Assessment of cell mediated immune response in rabbits immunized with affinity purified 35 kDa midgut antigen of *Rhipicephalus haemaphysaloides* ticks

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Received: 11 January 2013 / Accepted: 25 March 2013 / Published online: 3 April 2013
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Abstract Cell mediated immune response to immunoaffinity chromatography purified midgut antigen of *Rhipicephalus haemaphysaloides* ticks in rabbits was studied by using lymphocyte transformation test. This test was carried out by using 5-bromo-2'-deoxy-uridine kit method. The blastogenic response of peripheral blood lymphocytes of normal rabbit to different concentrations of antigen and mitogen (Con A) showed that 2 µg of antigen and 2 µg of mitogen gave maximum stimulation index. The antigen specific responsiveness of immunized rabbits with affinity purified 35 kDa midgut antigen was highly significant ($P \leq 0.01$) compared to mitogen. The maximum lymphocyte stimulation index (LSI) of 2.47 was observed on 49 day post immunization in immunized group. The lymphocytes separated from control animal cultured in RPMI1640 medium with 2 µg of antigen and 2 µg of mitogen (Con A) were never stimulated and their LSI values were below 2.0.

Keywords *Rhipicephalus haemaphysaloides* · Lymphocyte stimulation index and affinity chromatography · Midgut antigen

Introduction

Immunological control of ticks is attaining greater importance due to apprehension of emergence of acaricidal

resistant tick strains and environmental pollution. Animals can be protected against haematophagous parasites by immunization of hosts using concealed antigens (Willadsen and Kemp 1988). The principle of such immunization is uptake of blood meal, tissue fluid or wound exudates which inevitably carry antibodies, complements and other components of host immune system (Wikel 1979). There are many difficulties in the practical exploitation of this idea, the most important of which is the identification of appropriate antigen targets for the immune system.

The present study was carried out to assess the cell mediated immune response in rabbits immunized with affinity purified 35 kDa midgut antigen of *Rhipicephalus haemaphysaloides* ticks.

Materials and methods

Experimental animals

Tick colony maintenance and immunization were conducted using New Zealand white rabbits aged 3–4 months of both sexes with no previous exposure to ticks.

Ticks

Laboratory reared *R. haemaphysaloides* ticks were used for the preparation of antigens. The colonies were maintained in the laboratory at room temperature in moist sand trays until the stages moulted to the next instar. The unfed stages were placed in specimen vial and kept in desiccators containing saturated potassium chloride solution for maintenance of 85 % relative humidity. The desiccators were kept in incubator at $20 \pm 2^\circ\text{C}$.

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Preparation of midgut antigen

Midgut antigen was prepared as per the method of Opdebeeck et al. (1988) with minor modifications. Laboratory maintained semi-engorged adult female *R. haemaphysaloides* ticks were dissected individually under a Stereo zoom microscope (Leica, Germany). The midguts were isolated and placed in 0.15 M phosphate buffered saline (PBS) pH 7.2 and the harvested midguts were mixed with 1 mM disodium EDTA, homogenized at 1,500 rpm for 10 m in Potter S homogenizer (B. Braun Biotech international, Germany) in an ice bath. The homogenates were sonicated at 8 μ amplitude for 10 m, at 4 cycles per m, each cycle lasting for 2 m with an interval of 1 m each, using a sonicator (B. Braun. Labsonic, Germany) in an ice bath. The sonicated homogenate was centrifuged at 15,000 $\times g$ for 30 m at 4 °C in a refrigerated centrifuge (Sigma, USA), after which the supernatant was collected. The pellet was mixed with 0.0075 M sodium deoxycholate and the above mentioned steps of homogenization, sonication and centrifugation were followed. Finally, a second supernatant was collected. Both supernatants were dialyzed against PBS (pH 7.2) for 24 h, and then pooled. Phenyl methyl sulfonyl fluoride at 1 mM was added per mL of supernatant antigen to inhibit the proteolysis and stored at –20 °C in aliquots in 1.5 mL cryovials. This supernatant antigen was used as *R. haemaphysaloides* gut membrane antigen (RhGMAg). The protein concentration of antigen was determined as per the method of Smith et al. (1985) using bicinchoninic acid kit (Genei, Bangalore).

