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VACCINATION OF CATTLE AGAINST *RHIPICEPHALUS APPENDICULATUS* WITH DETERGENT SOLUBILIZED TICK TISSUE PROTEINS AND PURIFIED 20 kDa PROTEIN

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SUMMARY

Three groups of 4 cattle have been vaccinated with either detergent solubilized tick tissue proteins (SMP) of male and female *Rhipicephalus appendiculatus,* a 20 kDa soluble integumental antigen, a mixture of both SMP and 20 kDa. Two weeks after one booster injection all cattle were challenged by infestation with adult ticks.

Treatment had no influence on tick attachment but on cattle vaccinated by the 20 kDa 32.5 % fed ticks died ($p < 0.001$). Moreover, the mean weight of ticks fed on 7 out of 12 vaccinated cattle was significantly lower ($p < 0.05$ to $p < 0.001$). Individual differences could be seen where the mean weight reduction was up to 30 %. Moreover, ticks fed on 1 (group SMP) or 2 cattle (group 20 kDa) had some difficulties in converting their blood meal into eggs ($p < 0.05$ to $p < 0.001$).

RÉSUMÉ : **Vaccination de bovins contre *Rhipicephalus appendiculatus* : utilisation de protéines tissulaires de tiques solubilisées avec un détergent et d'une protéine purifiée de 20 kDa.**

Trois groupes de 4 bovins ont été vaccinés avec des protéines de tissus de tiques mâles et femelles *Rhipicephalus appendiculatus* extraites avec le détergent Triton X-100 (SMP), avec un antigène soluble de 20 kDa provenant du tégument ou avec un mélange des deux (SMP et 20 kDa). Deux semaines après une injection de rappel des bovins ont été infestés par des tiques adultes.

Les traitements n'ont pas eu d'influence sur la fixation des tiques, mais sur la survie, l'engorgement et la ponte des ectoparasites. Sur les bovins traités avec l'antigène de 20 kDa, 32,5 % des tiques nourries sont mortes dès leur détachement ($p < 0,001$). Le poids moyen des tiques nourries sur 7 des 12 bovins vaccinés, est significativement diminué jusqu'à 30 % ($p < 0,05$ à $p < 0,001$). De plus, les tiques nourries sur un bovin du groupe SMP ou sur deux du groupe 20 kDa convertissent avec difficulté le sang ingéré en œufs ($p < 0,05$ à $p < 0,001$).

INTRODUCTION

Rhipicephalus appendiculatus (Neumann, 1901) is the causative agent of East Coast Fever in East Africa. Beside the transmission of *Theileria parva,* this tick causes important economic losses by its massive infestation (Young *et al.,* 1988). To control tick populations, the use of acaricides is still the only efficient way, but the appearance of resistant strains to most acaricides available limits the use of this approach to tick control (Nolan, 1985; Riddles and Nolan, 1987).

Vaccination is envisaged to experimentally induce resistance. Since the work of Brossard (1976) and Allen and Humphreys (1979), several groups have shown that the injection of diverse tick extracts could induce resistance in laboratory animals and cattle (Wikel and Whelen, 1986; Willadsen *et al.,* 1988; Opdebeeck *et al.,* 1988*a*; 1988*b*; Wong and Opdebeeck, 1989; Jongejan *et al.,* 1989; Dhadialla *et al.,* 1990; Rutti *et al.,* 1991; Banerjee *et al.,* 1991).

In order to produce a vaccine, the identification of protective antigens is of crucial importance. Only few vaccination attempts have used defined antigens until now. Using biochemical techniques purified antigens derived from partially fed female *Boophilus microplus* have been used to induce resistance in cattle (Willadsen *et al.,* 1988; Jackson and Obdebeeck, 1990). The most impressive results have been obtained using a pure protein from the midgut of *B. microplus* (Willadsen *et al.,* 1989).

Recently, Rutti and Brossard (1989) identified an antigen of 20 kDa in the integumental extracts of *R. appendicu-*

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latus and of 25 kDa in *Ixodes ricinus*. A chromatographic fraction containing the 25 kDa protein was able to partially protect rabbits against *I. ricinus* (Rutti *et al.*, 1988; Aeschlimann *et al.*, 1989). Other experiments showed that tick insoluble material extracted with Triton X-100 was able to induce a high degree of protection against *R. appendiculatus* in rabbits (Dhadialla *et al.*, 1990).

In this paper we present results of a cattle vaccination experiment against *R. appendiculatus* using the integumental 20 kDa protein and material extracted with Triton X-100 from partially fed ticks. One group was injected with the purified 20 kDa protein. A second group has been injected with Triton X-100 solubilized proteins to confirm the presence of protective antigen for cattle in such crude fractions. A third group has been vaccinated with both antigens to see if a synergistic effect could be obtained.

