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Repetitive detection by immunoblotting of an integumental 25-kDa antigen in *Ixodes ricinus* and a corresponding 20-kDa antigen in *Rhipicephalus appendiculatus* with sera of pluriinfested mice and rabbits

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Abstract. Mice were pluriinfested with nymphs and rabbits, with adult *Ixodes ricinus*. As determined by immunoblotting, >50% of sera from these animals reacted against a tick antigen with a molecular weight of 25 kDa, which was detected in total extracts of partially fed *I. ricinus* females and in tick integumental extract. It was also found in engorged nymphs but was absent from larvae. Sera of *I. ricinus*-infested rabbits and mice or of rabbits infested with *Rhipicephalus appendiculatus* adults reacted with a 20-kDa antigen in total extracts of partially fed *R. appendiculatus* females and the integument of this species.

The effects and the immunological basis of host resistance to ticks have been reviewed by Willadsen (1980), Wikel (1982), Brown (1985), and Wikel and Whelen (1986). In our laboratory, the immunity of rabbits and its influence on the biology of *Ixodes ricinus* females has been the subject of several studies. Ticks feeding on resistant animals took smaller blood meals (Bowessidjaou et al. 1977), and their ability to convert the blood meal into eggs (egg conversion factor) was reduced (Brossard et al. 1982). Experiments involving the transfer of immune serum demonstrated the importance of humoral factors (Brossard 1977; Brossard and Girardin 1979). Cyclosporine A, an immunosuppressor acting on T lymphocytes, inhibited the resistance of rabbits; after this treatment, immediate and delayed-type hypersensitivity reactions against tick salivary antigens were reduced (Girardin and Brossard 1985; Girardin 1986). In addition, Brossard (1982) found that mepyramine, an antihistaminic-H_1, inhibited resistance in rabbits.

The tick antigens that initiate these immunological mechanisms are unknown. The use of the Western immunoblotting technique now permits better identification and characterization of antigens in parasite extracts. In the present study, we used this technique to detect and compare antigens of two species of ticks, *I. ricinus* and *Rhipicephalus appendiculatus*. We were particularly interested in a 25-kDa antigen of *I. ricinus* adults and nymphs, which elicited antibodies cross-reacting with a 20-kDa antigen of *R. appendiculatus* females. These antigens were abundant in the tick integumental extracts.

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Materials and methods

Infestation of mice or rabbits

I. ricinus were reared in our laboratory according to the method of Graf (1978). The different stages of *R. appendiculatus* were obtained from a laboratory colony maintained at the International Center of Insect Physiology and Ecology (ICIPE, Nairobi, Kenya). Adult BALB/c mice weighing approximately 30 g were used as hosts for *I. ricinus* and *R. appendiculatus* nymphs. Three successive infestations were carried out at 1-week intervals. For each infestation, 20 nymphs were placed on the head of a mouse anesthesized with 0.01 ml Nembutal (diluted 1:10 in a 0.9% NaCl solution)/g body weight. A collar prevented the mice from removing the ticks by scratching. The feeding period of the ectoparasites lasted 6–7 days. Himalayan rabbits of the genotype aac^Hc^H were used as the hosts for adult *I. ricinus* and *R. appendiculatus*. Ticks were fed on their ears and were protected by a nylon ear bag and a collar. Ten to fifteen males and females were used for each infestation.

Collection of sera

The blood needed for immunoblotting experiments was withdrawn from the retroorbital vein of mice before the first infestation (controls) and then 3 days after the blood meal of the

ticks. Before and after each infestation of rabbits, blood was collected from the marginal vein of the ear. The rabbit and mouse sera were separated by centrifugation at 1500 *g* for 15 min and stored in 50-µl aliquots at −20° C.