Raising of antibodies against 35 kDa midgut membrane antigen

The immunoreactive polypeptide at 35 kDa was identified by western blot analysis as per the method described by Towbin et al. (1979) with minor modifications. The 35 kDa polypeptide was extracted from SDS-PAGE gel bands from *R. haemaphysaloides* midgut protein as per the method of Amero et al. (1994) with minor modifications. Briefly, the gel bands were lyophilized and used to immunize rabbits. The immunized animals received 3 doses of 35 kDa polypeptide. On ‘0’ day, 250 μ g of antigen with Freund’s complete adjuvant (FCA) was given intramuscularly and subsequent doses of 125 μ g of antigen with Freund’s incomplete adjuvant (FIA) on 14 and 21 day were given subcutaneously to each rabbit. All rabbits were bled intracardially on 35th day after first injection and the sera were stored at –20 °C.

Immunoglobulin G (IgG) purification by the caprylic acid method

Serum was collected from 35 kDa midgut polypeptide immunized animals on 35th day of immunization. This

serum IgG was purified by the caprylic acid method described by Bhanushali et al. (1994), with minor modifications. Hyperimmune serum (20 mL) was added with acetate buffer (60 mM) at a ratio of 1:4 and the pH was adjusted to 4.5 with 1 N sodium hydroxide. Twenty-five micro litres of caprylic acid was slowly added per each mL of serum acetate buffer mixture. The solution was stirred for 30 m and insoluble material was removed by centrifugation at 10,000 $\times g$ for 30 m at 4 °C. The supernatant was mixed with EDTA buffer at 10:1 v/v and the pH was adjusted to 7.4 with 5 N sodium hydroxide. IgG was precipitated by addition of 50 % saturated ammonium sulphate (0.277 g/mL). The mixture was stirred for 30 m and centrifuged at 10,000 $\times g$ for 30 m at 4 °C. The pellet was resuspended in 1 mL of 1 $\times$ PBS (sodium chloride 0.8 g, potassium chloride 0.02 g, disodium hydrogen phosphate 0.115 g, and potassium dihydrogen phosphate 0.02 g) and dialyzed against PBS for 48 h. The amount of IgG was calculated, measuring absorbance value (OD) at 280 nm. Amount of IgG = (sample OD–blank OD) $\times$ 0.74, where 0.74 is the correction factor for IgG.

Immunoaffinity chromatography

Immunoaffinity chromatography was carried out as per the method described by Opdebeeck et al. (1989), with minor modifications. Cyanogen bromide activated Sepharose 4B, 1.5 g in 0.001 N HCl was allowed to swell for 15 m. The gel was washed with deionized water in a sintered glass funnel and collected in 50 mL falcon tube containing 20 mL of coupling buffer, pH 8.4 (0.1 M sodium bicarbonate +0.5 M sodium chloride) and 5 mL of the purified IgG. Excessive reactive sites were blocked by 0.1 M glycine buffer, pH 8.0. Prepared gel was washed with sodium acetate buffer, pH 4 (0.1 M sodium acetate +0.5 M sodium chloride) to remove unbound IgG. The gel was packed in a 1 cm diameter column and was washed with three bed volume of sodium acetate buffer and 0.1 M Tris HCl buffer, pH 8, three times each. The packed column was washed with PBS pH 7.2 to ensure OD value at 0.00. The crude midgut antigen was filtered through a 0.2 μ m filter, over equilibrated with equilibrating buffer pH 7.2 (10 mM sodium phosphate +5 mM disodium EDTA). The antigen was loaded into the column in batches. The column was washed with equilibrating buffer to remove unbound protein until UV absorbance returned to base line. The bound antigen fractions were eluted by 0.1 M glycine buffer pH 2.8 into Tris base, pH 8.5 containing vial to increase pH to 7.3. The eluted fractions were dialyzed extensively against PBS, pH 7.2 for 48 h and concentrated with polyethylene glycol. The purified 35 kDa midgut antigen (Aff-RhGM 35 kDa Ag) was used in immunization studies in New Zealand white rabbits.

Immunization trial

New Zealand white rabbits ($n = 4$ in each group) were immunized with affinity purified 35 kDa midgut antigen on day 0, 14 and 21. The first injection of 200 μg antigen with FCA was given I/M on day 0 followed by 100 μg antigen with FIA was given S/C on day 14 and 21. The control animals received only adjuvant with PBS on respective days. Blood was collected from all experimental animals from 0 to 56 days at weekly intervals from their ear veins. The cell mediated immune response was determined by performing lymphocyte transformation test (LTT).

