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Induction of host resistance to *Rhipicephalus appendiculatus* in rabbits: effects of immunizing with detergent-solubilized tick tissue proteins*

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Abstract. Resistance to the hard tick, *Rhipicephalus appendiculatus*, was induced in rabbits by immunizing them with tick tissue proteins extracted with a detergent, Triton X-100. There was 25% mortality in female ticks fed on immunized rabbits as compared with those fed on controls. Similarly, there was a 40% and 60% reduction in the engorged weight and the weight of egg batches, respectively, of ticks fed on immunized rabbits. Western blot analysis of detergent-solubilized tick tissue proteins, carried out using immune sera, recognized a complex pattern of proteins. A strong reaction was observed with proteins with apparent molecular weights of 94000 and 40000 daltons.

Ticks are important vectors of diseases affecting livestock. In East Africa, the brown ear tick, *Rhipicephalus appendiculatus*, is the major vector of *Theileria parva*, the causative agent for East Coast Fever (Young et al. 1988). Due to the great extent of economical loss inflicted due to infestation of cattle, various strategies to control ticks have been tried unsuccessfully. Vaccination against ticks could be one component of an integrated pest management.

It has been known for some time that host animals acquire immunologically mediated resistance after repeated infestations with ticks (Wikel and Allen 1982). The resistance is mediated by complex inflammatory and immunological phenomena. Although there is extensive literature on the basis of immune mechanisms (Brown 1985; Wikel and Allen 1982; Willadsen 1980), very little attention has been paid to characterizing the antigens involved in naturally acquired resistance (Brown et al. 1984; Rutti and Brossard 1989; Shapiro et al. 1987). It is conceivable that these secreted antigens could be used to vaccinate animals.

Another approach has been to immunize potential hosts or laboratory host animals with antigens of tick tissues (concealed antigens) that play no part in naturally acquired immunity (Wikel 1988; Willadsen and Kemp 1988). Hence, there have been a number of attempts artificially to immunize potential hosts or laboratory host animals against ticks using crude antigen preparations from whole ticks (Ackerman et al. 1980; Mongi et al. 1986a; Willadsen 1987), reproductive organs and gut tissues (Allen and Humphreys 1979) and salivary glands (Brossard 1976; Wikel 1981).

Of all these reports, the results reported by Allen and Humphreys (1979) were the most striking. They showed that extracts of the midgut and reproductive organs of partially fed *Dermacentor andersoni* females, when used to immunize guinea pigs, led to a drastic reduction in the engorgement weights of the ticks and to a reduction in or the abolition of egg laying. However, when these extracts were used to vaccinate cattle, less striking effects were produced, although both engorgement weights and egg laying were affected. In spite of the quite encouraging results, this study was not followed by identification of the target antigens involved in manifesting the effects.

We report the results of our experiment using non-ionic, detergent (Triton X-100)-solubilized proteins from *R. appendiculatus* females to immunize rabbits and describe the effects of immunization on ticks fed on these animals. By immunoblotting techniques, we also identified some tick antigens that might be responsible for inducing immunological responses deleterious to ticks.

Materials and methods

Ticks. Ticks used in this study were *R. appendiculatus* Neuman from a colony maintained at the International Centre of Insect

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Physiology and Ecology, Nairobi, Kenya. Ticks were maintained on rabbits using the rearing and feeding conditions previously described by Bailey (1960) and Irvine and Brocklesby (1970).

Animals. Male New Zealand rabbits weighing 2–3 kg that had had no previous contact with ticks were used in this study.

Extraction of detergent-solubilized antigens. Adult female ticks that had been allowed to engorge partially on rabbits for 6 days in the absence of adult males were used for the extraction of antigens. A total of 200–300 partially engorged females were homogenized in 10 ml PBSE buffer [10 m*M* sodium phosphate (pH 7.0), 100 m*M* NaCl, 1 m*M* EDTA containing 1 m*M* phenylmethylsulphonyl fluoride] on ice using a Kinematica polytron at a setting of 7–8. Homogenization was repeated three times for 1 min each at 1-min intervals. The homogenate was centrifuged at 5000 rpm for 10 min at 4° C in a Sorvall RC5 centrifuge using an SS34 rotor. The pellet was again homogenized in 5 ml PBSE and centrifuged as above. The pellet thus obtained was resuspended in PBSE and the cellular debris was allowed to settle. The brownish turbid supernatant was decanted and the cellular debris, resuspended in PBSE; this was repeated until the supernatant was clear. The pellet was finally resuspended in 3 ml PBSE containing 1% Triton X-100 and homogenized as above. The homogenate was centrifuged at 15000 rpm for 10 min at 4° C. The supernatant finally obtained was used for immunizing rabbits.

