J. Vet. Sci. (2005), **6**(1), 47–51

JOURNAL OF
Veterinary
Science

Immunization of mice with recombinant P27/30 protein confers protection against hard tick *Haemaphysalis longicornis* (Acari: Ixodidae) infestation

Myung-Jo You*

College of Veterinary Medicine and Bio-safety Research Center, Chonbuk National University, Jeonju 561-756, Korea

The success of immunological control methods is dependent upon the use of potential key antigens as tick vaccine candidates. Previously, we cloned a gene encoding 27 kDa and 30 kDa proteins (P27/30) of *Haemaphysalis longicornis*, and identified the P27/30 is a troponin I-like protein. In this study, the recombinant P27/30 (rP27/30) expressed in *Escherichia coli* was used to immunize mice and the mice were challenge-infested with ticks at different developmental stages of the same species. The rP27/30 protein stimulated a specific protective anti-tick immune response in mice, evidenced by the statistically significant longer pre-feeding periods in adult ticks. Furthermore, significantly longer feeding periods were noted in both larval and adult ticks. On the other hand, only larval ticks exhibited low attachment rates (31.1%). Immunization of mice with rP27/30 protein confers protection against hard tick *Haemaphysalis longicornis* infestation. These results demonstrated that the rP27/30 protein might be a useful vaccine candidate antigen for biological control of ticks.

Key words: ticks, troponin I, antigen, immunity, biological control

Introduction

The hard tick *Haemaphysalis longicornis* Neumann, 1901 (*H. longicornis*), is mainly distributed in East Asia and Australia. *H. longicornis* transmits pathogens, which causes deteriorative diseases in humans and animals [3,4]. A variety of methods have been employed for the suppression of tick vector populations, including the application of biological control agents [2] and the heavy reliance on the use of chemical acaricides [18]. However, the development of resistance to acaricides by ticks [23] and the increase in legislation to combat the detrimental effect of residues of acaricides on the environment [18] have emphasized the need to assess a variety of alternatives to tick vector control. The success of this method is dependent on identification and cloning of tick molecules involved in mediation of key physiological roles. Recently genes for two *Boophilus microplus* (*B. microplus*) midgut-associated molecules, Bm 86 and Bm 91, have been cloned and expressed [14,15]. These antigens have been shown to confer a significant protective immunity against *B. microplus* infestation in cattle [15,16]. Previously, we cloned *H. longicornis* P27/30 gene and anti-mouse sera against the recombinant P27/30 could react with native 27/30 kDa proteins from *H. longicornis* adult lysates using immunoblots, suggesting that the native P27/30 protein is a troponin I-like protein and may have two isoforms [22].

Troponin is a complex of three different subunits in vertebrates: troponin C which binds Ca^{2+}; troponin I which binds to actin and inhibits the actin-myosin interaction; and troponin T which binds to tropomyosin [10,25]. Troponin I can interact with all major proteins of the I filament, actin, tropomyosin, troponin C and troponin T. Troponin I plays a central role in the regulatory process in striated muscle [10]. A great deal of information, some of which is at times confusing, exists about the amino acids and peptide regions involved in these interactions. The most striking property of troponin I is to inhibit the magnesium activated ATPase of actomyosin [25]. This clearly indicates that in some way troponin I blocks the interaction of actin with myosin that is responsible for activation of the MgATPase [25].

Since the 1980s immunization against ticks with concealed antigens has been advocated over natural antigens because immunity induced by natural and conventional antigens was believed to be inferior to immunity induced by concealed antigens [20]. In addition, conventionally exposed antigens could have evolved under the pressure of host immunity and along the way may reduced their antigenicity that elicits the host immune response [20]. In the present

*Corresponding author
Tel: +82-63-270-3887; Fax: +82-63-270-3780
E-mail: tick@chonbuk.ac.kr

study, we report the immunization effects of a recombinant P27/30 protein against tick feeding on mice. In addition, we also discuss its possible use as a vaccine candidate antigen for tick control.

Materials and Methods

Tick

The parthenogenetic Okayama strain of the tick *H. longicornis* [3] has been maintained by feeding on rabbits and mice for several generations in our laboratory since 2003.

