promega, Madison, WI, USA). One microliter RNA was reverse transcribed in a 50-μl reaction mixture (1.5 mM $MgSO_4$, 1 X avian myeloblastosis virus (AMV) RT/*Thermus flavus* (*Tfl*) reaction buffer, 10 mM random hexamers, 0.2 mM each deoxynucleoside triphosphate (dNTP), 5 U AMV RT, 5 U *Tfl* DNA polymerase (Promega), 10 pmol of each primer) at 48 °C for 45 min. After 2 min incubation at 94 °C, PCR was performed in the same reaction mixture with specific primers (4F8, 4F8R5: 5′-GCGTCGTGTGGAGCATCAGCGAC-3′ and 4F8-R: 5′-TCGCAACGGACAACGGCAGGTTG-3′; 4D8, 4D8R5: 5′-GCTTGCGCAACATTAAAGCGAAC-3′ and 4D8-R: 5′-TGCTTGTTTGCAGATGCCCATCA-3′; 4E6, 4E6R5: 5′-GAAATATCTGTGAAACCAAGGCC-3′ and 4E6U-R: 5′-ATTGCACAACACATCATTAACTG-3′) and amplification conditions (4F8, 30 s at 61 °C and 2 min at 68 °C; 4D8, 30 s at 56 °C and 1 min at 68 °C; 4E6, 30 s at 52 °C and 1 min at 68 °C for annealing and extension steps, respectively). Control reactions were performed using the same procedures but without RT to control for DNA contamination in the RNA preparations and without RNA added to control contamination of the PCR reaction. Positive control reactions for the PCR were performed with plasmid DNA containing the cloned tick cDNAs and with genomic DNA extracted from *I. scapularis* IDE8 cells. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments (972, 577 and 138 bp for 4F8, 4D8 and 4E6 amplicons, respectively) by comparison to a DNA molecular weight marker (1 kb Plus DNA Ladder, Promega).

2.5. Immunohistochemistry of tick tissue sections

Adult *I. scapularis* and IDE8 tick cells derived originally from *I. scapularis* embryos [22] were fixed in formaldehyde and embedded in paraffin. Sections (4 μm) were prepared and mounted on microscope slides that were stored at 4 °C. For immunohistochemistry studies, tissue sections were deparaffinized and dehydrated twice for 5 min in xylene, 100% ethanol, 95% ethanol, followed by a 5-min wash in 80% ethanol. For antigen retrieval, slides were incubated with 0.05% pronase (DakoCytomation, Glostrup, Denmark) diluted in TBS, pH 7.2 during 15 min. The slides were incubated for 1 h with rabbit 4F8, 4D8 or 4E6 antisera prepared as described previously and diluted 1:400 in PBS, pH 7.2. A preimmune rabbit serum and a monospecific rabbit serum prepared with total IDE8 proteins as described previously for recombinant proteins were used as negative and positive controls, respectively. The slides were blocked in PBS/0.5% Tween 20, pH 7.2 (PBST) with 10% goat serum and 5% skim milk for 1 h and then incubated for 1 h with peroxidase-labeled goat anti-mouse IgG (KPL) diluted 1:3000 in PBST. To inactivate the endogenous peroxidase activity, slides were incubated with 3% H_2O_2 in PBS, pH 7 and 10% ethanol for 1 h prior to a 1 min incubation with the substrate 3′,3′-diaminobenzidine tetrahydrochloride Fast DAB set (Sigma) followed by staining with hematoxylin for 2 min. After each treatment, the slides were rinsed twice for 5 min in PBST, unless otherwise indicated. All

incubations were done at room temperature. For microscopic examination, the slides were rinsed with distilled water and dehydrated two times for 2 min each in 95% ethanol, 100% ethanol, and finally xylene, and mounted in permount.

2.6. Biological function of recombinant 4F8 putative tick nucleotidase

In order to characterize the biological function of the of recombinant 4F8 putative tick nucleotidase, an affinity purification experiment was conducted based on the nucleotidase's activity on 3′-phosphoadenosine 5′-phosphate (PAP) [23]. PAP-agarose resin (Sigma) was swelled in PAP-agarose buffer (50 mM HEPES, pH 7.5, 10 mM $CaCl_2$, and 50 mM KCl) and 3 ml were poured into a 4-ml glass chromatography column (Sigma). Calcium was added to satisfy the metal requirement for substrate binding while preventing the hydrolysis of immobilized PAP by homologous phosphomonoesterases. Purified recombinant 4F8 (0.4 mg) was resuspended in 1 ml PAP-agarose buffer and applied to the column by gravity flow. The column was washed with 10 column volumes of PAP-agarose buffer plus 0.5 M NaCl and then reequilibrated with 3 column volumes of PAP-agarose buffer containing no additional salt. The enzyme was eluted with 3 ml PAP-agarose buffer containing 300 μM 2′/3′,5′-PAP (Sigma). Protein fractions in the column pass flow (unbound protein) and after elution with 2′/3′,5′-PAP were collected and concentrated in Amicon Ultra-15 centrifugal filter devices, 10,000 nominal molecular weight limit (Millipore; Bedford, MA, USA). Both protein samples were then analyzed by Western blot using anti-FLAG M2 monoclonal antibodies (Sigma) as described above.

2.7. Protective properties of tick antigens

Three experiments were conducted to evaluate the effect of 4D8, 4F8 and 4E6 antigens on larval, nymphal and adult tick infestations. The first experiment was designed to compare the effect elicited by protein antigens with that previously obtained with tick cDNAS following the same protocol of mouse immunization and infestation with *I. scapularis* larvae [11]. The second experiment was conducted to obtain preliminary data on the inhibitory effect of the tick protective antigens on nymphal infestations by *I. scapularis*, *D. variabilis* and *A. americanum*. For this experiment, rabbits were chosen because they are a better host to support the infestation with nymphs of several tick species and were immunized following the protocol described above to elicit a strong antibody response with 4D8, 4F8 and 4E6 antigens. The third experiment was conducted in order to obtain preliminary data on the effect of the immune response to recombinant protective antigens against adult *I. scapularis* stages using an artificial feeding method followed by completion of the feeding period on a sheep, which supports adult tick infestations and is the host routinely used to feed *I. scapularis* adults at the Oklahoma State University Tick Rearing Facility.

