Characterization of *Haemaphysalis longicornis* Recombinant Cement-Like Antigens and Preliminary Study of Their Vaccination Effects

Thasaneeya HARNNOI1), Songwut WATCHABUNSOOK2), Takeshi SAKAGUCHI1), Xuenan XUAN3) and Kozo FUJISAKI1)*)

1) National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 0808555, Japan and 2) Parasitology Section, Eastern Veterinary Research and Development Center, Tambon Klongkeaw, Amphur Banbueng, Chonburi 20220, Thailand

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**ABSTRACT.** Two genes encoding immunodominant antigens, hlim2 and hlim3, were obtained from a salivary gland cDNA library of the hard tick, *Haemaphysalis longicornis*. The recombinant proteins were expressed in *Escherichia coli* as the GST fusion protein and used for immunization. We observed that the attachment rate of nymphal ticks fed on mice immunized with GST-hlim3 was significantly lower than that in the control group during the initial days of feeding. However, immunization with GST-hlim3 did not affect the engorgement rate of the ticks. In sharp contrast, GST-hlim2 did not influence the attachment rate and feeding period of ticks but had a significant reduction in the engorgement body weight. These data highlight the suitability of the 2 recombinant cement-like proteins for use in a cocktail vaccine.

**KEY WORDS:** cement-like antigen, *Haemaphysalis longicornis*, salivary gland, tick.

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Ticks are ectoparasites found in almost all parts of the world and surpass all other arthropods in the number of diseases they transmit to animals and humans. Tick-borne diseases in domestic animals are major constraints in livestock production, especially in developing countries. Although tick control strategies relied extensively on the use of chemical acaricides, this type of control is becoming less sustainable due to the development of resistance against acaricides among ticks and the undesirable contamination of the environment and animal food products [22]. The limitation in the current control measures against ticks and tick borne diseases has stimulated research in alternative method. Anti-tick vaccination may be an ideal, affordable, and sustainable way of controlling tick-borne diseases. There is currently only one commercially available tick vaccine against *Rhipicephalus (Boophilus) microplus* [5, 23]. This midgut membrane-bound protein antigen (BM86) can successfully induce protective immunity against tick feeding. Antibodies bind to epitopes on the midgut cells of the feeding tick causing damage and leakage of blood into the body cavity, killing the tick or reducing fecundity. Concealed antigens are those found on the tick gut wall and normally not presented to the host. Therefore, immunity to concealed antigens may not prevent the attachment of the ticks or the transmission of tick-borne infections. In addition, immunity from this vaccine is short-lived because natural infestation does not stimulate a response to the concealed antigen [2, 24].

Salivary gland proteins are exposed antigens that are injected into the host during blood feeding. Resistance to salivary gland proteins is naturally acquired by multiple tick infestation or immunization with crude tick extracts [18, 20]. As such, salivary gland antigens are potential candidates for a transmission blocking vaccine [12, 19] and the regulation of tick attachment and feeding [13]. Immune responses induced by exposed antigens are expected to be continually boosted by natural exposure of livestock to ticks in the field. Several exposed antigens have been identified, expressed as recombinant proteins, and evaluated as vaccine candidates [9, 15–17]. One such promising target molecule is the cement protein [15, 16]. The cement components are initially secreted to assist in the attachment to the host tissue. A cement protein of the tick *R. appendiculatus*, designated *Rhipicephalus* immunodominant molecule 36 (RIM36), is regarded as a strong antigenic molecule target for eliciting antibodies in cattle [1]. Furthermore, a putative tick cement protein (64P) from *R. appendiculatus* is another vaccine target used in clinical trials [16]. A cross-reactivity study of anti-sera raised against several truncated forms of recombinant 64P with several tissue extracts suggested that this cement protein may be a promising candidate targeting both exposed and concealed antigens for use as a broad-spectrum anti-tick vaccine [15].

The hard tick, *Haemaphysalis longicornis*, which commonly infests cattle and dogs, is a major vector that transmits pathogens, such as *Babesia*, *Theileria*, and *Borrelia* to domestic and wild animals in Japan and other parts of East Asia [3]. In a previous study, we identified immunodominant antigens from the salivary glands of *H. longicornis* by immunoscreening (Harnnoi et al., submitted). Several sequences that code for putative cement proteins were obtained. Further studies of recombinant expressed individual proteins were performed to clarify the role of these proteins for tick attachment and their possible use as a
recombinant cocktail vaccine. In the present study, 2 genes encoding cement-like antigens, hlim2 and hlim3, obtained from the salivary gland cDNA library of *H. longicornis*, were selected for expression as recombinant proteins. Their potential as anti-tick vaccine candidates is discussed herein.