Expression of recombinant P27/30 in *E. coli*

The P27/30 gene encoding *H. longicornis* P27/30 was inserted into the *Eco*RI site of pBluescript SK (+) vector. The P27/30 gene in pBluescript SK (+) vector was subcloned into the pGEMEX-2 (Promega, USA) plasmid of *E. coli* expression vector after digestion with *Eco*RI. The resulting plasmid pGEMEX-2/P27/30 was checked for accurate insertion by restriction enzyme analyses. pGEMEX-2/P27/30 was used to transform *E. coli* (JM109 (DE3), Promega, USA) by standard techniques. The recombinant P27/30 (rP27/30) was expressed as a gene 10 fusion protein, and designated gene 10-P27/30 protein.

Protein determination

The protein concentration was determined using the BCA protein assay reagent (Pierce, USA) with bovine serum albumin as a standard.

Immunization of rP27/30

Eighteen female mice (BALB/c, 8 weeks old) were immunized and challenged with *H. longicornis* ticks. Of the 18 mice, nine were immunized with rP27/30, and nine were immunized with control gene 10 proteins. One hundred micrograms of the rP27/30 and gene 10 proteins were injected into mice intraperitoneally after emulsifying each with Freund's complete adjuvant; first and second booster injections after emulsifying with Freund's incomplete adjuvant were each given at 2-week intervals.

Serological analysis

Host immune responses to the recombinant proteins were determined by immunoblot analysis for individual animals using methods described previously [22] on nitrocellulose strips with transferred rP27/30 and gene 10 proteins, and the antibody titer was expressed as the highest dilution showing immune reactive bands.

Challenge infestation

When antibody titers reached 1 : 5,000 to 1 : 8,000, the mice were challenged with the different developmental stages of *H. longicornis* ticks. Unfed larvae, nymphs and adults of *H. longicornis* were fed each on the shaved back of rP27/30 or gene 10-immunized BALB/c mice by using a polypropylene cap, which was fixed with a cement mass [7]. Infestations were carried on using 3 adult ticks, 10 nymphs and 30 larvae per mouse. Visual examinations of mice were performed post-tick infestation, and the following parameters were recorded: pre-feeding periods, feeding periods, attachment rates, engorged weights, molting periods and egg weights. Once the engorged ticks were obtained, they were transferred into individual glass flasks to an incubator at 25°C and approximately 85% of relative humidity to allow the egg-laying and molting. The time since infestation started and attachment occurred was named pre-feeding period. Feeding periods were assigned to the time since attachment was observed until engorgement was completed. Attachment rates were assigned to the rates from infestation to engorgement. Engorged weights instantly measured after dropping. Egg weights were measured 3 weeks after dropping.

Statistical analysis

All values are given as means with standard errors (mean ± SE). Comparisons between group means were carried out using the Student's *t*-test. Differences were considered significant at the 95% confidence level.

Results

The immunization effects of rP27/30 on mice for tick feeding are summarized in Figures 1, 2, and 3. There were no differences in attachment rates observed during the initial 24 h after nymphal and adult ticks were introduced onto mouse backs. On pre-feeding periods of larval, nymphal, and adult ticks from infestation to attachment, longer pre-feeding periods was observed in adult ticks that fed on rP27/30-immunized mice (8.0 ± 1.6 days) compared to the control (2.6 ± 1.2 days) ($p < 0.05$) (Fig. 1). No differences were observed in pre-feeding periods between nymphs (0.53 ± 0.13 days) and larvae (0.2 ± 0.07 days) compared to the control (0.46 ± 0.13, 0.13 ± 0.06 days, respectively) (Fig. 1). Both larval and adult ticks that fed on rP27/30-immunized mice showed a tendency to take longer feeding periods. The feeding periods of larvae were 4.1 ± 0.17 days ($p < 0.05$) and adult ticks 6.5 ± 0.5 days ($p < 0.05$) while the control showed 3.7 ± 0.07 days and 4.6 ± 0.3 days, respectively (Fig. 2). In the same Figure 2, it can be seen that nymphal ticks feeding periods on rP27/30-immunized mice ranged 4.6 ± 0.3 days compared to 4.3 ± 0.8 days in the control. On attachment rates of larval, nymphal, and adult ticks from infestation to engorgement, larval ticks that fed on rP27/30-immunized mice exhibited a low attachment rate (31.1%) compared to the control (98%), while no significant difference was observed in those of nymphs and adults. Engorged body weights of larval, nymphal, and adult ticks

