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REACTION IMMUNITAIRE DU LAPIN CONTRE LA TIQUE *IXODES RICINUS* L. (IXODOIDEA, IXODIDAE): EFFETS SUR LA COMPOSITION ET LA DIGESTION DU REPAS SANGUIN

Thèse présentée à la Faculté des Sciences de l'Université de Neuchâtel pour obtenir le grade de Docteur ès Sciences

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Ixodes ricinus L. (Ixodoidea, Ixodidae):
Effets sur la composition et la digestion du
repas sanguin

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UNIVERSITÉ DE NEUCHÂTEL

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Progressive sensitization of circulating basophils against *Ixodes ricinus* L. antigens during repeated infestations of rabbits

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Summary The sensitivity of rabbit basophils to antigens from *Ixodes ricinus* females has been studied by a degranulation test. Observations of basophil numbers and degranulation were made on the 6th day of each of four sequential infestations. Maximal degranulation of cells was observed after challenge of cells with antigen at a concentration of 10^6 and 10^7 pg/ml. At these concentrations, during a 1st infestation, 21·8 and 23·6% of cells degranulated. During a 2nd infestation, these percentages increased (34·8 and 33·8%) and reached 59·8 and 63·8% by the 4th infestation. A plasma factor which partially blocks basophil degranulation, is described. This was already present during the 1st infestation, since in its presence the percentage of degranulation was reduced by 2·8 and 4·0% respectively on challenge with 10^6 and 10^7 pg antigen/ml. Inhibition was maximal at the 4th infestation (difference: 16·5 and 20·5%). Basophil sensitization and inhibition of the degranulation are thus both progressive phenomena. After 10–15 infestations on four other rabbits, 75·0 and 79·8% degranulation was obtained. The inhibition of degranulation by plasma was also greater (difference: 25·5 and 27·4%). IgG specific anti-*I. ricinus* antibodies were identified by indirect immunofluorescence. In two animals, they were detected at the 6th day of the 1st infestation. Subsequently, they were generally present for all the animals.

Keywords: *Ixodes ricinus*, rabbit, immunity, basophils, antigens

Introduction

Rabbits infested by female *Ixodes ricinus* acquire a resistance which affects the nutrition and egg laying of this ectoparasite (Bowessidjaou, Brossard & Aeschlimann 1977). Experiments on the effects of immune serum transfer have shown that humoral factors participate in the expression of immunity (Brossard 1977, Brossard & Girardin 1979). Cell infiltration of the skin at the point of tick attachment occurs progressively during the course of infestation and changes in character during sequential infestations (Brossard & Fivaz, in press). For example, degranulated mast cells are more numerous at the beginning of re-infestation. However, degranulated basophils appear in the cutaneous

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lesion mainly towards the end of reinfestation, probably as a result of hypersensitivity phenomena. The reaction may be similar to the cutaneous basophil anaphylaxis described in other systems (Askenase *et al.* 1978).

In this work, we will show, by using a degranulation test (Benveniste *et al.* 1977), that circulating basophils are sensitized against the antigens of tick salivary glands. Further we will show the existence of a plasma factor blocking the specific degranulation of the cells.

We have also established for each infestation, the rate of egg production and the duration of preoviposition and embryogenesis. Thus, this study extends our knowledge (see Bowessidjaou *et al.* 1977) of the biological effects on *I. ricinus* of the resistance acquired by rabbits.

Materials and methods

I. ricinus ticks were bred in our laboratory. The infestation conditions were as described previously (Bowessidjaou *et al.* 1977). Four infestations were made on the ears of five Himalayan male rabbits (aa $c^H c^H$) of about 2 kg each. Four animals infested 10–15 times were also used. The rate of tick egg laying (mean weight of egg laying/ mean weight of fed females) was determined according to Graf (1978). The periods of preoviposition (time lapse from the end of feeding until the laying of the first eggs) and of embryogenesis (time separating the depositing of the first eggs and observation of the first larvae hatched) were also observed.