2.7.1. Immunization of mice and infestation with I. scapularis larvae

Five groups of six mice CD-1 female mice, 5–6 weeks of age at the time of first immunization, were used in this experiment. Experimental groups included immunization with 10 μg/dose of 4F8 or 4D8 recombinant proteins, 4E6 synthetic peptide, and recombinant *Anaplasma marginale* MSP1a [24], which did not affect tick feeding [25], or vehicle (TBS)/adjuvant alone to serve as controls. The mice were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals. Mice were injected subcutaneously with each antigen or TBS at weeks 0 and 2 with 100 μl/dose in FIA (Sigma) using a 1-ml tuberculin syringe and a $27\frac{1}{2}$ G needle as described previously [11]. In separate experiments we have shown that immunization with FIA did not have an effect on tick feeing (unpublished results). Two weeks after the last immunization, mice were infested with 100 *I. scapularis* larvae per mouse as described previously [11]. The unattached larvae were counted and removed 12 h after infestation, and engorged larvae were collected daily for 7 days from each mouse and counted. After tick feeding, the mice were euthanized by cervical dislocation, the blood was collected and the serum removed and stored. The engorged larvae were held in a humidity chamber for 34 days, after which molting was evaluated using a dissecting light microscope. The inhibition of tick infestation (*I*) and inhibition of molting (*M*) for each test group with respect to the MSP1a-immunized controls and the overall efficacy of the vaccine (*E*) were calculated as described previously [11]. The protection efficacy of the vaccine was calculated as $E = 100 \times [1 - (\mathrm{RI} \times \mathrm{RM})]$ where RI (average reduction in tick infestation) $= 1 - I/100$ and RM (average reduction in molting) $= 1 - M/100$.

2.7.2. Characterization of the immune response in the immunized mice by Western blot

Ten micrograms of 4F8 and 4D8 recombinant proteins and 4E6 synthetic peptide were loaded on a 12.5% polyacrylamide gel using a preparative comb for Western blot analysis of mouse immune response as described previously [11].

2.7.3. Characterization of the immune response in the immunized mice by ELISA

The antibody response against tick antigens in immunized and control mice was evaluated by ELISA. Antibody levels to *I. scapularis*recombinant 4F8, 4D8 and 4E6 were detected by indirect ELISA. Purified recombinant proteins were used to coat ELISA plates over night at 4 °C. Sera were serially diluted to 1:10 and 1:100 in PBST 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 sheep anti-mouse IgG-HRP conjugate (Sigma) for 1 h at 37 °C. The color reaction was developed with 3,3′,5,5′-tetramethylbenzidine (Sigma) and the $OD_{450\,nm}$ was determined. After incubations the plates were washed with PBST. Antibody titers were considered positive when yielded an OD value at least twice as

high as the negative control serum and were expressed as the geometric mean (mean ± S.D.) at the 1:100 serum dilution of the six immunized mice OD minus the OD of the control group.

2.7.4. Immunization of rabbits and infestation with I. scapularis, D. variabilis and A. americanum nymphs

Preliminary data on the inhibitory effect of the three tick protective antigens on nymphal infestations of *I. scapularis*, *D. variabilis* and *A. americanum*was obtained using immunized and control rabbits. One New Zealand White rabbit per group was each immunized with three doses (weeks 0, 4 and 7) containing 50 μg/dose purified 4F8 and 4D8 proteins or 4E6 synthetic peptide, a combination of 4F8 + 4D8 + 4E6 or bovine serum albumin (Sigma) as control in FIA (Sigma). Rabbits were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals. Rabbits were injected subcutaneously with 500 μl/dose using a 1-ml tuberculin syringe and a $27\frac{1}{2}$ G needle. Two weeks after the last immunization, each rabbit was infested in ear bags with 100 *I. scapularis* nymphs per rabbit in one ear and 110 nymphs of each *D. variabilis* and *A. americanum* on the other ear. The unattached nymphs were counted and removed 24 h after infestation. The ear bag was then removed from the *D. variabilis* and *A. americanum* infested ear and replete nymphs from each rabbit were collected on each of the next 7 days from either the ear bag left (for *I. scapularis*) or from water in the bottom of the cage (for *D. variabilis* and *A. americanum*), and were weighed and counted. *D. variabilis* and *A. americanum* engorged nymphs were separated under a dissecting light microscope. The inhibition of tick nymphal infestation was calculated in comparison with the control group as described above for larval tick infestations. Reduction in the weights of engorged nymphs was determined in experimental groups with respect to the nymphs collected from the control groups.

2.7.5. Capillary feeding of adult I. scapularis with rabbit immune sera against tick protective antigens

Twenty unfed female *I. scapularis* per group were used for capillary feeding (CF) experiments as described previously [26]. Five groups were included in the CF experiment that were fed tickmeals with rabbit immune sera against 4F8, 4D8, 4E6, 4F8 + 4D8 + 4E6, or rabbit preimmune serum as control. The 4F8 + 4D8 + 4E6 serum contained 4F8, 4D8 and 4E6 sera combined in equal amounts prior to preparing the tickmeal for CF. The quantity of serum consumed per tick was determined by measuring the difference in level of serum in the micropipets when first filled and prior to changing of the meal. The height of the ingested serum meal column (mm) was determined daily and used to calculate the total volume of ingested serum per tick. After 2 days of CF, the ticks were removed from the tape and placed with 10 unfed males per group in separate orthopedic stockinettes glued to the side of a sheep and allowed to attach and feed for 11 days. Replete ticks were collected daily and the number recorded, and the ticks were weighed prior to incubation in a humidity chamber and held until oviposition was completed (40 days). The egg mass from each tick was weighed and recorded. The number of dead ticks during feeding and oviposition was also recorded.

2.7.6. Statistical analysis

The ratio of the number of engorged larvae recovered per mouse 7 days after infestation (RL) and the number of larvae attached per mouse (RLi) and the ratio of the number of nymphs (ML) and the number of engorged larvae per mouse (RL) were compared by Student's *t*-test between tick antigen-immunized and control MSP1a-immunized mice in the mouse vaccination experiment. In the CF experiment, ingested serum volume, tick weights and weight of egg mass of ticks capillary fed on immune sera were compared using a Student's *t*-test with the control ticks fed on preimmune serum. The ratios of dead to fed ticks were compared by χ^2-test. The number of ticks on the sheep was recorded daily and compared using a Wilcoxon signed rank test. Wilcoxon signed rank and χ^2-tests were implemented using Mstat 4.01.