MATERIAL AND METHODS

Host

Sixteen male *Bos taurus* cattle (Simenthal, Brown Swiss or Aberdeen Angus-Simenthal) approximately 6 months old and weighing about 150 kg each were used. They were randomly allocated to four groups (I to IV). These cattle were kept in a tropical stable at 25-27° C and 70-80 % relative humidity. Food and water were provided ad libidum.

Ticks

R. appendiculatus ticks (Muguga strain) were reared and maintained at the International Center of Insect Physiology and Ecology (Nairobi). They were fed for five days on cattle for antigen preparation.

For cattle infestation, ticks (Pietermaritzburg strain, Ciba-Geigy) were obtained from Ciba-Geigy, Saint-Aubin, Switzerland.

Antigen preparation

Two hundred grams of *R. appendiculatus* ticks, partially fed on cattle for 5 days were homogenized in 50 mM phosphate buffered saline (PBS) pH 7.4 containing 1 mM ethylene diaminetetraacetic acid (PBSE). One mM of a proteinase inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added to this buffer. We used a 5 : 1 ratio of this buffer to homogenize the ticks. The extract was centrifuged for 30 min at 12,000 g. The supernatant was kept on ice for treatment and the pellet reextracted as described before. After centrifugation under the same condition, the two supernatants were pooled.

The pellet was washed several time in PBSE. Cell debris and membranes were centrifuged at 5,000 g and the brownish supernatant discarded. This washing procedure was repeated until the supernatant was clear. The pellet was then extracted in PBSE in presence of Triton X-100 1 % (2 ml/g of precipitated material). After centrifugation at 20,000 g for 30 min at 4° C the supernatant, now referred to as solubilized membrane proteins (SMP), was removed and kept at – 20° C until used for subsequent analysis.

The supernatant containing soluble proteins was fractionated with ammonium sulfate 50-80 % (S2). The fraction which contained the 20 kDa antigen (Rutti and Brossard, 1989) was dialysed against 10 mM citrate phosphate buffer, pH 5.0 and loaded onto a carboxymethyl Sepharose column (Pharmacia Fine Chemicals). The proteins were eluted with a sodium chloride gradient of 10 to 200 mM. Fractions containing the antigen were pooled and concentrated on an Amicon PM 10 membrane then separated on a gel filtration column Sephadex G-50 fine (Pharmacia Fine Chemicals). Purity of the 20 kDa protein was monitored by SDS-PAGE (Laemmli, 1970).

Vaccination

The cattle received two injections in the prescapular lymph nodes at a 3 weeks interval. The first dose was prepared in complete Freund's adjuvant in a 1 : 1 ratio. The second dose was in incomplete Freund's adjuvant.

The first group, the control group, was injected with a solution of PBS, the second group received 0.4 mg of SMP per animal and per dose, the third group 0.5 mg of the 20 kDa antigen and the fourth group a mixture of SMP (0.4 mg) and of 20 kDa antigen (0.5 mg).

Challenge infestations

Fifteen days after the second injection, cattle were infested with 30 females and 35 males of *R. appendiculatus* per animal. A nylon sac, about 60 cm long was placed over the length of the tail, and was fixed to the region of the anus by adhesive tape. Ticks were introduced into the sac through the open end which was then closed off with adhesive tape. Movements of the tail were restricted by an elastic tied to the tail and to a bracket. After the drop off female *R. appendiculatus* were weighed and placed in saturated humidity at 28° C for egg laying and hatching.

Immunoblotting

A 10 ml blood sample was collected prior to immunizing the animals (day 0) and then one week before infestation. One more blood sample was taken one week after the last tick drop off. For immunoblotting we used the procedure described in Rutti and Brossard (1989). Five µg of SMP extract and 0.2 µg of the 20 kDa antigen were loaded and separated on SDS-PAGE 12 %.

Statistical analysis

Statistical analysis of the biological data was done using the non-parametric test of Mann-Whitney (Siegel, 1956).

RESULTS

Serological analysis

Tick antigens recognized by vaccinated cattle have been characterized by immunoblot analysis *(Fig. 1)*. Sera of cattle vaccinated with SMP extract mainly recognized antigens of high molecular weight *(Fig. 1C)*. This pattern seemed identical for all cattle sera in the same group (not shown). Three high molecular weight proteins or subunits of apparent molecular weight of 94, 90 and 89 were detected both in a soluble (S2) and solubilized extract (SMP) derived from partially fed *R. appendiculatus* females. A weak reaction was detected on the purified 20 kDa antigen.

