Acquisition of protective immunity in *Geochelone pardalis* against *Amblyomma marmoreum* (Acari: Ixodidae) nymphal ticks

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ABSTRACT

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Significant increases in serum globulins (α_1, α_2, β and γ) were observed in mountain leopard tortoises (*Geochelone pardalis*) after they had been immunized with nymphal homogenates of *Amblyomma marmoreum.* There was a concomitant significant increase in the numbers of leukocytes (lymphocytes, basophils, monocytes and eosinophils).

Resistance to nymphal-challenge infestations was manifested by reduced feeding time, lower engorgement masses, and significantly fewer ($P < 0,0001$) numbers of nymphs that moulted.

These findings are contrary to the generally reported phenomenon, that ticks do not induce resistance in their natural hosts.

Keywords: *Amblyomma marmoreum*, *Geochelone pardalis*, homogenates, immunity, nymphal ticks, tortoises

INTRODUCTION

Amblyomma marmoreum Koch, 1844 occurs over much of central and southern Africa and, in South Africa, the tick is particularly common in the eastern Cape (Theiler & Salisbury 1959). Adults infest reptiles such as lizards, snakes and tortoises (Norval 1975; MacLeod, Colbo, Madouly & Mwanaumo 1977; Ghirroti & Mwanaumo 1989; Walker 1991).

Immature stages of *A. marmoreum* are known to infest a wide variety of bird, reptile and mammal hosts (Horak & Williams 1986; Horak, MacIvor, Petney & De Vos 1987; Petney, Horak & Rechav 1987). *A. marmoreum* has specific attachment sites on many of their host species (Nelson, Keirans, Bell & Clifford

1975; Andrew & Petney 1981; Bauwens, Strijbosch & Stumpel 1983).

Earlier studies have shown that *A. marmoreum* are experimental vectors of *Cowdria ruminantium* (Walker & Schulz 1984; Oberem & Bezuidenhout 1987). Bezuidenhout (1987, 1988) showed *A. marmoreum* to be suitable laboratory carriers of *C. ruminantium.* Petney & Horak (1988) demonstrated the transmission of heartwater by *A. marmoreum*, and indicated that the immature stages of the tick could also transmit *C. ruminantium* to livestock in South Africa.

Further studies need to be conducted on how homogenates can induce specific protective immunity against ticks in their natural hosts, such as *A. marmoreum* in *Geochelone pardalis* (Norval 1975). It has been argued that repeated tick infestations do not induce protective immunity in their natural hosts (Chabaud 1950; Randolph 1979; Fielden, Rechav & Bryson 1992). It was for this reason that *A. marmoreum* and its host, *G. pardalis*, were selected for the study on homospecific resistance to ticks in their natural hosts.

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The acquisition of resistance to *A. marmoreum* nymphs in rabbits immunized with crude *A. marmoreum* nymphal homogenates, was reported by Tembo & Rechav (1992).

The present paper reports the findings on the homospecific, protective immune responses in tortoises immunized with nymphal homogenates of *A. marmoreum*.

MATERIAL AND METHODS

Ticks

A. marmoreum, originally collected from mountain leopard tortoises in Pretoria Zoological Gardens, were reared on Himalayan rabbits in the Biology Department of the Medical University of Southern Africa. All tick stages were kept and maintained as previously described (Winston & Bates 1960; Tembo & Rechav 1992).

Tortoises

Six adult mountain leopard tortoises of both sexes (average age: 6 years) were obtained from the Pretoria Zoological Gardens. They were de-ticked and left to acclimatize at 25°C at the Biology Department for 2 months. Throughout the experiment, tortoises were kept in pairs in plastic containers (40 x 22 x 25 cm). They were fed *ad libitum* on vegetables such as carrots, lettuce, tomatoes, lucerne and cabbage, supplemented with pumpkin.

Nymphal homogenates

A. marmoreum nymphal homogenates were prepared in batches. Three-week-old ticks were used, as previously described (Tembo & Rechav 1992).

Protein estimation in nymphal homogenates

The Biuret-rate method was used (Laurell 1972).

Blood sampling

One and 2 mℓ of blood for serological and haematological analyses, respectively, were collected under sterile conditions at 3-week intervals by intercardial puncture through a hole (1 mm in diameter) drilled in the sternal carapace. Blood was withdrawn with a 23-G needle and syringe. After each bleeding, the holes in the carapace were sealed by the application of tipp-ex fluid (Tipp-Ex Vertrieb GmbH & Co KG, POB 940290 D-6000 Frankfurt M94, Germany).

Serum-protein analysis

Albumin, α_1, α_2, β and γ globulins were analysed by electrophoresis by means of a modified Laurell (1972) method.

Blood-cell counts

Blood samples for differential cell counts were collected by means of pyrogen-free needles and transferred into vials previously coated with EDTA. Blood-cell-count percentages were performed on thin blood smears stained with May-Grünwald and Giemsa (Meyday, Prier & Wilkinson 1969).

Immunization protocol

Nymphal homogenates were inoculated three times intramuscularly in the forelimb, at 3-week intervals. A dosage of 1,5 mg of homogenate in 2 mℓ of 9 mM phosphate-buffered saline (PBS) was given at each inoculation. All subsequent inoculations also contained 1,0 mg of Quil "A" saponin (QAS). Three control tortoises were given a dosage of 1 mg of QAS dissolved in 2 mℓ of sterile PBS.

Nymphal challenge

On day 63, each tortoise was placed in a plastic container (30 x 20 x 20 cm) coated with grease at the open edges to avoid ticks from escaping, and challenged by infestation with 450 *A. marmoreum* applied on the head, forelimbs and hind limbs. Nymphs that had not attached after 7 h were removed. Engorged nymphs were collected, weighed and kept in humidity chambers to moult. The duration of feeding as well as moulting percentages were recorded.