3. Results

3.1. Characterization of protective tick cDNAs

The *I. scapularis* cDNA clones 4F8, 4D8 and 4E6 that caused inhibition of tick infestation in ELI experiments were sequenced and the ORFs identified. The cDNA clone 4F8 contained a cDNA insert of 1791 bp plus a poly-A tail of 30 bases. An ORF of 951 bp was identified encoding for a protein of 316 amino acids with a predicted molecular weight of 34.7 kDa. The protein was predicted to be a soluble polypeptide localized in the cytoplasm of the cell. Protein domain analysis resulted in identity to highly conserved protein families KOG3099, bisphosphate 3′-nucleotidase BPNT1/Inositol polyphosphate 1-phosphatase involved in nucleotide transport and metabolism ($E = 4e{-}84$) and KOG3853, inositol monophosphatase involved in signal transduction mechanisms ($E = 8e{-}38$). The 4F8-encoded protein contained the consensus for the sequence involved in metal binding and catalysis (D-X_n-EE-X_n-DP(i/l)D(s/g/a)T-X_n-WD-X_{11}-GG; ref. [23]) and was similar to *A. variegatum* (TIGR gene index TC259; 64%), mouse nucleotidase (Genbank accession number AAH11036; 61%), human nucleotidase (AAD17329; 60%), *A. gambiae* (XP_316740; 61%), *D. melanogaster* (AAF56941; 58%), *C. elegans* (NP_494780; 46%) and *X. laevis* (AAH59974; 16%) sequences. Sequence analysis suggested that 4F8 encoded for a tick nucleotidase. The putative function of 4F8 as a nucleotidase was then analyzed in a biological assay showing that the recombinant protein bound to the immobilized substrate PAP and specifically eluted with 2′/3′,5′-PAP (data not shown).

The cDNA clone 4D8 contained a cDNA insert of 2664 bp plus a poly-A tail of 79 nucleotides. This cDNA contained

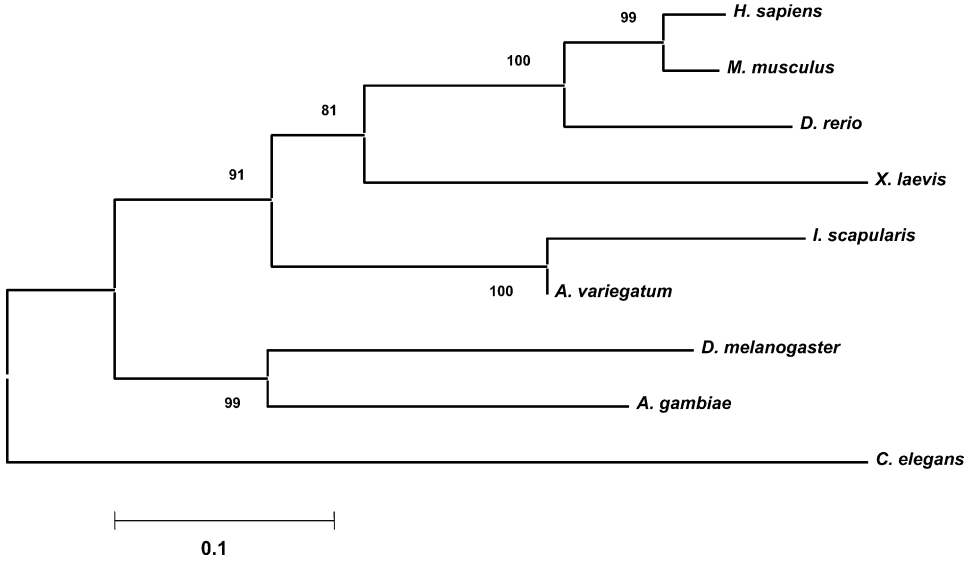

Fig. 1. Phylogenetic relationships of 4D8 protein sequences using the minimum-evolution criterion. Bootstrap values for 1000 replicates are shown.

a large 3′ untranslated region (UTR) of 2030 bp excluding the poly-A tail and a 5′ UTR of 79 bp. An ORF of 555 bp was identified encoding for a putative soluble cytoplasmic protein of 184 amino acids with a predicted molecular weight of 20.7 kDa. A conserved protein domain (KOG4330) was localized in the 4D8-encoded protein that was present in uncharacterized conserved proteins of unknown function. The protein encoded by 4D8 was similar to *A. variegatum* (T10865; 32%; 100% similarity in the 40 amino acids reported for *A. variegatum*), *D. rerio* (AAQ94594; 56%), mouse (XP_131324; 51%), human (NP_060534; 49%), *X. laevis* (AAH43949; 48%), *A. gambiae* (EAA04195; 47%), *D. melanogaster* (AAF50569; 46%) and *C. elegans* (NP_491304; 24%) sequences. Phylogenetic analysis of 4D8 protein sequences revealed similar tree structures for MP, ME and NJ algorithms (Fig. 1 and data not shown). Tick sequences clustered together, separated from insect and vertebrate sequences (Fig. 1).

The cDNA clone 4E6 contained a small insert of 320 bp excluding a 29 bases poly-A tail. This insert contained an ORF of 117 bp encoding for a soluble peptide of 38 amino acids with a predicted molecular weight of 4.4 kDa or corresponding to a truncated protein from an incomplete cDNA. The only sequence found in the databases with homology to 4E6 corresponded to a *D. melanogaster* protein of unknown function (AAL90160; 39% similarity in the region comprising the 38 amino acids of 4E6).

3.2. Expression of recombinant proteins

Recombinant 4F8 and 4D8 proteins were expressed in *E. coli* fused on the C-terminal to a FLAG marker octapeptide for purification by FLAG-affinity chromatography. Expression levels of recombinant proteins after induction reached approximately the 5–10% of total cellular proteins (Fig. 2, lanes 2 and 4). Rabbit immune sera were prepared with recombinant proteins 4F8 and 4D8 and with the synthetic peptide 4E6. Rabbit sera specifically recognized 4F8 and 4D8 proteins in induced *E. coli* protein extracts (Fig. 3A, lanes 2 and 4) and in purified protein preparations (Fig. 3A, lanes 3 and 5). The rabbit serum against the 4E6 peptide recognized the peptide in Western blots (Fig. 3A, lane 6). The anti-FLAG M2 monoclonal antibody specifically recognized the FLAG peptide fused to the C-terminal of 4F8 and 4D8 proteins. Combined analysis of Western blots of *E. coli*-induced and

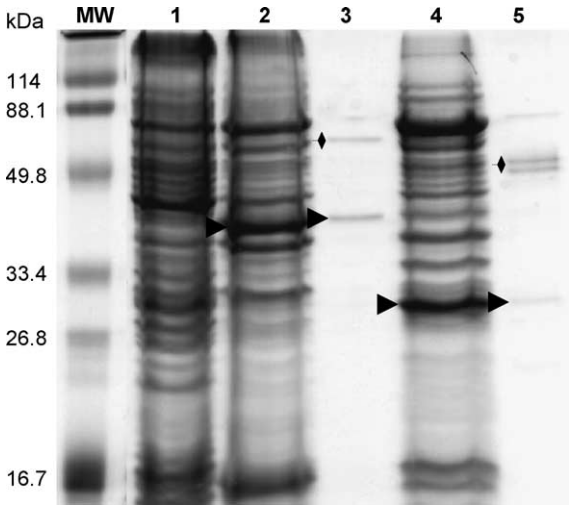

Fig. 2. PAGE of recombinant 4F8 and 4D8 proteins. Protein samples were loaded on 12.5% polyacrylamide gels that were stained with Coomassie Brilliant Blue. Lane 1, induced *E. coli* cells (control); lane 2, induced *E. coli* expressing 4F8; lane 3, purified 4F8; lane 4, induced *E. coli* expressing 4D8; lane 5, purified 4D8. Arrows indicate the size of the proteins while diamonds indicate the presence of higher molecular weight products. MW, molecular weight markers (TriChromRanger marker, Pierce Biotechnology, Inc., Rockford, IL, USA).