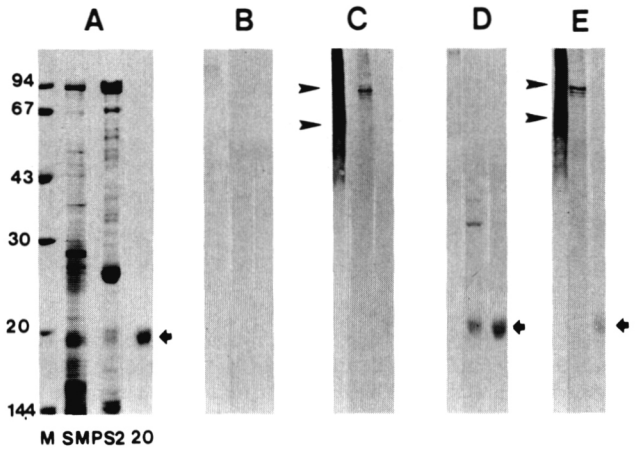

FIG. 1. — SDS-PAGE and immunoblot analysis of *R. appendiculatus* protein extracts with sera of vaccinated cattle.

Tick solubilized proteins (SMP), soluble proteins (S2) or pure 20 kDa integumental protein were run on SDS-PAGE 12 %. Silver staining (A) of each protein sample used to assess the antibody response obtained after vaccination of cattle with saline solution (B) (control); SMP (C); 20 KDa (D) or SMP + 20 kDa (E). Reference proteins (M) : phosphorylase *b* (94 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitore (20 kDa) and lysozyme (14.4 kDa).

Arrows (→) show purified integumental 20 kDa protein recognized by sera of vaccinated cattle with that antigen. Arrows (→) show high molecular weight antigens recognized by sera of vaccinated cattle with SMP.

All four cattle vaccinated with the 20 kDa antigen developed antibodies against that protein. *Figure 1D* shows that only a few other proteins were detected in the soluble extract. A strong reaction was observed both on the 20 kDa pure protein or on that present in the soluble extract.

Sera from cattle vaccinated with both the SMP extract and the purified 20 kDa antigen *(Fig. 1E)* produced a similar pattern in the immunoblot as those in *Figures 1C* and *D*.

EFFECT OF VACCINATION ON TICKS

Table I summarizes the effect of vaccination on the biology of *R. appendiculatus* females after challenge.

The mean weight of fed ticks on the four control animals was between 511.9 ± 112.8 and 568.9 ± 82.9 mg. Ticks applied to a host have approximately the same ability to feed on it.

The mean weight of fed ticks on 7 out of 12 vaccinated animals was significantly lower. This mean weight was affected in some cattle by more than 30 % (cattle 633, 636 and 640). Consequently the eggs laid were also affected.

In that experiment, the better and more regular protection has been obtained with the 20 kDa antigen. Three animals out of 4 belonging to that group have developed some resistance as shown by decrease of the mean weight of fed females ($p < 0.05$ to $p < 0.001$). The weight of eggs laid by ticks fed on these animals was also affected by the treatment ($p < 0.05$ to $p < 0.001$). The egg conversion factor was lower for ticks fed on 2 animals of that group ($p < 0.05$ to $p < 0.001$). In the two other groups vaccinated with SMP extract or with SMP extract and the 20 kDa antigen, the response was less uniform and only 2 animals out of 4 in each group have developed some resistance against *R. appendiculatus.*

In *Table II* some other biological criteria have been considered. Except for the group of cattle vaccinated with the 20 kDa antigen, no statistically detectable differences were observed on the number of live fed ticks and ticks laying eggs between control and vaccinated animals.

In cattle treated with the 20 kDa antigen, only about 40 % of female ticks deposited on cattle laid eggs (about 60 % for the control and for cattle immunized with the SMP antigen, 47 % in cattle immunized with SMP and the 20 kDa).

DISCUSSION

We have shown in this experiment that cattle vaccinated with a tick 20 kDa protein acquire a significant degree of resistance against *R. appendiculatus.* For the first time a purified antigen has been utilized with some success against a three host tick in cattle. By immunoblot, using sera of infested rabbits, this antigen has been detected in the tick integument extract (Rutti and Brossard, 1989). Such sera also recognize proteins of about 20 kDa in total extract from partially fed female *Amblyomma variegatum* and *B. microplus,* indicating common epitopes (Brossard *et al.,* 1991). These antigens are certainly related to the 25 kDa protein of *I. ricinus* which is a major constituent of the soluble proteins in the integument extracts of partially engorged adult female ticks. The relative abundance of that protein increases dramatically during the growth of the integument. It is probably involved in the formation of new alloscutum material, which is a prerequisite to a normal blood feeding. Some resistance has also been obtained with detergent solubilized tick tissue proteins (SMP). These observations confirm previous results with, either female *I. ricinus* ticks fed on rabbits vaccinated with the 25 kDa integumental protein (Rutti *et al.,* 1988; Aeschlimann *et al.,* 1989) or with female *R. appendiculatus* ticks fed on rabbits vaccinated with detergent solubilized tick tissue proteins (Dhadialla *et al.,* 1990). The combination of the 20 kDa antigen and crude solubilized material did not improve the protection.

