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IMMUNITY AGAINST FEMALE *IXODES RICINUS* L.: EFFECT ON FEEDING AND HAEMOGLOBIN DIGESTION

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SUMMARY

Rabbits have been infested 3 times with 10 females and 10 males *Ixodes ricinus.* Immunity which is acquired when ticks feed on naive rabbits (first infestation) perturbs tick feeding on reinfested animals (third infestation). Then ticks ingest less blood ($p < 0.001$). Blood meal digestion in also altered. It was estimated by measuring haemoglobin concentration in ixodid midgut during 20 days after their drop off. After the first infestation this concentration decreases linearly with time ($r_1^2 = 46.14$ %, $n_1 = 63$, $p < 0.001$). After 3 infestations it is no longer correlated with time, indicating an impaired digestive process ($r_3^2 = 7.15$ %, $n_3 = 49$, $p > 0.05$).

This observation was corroborated by an analysis of multiple regression. Haemoglobin concentration of tick midgut only correlates with time after a first infestation ($r_1^2 = 45.25$ %). In ticks fed on immune animals this concentration is predicted with the quantity of midgut C_3 and the weight of fed ticks and not with time ($r_3^2 = 60.99$ %).

RÉSUMÉ : **Immunité contre les tiques *Ixodes ricinus.* L. femelles : effet sur le repas et la digestion de l'hémoglobine.**

Des lapins ont été infestés à 3 reprises par 10 femelles et 10 mâles d'*Ixodes ricinus.* L'immunité acquise durant la première infestation pertube le repas des tiques nourries lors d'une réinfestation (3e infestation). Les ectoparasites prélèvent alors moins de sang ($p < 0{,}001$) et le digèrent plus difficilement. Ainsi la concentration de l'hémoglobine intestinale décroît linéairement après une primo-infestation ($r_1^2 = 46{,}14$ %, $n_1 = 63$, $p < 0{,}001$). Ce n'est plus vrai après une 3e infestation ($r_3^2 = 7{,}15$ %, $n_3 = 49$, $p > 0.05$), à la suite d'une digestion perturbée.

Cette constatation est corroborée par les résultats d'une analyse de régression multiple. La concentration d'hémoglobine intestinale ne corrèle avec le temps qu'après une primo-infestation ($r_1^2 = 45{,}25$ %). Pour des Ixodides nourris sur des animaux immuns, celle-ci dépend de la concentration en C_3 intestinale et du poids des tiques gorgées ($r_3^2 = 60{,}99$ %), le temps n'intervenant plus dans la régression.

INTRODUCTION

Skin sensitivity (immediate and delayed type) against *Ixodes ricinus* salivary gland antigens develops during successive infestations of rabbits (Girardin and Brossard, 1985). Treatment of immune animals with cyclosporin A, an immunosuppressive drug acting specifically on T cells, inhibits these phenomena and allows a better tick blood meal and egg laying (Girardin and Brossard, 1987; Girardin and Brossard, 1989). As shown by passive transfer of immune serum, humoral factors also participate in this immunity (Brossard, 1977; Brossard and Girardin, 1979). Titres of anti-tick salivary glands antibodies increase progressively during successive infestations of rabbits (Brossard *et al.,* 1982). As shown by a degranulation test, sensitization of circulating basophils against tick antigens and the concentration of the acute phase reactant C_3 are also higher in resistant animals (Brossard *et al.,* 1982; Papatheodorou and Brossard, 1987).

These inflammatory and immunological responses developed by hosts against ticks affect the tick nutrition and reproductive mechanisms (Allen, 1989). Female *I. ricinus* feed and lay fewer eggs after infestation on immune rabbits (Bowessidjaou *et al.,* 1977). They also convert their blood meal less effectively into eggs as indicated by the reduction of the egg conversion factor (weight of eggs laid/weight of fed tick; Brossard *et al.,* 1982). This may indicate that digestion of the blood meal might be disturbed in ticks fed on immune animals.

Here, this hypothesis has been verified by following the change in midgut haemoglobin concentration during the 20 days after drop off in female *I. ricinus* fed on naive (1st infestation) and immune rabbits (3rd infestation). Ticks also ingest C_3 during their blood meal (Papatheodorou and Brossard, 1987). Using a multiple regression analysis, midgut C_3 as well as duration of the blood meal, weight

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of fed ticks and time after the drop off have also been considered to predict the concentration of midgut haemoglobin in ticks fed on naive or immune rabbits.