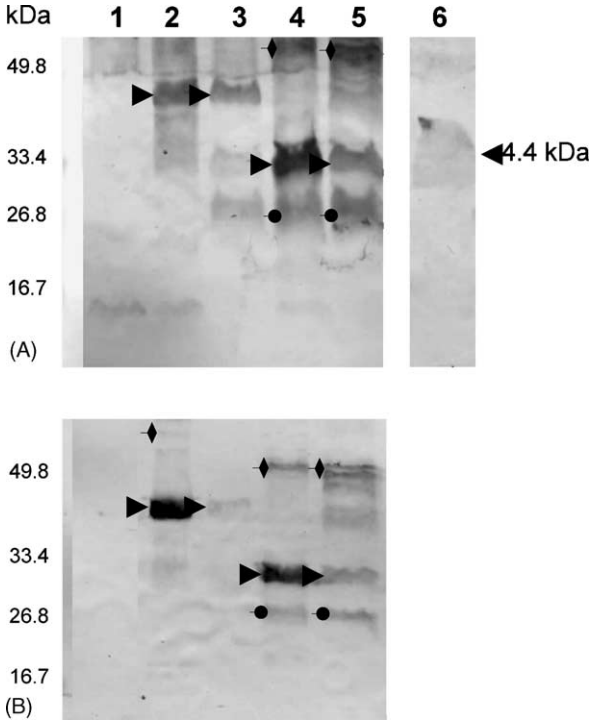

Fig. 3. Western blot analysis of recombinant 4F8 (lanes 2 and 3) and 4D8 (lanes 4 and 5) proteins and synthetic peptide 4E6 (lane 6). Samples of induced *E. coli* proteins, recombinant proteins expressed in *E. coli* (lanes 2 and 4) and after purification (lanes 3 and 5) and the synthetic peptide were separated by SDS-PAGE and reacted with (A) monospecific rabbit immune sera or (B) anti-FLAG M2 monoclonal antibody. Arrows indicate the size of the proteins while diamonds and circles indicate the presence of higher and lower molecular weight products, respectively. MW, molecular weight markers (TriChromRanger marker, Pierce).

purified proteins with the anti-FLAG M2 monoclonal antibody and the rabbit immune sera evidenced the presence of higher molecular weight proteins in 4F8 (Fig. 3B, diamond in lane 2) and 4D8 (Fig. 3A and B, diamond in lanes 4 and 5), also present in the PAGE (Fig. 2, lines 3 and 5), and lower molecular weight products in 4D8 (Fig. 3A and B, circle in lines 4 and 5). Higher molecular weight products corresponded in size to protein dimers and lower molecular weight polypeptides could represent degradation products. After purification, the purity of recombinant proteins was estimated to be ≥90% as assayed by densitometry scanning of protein gels (Fig. 2, lanes 3 and 5) considering all products specifically recognized in Western blot analyses (Fig. 3A and B).

3.3. Expression of protective cDNAs

Expression of genes encoding for tick protective antigens was analyzed at mRNA and protein levels by RT-PCR and immunohistochemistry, respectively. Expression of 4F8, 4D8 and 4E6 mRNA was detected in *I. scapularis* eggs, larvae and nymphs and in guts and salivary glands from adult ticks (Fig. 4). Control reactions ruled out contamination with genomic DNA or during the PCR (Fig. 4). Furthermore, PCR of tick genomic DNA showed that the size of the amplified DNA fragments was higher than the size corresponding to cDNA fragments, probably due to the presence of intron sequences in the analyzed genes (data not shown).

Protective polypeptides were detected by immunohistochemistry in *I. scapularis* IDE8 cells (Fig. 5). Furthermore, proteins 4D8 and 4E6 were also detected in *I. scapularis* gut sections (Fig. 5). The positive control anti-IDE8 proteins serum labeled IDE8 cells and tick gut sections (Fig. 5). Labeling was not seen in sections reacted with the negative control rabbit preimmune serum (Figs. 5).

Sequence conservation and expression of 4F8, 4D8 and 4E6 were analyzed in *I. scapularis* related species, *I. pacificus* and *I. ricinus*, and in *D. variabilis*, *R. sanguineus*, *B. microplus* and *A. americanum* by RT-PCR using the primers derived from *I. scapularis* sequences. Expression was detected in *I. ricinus* for all three genes and in *I. pacificus* and *A. americanum* for 4D8 and 4E6 (Fig. 4). Expression of 4D8 was also detected in *D. variabilis*, *B. microplus* and *R. sanguineus* (Fig. 4).

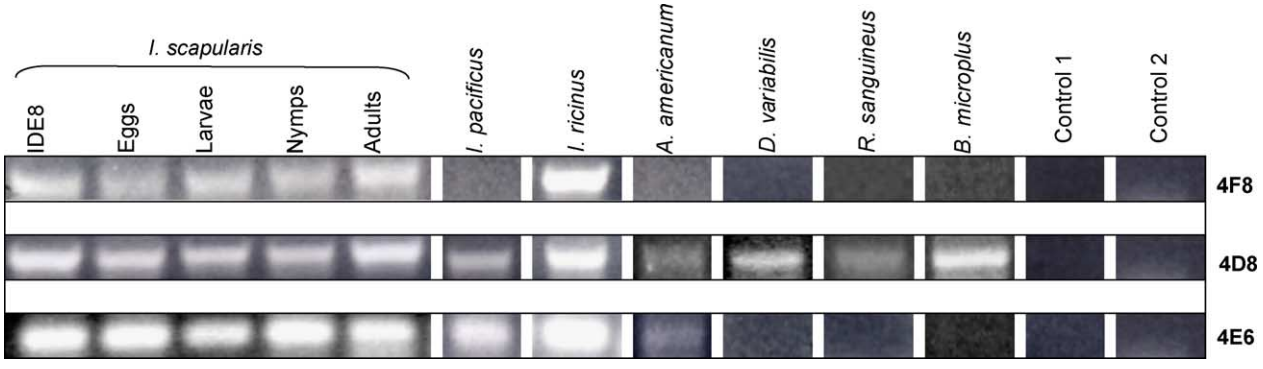

Fig. 4. Expression of tick protective antigen mRNAs in *I. scapularis* developmental stages and adult tissues and in other tick species. RT-PCR reactions were done with primers specific for 4F8, 4D8 and 4E6 *I. scapularis* sequences with conditions described in Section 2. Control reactions were performed using the same procedures but without RT to control for DNA contamination in the RNA preparations (control 1) and without RNA added to control contamination of the PCR reaction (control 2).