Tick antigens

For preparation of the protein extracts, *I. ricinus* larvae were fed on BALB/c mice and either *I. ricinus* nymphs or *I. ricinus* or *R. appendiculatus* adults were fed on rabbits. A total of 500 larvae were placed on the head of an anesthesized mouse and allowed to engorge fully. In all, 50 nymphs were fed to repletion and 100–150 adult ticks per rabbit were partially fed for 5 days. Approximately 250 mg tick weight/ml 50 m*M* phosphate-buffered saline (PBS, pH 7.4) containing 1 m*M* proteinase inhibitor phenylmethyl-sulfonyl fluoride (PMSF) and 1 m*M* ethylene diaminetetraacetic acid (EDTA) was homogenized twice with a Polytron (Kinematica) set at position 10 for 1 min at 4° C. The homogenate was centrifuged at 12,000 *g* for 20 min, and the supernatant was dialyzed overnight in 10 m*M* PBS (pH 7.4). The protein concentration was determined by the micromethod of BioRad, using bovine serum albumin as a standard. A total of 50 partially engorged female *I. ricinus* or *R. appendiculatus* were used to prepare the different tissue extracts. After the salivary glands and midguts were dissected, all other internal organs, muscles, and trachea were removed. The integumental extract of the ticks was homogenized as described above.

Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as previously described by Laemmli (1970), was used with a 10% or 12% polyacrylamide separation gel and a 6% polyacrylamide concentration gel. Tick extracts (20–50 µg) were boiled for 3 min in 2.5% SDS containing 0.7 *M* 2-mercaptoethanol, 6% glycerol, and 0.001% bromophenol blue before loading on the concentration gel. A 20-mA current was applied until the bromophenol blue migrated to the interface of the concentration gel; the current was then increased to 30 mA until the bromophenol blue migrated to the bottom of the separating gel.

The transfer was done according to the method of Burnette (1981) at 30 V for 14 h or 50 V for 4 h. The preparation of the blot and the immunological reactions were carried out as previously described by Tsang et al. (1983). The primary antibodies were diluted 1:200 in TRIS-buffered saline (TBS) with 1% milk and incubated overnight at room temperature. After several washes, the blots were incubated with the secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Cappel Laboratories), diluted 1:1000 in TBS-1% milk for 2 h at room temperature. Incubation of the blots with a solution of 0.6% 4-chloro-1-naphthol in 10 m*M* TRIS-HCl (pH 6.8) and 0.01% H_2O_2 for 5–15 min was sufficient to reveal the immunological reactions.

Results

During a preliminary experiment, we demonstrated that sera from *I. ricinus*-infested mice and *I. ricinus*- or *R. appendiculatus*-infested rabbits reacted with tick antigens of different molecular weights. However, >50% of the sera from rabbits (11 of 19) or mice (3 of 5) reacted with an antigen