Immunization of rabbits. Before the immunization was started, both the experimental and the control rabbits were bled from the ear margins to obtain pre-immune sera. At 1 week after the second booster injection, rabbits were bled again to collect sera. Each experimental rabbit was immunized with 1 ml antigen (1.2 mg protein/ml) emulsified with an equal volume of Freund's complete adjuvant (FCA). Rabbits were injected at three subcutaneous and two intramuscular sites. Control rabbits were treated the same way except that instead of antigen extract, PBSE was used to make an emulsion with FCA. At 3 and 5 weeks later, both the experimental and the control rabbits were given booster injections. Emulsions were prepared with the same concentration of antigen extract (or volume of PBSE), but with Freund's incomplete adjuvant (FIA).

Tick challenge and collection of data. At 2 weeks after the second booster, rabbits (six per group) were challenged with adult *R. appendiculatus*. A total of 20 females and 30 males were applied to each rabbit (half of the numbers on each ear, enclosed in a fine-mesh nylon bag). The engorged weights of ticks were recorded on the day of the drop. Each tick was kept in an individual tube for oviposition. At 20 days after the ticks had dropped from the rabbits (i.e. at the end of the oviposition period), the weight of the deposited egg batches was also recorded. Statistical analysis of the data was done using the non-parametric test of Mann-Whitney (Siegel 1956).

Electrophoresis and immunoblotting. Sodium dodecyl sulphate electrophoresis was carried out on 12% polyacrylamide slab gels (SDS-PAGE) according to Laemmli (1970). While part of the gel was being used for silver staining, proteins on the other part were electrophoretically transferred onto nitrocellulose paper. To characterize antigens recognized by the immune sera, immunological reactions were carried out on the paper as previously described by Towbin et al. (1979), with slight modifications. Instead of bovine serum albumin to block unoccupied sites on nitrocellulose paper, a 5% fat-free milk solution in TRIS-buffered saline [20 m*M* TRIS-HCl (pH 7.5) 0.5 *M* NaCl] was used. During incubation with primary and secondary antibodies, the concentration of the milk powder was reduced to 1%. Primary antibody was used at a dilution of 1:400 and the secondary antibody, goat-anti-rabbit IgG conjugated to horseradish peroxidase (Cappel Laboratories), was used at a 1:1000 dilution. Antigen-antibody reaction was visualized with the substrate 4-chloro-1-naphtol.

Results

Feeding and oviposition performance of ticks

Of the 120 female ticks applied to each of the control and immunized groups of rabbits, only 87 (73%) attached to the immunized animals vs 104 (87%) on the control rabbits (Table 1). The effect of feeding on immunized rabbits was further manifested by only 63% of the ticks' being able to feed and about 54% being able to lay eggs (on control rabbits: 85% and 83%, respectively). The number of ticks that could feed on immunized rabbits and deposit eggs was significantly different ($P<0.01$) from that on control rabbits.

The effects of immunizing rabbits with solubilized tick-membrane proteins were also observed in the amount of blood ingested, the weight of egg batches deposited by ticks and the egg conversion factor (Table 1). The mean engorged weight of ticks from immunized rabbits was about 231 mg as compared with 361 mg for ticks from control rabbits ($P<0.001$). The mean egg batch weight (90 mg) of ticks that fed on immunized rabbits was only half that of ticks fed on control rabbits ($P<0.001$). That the effect on egg production was not merely due to the reduced blood meal taken by ticks fed on immunized rabbits could be demonstrated by differences in the egg conversion factor (ECF; calculated by dividing the egg batch weight by the weight of the engorged tick). The ECF for ticks fed on immunized rabbits (0.36) was significantly lower ($P<0.001$) than that for ticks from control rabbits (0.49).

The data on the weights of engorged ticks and their egg batches were further analysed by weight frequency histograms (Fig. 1). It is quite clear that although most of the ticks from control rabbits had engorgement weights of ≥ 300 mg, ticks that dropped from immunized rabbits weighed ≤ 300 mg. A similar trend in the shift to lower weight classes was seen in the weight of egg batches of ticks fed on immunized rabbits (Fig. 1 B). Data for egg conversion factors is also presented as above to show the reduced egg-producing ability of ticks from immunized rabbits (Fig. 1 C).