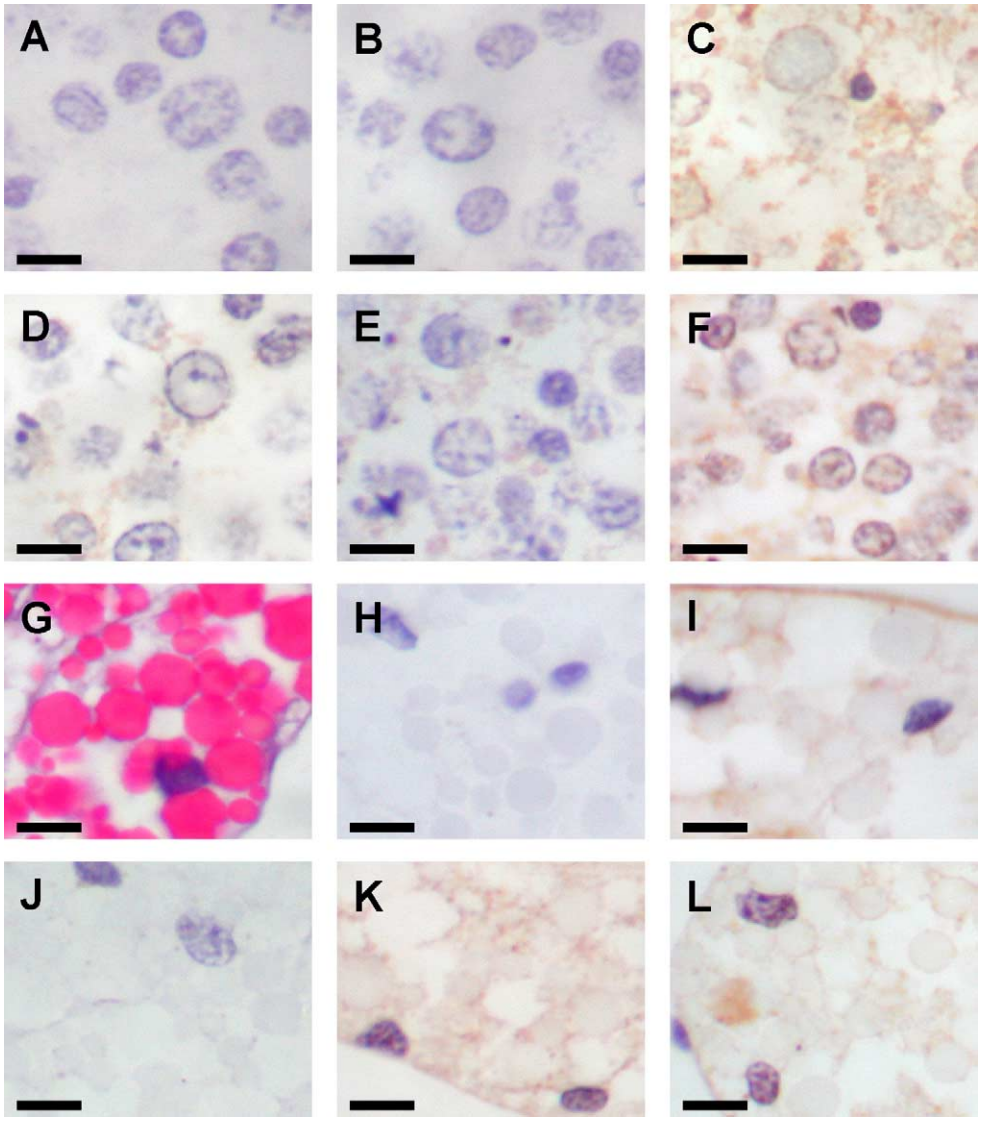

Fig. 5. Localization of tick protective antigens by immunohistochemistry in paraffin sections of IDE8 tick cells (A–F) and adult *I. scapularis* guts (G–L). Sections A and G, stained with hematoxylin and eosin (bar 6.7 μm (A) and 6.9 μm (G)); B and H, negative controls reacted with rabbit preimmune serum (bar 6.7 μm (B) and 6.9 μm (H)); C and I, positive controls reacted with anti-IDE8 antibodies (bar 6.9 μm (C) and 6.7 μm (I)); D and J, reacted with anti-4F8 antibodies (bar 7.0 μm); E and K, reacted with anti-4D8 antibodies (bar 6.8 μm (E) and 6.9 μm (K)); F and L, reacted with anti-4E6 antibodies (bar 6.7 μm (F) and 6.8 μm (L)). Original magnification, 600×. All sections except A and G were counterstained with hematoxylin.

3.4. Protective properties of tick antigens against I. scapularis larval infestations

The first vaccination experiment was designed to evaluate the effect of recombinant or synthetic proteins on larval *I. scapularis* infestations as evaluated previously for cDNA immunizations [10]. Mice were immunized with 10 μg/dose of purified recombinant 4F8 and 4D8 proteins and with the synthetic 4E6 peptide. Control mice received 10 μg/dose of the unrelated recombinant MSP1a protein or saline/adjuvant alone. Antibody response against protective antigens was confirmed in mice after immunization by ELISA (mean OD ± S.D. of 1.0 ± 0.5, 0.9 ± 0.6 and 0.4 ± 0.04 for 4D8, 4F8 and 4E6 immunized mice, respectively) and Western blot (data not shown). Sera from mice immunized with adjuvant/vehicle alone did not recognize 4F8, 4D8, 4E6 or MSP1a (data not shown). Vaccination with recombinant proteins 4F8 and 4D8 and with the synthetic peptide 4E6 resulted in protection of mice against tick larval infestations. Mice vaccinated with tick protective antigens showed a decrease in tick infestations (RL/RLi) when compared to controls, although it was statistically significant for 4F8 and 4D8 only (Table 1). The inhibition of tick infestations (*I*) was ≥46% for all three tick antigens (Table 1). A decrease in the molting of tick larvae to nymphs was found in ticks that fed on mice immunized with 4D8 and 4E6 proteins, while molting of larvae that fed on 4F8-immunized mice was similar to controls (Table 1). Differences in tick infestations