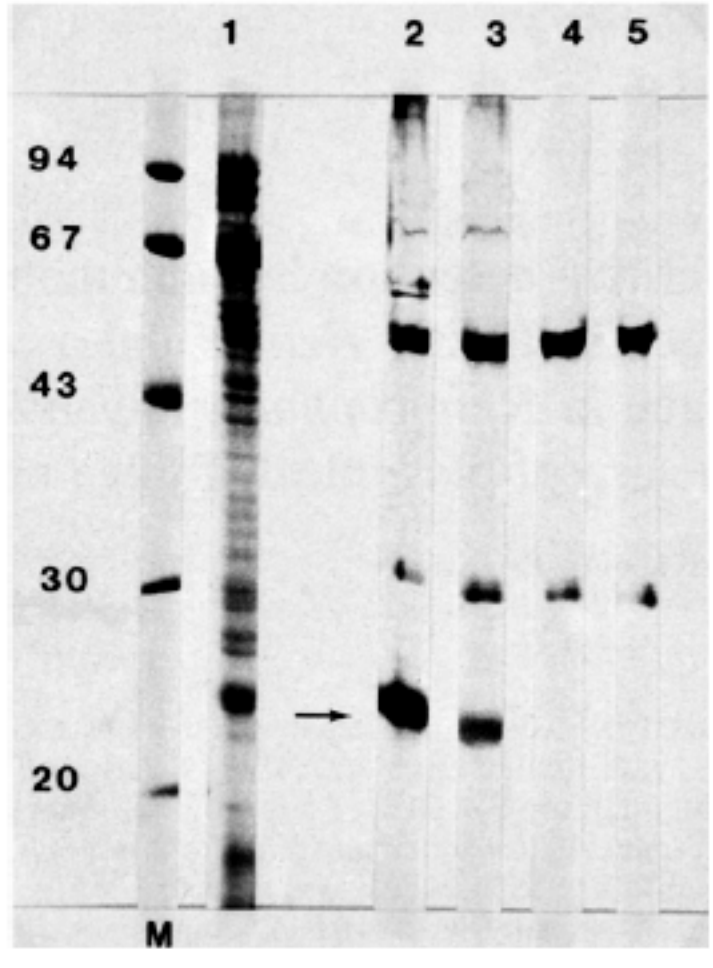

Fig. 1. Detection of a 25-kDa antigen (*arrow*) in total extracts from partially engorged *I. ricinus* using sera from rabbits pluriinfested with *I. ricinus* or *R. appendiculatus*. In all, 20 µg total extract from partially engorged *I. ricinus* females was separated on 10% SDS-PAGE and stained with Coomassie blue (*1*). Antigens were detected using sera from rabbits pluriinfested with *I. ricinus* (*2*) or *R. appendiculatus* (*3*). Control of immunological reactions was obtained with tick naive rabbit serum (*4*) or horseradish peroxidase-conjugated goat anti-rabbit IgG (*5*). Reference proteins (*M*): phosphorylase b (94 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa)

with an approximate molecular weight of 25 kDa in total extracts of partially fed female *I. ricinus*. Figure 1 demonstrates a typical immunological reaction obtained from such rabbit sera after SDS-PAGE and immunoblotting of proteins extracted from female *I. ricinus* (*1*). The 25-kDa antigen (*arrow*) was detected using sera from 9 of 16 rabbits pluriinfested with *I. ricinus* (*2*) or sera from 2 of 3 rabbits pluriinfested with adult *R. appendiculatus* (*3*). The control sera, obtained from tick naive rabbits, did not react with tick antigens, as demonstrated by the blot presented in (*4*). Only two bands corresponding to the heavy and light chains of ingested IgG were detected by using peroxidase-labelled anti-IgG for the immunoblotting reaction (*5*).

In Fig. 2, the 25-kDa antigen detected in the total extract of partially engorged *I. ricinus* females (A) was also detected in the extract of engorged

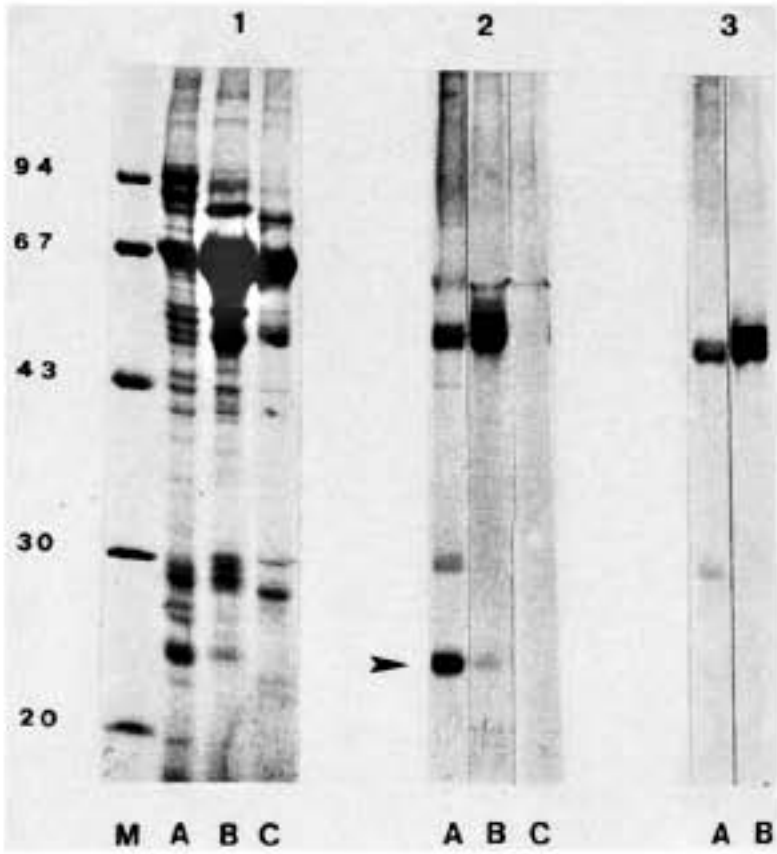

Fig. 2. Search for the 25-kDa antigen (*arrowhead*) in total extracts of adults, nymphs, and larvae of *I. ricinus*. Proteins from total extracts of adults, nymphs, and larvae of *I. ricinus* (*1: A, B, C*, respectively) were separated on 10% SDS-PAGE and stained with Coomassie blue. Antigens common to adult, nymphal, and larval stages were detected with serum from a rabbit pluriinfested by ticks (*2: A, B, C*, respectively). Control of immunological reactions was obtained with tick naive rabbit serum (*3: A, B*)