Lymphocyte transformation test (LTT)

Lymphocyte transformation test was carried out as per the method described by Wagner et al. (1999) with minor modifications. Five milliliters of heparinised blood collected from affinity purified 35 kDa immunized and control rabbit. This blood sample was gently overlaid (100 IU/mL) on 5 mL of cold Biocoll separating solution and centrifuged at $3,000 \times rpm$ for 30 min to separate buffy coat layer. Lymphocytes collected at Biocoll and plasma interphase were washed twice with cold RPMI media and resuspended in the media. The viable cells were counted using haemocytometer by Trypan blue dye exclusion technique and were suspended at a concentration of 5×10^5 lymphocytes per 100 μL of culture medium. The cell suspension was distributed in triplicates containing 5×10^5 cells in 100 μL per well in a flat bottom tissue culture plate. 2 μg of ConA and 2 μg of affinity purified 35 kDa antigen were added separately in wells maintained for mitogenic stimulation. Control wells were maintained without addition of stimulants and the plate was incubated at 37 °C for 72 h in a 5 % CO_2 incubator (Forma Scientific Inc, USA).

BrdU-incorporation and proliferation assay

In vivo proliferation was measured using 5-bromo-2'-deoxy-uridine (BrdU) labeling and detection and kit III (Roche Molecular Biochemicals, Germany). Quantification of in vitro lymphocyte proliferation was based on the measurement of BrdU incorporated into the cellular DNA. Cells were pulsed with 110 μM /well of BrdU labeling reagent during the last 18 h of antigenic stimulation. 72 h After initial stimulation, plates were centrifuged at $300 \times g$ for 10 min at 20 °C. Media was carefully aspirated and cells were air dried at 60 °C for 2 h. Cells were fixed in 70 % acid ethanol (0.5 M HCl) at –20 °C for 30 min. Cells were washed thrice with PBS (pH 7.2) supplemented with 10 % bovine serum albumin (BSA; fraction V). The cells were incubated with 100 μL of 1:100 diluted anti-

BrdU peroxidase conjugate (Fab fragments) for di-ethyl-benzothiazoline-sulfonic acid (ABTS) dye substrate solution. Absorbance was measured within 20–30 min using a plate reading spectrophotometer (Bio-Tek instruments, USA) at 405 nm with reference wavelength of 490 nm. All data points were measured in triplicate. LSI was calculated as the ratio (R) of the absorbance signal in control and stimulated cells.

$$R = S - B/B.$$

S = OD values for stimulated cells (either with antigen or Con A).

B = OD values for mitogen negative control cell.

The statistical analysis of results was done using two way analysis of variance.

Results and discussion

The blastogenic response of peripheral blood lymphocytes of normal rabbit to different concentrations of antigen and mitogen (ConA) showed that 2 μg of antigen and 2 μg of mitogen gave maximum stimulation index. The results of LTT are presented in Tables 1 and 2. The affinity purified 35 kDa antigen specific responsiveness of immunized rabbits was highly significant ($P \leq 0.01$) compared to mitogen. The maximum LSI of 2.47 was observed on 49 day post immunization (DPI) in immunized group. The lymphocytes separated from control animal cultured in RPMI1640 medium with 2 μg of antigen and 2 μg of mitogen were never stimulated and their LSI values were below 2.0.

In immunization studies using tick antigens, antigen specific lymphocyte blastogenesis was demonstrated by Wikel and Osborn (1982) in *Bos taurus* cattle infested with *D. andersoni*. Manohar and Banerjee (1992) observed

Table 1 Lymphocyte stimulation index (LSI) in rabbits immunized with affinity purified midgut antigen of *Rhipicephalus haemaphysaloides* ticks (mean $\pm$ SE)

Days PI	Immunized group		Control group	
	Purified antigen	Mitogen	Purified antigen	Mitogen
0	1.06 $\pm$ 0.00	0.97 $\pm$ 0.01	1.00 $\pm$ 0.00	0.93 $\pm$ 0.01
14	1.69 $\pm$ 0.00	1.26 $\pm$ 0.00	1.08 $\pm$ 0.00	1.02 $\pm$ 0.00
21	1.73 $\pm$ 0.00	1.38 $\pm$ 0.00	1.10 $\pm$ 0.00	1.11 $\pm$ 0.00
28	1.82 $\pm$ 0.00	1.64 $\pm$ 0.00	1.17 $\pm$ 0.00	1.12 $\pm$ 0.00
35	1.98 $\pm$ 0.00	1.87 $\pm$ 0.00	1.22 $\pm$ 0.00	1.17 $\pm$ 0.00
42	2.29 $\pm$ 0.00	1.91 $\pm$ 0.00	1.28 $\pm$ 0.00	1.19 $\pm$ 0.00
49	2.47 $\pm$ 0.00	1.95 $\pm$ 0.00	1.31 $\pm$ 0.00	1.12 $\pm$ 0.00
56	2.32 $\pm$ 0.00	2.01 $\pm$ 0.00	1.29 $\pm$ 0.00	1.28 $\pm$ 0.00