Table 1
Results of vaccination with recombinant tick protective antigens on *I. scapularis* larvae

Experimental group	RL/RLi	*I* (%)	ML/RL	*M* (%)	*E* (%)
4F8	0.14 ± 0.07[a]	64	0.84 ± 0.05	−5	62
4D8	0.15 ± 0.10[a]	61	0.59 ± 0.30[a]	26	71
4E6	0.21 ± 0.17	46	0.54 ± 0.29[a]	32	63
Control (MSP1a)	0.39 ± 0.33	–	0.80 ± 0.17	–	–

The inhibition of tick larval infestation (*I*) for each test group was calculated with respect to controls as $[1 - (RL/RLc \times RLic/RLi)] \times 100 = [1 - (RL/RLi \times RLic \times RLc)] \times 100$, where RL is the average number of replete larvae recovered per mouse in test groups, RLc is the average number of replete larvae recovered per mouse in controls, RLic is the average number of larvae attached per mouse in controls, and RLi is the average number of larvae attached per mouse for each test group. The inhibition of molting (*M*) for each test group was calculated with respect to controls as $[1 - (ML/MLc \times RLc/RL)] \times 100 = [1 - (ML/RL \times RLc/MLc)] \times 100$, where ML is the average number of nymphs per mouse for each test group and MLc is the average number of nymphs per mouse for the control group. The protection efficacy of the vaccine was calculated as $E = 100 \times [1 - (RI \times RM)]$ where RI (average reduction in tick infestation) $= 1 - I/100$ and RM (average reduction in molting) $= 1 - M/100$.

[a] Significantly different from controls ($P < 0.05$).

were not observed between MSP1a-immunized mice and mice that received saline/adjuvant alone (data not shown). The overall efficacy of immunization was calculated for each tick antigen considering the effect on larval infestations and on molting to nymphs and ranged from 62 to 71% (Table 1).

3.5. Protective properties of tick antigens against *I. scapularis*, *D. variabilis* and *A. americanum* nymphal infestations

The detection of 4D8 and 4E6 expression in non-*Ixodes* tick species and the evaluation of the effect of tick protective antigens on tick nymphal infestations were the basis for an experiment in which rabbits were immunized and challenged with *I. scapularis*, *D. variabilis* and *A. americanum* nymphs. Although viewed as preliminary data because of the use of one rabbit per group only, the results demonstrated an effect of 4D8 immunization on the inhibition of nymphal infestations in all three tick species. In addition, a notable effect of immunization with 4D8 was observed on *D. variabilis* nymphs by the number of visibly damaged ticks (Fig. 6) and a reduction in the weight of engorged nymphs (Table 2). Immunization with 4F8 affected *I. scapularis* nymphal infestations and immunization of rabbits with 4E6 affected *A. americanum* infestations and the weights of *D. variabilis* engorged nymphs (Table 2).

3.6. Effect of rabbit immune sera against recombinant proteins on adult *I. scapularis*

Capillary feeding experiments were conducted in order to obtain preliminary data on the effect of the immune response to recombinant protective antigens against adult *I. scapularis* stages. Female ticks that were capillary fed for 2 days with rabbit immune sera prepared against recombinant proteins and were then allowed to feed on a sheep for 11 days had reduced feeding periods as compared to controls ($P < 0.05$; Fig. 7). In addition, the weight of these ticks at repletion

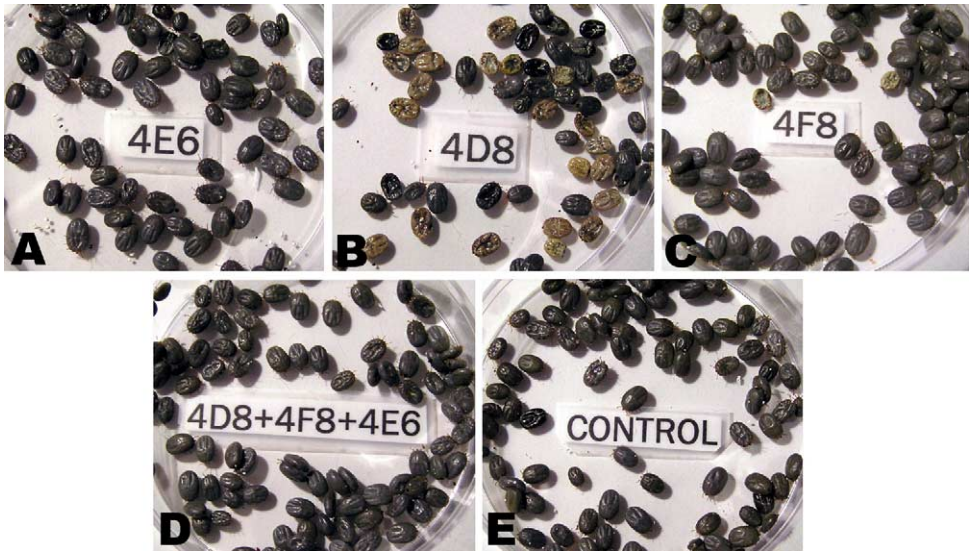

Fig. 6. Effect of vaccination with recombinant *I. scapularis* antigens on *D. variabilis* nymphs. Engorged nymphs were collected from rabbits immunized with recombinant tick antigens and inspected for morphological changes in comparison with nymphs recovered from the control group. Nymphs visibly damaged were observed in the group that fed on the 4D8-vaccinated rabbit.

Table 2
Results of vaccination with recombinant tick protective antigens on *I. scapularis*, *D. variabilis* and *A. americanum* nymphs

Tick species	Inhibition of tick nymphal infestation (%)[a]				Reduction in the weight of engorged nymphs (%)[b]			
	4D8	4F8	4E6	All[c]	4D8	4F8	4E6	All[c]
I. scapularis	35	39	0	63	0	0	0	0
D. variabilis	22	0	5	8	32	0	27	0
A. americanum	17	9	29	12	3	1	0	16

[a] Determined with respect to the number of engorged nymphs recovered from the control group as described above for tick larval infestations.
[b] Average weight per engorged nymph with respect to the weight of nymphs collected from the control group.
[c] Rabbit in this group was immunized with a combination of 4D8, 4F8 and 4E6 antigens.

was reduced in comparison with the control group, with significant differences in the weights of ticks capillary fed on 4D8 and 4E6 immune sera ($P < 0.05$; Table 3). All ticks survived CF, but tick mortality during 11 days of feeding on the sheep was higher in ticks fed on 4D8, 4E6 and the combined immune sera ($P < 0.05$; Table 3). For ticks that survived feeding and completed engorgement, a significant reduction in weight was observed in ticks that fed on 4D8 immune serum ($P < 0.05$; Table 3). When egg mass weight and tick mortality were determined at 40 days post-oviposition, the reduction in oviposition observed for ticks fed on 4D8 immune serum was statistically significant ($P < 0.05$; Table 3). Furthermore, the overall tick mortality was significantly higher for ticks that fed on 4D8 immune serum ($P < 0.01$; Table 3). Although ticks capillary fed on 4F8, 4D8 and 4E6 immune sera ingested more serum than the controls fed on preimmune serum or those fed on the combined immune sera ($P < 0.05$; Table 3), a correlation was not observed between the volume of ingested serum by CF and the effect on ticks. Notably, the effect of the 4D8 immune serum had the most pronounced effect on tick feeding, oviposition and survival (Table 3).