**MATERIALS AND METHODS**

**Ticks:** The parthenogenetic Okayama strain of the tick *H. longicornis* has been maintained by feeding on rabbits and mice for several generations in our laboratory since 1997. The adults and nymphs were used in this experiment.

**Animals:** All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (Allowance No. 17–77).

**Nucleotide sequence analysis:** The nucleotide sequences of cDNA fragments were determined using a BigDye terminator cycle sequencing kit (PerkinElmer Life Sciences, Tokyo, Japan) and an automated sequencer (ABI PRISM 310 Genetic Analyzer, Tokyo, Japan). The sequences were obtained by using vector-specific T3 and RPV primers and gene-specific primers. The nucleotide sequences were translated into amino acid sequences using an ExPASy server (http://www.expasy.org) [4].

**Analysis of hlim2 and hlim3 mRNA expression in different tick tissues:** Total RNA was prepared from salivary glands, midguts, ovaries, synganglions, and carcasses (remnants of ticks after the removal of salivary glands, midguts, ovaries, and synganglions) of partially fed ticks using the TRI reagent, as previously described (Harnnoi et al., submitted). RT-PCR was performed using the Takara one-step RNA PCR kit (Takara, Tokyo, Japan) according to the manufacturer’s instructions. Primers specific for the *H. longicornis* actin gene were used as an internal control for the RT-PCR reaction. A negative-control reaction was performed without adding reverse-transcriptase to exclude some contamination of genomic DNA.

**Subcloning of hlim2 and hlim3 cDNA sequences into the pGEX-4T-3 expression vector:** The PCR amplification of the pBluescript phagemid harboring the hlim2 and hlim3 cDNA sequences for expression was performed using primers flanking the coding sequences of these genes. A truncated hlim2 was amplified using the hlim2 forward primer fused in-frame with the GST gene (5’AGGAATTCCGACGAGGGGA3’) and the hlim2 reverse primer (5’GGCAATTCCTATGACGGCCTGA3’). The full-length hlim3 was amplified using the hlim3 forward primer (5’GGGAATTCCATCATGGAGATA3’) and the hlim3 reverse primer (5’GGCAATTCCTATGACGGCCTGT3’). The start codon (ATG) and stop codon (TAT, TCA) are indicated in bold. The *EcoRI* restriction sites are indicated in italics. The PCR reaction conditions were as follows: 94°C for 10 min, 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and 1 cycle of 72°C for 7 min. The expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Tokyo, Japan) was digested with *EcoRI*, treated with calf intestinal alkaline phosphatase (CIAP), and purified using the GeneClean II kit (Q-BIO gene®, Bio101 system, MP Biomedicals, CA, U.S.A.). The insert DNA was digested with *EcoRI*, purified using GeneClean, and ligated into the compatible ends of pGEX-4T-3 using the DNA ligation kit version 2.0 (Takara, Tokyo, Japan) according to the manufacturer’s instructions. The ligation product was transformed into *E. coli* DH5α. Transformants grown on the plate were selected for plasmid DNA mini-preparation using alkaline lysis methods. After restriction digestion analysis, positive clones with correct orientation were selected for sequencing. The recombinant constructs were transformed into *E. coli* BL21 cell for expression.

**Expression and purification of the GST-hlim2 fusion protein:** A single colony of *E. coli* BL21 harboring the recombinant pGEX-hlim2 plasmid was grown overnight in 5 ml LB-ampicillin broth. The culture was diluted 1:50 in 200 ml of a fresh LB-ampicillin medium and grown until O.D.600 reached 0.4. Then, isopropyl-beta-D-thiogalactopyranoside (IPTG) induction was performed with a final concentration of 0.5 mM at 25°C overnight. The cells were pelleted by centrifugation at 4,000 × g for 20 min at 4°C. The pellet was resuspended in a 10 ml TNE buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 0.1 M NaCl) and further incubated at room temperature for 10 min in the presence of a 20 µg/ml final concentration of lysozyme. The cell suspension was lysed by sonication 3 times, 2 min each time, and incubated on ice for 1 hr with 1% Triton-X 100 in PBS. After repeat sonication, the suspension was centrifuged at 15,000 × g for 20 min at 4°C. The supernatant containing soluble GST-hlim2 was collected for further purification by glutathione sepharose 4B according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Tokyo, Japan).