The IgG anti-*I. ricinus* antibodies were detected by an indirect immunofluorescence technique. Samples were taken on the 6th day of each infestation (Ambroise-Thomas 1969, Brossard 1976). Five micron histological sections of salivary glands taken from ticks fed for 3 days were used as antigen. Fluorescein-labelled, goat anti-rabbit IgG (Miles) was diluted (5%) in a counter colour (Evans Blue 1/10 000).

To carry out the basophil degranulation test according to Benveniste *et al.* (1977), the antigen was prepared with the salivary glands of female *I. ricinus* which had been fed 3–5 days. After dissection, these glands were washed three times at 500*g* in PBS pH 7·2, then homogenized and centrifuged ($13 \times 10^3 g$, 30 min at 4°C). The supernatant was dialysed against distilled water overnight. The dialysate was frozen at −20°C in aliquots sufficient for an experiment.

Briefly, the test procedure is the following: 10 ml of rabbit blood are collected in a test tube containing heparin and centrifuged (500*g*, 10 min at 4°C). Plasma is collected and stored at 4°C. Cells are washed twice in Tyrode's solution without Ca^{2+} and Mg^{2+}, containing 1×10^{-4} M EDTA. Half of the sample is resuspended with plasma to the original blood volume (5 ml) and the other half with complete Tyrode's solution. Then 0·5 ml of those solutions (with or without plasma) are mixed with increasing quantities of antigen (0–10^7 pg/ml). The mixtures are incubated at 37°C for 15 min. The reaction is stopped with 25 μl 0·2 M EDTA, pH 7·4. To an aliquot of 10 μl, 90 μl of toluidine blue were added. Basophils are counted in a Malassez haemocytometer. The results are expressed as average percentage of degranulated cells after comparison of mixtures incubated with or without antigen:

$$\frac{\text{No. of basophils in mixtures without antigen} - \text{No. of basophils in mixtures with antigen}}{\text{No. of basophils in mixtures without antigen}} \times 100$$

Results

TICK BIOLOGY

We have calculated the rate of egg laying for the four infestations considered (Table 1). This decreases progressively from 0·52 to 0·29 going from the 1st to the 4th infestation. The duration of preoviposition and embryogenesis were also measured. Both increase by 3 days from the 1st to the 4th infestation. The evolution of the weight of fed females and the period of nutrition is comparable to that observed by Bowessidjaou *et al.* (1977).

Table 1. Biology of *I. ricinus* females

Infestations	Mean weight of fed females (mg)	Rate of egg laying	Mean duration of blood meal (h)	Mean duration of preoviposition (d)	Mean duration of embryogenesis (d)
1	196·2 ± 97·9 (*n* = 36)	0·52	163 ± 27 (*n* = 36)	12·6 ± 3·0 (*n* = 32)	48·4 ± 3·3 (*n* = 28)
2	98·7 ± 65·6 (*n* = 32)	0·35	195 ± 63 (*n* = 32)	13·0 ± 3·1 (*n* = 20)	46·6 ± 5·8 (*n* = 20)
3	76·5 ± 58·8 (*n* = 27)	0·22	200 ± 47 (*n* = 27)	17·7 ± 5·3 (*n* = 13)	50·1 ± 6·9 (*n* = 9)
4	101·9 ± 80·0 (*n* = 32)	0·29	221 ± 60 (*n* = 32)	15·4 ± 3·9 (*n* = 15)	51·1 ± 4·4 (*n* = 12)

h = hours; d = days; *n* = number of ticks.

EVOLUTION OF IgG ANTIBODIES AGAINST *I. RICINUS* SALIVARY GLANDS

All the serological tests were made on the 6th day of each infestation. Antibodies appeared from the 1st infestation in two cases out of five only (titre 1/20). From the 2nd infestation on they were always detected. Average reciprocal titres increased from then on progressively, from 47·6 to 269·1 (Table 2).