Presentation of data

The student T-test was employed in the analyses of serum globulin and leukocyte numbers, and the χ^2 test, in interpreting the number of nymphs that did not moult.

RESULTS

Humoral response to nymphal homogenates in immunized tortoises were generally demonstrated by significant increases in levels of α_1, α_2, the β serum globulins and the γ globulins (Table 1).

The cellular immune response was demonstrated by highly significant ($P < 0,001$) increases in monocytes and increases in lymphocytes ($P < 0,05$), basophils ($P < 0,025$) and eosinophils ($P < 0,05$) (Table 1).

Nymphs that fed on immunized and control tortoises engorged in a significantly shorter time (26,5 ± 5,21 d) than those that fed on control tortoises (36,1 ± 8,27 d) and mean engorgement masses were also significantly ($P < 0,001$) lower (Table 2).

The number of nymphs from immunized tortoises that moulted, was significantly ($P < 0,001$) less than those from the control tortoises (Table 2).

TABLE 1 Mean concentrations of blood components in three tortoises (1) immunized with nymphal homogenates of *Amblyomma marmoreum* and plus Quil "A" saponin, and (2) three controls with adjuvant only

Blood component	Group	Mean engorgement masses (± S.D)	T-test*	P
Albumin	1	12,30 ± 0,62	−2,40	−
	2	13,63 ± 1,37		
α_1 globulin		32,67 ± 2,80	0,71	0,500
	2	32,63 ± 1,27		
α_2 globulin	1	19,57 ± 1,12	0,54	0,050
		219,27 ± 0,61		
β globulin	1	22,87 ± 1,75	1,72	0,001
	2	21,27 ± 0,91		
γ globulin	1	14,43 ± 1,26	1,22	0,001
	2	12,90 ± 1,18		
Lymphocytes	1	54,00 ± 6,38	0,54	0,050
	2	55,75 ± 1,26		
Monocytes	1	10,00 ± 1,91	7,00	0,001
	2	8,00 ± 0,75		
Basophils	1	3,25 ± 0,96	0,77	0,025
	2	2,75 ± 0,50		
Eosinophils	1	5,00 ± 1,82	0,52	0,050
	2	4,75 ± 1,15		

* $n = 9$
degrees of freedom = 7

TABLE 2 Feeding performance of *Amblyomma marmoreum* nymphs on (1) three tortoises immunized with nymphal homogenates of *Amblyomma marmoreum* plus adjuvant, and (2) three with adjuvant only. Means are given ± S. D.

A. marmorium	Immunized	Control	T-test	P
Nymphs recovered	930	1 036	−	−
Mean feeding period (d)	26,50 ± 5,21	36,10 ± 8,27	< 8,71	0,01
Mean engorgement masses	77,99 ± 7,71	86,53 ± 8,23	< 3,60	0,05
No. and % moulting	478 (51,40)	632 (61,00)	−	−

DISCUSSION

Schneider, Rotj & Lehman (1971) reported that inoculation of tortoises with extracts of *Amblyomma testudinis* prevented successful propagation of the tick, because most nymphs failed to moult to the adult stage after feeding on the immunized reptile hosts. No observations were made on the effect of immunization on the induction of both humoral and cellular immune responses. In the present study, changes in the amount of all serum globulins were monitored (Table 1). There were significant changes in the α_1, and α_2 and β and γ globulins. Leukocytes also increased significantly. These findings in tortoises are similar to those observed in the immunized mammal hosts (Varma, Heller-Haupt, Trinder & Langi 1990; Tembo & Rechav 1992).

The argument that ticks do not induce resistance in their natural hosts because through co-evolution they developed mechanisms to circumvent the host's immune responses (Chaubad 1950; Randolph 1979; Fielden, Rechav & Bryson 1992), does not seem to hold in the case of *A. marmoreum* and its natural host, *G. pardalis*. Recently, Randolph (1994) also showed that bank voles (*Cleothrionomys gla–reolus*), a natural host of *Ixodes trianguliceps,* do acquire protective immunity against the tick.

Increases in the β and γ globulins in sensitized animals, and the reduction in mean engorgement masses of ticks that subsequently feed on them, serve as indicators of antigenicity and immunogenicity, respectively. Resistance in sensitized animal hosts can best be measured by the number of immature ticks that fail to moult to the next stage and, in the case of adult ticks, the reduced number of viable eggs oviposited by the females, serves as a good measure of resistance against ticks in animal hosts.

Ribeiro (1987) pointed out that various host species may differ in their physiological tolerance and defences against tick feeding. This could explain the differences in mean engorgement masses and feeding duration between the nymphs of *A. marmoreum* ticks that fed on tortoises and those that fed on rabbits (Tembo & Rechav 1992). Generally, *A. marmoreum* nymphs feed on tortoises for longer periods than they do on rabbits. Norval (1975) observed that under natural conditions, *A. marmoreum* nymphs fed for 8–20 d on sheep, and 51 d on tortoises. In this study, it took *A. marmoreum* nymphs 26,5 ± 5,21 d to feed to repletion on immunized tortoises.

A. marmoreum ticks are of great economic importance because they commonly use tortoises as hosts, together with immature stages of *Amblyomma hebraeum* which are the major vectors of *Cowdria ruminantium*. *A. marmoreum* feeding at the same time as infected *A. hebraeum* do, could become infected (Walker & Schulz 1984; Oberem & Bezuidenhout 1987).

This study has shown that nymphal homogenates of A. *marmoreum* induced a protective immune response in tortoises, that was demonstrated by reductions in both the duration of nymphal-challenge feeding periods and in mean engorgement masses. The immune reaction was illustrated by decreased moulting percentages of nymphs that fed on the immunized tortoises.

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