**Expression of GST-hlim3 fusion protein:** The expression of *E. coli* BL21 harboring the recombinant pGEX-hlim3 plasmid was performed in a 1 L culture at 37°C for 4 hr with a 1 mM final concentration of IPTG. After the cells were lysed by sonication, the suspension was centrifuged at 15,000 × g for 10 min at 4°C, and the supernatant was discarded. The pellet was washed 3 times by sonication in 1% Triton-X-PBS and one time in PBS. The pellet was resuspended with 10 ml of 8 M urea in a urea-free buffer (50 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM EDTA). The mixture was incubated at room temperature for 1 hr and centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was transferred to a dialysis tube, and slow removal of urea was performed by stepwise dialysis against 1 L of a 4 M and 2 M urea solution, one hour each, and with a urea-free buffer 3 times, 30 min each time. The supernatant was further dialyzed against 2 L of a urea-free buffer overnight at 4°C. After centrifugation at 15,000 × g for 20 min at 4°C, the supernatant was collected. Determination of the fusion protein concentration was performed by SDS-PAGE in comparison to the known concentration of the standard BSA protein.

**Immunization of mice using GST-hlim2 and the GST-
**hlim3 fusion protein**: Nine DDY mice were divided into 3 groups for the immunization and challenge infestation experiment. The animals were immunized with the control GST protein, GST-hlim2, and the GST-hlim3 fusion protein. First, immunization was performed by injecting 100 µg of the protein mixed with Freund’s complete adjuvant intraperitoneally. The booster was given at 2-week intervals with the same amount of protein mixed with Freund’s incomplete adjuvant. Blood samples were collected from the mice tail veins after the third booster. The host immune response to the immunization was analyzed by Western blot analysis of immunized mouse sera with the immunoblots of recombinant proteins.

**Analysis of native hlim2 and hlim3 in salivary glands by Western blotting**: Salivary glands of 4-day fed *H. longicornis* were dissected, washed with ice-cold PBS, and kept at −80°C until used. The tissue was homogenized using a tissue homogenizer, sonicated for 15 ± 3 times, and centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was collected, and the protein concentration was measured using a BCA kit (Pierce, Rockford, IL). A salivary gland extract was used as an antigen for Western blotting by electrophoresing on a 12% polyacrylamide gel. The protein was transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in 3% skim milk in PBS for 1 hr at room temperature and further incubated with mouse sera (1:100) and goat anti-mouse-conjugated horseradish peroxidase (Dako Cytomation, Tokyo, Japan, 1:2,000). The signal was detected by 3,3-diaminobenzidine tetrahydrochloride (DAB) staining.

**Challenge infestation**: When the antibody titer reached 1:5,000 to 1:8,000, the mice were challenged with nymphal ticks. Three DDY mice per group were used for the challenge infestation. Ten days after the third booster, 12 *H. longicornis* nymphs per mouse were applied to the backs of the mice with the help of a polypropylene cap fixed with cement mass as described elsewhere [6]. The vaccine effect was determined 24 hr later by visual examination of the attachment rate (number of tick attach on host) and duration of feeding (the time since attachment was observed until engorgement was completed). The engorged body weights, molting rate, and mortality rates were determined post-engorgement.

**Statistical analysis**: All data are presented as a mean ± standard error or percentages where applicable. Differences are considered to be statistically significant if the *P* value was less than 0.05 in the Student’s *t* test.

**RESULTS**

**Sequence analysis of hlim2 and hlim3**: The nucleotide sequences and deduced amino acid sequences of hlim2 and hlim3 are shown in Fig. 1. The hlim2-truncated sequence (Fig. 1a) nucleotide accession number AB259292) is 696 bp in length, with an open reading frame (ORF) of 539 bp. The deduced amino acid is 179 bp with an expected molecular mass of 16.5 and a pI of 8.85. This protein has no similarity with any sequence reported in the database. The hlim2 protein has high glycine (34.6%) and serine (14.53%) contents. Several glycine-rich repeats (GGXG, GXGG) were observed, as reported for the *R. appendiculatus* glycine-rich protein [8]. The presence of GLX repeats, the common features of cement component were shown.

The hlim3 full-length sequence (Fig. 1b) nucleotide accession number AB252633) is 1,089 bp, has an ORF extending from position 40 to position 1,003, and codes 321 amino acid residues with a predicted molecular mass of about 33 kDa and a pI of 9.1. This deduced amino acid has a secretory signal peptide signature. The hlim3 sequence shared 41% and 83% identities with previously reported *H. longicornis* salivary gland proteins, HL34 and HL35 antigen U, respectively [17]. The common features of the adhesive protein, YPG motifs, within the tyrosine-rich domain or PXE motifs in the c-terminal proline-rich domain were observed as described by Tsuda et al. [17].