Table 2 Analysis of variance of lymphocyte stimulation index (LSI)

Source	D.F.	Sum of squares	Mean sum of squares	F ratio
Replications	7	3.46	0.49	12.45**
Treatments	3	1.82	0.60	15.11**
Error	21	0.83	0.04	
Total	31	6.11	–	

** Highly significant

antigen specific responsiveness in immunized rabbits *Hyalomma anatolicum anatolicum* was significant from 21 to 42 DPI. The maximum stimulation was observed on 35 DPI.

Similar to the current study, the blastogenic response of the lymphocytes to purified midgut antigen of *Boophilus microplus* was evident with significant stimulation index values till 119 DPI with a peak on 42 DPI using methyl ³H thymidine (Raman 2000). The maximum stimulation index was observed on 42 DPI in response to purified antigen of *R. haemaphysaloides* and significant stimulation index values till 63 DPI using MTT dye (Aruljothi 2003). Kumar (2004) using BrdU kit and recorded significantly higher stimulation index values from day 28 DPI which was maintained up to 56 DPI in response to aff-29 kDa *Haemaphysalis bispinosa* midgut antigen. The present study revealed that significant blastogenic response to affinity purified 35 kDa midgut antigen by sensitized lymphocytes was evident from 14 to 56 DPI with a peak in LSI values on 49 DPI.

Conclusion

Based on the results of the present study, we concluded that the rabbits immunized with affinity purified 35 kDa midgut antigen of *R. haemaphysaloides* ticks showed higher lymphocyte stimulation index compared to unimmunized rabbits. Therefore the affinity purified 35 kDa protein can be used as candidate antigen for control of *R. haemaphysaloides* ticks.

Acknowledgments Sincere thanks are due to the TANUVAS for providing TANUVAS merit scholarship and thanks are due to the Head of Department of Veterinary Parasitology for facilities provided.

References

- Amero S, James TC, Elgin CR (1994) Production of antibodies using proteins in Gel Bands. In: Walker JM (ed) Methods in molecular biology, basic protein and peptide protocols, vol 32. Humana, Totowa, pp 401–406
- Aruljothi A (2003) Immunological control of *Rhipicephalus haemaphysaloides*. M.V.Sc., thesis submitted to TANUVAS
- Bhanusali JK, Gilbert JM, Mc Dougald LR (1994) Simple method to purify chicken immunoglobulin G. Poultr Sci 73:1158–1161
- Kumar BVD (2004) Immunoprophylaxis against haemaphysalid ticks in sheep. Ph.D., thesis submitted to TANUVAS
- Manohar GS, Banerjee DP (1992) Immune responses in rabbits immunized against the tick, *Hyalomma anatolicum anatolicum*. Ind J Anim Sci 62:505–512
- Opdebeeck JP, Wong JYM, Jackson LA, Dobson C (1988) Hereford cattle immunized and protected against *Boophilus microplus* with soluble and membrane-associated antigens from the midgut of ticks. Parasite Immunol 10:405–410
- Opdebeeck JP, Wong JYM, Jackson LA, Dobson C (1989) Vaccines to protect Hereford cattle against *Boophilus microplus*. Immunology 67:363–367
- Raman M (2000) Immunoprophylaxis against Boophilid ticks. Ph.D., thesis submitted to TANUVAS
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
- Wagner U, Burkhardt E, Failing K (1999) Evaluation of canine lymphocyte proliferation: comparison of three different colorimetric methods with the H3-thymidine incorporation assay. Vet Immunol Immunopathol 70:151–159
- Wikel SE (1979) Acquired resistance to ticks. Expression of resistance by C4 deficient guinea pigs. Am J Trop Med Hyg 28:586–590
- Wikel SK, Osburn RL (1982) Immune responsiveness of the bovine host to repeated low level infestations with *Dermacentor andersoni*. Ann Trop Med Parasitol 76:405–414
- Willadsen PD, Kemp H (1988) Vaccination with concealed antigen for tick control. Parasitol Today 4:196–198