4. Discussion

The feasibility of controlling tick infestations through immunization of hosts with tick antigens has been demonstrated for *Boophilus* spp. (reviewed by [4,5]). Although proteins with the capacity to control tick infestations have been described in various tick species, identification of tick protective antigens has continued to be the limiting step in the process of developing new effective tick vaccines (reviewed by [4,5]). Recently, we used a high throughput screening method for the identification of tick protective antigens in *I. scapularis* by using cDNA ELI and EST analysis [11,12]. Immunization of mice with the *I. scapularis* 4F8 and 4D8 cDNAs resulted in 50 and 40% inhibition of tick infestations, respectively [11,12], and these cDNAs were therefore selected for further characterization. 4E6 cDNA, although inhibited tick infestations at a lower level (20%) compared to 4D8 and 4F8 [12], was selected for further evaluation because it encodes a small protein of 38 amino acids which could be interesting to use in chimeric polypeptides or in combination with other antigens for vaccination against ticks.

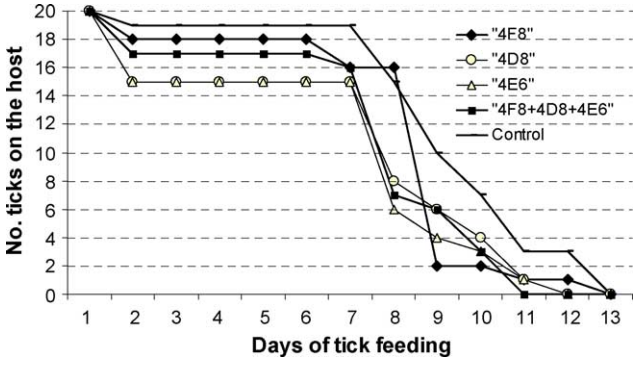

Fig. 7. Daily number of female ticks feeding on sheep after capillary feeding on rabbit immune sera prepared against recombinant tick protective antigens.

Recombinant polypeptides were produced for 4F8 and 4D8 in *E. coli* and a synthetic peptide was prepared for the short 4E6 protein. Vaccination of mice with recombinant 4F8 and 4D8 proteins and with the synthetic peptide 4E6 resulted in an overall vaccine protection efficacy of >60% against *I. scapularis* larval infestations. Previous pen vaccine trials using Bm86 and Bm95 recombinant antigens with the one-host cattle tick *B. microplus* showed that a vaccine efficacy higher than 50% resulted in control of tick populations in the field (reviewed by [4]). However, in the three-host tick, *I. scapularis*,the efficacy of immunization with 4F8, 4D8 and 4E6 on all tick developmental stages is unknown. Nonetheless, preliminary immunization trials in rabbits and CF studies suggested that immune sera against 4F8, 4D8 and 4E6 have an inhibitory effect on *I. scapularis* nymphs and adults. Although statistical analyses could not be done in the rabbit immunization trial because of the use of one rabbit per group, the results suggested an effect of 4D8 and 4F8 immunizations on *I. scapularis* nymphs. The lack of statistical significance in some analyses of the CF experiment may reflect differences in the antibody concentration between the different immune sera, differences in the activity of immune sera against adult ticks or the result of the small number of ticks used in the CF experiment. Nevertheless, these results are particularly encouraging because adult ticks were exposed to specific antibodies for two days only, which represents 15% of the total feeding time. Ticks feeding on artificial membrane systems or on ascitic mice producing tick-specific IgGs and inoculation of female ticks with immune sera have been used before to test the inhibitory effect of antibodies

Table 3
Effect of capillary feeding with rabbit immune sera prepared against recombinant protective antigens on adult female *I. scapularis*

Immune serum	Volume of serum ingested by CF (μl) ($N = 20$)	Weight of ticks (mg) after 13 days of feeding ($N = 20$)	No. dead ticks during the 13 days of feeding	Ratio of dead/fed ticks (%)[a]	Weight of engorged ticks (mg) that survived after 13 days of feeding	Weight of egg mass (mg) after 40 days of oviposition	No. dead ticks during the 40 days of oviposition	Ratio of total dead/fed ticks[b]
4F8	4.9 ± 0.8[c]	183.8 ± 93.7	2	10	208.9 ± 72.0	105 ± 39	1	15
4D8	4.7 ± 0.6[c]	141.7 ± 97.5[c]	5	25[d]	188.9 ± 57.8[c]	103 ± 24[c]	4	45[d]
4E6	4.5 ± 0.7[c]	164.5 ± 105.9[c]	5	25[d]	219.3 ± 48.4	103 ± 34	1	30
4F8 + 4D8 + 4E6	4.2 ± 0.8	212.9 ± 102.7	3	15[e]	250.5 ± 50.3	116 ± 39	2	25
Control[f]	4.2 ± 0.5	219.9 ± 90.5	1	5	231.4 ± 76.3	120 ± 28	3	20

[a] Ticks that died during feeding with respect to the 20 ticks that were fed.
[b] Ticks that died during the entire experiment (feeding + oviposition) with respect to the 20 ticks that were fed.
[c] Different from control ($P < 0.05$; Student's *t*-test).
[d] Different from control ($P < 0.01$).
[e] Different from control ($P < 0.05$; χ^2-test).
[f] Control ticks were capillary fed on rabbit preimmune serum.

against tick antigens in *B. microplus* [27,28], *Ornithodoros moubata* [29] and *Haemaphysalis longicornis* [30].