MATERIEL AND METHODS

Ticks

I. ricinus ticks were bred in our laboratory. The infestation conditions are as described previously (Bowessidjaou *et al.*, 1977). Three infestations of 10 females and 10 males are made alternately on each ear of Himalayan male rabbits (aac^H c^H) of about 2 kg each. Only females of this species engorge, but copulation is necessary for an optimal blood meal (Graf, 1978).

Midgut extracts

To measure midgut haemoglobin and C_3 levels, female *I. ricinus* engorged during the first or third infestations were weighed and dissected immediately after the drop off and every 2 days of the preoviposition and oviposition periods. The last dissection was carried out 20 days after the drop off. Each midgut was sonicated (Labsonic 1510 100 W) in 150 mM PBS pH 7.2 at 4° C. After centrifugation at 25.000 G for 15 minute at 4° C, the supernatant was made up to 5 ml with PBS, lyophilised in aliquot of 1 ml and stored at 4° C. Before use, lyophilised aliquots were reconstituted with distilled water according to the respective tick weight: 60-120 mg with 0.2 ml, 120-240 mg with 0.4 ml, 240-360 mg with 0.6 ml, > 360 mg with 0.8 ml. Ticks weighing less than 60 mg were not taken into account in this study. The sensitivity of the tests was insufficient to detect C_3 and only scarcely just sensitive enough to detect haemoglobin in these ticks.

Determination of midgut haemoglobin

In a preliminary assay the absorption spectrum of rabbit haemoglobin (Sigma Chemicals Company) and tick midgut extracts has been compared (results not shown). Both spectra are similar with the two characteristic peaks of absorption at 540 and 578 nm. Consequently haemoglobin of midgut extracts has been measured by a photometrical test routinely utilized for red cells haemoglobin determination (Roche). Twenty μl of reconstituted midgut extract is converted by potassium ferricyanate and potassium cyanate (200 μl of Roche reactant) into cyanmethaemoglobin. After an incubation period of 3 min at room temperature, haemoglobin was measured at 570 nm (Microelisa autoreader MR580, Dynatech). Haemoglobin concentration was determined (mean of 3 measurements) according to a standard curve prepared with rabbit haemoglobin. Results are expressed as percentages of haemoglobin out of tick weight (mg haemoglobin/mg fed tick × 100).

Single radial immunodiffusion test for C_3 determination

Anti-rabbit-C_3 (0.75 μl/ml, Cappel) in barbitone buffer pH 8.6 (5′5 diethyl barbituric acid 4.3 mM, 5′5 diethyl sodium barbiturate 20 mM, NaN_3 15 mM) was mixed with agarose (1 % in barbitone buffer) at 50° C. This mixture was poured as a 1.5 mm layer onto a glass plate. Five μl of reconstituted midgut extracts were deposited into wells punched in the gel. After an incubation period of 48 h in a humid atmosphere at room temperature the diameter of precipitates was measured. The logarithm of midgut C_3 level is proportional to the diameter of the precipitates. A standard curve was made using a reference serum. For each tick, results were expressed as equivalent dilutions of a standard serum per 100 mg of fed tick (C_3 equivalent dilution/mg fed ticks × 100).

Statistical analysis

Haemoglobin content in the tick gut has been analysed in time by simple linear regression (Scherrer, 1984). The following parameters have also been considered in a multiple regression analysis to express midgut haemoglobin: blood meal duration, weight of fed ticks and concentration of midgut C_3. For each infestation (first and third infestations), a comparison between the concentration of midgut haemoglobin after the drop off and 20 days later has been realized with the non parametric Mann-Whitney test. Comparison between feeding duration and weight of fed ticks on naive and immune rabbits has been done using the same test.

RESULTS

1 — Tick biology

Only duration of tick blood meal and weight of fed ticks are considered in this study *(table I)*. Indeed all ticks were dissected after their drop off in order to measure the concentration of midgut haemoglobin and C_3. Ticks fed on immune rabbits (3rd infestation) weigh less than those fed on naive animals ($p < 0.001$). The duration of their blood meals is longer ($p < 0.001$).

Table I. — *Tick biology.*

Infestations	*n*	*Mean weight of fed ticks (mg)*	*Mean duration of blood meal (d)*
1	63	254 ± 80.7	6.6 ± 0.8
		*	*
3	49	171.6 ± 84.8	8.2 ± 2.8

(*d*) days; *n* = number of fed female *I. ricinus;* * $p < 0.001$.

These results are analogous to those observed previously (Bowessidjaou *et al.*, 1977; Brossard *et al.*, 1982).

2 — Evolution of midgut haemoglobin of ticks

The haemoglobin content in the midgut of fed female *I. ricinus* has been measured from their drop off until 20 days after (i.e. during preoviposition and oviposition). Results have been compared between 2 infestations (1st and 3rd, *fig. 1 and fig. 2)*.