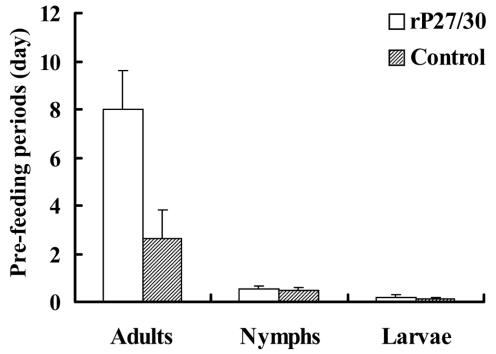

Fig. 1. Comparison of pre-feeding periods after application of larva, nymph, and adult *H. longicornis* ticks on rP27/30-immunized mice and gene 10 protein-immunized control mice (*, $p < 0.05$). Data are presented as mean ± SE.

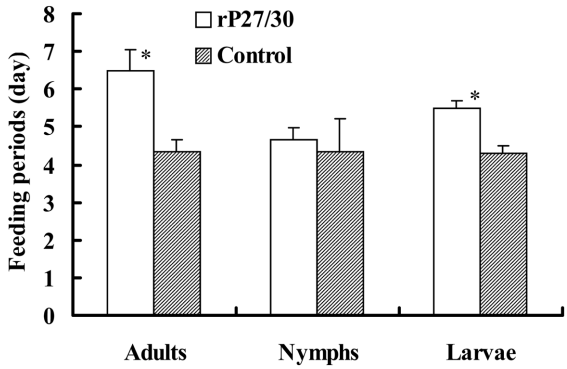

Fig. 2. Comparison of feeding periods after attachment of larva, nymph, and adult *H. longicornis* ticks on rP27/30-immunized mice and gene 10 protein-immunized control mice (*, $p < 0.05$).

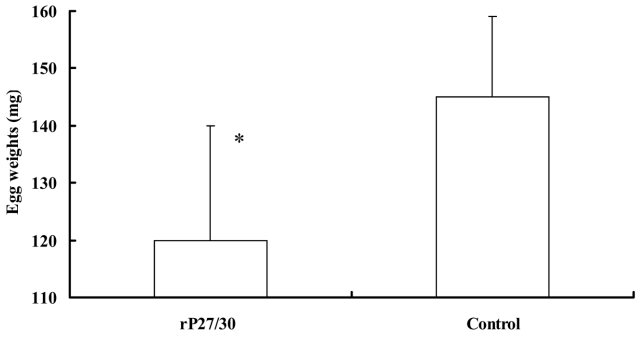

Fig. 3. Comparison of egg weights after 3 weeks after dropping of adult *H. longicornis* ticks on rP27/30-immunized mice and gene 10 protein-immunized control mice (*, $p < 0.05$). Data are presented as mean ± SE.

following feeding on rP27/30-immunized mice were 0.63 ± 0.05, 4.2 ± 0.1, and 258 ± 11.5 mg compared to the controls, 0.68 ± 0.05, 4.1 ± 0.1, and 262 ± 18.8 mg, respectively, and no significant reduction of engorged weights was observed in immunized mice. Molting periods of larval and nymphal ticks from dropping to molting following feeding on rP27/30-immunized mice were 9.3 ± 0.2 and 10.8 ± 0.3 days compared to 9.1 ± 0.3 and 10.6 ± 0.3 days in the controls, respectively. There was an apparent decrease in egg weight in ticks fed on rP27/30-immunized mice (120 ± 22 mg) compared to the control (147 ± 12 mg) (Fig. 3).