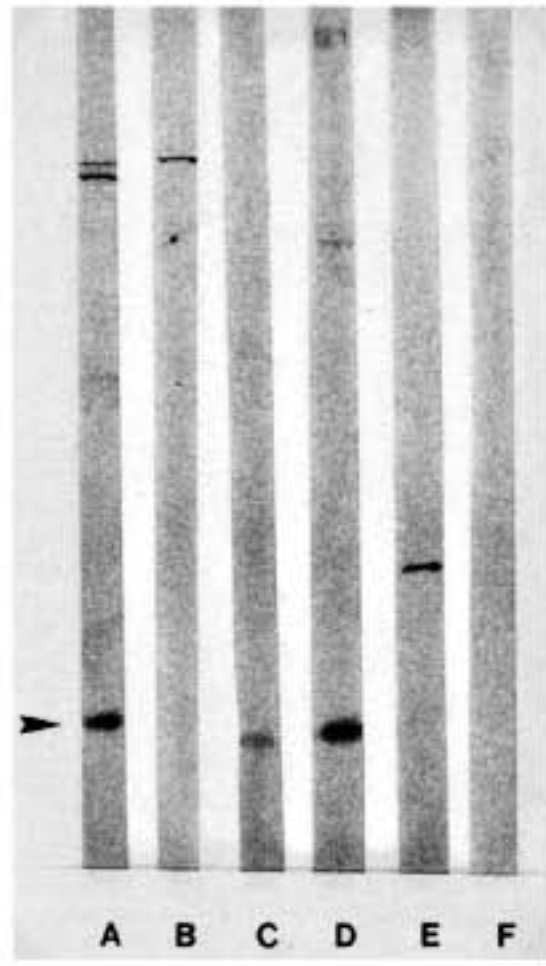

Fig. 3. Antigens from *I. ricinus* were detected by sera from BALB/c mice pluriinfested with nymphs of this species. Proteins from total extracts of partially fed *I. ricinus* females were separated on 10% SDS-PAGE. *A–E*, immunological reactions were obtained with five different sera; *F*, control using serum from a tick naive mouse

nymphs (B). In contrast, the same antigen could not be detected in the extract of engorged larvae of this ectoparasite (C). The intensity of the staining obtained after the immunological reaction may reflect the abundance of the antigen in the adult compared with that in the immature stages. However, due to the fact that nymphs and larvae had completely engorged, the ratio of tick antigens to blood proteins is much lower in these extracts. In the larval extract, the two bands corresponding to heavy and light chains of immunoglobulins were not detected, because the larvae were fed on mice and not on rabbits as were the nymphs and adults (A, B).

Five BALB/c mice pluriinfested with *I. ricinus* nymphs produced antibodies that also recognized antigens in the total extracts of partially engorged *I. ricinus* females (Fig. 3, A–E). The number of antigens detected varied with sera from one mouse to another, as was also the case for rabbits. In addition, the antigens also differed in their relative molecular weights. However, sera from three of five mice recognized a protein of approximately 25 kDa (A, C, D), which was also detected in the total extracts of *I. ricinus* females using sera from mice pluriinfested with *R. appendiculatus* nymphs (results not shown).

The repeated detection of this antigen in the adult and nymphal protein extracts of *I. ricinus* and its immunogenicity to two different hosts encouraged us to investigate its tissue source. Whole tick extracts were compared with salivary gland, midgut, and integumental extracts of adult *I. ricinus* that were considered to be potential sources of the 25-kDa antigen (Fig. 4A). Sera from a rabbit pluriinfested with adult *I. ricinus*, which contained antibodies to the 25-kDa antigen was used in immunoblotting experiments (Fig. 4B). A strong reaction was revealed in both the whole tick and integumental extracts. Only a weak reaction with a protein with similar molecular weight was detected in the midgut extract. The 25-kDa antigen was not detected in the salivary gland extract under our experimental conditions. In a similar experiment using *R. appendiculatus* extract, we could detect an abundant antigen weighing 20 kDa in both