A pronounced decrease of haemoglobin concentration occurs only after the first infestation *(fig. 1)*. Diminution is here linear ($r_1^2 = 46.14$ %, $n_1 = 63$, $p < 0.001$). In contrast, there is no correlation between haemoglobin concentration and time after the 3rd infestation *(fig. 2;*

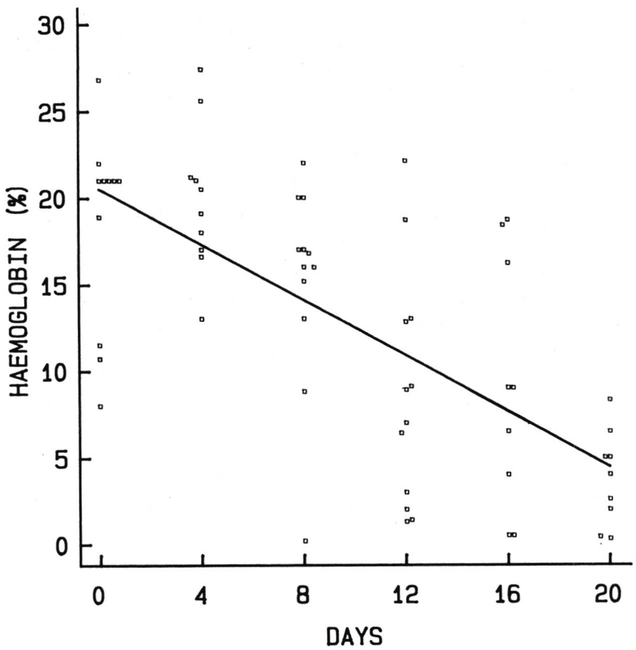

FIG. 1. — Relation between haemoglobin concentration of tick midgut and time after drop off (during 20 days) in a first infestation. Haemoglobin is expressed in mg per 100 mg of fed ticks. $y = -0.80\,x + 20.44$ ($r_1^2 = 46.14$ %, $n_1 = 63$, $p < 0.001$).

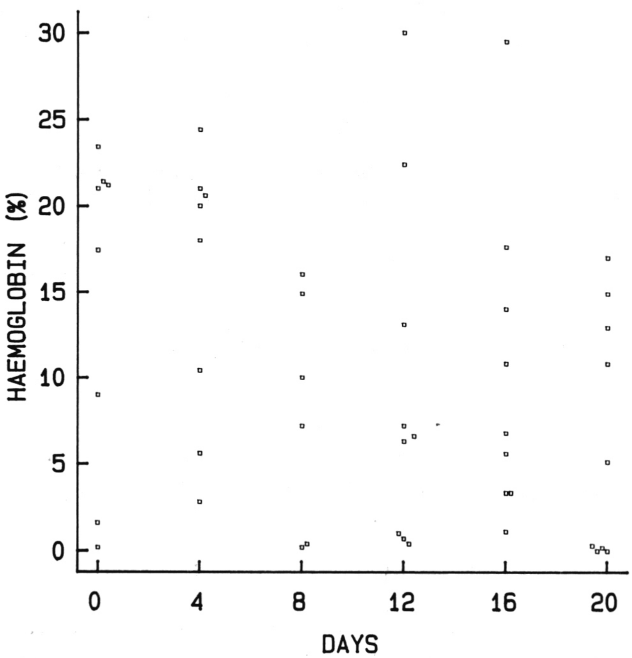

FIG. 2. — Relation between haemoglobin concentration of tick midgut and time after drop off (during 20 days) in a third infestation. $y = -0.36\,x + 14.86$ ($r_3^2 = 7.15$ %, $n_3 = 49$, $p > 0.05$).

($r_3^2 = 7.15$ %, $n_3 = 49$, $p > 0.05$). Moreover haemoglobin quantities in tick midgut after the drop off and 20 days later only differ for the 1st infestation ($p < 0.001$,

TABLE II. — *Midgut haemoglobin after the tick drop off.*

	Haemoglobin concentration in mg %		
Infestations	*Day 0*		*Day 20*
1	19.7 ± 6.9 (n = 12)	*	3.9 ± 2.6 (n = 10)
3	14.4 ± 9.4 (n = 8)		6.8 ± 7.1 (n = 9)

n = number of fed female *I. ricinus*; * $p < 0.001$.

table II). After the third, no difference is observed ($p > 0.05$). The quantity of ingested haemoglobin seems to differ from tick to tick particularly during that latter infestation (*fig. 2,* day 0). At the end of the preoviposition and oviposition periods, the midgut content of haemoglobin for some ticks is still high (*fig. 2*), day 20). Blood meal composition and haemoglobin digestion are then altered.