**The expression of hlim2 and hlim3 mRNA transcripts in different tick tissues**: The expression of the mRNA transcripts of hlim2 and hlim3 was studied using RT-PCR (Fig. 2). Both mRNA transcripts were expressed predominantly in salivary glands. The hlim2 transcript was expressed in every organ except the midgut (Fig. 2 a). For hlim3, the mRNA expression was observed in the salivary gland, synanglion, and carcass (Fig. 2 b). The RT-PCR internal control was performed using the actin gene, as shown in Fig. 2 c).

**Expression and purification of recombinant hlim2 and hlim3**: The heterologous expression of recombinant hlim2 in *E. coli* was performed. The GST fusion protein with an expected size of 42 kDa was obtained. The recombinant hlim3 was expressed with the expected size of 59 kDa. Although the expression conditions were optimized with various concentrations of IPTG at different temperatures, the protein did not become soluble (data not shown). For this reason, hlim3 was expressed as the inclusion body, solubilized using urea, and refolded by stepwise dialysis as described in materials and methods. The purification of the refolded hlim3 gave a low yield (less than 300 µg from 1 L culture). Therefore, the hlim3 inclusion body was used for immunization.

**Analysis of native hlim2 and hlim3 in salivary glands by Western blotting**: The Western blotting of a salivary gland extract and mouse anti-hlim2 and anti-hlim3 is shown in Fig. 3. The mouse anti-hlim2 reacted with 64 kDa and 26 kDa proteins, whereas anti-hlim3 reacted with a 33 kDa protein from a salivary gland extract. The reaction with the 26 kDa protein possibly resulted from a cross-reaction between the anti-GST antibody and the GST protein in the salivary glands of ticks. It is not known whether the molecular mass of the native hlim2 is 64 kDa, since the full-length sequence of this clone is not yet available.

**Challenge infestation**: The challenge infestation was performed as described in Materials and Methods. The attachment rate of ticks fed on GST-hlim3-immunized mice (34.2 ± 17.6%) was significantly lower (*p*<0.05) than that in the
Fig. 1. Nucleotide sequences of genes encoding hlim2, hlim3 and their deduced amino acid sequences. a) Truncated nucleotide sequence and deduced amino acid sequence of hlim3. The stop codon (TAA) is underlined with a thick line. Several glycine-rich repeats (GGXG, GXGG), as reported for the R. appendiculatus glycine-rich protein motifs are underlined. The GLX repeats, the common features of cement component are highlighted by boxes. b) Full-length nucleotide and deduced amino acid sequence of hlim3. The signal peptides are underlined with a double line. The start (ATG) and stop (TGA) codons are underlined with a thick line. The common features of the adhesive protein, YPG motifs, within the tyrosine-rich domain or PXP motifs in the c-terminal proline-rich domain are shown. The tyrosine-rich and proline-rich regions are underlined with a broken line. The YPG motifs are indicated in bold, and the PXP motifs are in italics. The GLX repeats within the deduced amino acid are highlighted by boxes.
control group immunized with GST (100%) after the first day of tick application (Fig. 4). The feeding duration was prolonged, as indicated by the significantly lower engorgement rate \((p<0.05)\) on day 3 \((4.8 \pm 3.9\%)\) and day 4 \((23.8 \pm 3.8\%, p<0.01)\) of feeding (Fig. 5). Most of ticks (95%) were engorged after 6 days of feeding. In contrast, GST-hlim2-immunized mice exhibited no apparent differences in attachment and feeding periods from those observed in the control group. However, the body weights of engorged ticks fed on GST-hlim2-vaccinated mice \((3.93 \pm 3.9 \text{ mg})\) were significantly lower \((p<0.01)\) than that of the control group \((4.37 \pm 0.05 \text{ mg})\) as shown in Fig. 6. The vaccination was found to have no effect on tick molting, since all ticks were able to progress to the adult stage.