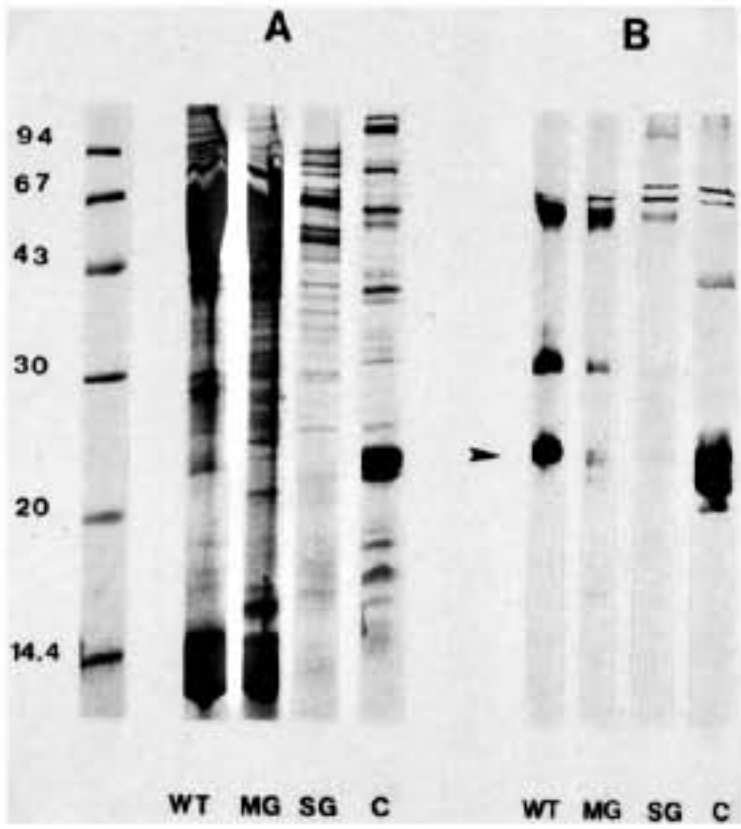

Fig. 4A, B. Search for the 25-kDa antigen (*arrowhead*) in whole tick (*WT*), midgut (*MG*), salivary gland (*SG*), and integumental extracts (*C*) from partially fed female *I. ricinus*. Proteins from the diferent extracts were separated by 12% SDS-PAGE and stained with Coomassie blue (**A**). Immunological reactions on these extracts were obtained with serum from a rabbit pluriinfested by ticks