3 — EVOLUTION OF C_3 IN MIDGUT OF TICKS

Ticks ingest immunological and inflammatory factors with their blood meal (Papatheodorou, 1985; Papatheodorou and Brossard, 1987). In this study, midgut evolution of C_3 has been measured during 20 days after the tick drop off on naive and immune rabbits (*fig. 3 and fig. 4*).

The concentration of C_3 in tick midgut decreases in a linear way after each rabbit infestation ($r_1^2 = 43.18$ %, $n_1 = 63$, $p < 0.001$ for the first infestation;

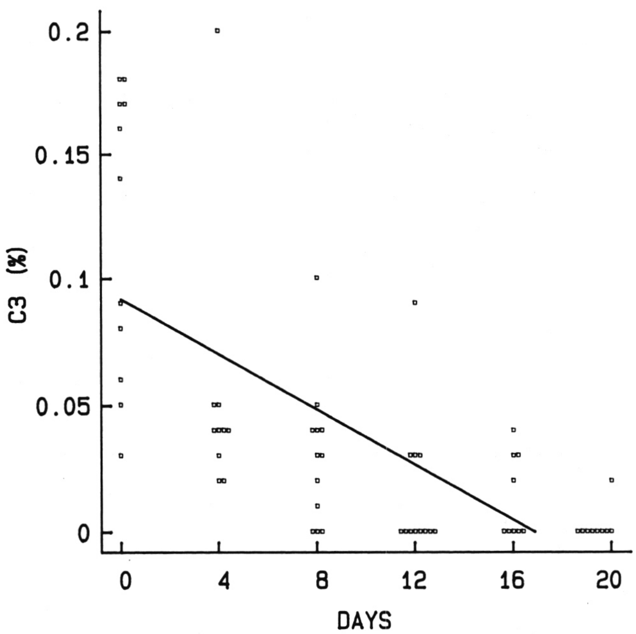

FIG. 3. — Relation between C_3 concentration of tick midgut and time after drop off (during 20 days) in a first infestation. $y = -0.005\,x + 0.091$ ($r_1^2 = 43.18$ %, $n_1 = 63$, $p < 0.001$).

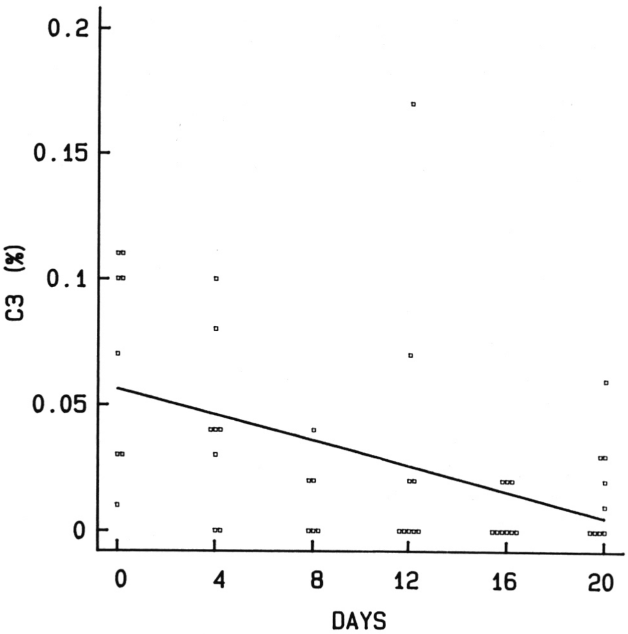

FIG. 4. — Relation between C_3 concentration of tick midgut and time after drop off (during 20 days) in a third infestation. $y = -0.002\,x + 0.056$ ($r_3^2 = 20.51$ %, $n_3 = 49$, $p < 0.01$).

($r_3^2 = 20.51$ %, $n_3 = 49$, $p < 0.01$ for the 3rd infestation). The correlation is weaker for the reinfestation.

4 — MULTIPLE REGRESSION ANALYSIS *(tableau III)*

After the 1st infestation, haemoglobin concentration is only correlated with time ($r_1^2 = 45.26$ %). This is certainly the expression of a normal tick digestion. After the third, midgut haemoglobin is expressed by the concentration of C_3 and the weight of fed ticks ($r_3^2 = 60.99$ %). Time elapsing from the tick drop off does not influence haemoglobin concentration. This observation confirms that the blood meal and its digestion is altered in ticks fed on immune rabbits. Moreover blood meal duration was never introduced into the regressions.