In our experiment, similar protection with SMP against *R. appendiculatus* was obtained compared with that produced by immunization of cattle with crude extracts of *B. microplus* (Johnston *et al.,* 1986). Immunosuppressive

TABLE I. — *Biology of* Rhipicephalus appendiculatus *fed on vaccinated cattle.*

Treatment	*Cattle N°*	*Weight of fed ticks*	*Weight of eggs*	*Conversion factor*
CONTROLS	645	511.9 ± 112.8 (n = 15)	266.1 ± 69.3 (n = 15)	0.52
	646	568.7 ± 88.2 (n = 19)	270.0 ± 90.0 (n = 19)	0.48
	641	539.9 ± 122.4 (n = 21)	262.1 ± 117.9 (n = 19)	0.49
	642	568.9 ± 82.9 (n = 19	262.7 ± 103.0 (n = 18)	0.46
SMP EXTRACT	631	559.6 ± 95.0 (n = 19)	262.1 ± 88.7 (n = 18)	0.46
	632	502.5 ± 92.8* (n = 19)	249.8 ± 70.6 (n = 18)	0.51
	633	320.6 ± 138.8*** (n = 10)	144.4 ± 112.8** (n = 9)	0.40*
	634	544.4 ± 89.8 (n = 25)	294.9 ± 70.6 (n = 25)	0.54
20 kDa	635	520.3 ± 98.7 (n = 13)	267.1 ± 72.3 (n = 13)	0.51
	636	373.5 ± 83.7*** (n = 11)	181.3 ± 64.2** (n = 11)	0.47
	637	489.2 ± 90.7* (n = 13)	220.4 ± 72.0* (n = 10)	0.44*
	638	427.6 ± 117.5*** (n = 17)	192.8 ± 95.8*** (n = 9)	0.43***
SMP EXTRACT + 20 kDa	639	495.6 ± 149.0 (n = 13)	265.5 ± 106.3 (n = 11)	0.50
	640	374.6 ± 190.6*** (n = 11)	168.6 ± 74.0*** (n = 9)	0.44
	643	475.2 ± 96.2*** (n = 19)	264.4 ± 71.9 (n = 16)	0.53
	644	577.7 ± 88.8 (n = 21)	295.4 ± 97.7 (n = 20)	0.50

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

TABLE II. — *Biology of* Rhipicephalus appendiculatus *fed on cattle.*

	I *Controls*	*II* *SMP*	*III* *20 kDa*	*IV* *SMP + 20 kDa*
Infestation (I)	120	120	110[a]	120
Fed Ticks (FT)	82	85	80	73
Alive Fed Ticks (AFT) (AFT/FT × 100)	74 (90.2 %)	73 (85.9 %)	54** (67.5 %)	64 (87.7 %)
Ticks Laying Eggs (TLE) (TLE/AFT × 100)	71 (95.9 %)	70 (95.9 %)	43* (79.6 %)	56 (87.5 %)
Summary (TLE/I × 100	71/120 (59.2 %)	70/120 (58.3 %)	43/110* (39.1 %)	56/120 (46.7 %)

* $p < 0.01$; ** $p < 0.001$.

[a] : one animal was infested with 20 females and 30 males *R. appendiculatus* only.

substances present in these extracts, as those demonstrated in the salivary glands of *R. appendiculatus* (Fivaz, 1989), could depress the induced immunity. Using a crude midgut antigen (Opdebeeck *et al.*, 1988*b*) or the highly enriched antigen Bm86 (Willadsen *et al.*, 1989), more dramatic effect on the biology of ticks have been achieved. According to Willadsen *et al.* (1989) the induced immunity with midgut antigen provokes direct damage to the *B. microplus* midgut and is therefore different from that acquired naturally. The latter depends on the development of an immediate skin reaction and interferes essentially with the fixation of tick larvae.

Immunoglobulins can cross the midgut epithelium and still retain their immunological activity (Brossard and Rais, 1984). Using an efficient midgut-derived antigen instead of SMP could improve the passage of antagonist antibodies against other tick tissues like integument and consequently produce more relevant protection.

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