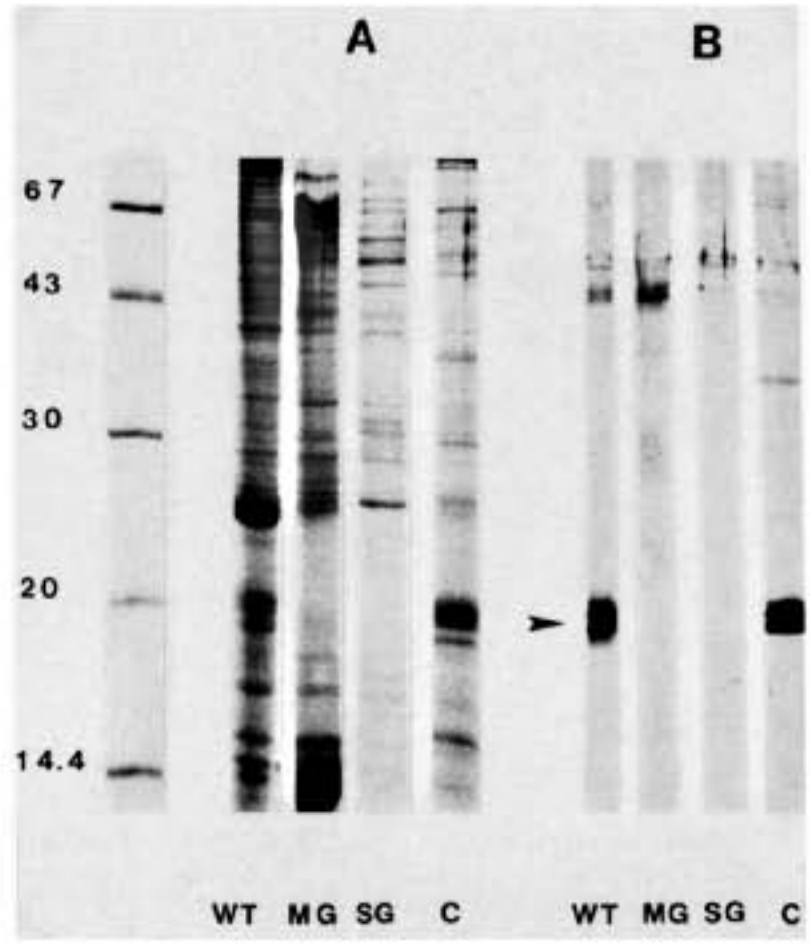

Fig. 5A, B. Antigens from *R. appendiculatus* detected with serum from a rabbit pluriinfested by *I. ricinus*. Whole tick (*WT*), midgut (*MG*), salivary gland (*SG*), and integumental extracts (*C*) from partially fed female *R. appendiculatus* were separated by 12% SDS-PAGE and stained with Coomassie blue (**A**). Immunological reactions on these extracts were obtained with serum from a rabbit pluriinfested by *I. ricinus*

the whole tick and integumental extracts (Fig. 5A, B).

Discussion

There are a number of reports on the effects of acquired resistance on the biology of ticks and on the implicated immunological mechanisms (see reviews by Willadsen 1980; Wikel 1982; Brown 1985). However, little is known about the antigenic molecules responsible for immunity; it has rarely been possible to assign a biochemical function to these immunogenic proteins. However, an esterase and a proteinase inhibitor have been purified from *Boophilus microplus* larvae (Willadsen and Williams 1976; Willadsen and Riding 1979). These proteins are allergens that initiate cutaneous reactions (immediate type) when injected into the skin of resistant bovines (Willadsen et al. 1978). Reich and Zorzopulos (1980) detected antibodies in sera from bovines infested with this species that inhibited in vitro a phosphomonoesterase extracted from larval ticks.

Antigens of different tick species (*Dermacentor andersoni, Amblyomma americanum, Hyalomma anatolicum,* and *R. appendiculatus*) have been described using the immunoblotting technique (Wikel and Whelen 1986). Gill et al. (1986) have also characterized salivary gland antigens in *H. anatolicum*. At least 17 different antigenic proteins have been detected by sera from infested rabbits. Cross-reactions between *D. andersoni* and *A. americanum* have also been demonstrated (Whelen et al. 1984). With sera of highly resistant guinea pigs, a 20-kDa protein was detected in the salivary gland as well as cement extracts of *R. appendiculatus* (Shapiro et al. 1986) and *A. americanum* (Brown et al. 1984).

The present study presents interesting and comparative information on the antigens of two species of ticks that are disease vectors of economic importance: *I. ricinus* in Europe and *R. appendiculatus* in Africa. The use of sera from infested rabbits and mice permitted the identification of antigenic macromolecules by the immunoblotting technique. The variability of response from one host to another makes the interpretation of the results difficult. Nevertheless, our study demonstrates that one *I. ricinus* antigen with a molecular weight of 25 kDa was detected in >50% of the sera from infested animals. It was found in the total extracts

of nymphs and partially fed females of *I. ricinus*. In partially fed female *R. appendiculatus*, a 20-kDa antigen was detected using the same sera. In both species, these antigens were abundant in integumental extracts. Using the indirect immunofluorescence and protein A-gold technique with antisera against the 25-kDa and 20-kDa proteins, specific labelling has been detected mainly in the epidermis and cuticle of the alloscutum of female *I. ricinus* and *R. appendiculatus* (Haug, personal communication). Immunolabelling has also been revealed in hemocytes, nephrocytes, and cells that provide contact between the cuticle and muscle cells.

To stimulate the rabbit immune system, the tick saliva might contain quantities of these proteins too law to be detected under our test conditions. It is also possible that they possess an antigenic determinant in common with a saliva protein with a different molecular weight.

In conclusion, the 25-kDa and 20-kDa antigens might be important in the process of material deposition in the cuticle during feeding of ixodid ticks. As intact immunoglobulins pass across the gut wall of *I. ricinus* (Brossard and Rais 1984), specific antibodies to these antigens could interfere with the cuticle formation. At present, the 25-kDa and 20-kDa proteins are the subject of more in-depth biochemical and immunological investigations.

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