DISCUSSION

Blood meal digestion in ticks differ from haematophagous insects. In blood-sucking insects, digestion occurs extracellularly in the lumen of the intestine. In contrast it is a slow intracellular process in ticks (Balashov, 1972; Araman, 1979; Raikhel, 1983). Like in other haematophagous arthropods, the tick diet consists for 90 to 95 % of proteins (Diehl *et al.,* 1983). Among these, haemoglobin is the major constituent (Papatheodorou, 1985). In Argasids digestion of the blood meal begins only after the drop off (Tatchell, 1964; Arthur, 1965; Balashov, 1972). It can be divided into three stages: *a*) blood meal concentration, *b*) intense digestion and *c*) slow digestion phase (Galun and Warburg, 1967; Tatchell *et al.,* 1972; Aeschlimann and Grandjean, 1973). The ixodid digestion also displays three phases, but here two occur on the host and only the last, after tick drop off. There are : 1) A continuous-digestion phase which is initiated by feeding and corresponds with the slow-feeding period during several days (Tarnowski and Coons, 1989). During that time ticks utilize nutrients to synthesize new cuticle and to allow the growth of internal organs (Balashov, 1972; Araman, 1979). 2) A phase of reduced-digestion during the rapid-engorgement period which is generally initiated by mating, except for *I. ricinus* (Graf, 1978). During that period (12 to 24 hours before the drop off) the tick weight increases by about 150 times. 3) A further phase of continuous-digestion during the post-feeding period of preoviposition and oviposition. It involves the digestion of the blood meal taken up during the rapid-engorgement period. The majority of the digested blood meal is now used to produce the female-specific protein vitellogenin and consequently the eggs (Snow and Arthur, 1966; Araman, 1979).

As shown in this study, host immunity impairs the feeding and digestion of female *I. ricinus* during the periods of preoviposition and oviposition. In ticks fed on naive rabbits, the quantity of midgut haemoglobin diminishes continuously after their drop off. After twenty days haemoglobin in scarcely detectable. This observation is in accordance with the findings in female *Rhipicephalus sanguineus* fed on naive rabbits (Araman, 1979). In contrast, after a 3rd infestation, there is no longer a linear correlation between midgut haemoglobin concentration and time. Accordingly there is no statistically significant difference between haemoglobin concentration at the end of feeding and 20 days later. Numerous ticks feed and digest their blood meal with difficulty. This observation could explain the deleterious effect of immunity on tick reproduction,

TABLE III. — *Equations of multiple regression predicting haemoglobin concentration in tick midgut.*

Infestations			
1	$H = -0.80\,x + 20.44$	$r_1^2 = 45.26$ %	$n_1 = 63$
3	$H = 1.64\,y + 0.03\,z + 1.04$	$r_2^3 = 60.99$ %	$n_3 = 49$

H = midgut haemoglobin (mg %); x = days after drop off; y = midgut C_3 (%); z = weight of fed tick (mg); r^2 = squared correlation coefficient; n = number of fed ticks.

particularly the bad conversion of the blood meal into eggs (Brossard *et al.*, 1982).

Using multiple regression analysis haemoglobin content of the tick midgut has been predicted, confirming previous observations. After the first infestation, haemoglobin concentration correlates only with time suggesting normal digestive behaviour. After the third, only the quantity of C_3 and the weight of fed ixodids enter into the regression. With the present state of our knowledge, it is difficult to interpret such mathematical analyses biologically. Cytological and biochemical studies have shown that the development as well as the protease activity of midgut epithelium are delayed in female *I. ricinus* fed on immune animals (Girardin, 1987). Some disorders of that epithelium have also been observed. The peritrophic membrane which is also present in female *I. dammini* (Rudzinska *et al.*, 1982) is thickened and midgut microvilli are in a degenerate state (Girardin, 1987). In contrast to the intestine content of ticks fed on naive animals, which is clear, that of ixodids fed on immune animals is filled with a granular material containing unlysed leucocytes. Hemolysin activity which has been described in *I. dammini* (Ribeiro, 1988) and endocytic mechanisms could be inhibited. Moreover in Western blot analysis, antibodies of infested rabbits react with antigens extracted from female *I. ricinus* salivary glands, integument and midgut too (Rutti and Brossard, 1989). In an other system (guinea pigs and female *Amblyomma americanum*) midgut antigens were also displayed (Brown, 1988). *In vivo* ingested antibodies and complement, in association with inflammatory cells, could delay the development of midgut epithelium and alter the structure of that tissue. Blood meal digestion and the transformation of nutrients into eggs could then be impaired.

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