Discussion

Troponin I is a principal component of thin filaments-linked system of regulatory proteins and binds to actin and inhibits the actin-myosin interaction [10,25]. This unique role requires that it must interact with each of the proteins involved in this process, directly with actin, troponin C, troponin T and possibly indirectly with tropomyosin and myosin [13]. In the present study, we demonstrated that recombinant troponin I-like protein expressed in *E. coli* stimulated a specific protective anti-tick immune response in mice, as shown the statistically significant longer pre-feeding periods for adult ticks, the statistically significant longer feeding periods for larval and adult ticks, and the low attachment rates in larval ticks (31.1%). These results suggested that mice immunized with rP27/30 protein acquired a significant level of resistance against *H. longicornis* infestations. No apparent differences were observed in the engorged body weights and molting periods for ticks that applied on rP27/30-immunized mice compared to those in the control. Egg weights in ticks fed rP27/30-immunized mice compared to the control exhibited an apparent slight difference. Kemp *et al.* [8,9] provided evidences which may support the hypothesis that immature and mature ticks of *B. microplus* have different sensitivities to host acquired resistance against tick molecules. In a series of experiment, the authors showed that there was evidence of severe gut damage in both adult female and male *B. microplus* ticks feeding on cattle immunized with *B. microplus*-derived extracts. However, there were no effects observed in larval ticks feeding on the same protected animal. These data are consistent with our present result demonstrating the vaccine effect in immature and mature *H. longicornis* ticks following feeding on rP27/30-immunized mice.

The evidence from affinity chromatography [12] from fluorescence studies on pyrene-labelled tropomyosin [24] indicates that the direct interaction between troponin I and tropomyosin is weak. In the presence of troponin T and its tropomyosin binding fragments, troponin I is bound more strongly in the ternary complex [24]. This unique role requires that it must interact with each of the proteins involved in this process, with troponin I, tropomyosin, troponin T. Tropomyosin induces acquired immunity in several helminthic infections of laboratory and domesticated animals [1,5,11]. Vaccination with recombinant tropomyosin of filarial parasite, *Onchocerca volvulus*, induces a 48-62%

reduction in parasite recoveries compared to controls [19]. Recent studies in tropomyosin, have revealed that tropomyosin may be implicated in host protective responses to microfilariae in onchocerciasis [6]. In this study, mice that were immunized with troponin I-like protein showed considerable resistance to tick infestation, indicating that the rP27/30 protein implicated in host protective responses to *H. longicornis* may be identical to tropomyosin. Our data also indicate that rP27/30 protein may play an important role during bloodsucking in *H. longicornis* ticks, suggesting the possibility of the *H. longicornis* rP27/30 protein being a potential candidate antigen for a tick vaccine.

An effective tick vaccine will require a combination of several target antigens and each of them needs to mediate a physiological function either independently or synergistically [17]. Previous studies by Riding *et al*. [15] and Willadsen *et al*. [21] provided evidence that anti-tick immunity induced by a combination of vaccine antigens is more effective than exposed single antigen vaccine. Immunological and biological control strategies in the tick-host interface are currently the only sustainable and practical alternative methods to the current use of chemical acaricides that have been shown to have serious limitations. Our efforts are currently directed toward characterization and *in vitro* expression of other concealed tick molecules for using in vaccine trials in combination with *H. longicornis* rP27/30 protein.

Acknowledgments

This paper was supported by Research funds of Chonbuk National University. We thank Dr. K. Fujisaki for advice this paper.