The degree of protection obtained with 4F8, 4D8 and 4E6 protein vaccine formulations was higher than that obtained with cDNA vaccination [11,12]. The inhibition of tick infestation for protein versus cDNA vaccination was 64% versus 50% for 4F8, 61% versus 40% for 4D8 and 46% versus 20% for 4E6. The inhibition of molting was assayed with cDNA immunization for 4F8 ($M = 17\%$) and 4D8 ($M = 7\%$) in previous experiments [11]. Although inhibition of molting was not observed in the experiments described herein for the group immunized with recombinant 4F8, the vaccination efficacy observed was higher for 4D8 protein formulation when compared to cDNA vaccination (71% versus 44%). Additionally, antibodies against protective antigens were detected in all protein-immunized mice, a finding that contrasted with the poor antibody response observed in cDNA-vaccinated mice [11]. The higher protection efficacy obtained with protein vaccine formulations probably reflects a higher stimulation of the host antibody response by protein immunization (reviewed by [31]) and suggests a role for antibodies in the mechanism of protection with tick vaccine formulations [32]. Immunoglobulins trigger a variety of effector mechanisms and are specifically transported into the tick hemolymph during feeding [33,34]. Therefore, immunization with tick antigens may target a variety of tick cell proteins.

The expression of 4F8, 4D8 and 4E6 in all *I. scapularis* developmental stages suggested that these genes are constitutively expressed throughout the life cycle of the tick. In addition, expression was detected for all three genes in *I. ricinus* and for 4D8 and 4E6 in *I. pacificus*, also members of the *I. ricinus* complex. Recent analyses indicate that members of the *I. ricinus* species complex are closely related despite the fact that they are distributed in different regions of the world [35]. These results strongly suggest the possibility for *I. scapularis* protective antigens to be cross-protective against other tick species of the *I. ricinus* complex.

Expression of 4F8, 4D8 and 4E6 mRNA was detected in tick guts and salivary glands and the expression of 4D8 and 4E6 was confirmed at the protein level in tick gut sections. Vaccine trials with tick recombinant proteins have demonstrated the feasibility of controlling tick infestations by use of gene products that are expressed in different tick tissues and are involved in various physiological processes (reviewed by [4,5]). Targeting gut antigens may result in impairment of tick development by interfering with uptake of the blood meal and digestion, which may cause the visible damage observed in *D. variabilis* nymphs fed on the 4D8-immunized rabbit as previously noted on *I. scapularis* [11] and *B. microplus* [36,37] fed on mice and cattle immunized with tick cDNAs and Bm86 and Bm95 recombinant proteins, respectively. The use of antigens expressed in tick salivary glands may affect the production of factors directed to overcome the immune responses of the host, thus affecting tick engorgement (reviewed by [5]). The identification of tick antigens such as 4F8, 4D8 and 4E6 that are expressed in various tissues may

indicate a conserved function for these proteins and the possibility of targeting several tick physiological processes that affect tick survival and development (reviewed by [4,5]).

The protein encoded by 4D8 was found to be highly conserved and tracked ancestors to the bilateria (Taxblast threshold 10^{-3}). With the exception of the position of fish and amphibian sequences, 4D8 trees were in accordance with the phylogenetic relationships proposed for eukaryotes [38]. Expression of 4D8 mRNAs were detected in all *I. scapularis* developmental stages (eggs, larvae, nymphs and adults). Furthermore, the detection of 4D8 mRNA expression using primers identical to the *I. scapularis* sequence in related tick species, *I. ricinus* and *I. pacificus*, and in the more phylogenetically distant species, *A. americanum*, *B. microplus*, *R. sanguineus* and *D. variabilis* [39], suggests that the function of 4D8 is widely conserved among tick species. Expression of the human 4D8 homologue has been detected in a variety of adult and fetal tissues (expression information for NCBI UniGene Cluster Hs.201864 *Homo sapiens*) and in *C. elegans* the 4D8 homologue was expressed in all developmental stages [40]. Interestingly, the biological function of 4D8 is unknown, but may be involved in the control of developmental processes as deduced from the possible involvement of the *D. melanogaster* homologue in the dorsal-thorax formation of the embryo [41] and the embryonic lethal phenotype obtained by RNA interference with the *C. elegans* homologue [40]. Importantly, conservation of 4D8 in different tick species suggests that this antigen may be useful in vaccine formulations designed for the control of multiple tick species, as evidenced by our preliminary experiments in which immunized rabbits were challenged with both *D. variabilis* and *A. americanum* nymphs.

The cDNA clone 4F8 encodes for a metal-dependent tick nucleotidase, a member of the phosphomonoesterase protein family that is involved in nucleotide transport and metabolism and signal transduction [23]. In general, 5′-nucleotidase has been considered as a marker enzyme for the plasma membrane, and is considered to be a key enzyme in the generation of adenosine, a potential vasodilator [42]. However, from its wide range of localization in tissues it is also considered to be related to the membrane movement of cells in the transitional epithelium, cellular motile response, transport process, cellular growth, synthesis of fibrous protein and calcification, lymphocyte activation, neurotransmission, and oxygen sensing mechanisms [42]. The inhibition of nucleotidases results in the accumulation of PAP and 3′-phosphoadenosine 5′-phosphosulfate (PA) leading to cell toxicity [23]. Although a 5′-nucleotidase (AAB38963) with 6% similarity to 4F8 was identified and characterized in *B. microplus* by Liyou and coworkers [43,44], the protection capacity of this antigen has not been reported.

Presently, little is known about 4E6, but immunization with this short 38 amino acid peptide resulted in the inhibition of tick infestations. Immunizations with Bm86-derived peptides have resulted in control of *B. microplus* infestations ([45]; de la Fuente J and Garcia-Garcia JC, unpublished results). These results suggest that chimeric vaccine antigens containing protective epitopes derived from multiple tick proteins may enhance development of new tick vaccines.

The tick protective antigens characterized in this work were identified by ELI in the mouse model of *I. scapularis* larval infestations [11]. The cDNA library for ELI screening was constructed from *I. scapularis* IDE8 cells in order to target the immature stages of *I. scapularis* that are involved in transmission of tick-borne pathogens to humans, dogs and other animal species [2,11]. However, the use of cultured embryonic tick cells to prepare the cDNA library for ELI, which should have a large repertoire of expressed genes usually found in not fully differentiated cells, had the goal to identify antigens expressed in all tick developmental stages. The characterization of 4F8, 4D8 and 4E6 have demonstrated that these antigens are expressed in immature as well as adult stages of several tick species and preliminary results support their use for the control of adult tick infestations.

In summary, we identified and characterized three *I. scapularis* tick protective antigens, 4D8, 4F8 and 4E6. These antigens, and especially 4D8, appear to be good candidates for continued development of a commercial product for control of tick infestations. Although these antigens are expressed in all tick developmental stages and preliminary immunization trials and capillary feeding experiments have provided encouraging results, further studies are needed to test the efficacy of these proteins for the control of all *I. scapularis* stages and of other tick species. Experiments are in course in our laboratory to evaluate the protection capacity of these recombinant antigens against adult *I. scapularis* infestations.

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