Characterization of tick antigens

Sera obtained from the immunized rabbits was used to characterize tick antigens in the solubilized tick-mem-

Table 1. Effects of attachment, feeding and oviposition performance of *R. appendiculatus* females applied to immunized and control rabbits

	Control ($n=6$)	Immunized ($n=6$)
Number of ticks applied	120	120
Number of ticks attached	104 (87%)	87 (73%)*
Number of fed ticks	102 (85%)	76 (63%)*
Number of ovipositing ticks	100 (83%)	65 (54%)*
Weight of ticks (mg)	361.1 ±86.3	230.9 ±91.8**
Weight of eggs (mg)	179.4 ±57.3	90.2 ±53.7**
Egg conversion factor	0.49± 0.09	0.36± 0.12**

* $P<0.01$
** $P<0.001$

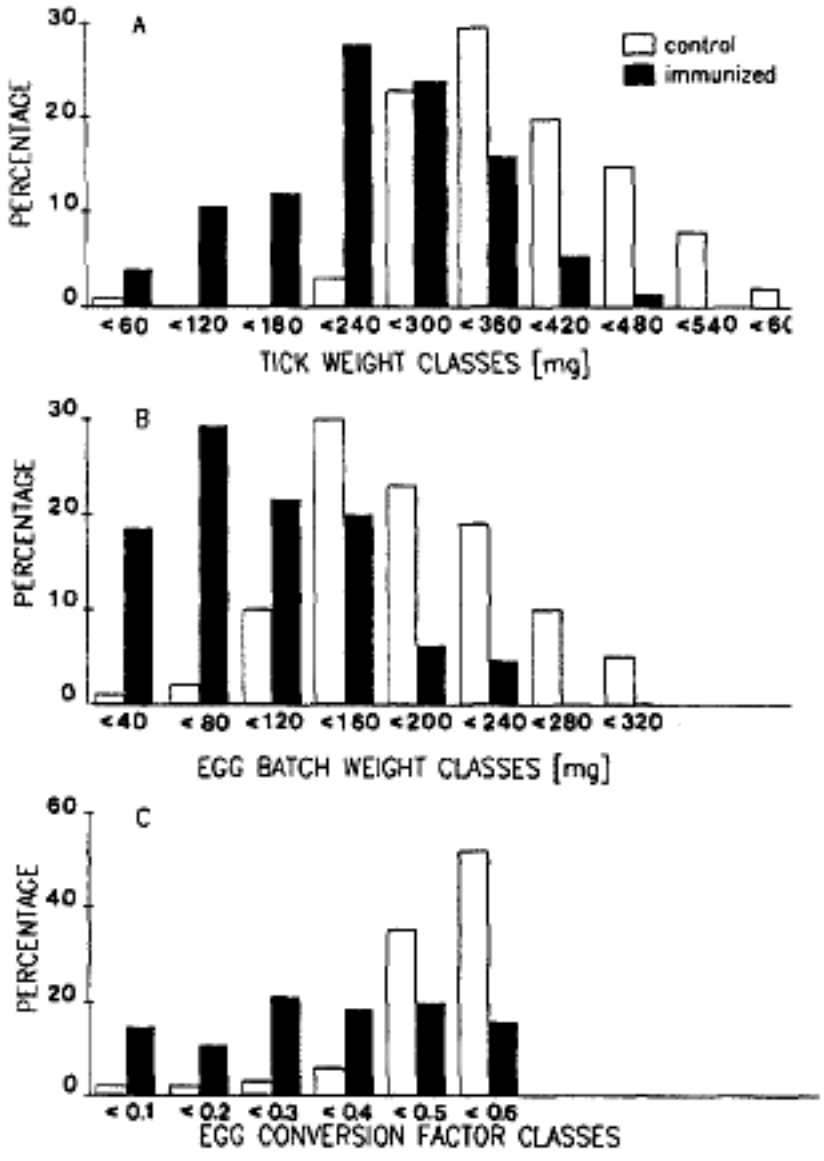

Fig. 1. Frequency histogram of weight of fed ticks, weight of eggs laid and egg conversion factors. Ticks in each class are presented as a percentage of the total number of ticks that completed engorgement on either immunized (*black bars*) or control (*grey bars*) rabbits. The weights of **A** ticks or **B** egg batches in each class are separated by increments of 60 and 40 mg, respectively. **C** Egg conversion factors are distributed in classes of 0.1 increments

brane protein extract. By immunoblotting, the pattern of antigens recognized by sera of the six immunized rabbits was seen to be similar (not shown). Besides a faint reaction with proteins in the high-molecular-weight region of the resolving gel, intense reactions with antigens with an approximate molecular weight of 90 kDa and one weighing 40 kDa were obtained with sera from immunized animals (Fig. 2). With sera from control rabbits, only a faint, nonspecific reaction with a 90-kDa protein was detected.