References

1. **Emery DL, Wagland BM.** Vaccines against gastrointestinal nematode parasites of ruminants. Parasitol Today 1991, **7**, 347-349.
2. **Frazzon AP, Da Silva Vaz Junior I, Masuda A, Schrank A, Vainstein MH.** *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus*. Vet Parasitol 2000, **94**, 117-125.
3. **Fujisaki K.** Development of acquired resistance and precipitating antibody in rabbits experimentally infested with females of *Haemaphysalis longicornis* (Ixodoidea: Ixodidae). Natl Inst Anim Health Q (Tokyo) 1978, **18**, 27-38.
4. **Fujisaki K, Kawazu S, Kamio T.** The taxonomy of the bovine *Theileria* spp. Parasitol Today 1994, **10**, 31-33.
5. **Hartmann S, Adam R, Marti T, Kirsten C, Seidinger S, Lucius R.** A 41-kDa antigen of the rodent filaria *Acanthocheilonema viteae* with homologies to tropomyosin induces host-protective immune responses. Parasitol Res 1997, **83**, 390-393.
6. **Jenkins RE, Taylor MJ, Gilvary NJ, Bianco AE.** Tropomyosin implicated in host protective responses to microfilariae in onchocerciasis. Proc Natl Acad Sci USA 1998, **95**, 7550-7555.
7. **Kabamoto S, Bhagat NA, Suzuki H, You M, Kamio T, Tsuji N, Claveria FG, Nagasawa H, Igarashi I, Toyoda Y, Fujisaki K.** Comparison of macrophage scavenger receptor-A knockout mice with wild type ones in the immune response against repeated infestation with *Haemaphysalis longicornis*. J Vet Med Sci 2002, **64**, 355-359.
8. **Kemp DH, Agbede RIS, Johnston LAY, Gough JM.** Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: feeding and survival of the parasite on vaccinated cattle. Int J Parasitol 1986, **16**, 115-120.
9. **Kemp DH, Pearson RD, Gough JM, Willadsen P.** Vaccination against *Boophilus microplus*: localization of antigens on the tick gut cells and their interaction with the host immune system. Exp Appl Acarol 1989, **7**, 43-58.
10. **Leavis PC, Gergely J.** Thin filament proteins and thin filament- linked regulation of vertebrate muscle contraction. CRC Crit Rev Biochem 1984, **16**, 235- 305.
11. **O'Donnell IJ, Dineen JK, Wagland BM, Letho S, Werkmeister JA, Ward CW.** A novel host-protective antigen from *Trichostrongylus colubriformis*. Int J Parasitol 1989, **19**, 327-335.
12. **Pearlstone JR, Smillie LB.** Effects of troponin I plus-C on the binding of troponin T and its fragments to a tropomyosin. J Biol Chem 1983, **258**, 2534-2542.
13. **Perry SV.** Troponin I: inhibitor or facilitator. Mol Cell Biochem 1999, **190**, 9-32.
14. **Rand KN, Moore T, Srikantha A, Spring K, Tellam R, Willadsen P, Cobon GS.** Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus*. Proc Natl Acad Sci USA 1989, **86**, 9657-9661.
15. **Riding GA, Jarmey J, McKenna RV, Pearso R, Cobon GS, Willadsen P.** A protective concealed antigen from *Boophilus microplus*: purification, localization and possible function. J Immunol 1994, **153**, 5158-5166.
16. **Rodriguez M, Penichet ML, Mouris AE, Labarta V, Luaces LL, Rubiera R, Cordoves C, Sanchez PA, Ramos E, Soto A.** Control of *Boophilus microplus* populations in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. Vet Parasitol 1995, **57**, 339-34.
17. **Shapiro SZ, Voigt WP, Fujisaki K.** Tick antigens recognized by serum from a guinea pig resistant to infestation with the tick *Rhipicephalus appendiculatus*. J Parasitol 1986, **72**, 454-463.
18. **Solomon KR.** Acaricide resistance in ticks. Adv Vet Sci Comp Med 1983, **27**, 273-296.
19. **Taylor MJ, Jenkins RE, Bianco AE.** Protective immunity induced by vaccination with *Onchocerca volvulus* tropomyosin in rodents. Parasite Immunol 1996, **18**, 219-225.
20. **Willadsen P.** Immunological approaches to the control of ticks. Int J Parasitol 1987, **17**, 671-677.
21. **Willadsen P, Smith D, Cobon G, McKenna RV.** Comparative vaccination of cattle against *Boophilus microplus* with recombinant Bm86 alone or in combination with recombinant Bm91. Parasite Immunol 1996, **18**, 241-

246.

22. **You M, Xuan X, Tsuji N, Kamio T, Igarashi I, Nagasawa H, Mikami T, Fujisaki K.** Molecular characterization of a troponin I-like protein from the hard tick *Haemaphysalis longicornis*. Insect Biochem Mol Biol 2001, **32**, 67-73.
23. **Zaim M, Guillet P.** Alternative insecticides: an urgent need. Trends Parasitol 2002, **18**, 161-163.
24. **Zhou X, Morris EP, Lehrer SS.** Binding of Troponin I and the troponin I-troponin C complex to actin-tropomyosin. Dissociation by myosin subfragment 1. Biochemistry, 2000, **39**, 1128-1132.
25. **Zot AS, Potter JD.** Structural aspects of troponin-tropomyosin regulation of skeletal muscle contraction. Annu Rev Biophys Biophys Chem 1987, **16**, 535-559.