**DISCUSSION**

Several studies have demonstrated that vaccination with defined protein antigens is able to induce significant immunity to tick infestation [21]. As a vaccine target, salivary glands are a rich source of exposed antigens that can stimulate the host immune response following natural infestation. In a previous study, we identified 17 genes encoding immunodominant antigens from the salivary glands of *H. longicornis* (Harnnoi et al., submitted). These genes were obtained by immunoscreening of a salivary gland cDNA library using a tick-sensitized rabbit serum. Of 17 positive clones obtained, 8 sequences did not yield any similarities...
with known proteins and one sequence shared similarity with the HL35 antigen U. These 9 sequences were found to possess characteristics in common with cement proteins that may assist ticks in their attachment to the host during blood feeding. To test whether or not this immune response was protective, individual genes were selected for expression as recombinant proteins and the vaccine potency was evaluated. In this study, 2 genes that gave a strong positive reaction with a rabbit serum, hlim2 and hlim3, were selected for further study. The hlim2 mRNA transcript was equally expressed in the salivary gland of unfed and fed ticks, whereas hlim3 was upregulated during blood feeding (Harnnoi et al., submitted). The analysis of mRNA expression in various tick organs suggested that both genes were also expressed in tissues other than the salivary glands. These 2 genes were subcloned and expressed as a recombinant GST fusion protein using a bacterial expression system. The recombinant GST-hlim2 was expressed as a soluble protein at 25°C, whereas GST-hlim3 was expressed as an inclusion body. The recombinant GST-hlim2 and GST-hlim3 were used for the immunization of mice. Challenge infestation was performed using H. longicornis nymphs fed on vaccinated mice. In comparison to control GST, the ticks that fed on GST-hlim2-immunized mice had significantly lower engorged body weight, although the attachment and engorgement process was unaltered. On the other hand, ticks fed on mice vaccinated with GST-hlim3 showed a significantly lower attachment rate 24 hr post-infestation, which prolonged the engorgement rate on day 3 and day 4. However, those ticks were later attached and fed until repletion with no significant difference in their engorged body weight. These results suggested that antibodies against immunodominant cement-like antigens in vaccinated animals could interfere with tick feeding in some way.

Both hlim2 and hlim3 proteins contain glycine repeats and a glycine-rich region. The glycine-rich proteins have been found to be expressed in the salivary glands of R. appendiculatus [1, 10, 16] and A. variegatum [11] and are believed to be a component of tick cement, the proteins of which are rich in glycine, serine, leucine, tyrosine, and proline [7]. A tick attachment site usually consists of a cement cone that protects the tick hypostome during feeding and secures the site of skin penetration [7]. Since cement proteins are essential for tick attachment and feeding on the host, there is interest in using cement proteins or their derivative as anti-tick vaccine components. Immunization of rabbits with purified cement proteins elicits delayed-type hypersensitivity to the antigen [14]. The hypersensitive rabbit demonstrated resistance to feeding of the R. appendiculatus tick but a slightly enhanced feeding of R. pulchellus ticks. Vaccination with recombinant p29, glycine-rich extracellular matrix protein from H. longicornis led to a significant reduction of the engorged body weight in adult ticks and the mortality of both larvae and nymphs [9]. HL34, another candidate, was reported to induce morbidity and mortality in adult and nymphal stages of the same tick species [17]. The recombinant hlim3 studied in this work shared 41% amino acid identity with HL34. However, no mortality of ticks fed on vaccinated mice was observed. Instead, this recombinant protein seemed to interfere with tick attachment with an unknown mechanism. Visual observation indicated that ticks fed on vaccinated animals accumulated on the polypropylene cap instead of seeking a host. A further challenge infestation experiment using a higher number and different stages of ticks fed on vaccinated rabbits is now ongoing to confirm this phenomenon. A putative tick cement protein (64P) from R. appendiculatus is currently a promising vaccine candidate that was used for vaccine targeting for both exposed and concealed antigens [16]. As reported with regard to the vaccination effects of cement protein 64TRP, most adult R. sanguineus on vaccinated guinea pigs were unattached and observed to be crawling on the retaining gauze as if attempting to leave the host 24 hr post-infestation [15]. As discussed by the author, if the ticks had not been restrained within the feeding chambers, they would most likely have abandoned the immunized animals and sought alternative hosts.

Cement is among the initial chemical compounds ixodid ticks secreted and inject into feeding site after proboscis penetrated host skin [1]. Cement is critical for tick attachment and prevention of host immune response molecules for interacting with proboscis. Since the 9 sequences including hlim2 and hlim3 identified in the present study possessed common characteristic with cement protein, it is conceivable to speculate the mice immunized with hlim3 mounted a strong immunity against cement proteins that prevented tick attachment and subsequent feeding.

In the present study, we have expressed, characterized, and preliminarily evaluated the vaccine potency of the recombinant putative cement protein from H. longicornis. The complementary effects of hlim2 on tick engorged body weight and hlim3 on tick attachment indicate the suitability of the 2 recombinant proteins for use in a cocktail vaccine.

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REFERENCES