Discussion

Rabbits immunized with proteins extracted from whole ticks in the presence of Triton X-100 became resistant to an artificial infestation by the hard tick *R. appendiculatus*. The resistance was manifested by only 63% of the applied ticks' being able to engorge and 85% of those ticks' being able to lay eggs. The ticks that did oviposit produced eggs at a 30% reduced efficiency as compared with that of ticks applied to control rabbits. Although not as dramatic as those reported by Allen and Humphreys (1979), these results nevertheless reinforce previous indications that the possibility of vaccination of host animals against ticks is a real one (see review by Willadsen 1987).

Fig. 2. SDS-PAGE and immunoblot analysis of *R. appendiculatus* protein extracts. In all, 20 µg detergent-solubilized whole-tick extract of partially fed females was separated by SDS-PAGE 12% and proteins were visualized by silver staining. *A*, silver-stained gel; *M*, molecular-weight markers; *B*, immunoblot of the SDS gel, demonstrating the tick antigens recognized by immune sera (*1*). Control (*0*) represents tick proteins transferred onto nitrocellulose paper and reacted with horseradish peroxidase-conjugated anti-rabbit IgG only

Although we extracted antigens from pelleted cell debris of whole ticks obtained after the extraction of aqueous buffer-soluble proteins and used the whole extract for immunization instead of using either extracts from dissected tissues or fractionated extracts, electrophoresis and immunoblotting experiments revealed very few identifiable antigens. This contrasts with previous work in which sera from immune animals recognized a number of antigens (McGowan et al. 1980; Mongi et al. 1986b; Wikel 1981). One of the assumptions for a vaccine is that probably only one or a few antigens from a crude extract (aqueous or detergent-soluble) would be critical for its efficacy. On the other hand, if more than one antigen is critical, then a combination of the critical antigens would be required to increase the potency of the vaccine.

A number of investigators have demonstrated that immunoglobulins can cross the midgut of arthropods. The passage of IgG from the gut into the hemolymph has been demonstrated by immunocytochemical techniques in flies (Schlein and Lewis 1976), enzyme-linked immunosorbent assay in ixodid ticks (Ben-Yakir and Barker 1987) and immunoblotting methods in argasid ticks (Chinzei and Minoura 1987). However, all of these techniques are quite sensitive and are particularly useful for the detection of minute quantities of the molecule sought for. This indicates that antibodies do cross the midgut to enter the hemocoele, but in very small quantities. Nonetheless, the antibodies not only cross the midgut but also reach the target organs (Fujisaki 1978;

Schlein and Lewis 1976) and retain their specificity for the antigen to which the host had been exposed (Brossard and Rais 1984; Fujisaki et al. 1984).

The strength of this effect could perhaps be augmented by increasing the passage of immunoglobulins as a result of increased permeability of the midgut. In the present experiments, we extracted membrane-bound proteins from whole ticks (although the possibility of low quantities of buffer-soluble proteins in our extracts cannot be ruled out). If our extracts contained receptor proteins to any of the receptor-mediated endocytotic processes (for example, blood meal in the midgut or the major yolk protein in the ovary), then these would be ideal targets for the disruption of egg production, which we achieved to some extent. Moreover, a few of the ticks that engorged to repletion on immunized rabbits failed to digest the blood meal and died without producing any eggs, indicative of interference with digestion of the blood meal. It is noteworthy that individual ticks reacted differently to the induced host immune responses instead of host resistance having an all-or-none effect on the engorging ticks.

In this study we made use of only one commonly used non-ionic detergent. Other groups of investigators have used NP-40 (Willadsen and Kemp 1988). It would be interesting to use different concentrations of different detergents such as Triton X-114 (Bordier 1981) or beta-octyl-glucoside (Baron and Thompson 1975; Chattopadhyay and London 1984). With this approach it might be possible to extract membrane-bound antigens more selectively. Experiments along these lines are in progress in our laboratories.

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