EVOLUTION OF THE NUMBER OF CIRCULATING BASOPHILS AND OF THEIR SENSITIZATION AGAINST THE ANTIGENS OF *I. RICINUS* SALIVARY GLANDS

According to Spector (1956), the normal range for the concentration of rabbit basophils lies between 120 and 310 per mm^3. With the exception of the counts for the 1st infestation,

Table 2. Evolution of IgG antibodies to *I. ricinus* salivary glands

	Negative	1/20	1/40	1/80	1/160	1/320	1/640	*n*	GMRT
1st infestation	3	2	0	0	0	0	0	5	13·2
2nd infestation	0	0	3	1	0	0	0	4	47·6
3rd infestation	0	0	0	2	2	0	1	5	160·0
4th infestation	0	0	0	1	0	2	1	4	269·1

n = number of tests; GMRT = geometrical mean of reciprocal titres.

Table 3. Evolution of the number of basophils and results of degranulation tests for four successive infestations (a: without plasma; b: with plasma). The results are calculated as average percentage degranulation seen with five rabbits (see Materials and methods)

	Mean number of basophils (mm^3)	Concentration of antigen (pg/ml)			
		a		b	
		10^6	10^7	10^6	10^7
Controls	129±27	7·0±3·0	5·0±1·4	not done	not done
1st infestation	103±29	21·8±7·4	23·6±4·0	19·0±5·2	19·6±4·2
2nd infestation	260±110	34·8±7·4	33·8±7·9	23·3±6·2	22·8±5·4
3rd infestation	248±73	56·8±5·2	57·7±6·9	46·7±5·4	47·5±4·8
4th infestation	166±31	59·8±7·8	63·8±4·9	43·3±7·1	43·3±5·7

our results were always within these limits (Table 3a). From the 1st to the 2nd infestation, there is a considerable increase (103 to 260). Subsequently, at the moment of observation considered, counts were always decidedly higher than at the 1st infestation.

The specificity threshold of the basophil degranulation test for the tick antigen has been fixed at 10%. Indeed for four rabbits observed, the degranulation average varied between 1·3 and 7·0 (means: 7·0 and 5·0 respectively) for antigen concentrations of 10^6 and 10^7 pg/ml (Table 3a).

One observes a degranulation maximum for antigen concentrations of 10^6 and 10^7 pg/ml (Figure 1). Thus one can compare the results obtained at these rates of antigen during sucessive infestations (Table 3a). At the 1st infestation, 21·8 and 23·6 of the cells degranulated. From the 2nd infestation, these percentages increased (34·8 to 33·8%) to reach 59·8 and 63·8% at the 4th infestation. Clearly during the series of infestations, basophils became sensitized progressively towards the antigens of tick salivary glands.

The presence of a plasma factor partly blocking the degranulation of basophils was also demonstrated. Replacement of Tyrode's buffer by plasma in the test, with other factors kept constant, resulted in less degranulation of the cells at the same concentrations of antigen (Table 3, Figure 1). This factor was probably present during the primary infestation, since the percentage of degranulation was reduced by 2·8 and 4·0% (see concentrations 10^6 and 10^7 pg/ml, Table 3a & b). The blockage of the reaction then increased progressively during reinfestations. It was maximal at the 4th reinfestation (difference: 16·5 and 20·5).

After 10–15 infestations carried out on four other animals, the sensitization of basophils was very strongly marked. We obtained 75·0 and 79·8% degranulation for 10^6 and 10^7 pg/ml antigen (Table 4a). Application of the test before the last of the reinfestations showed that the values were already high (69·0 and 74·8%) and greater than those obtained at the 6th day of the 4th infestation (Table 3a). Furthermore, blockage of the reaction was also more marked. A difference of 25·5 to 27·4% between the tests carried out with and without plasma was observed (Table 4b, observations carried out after the last infestation).

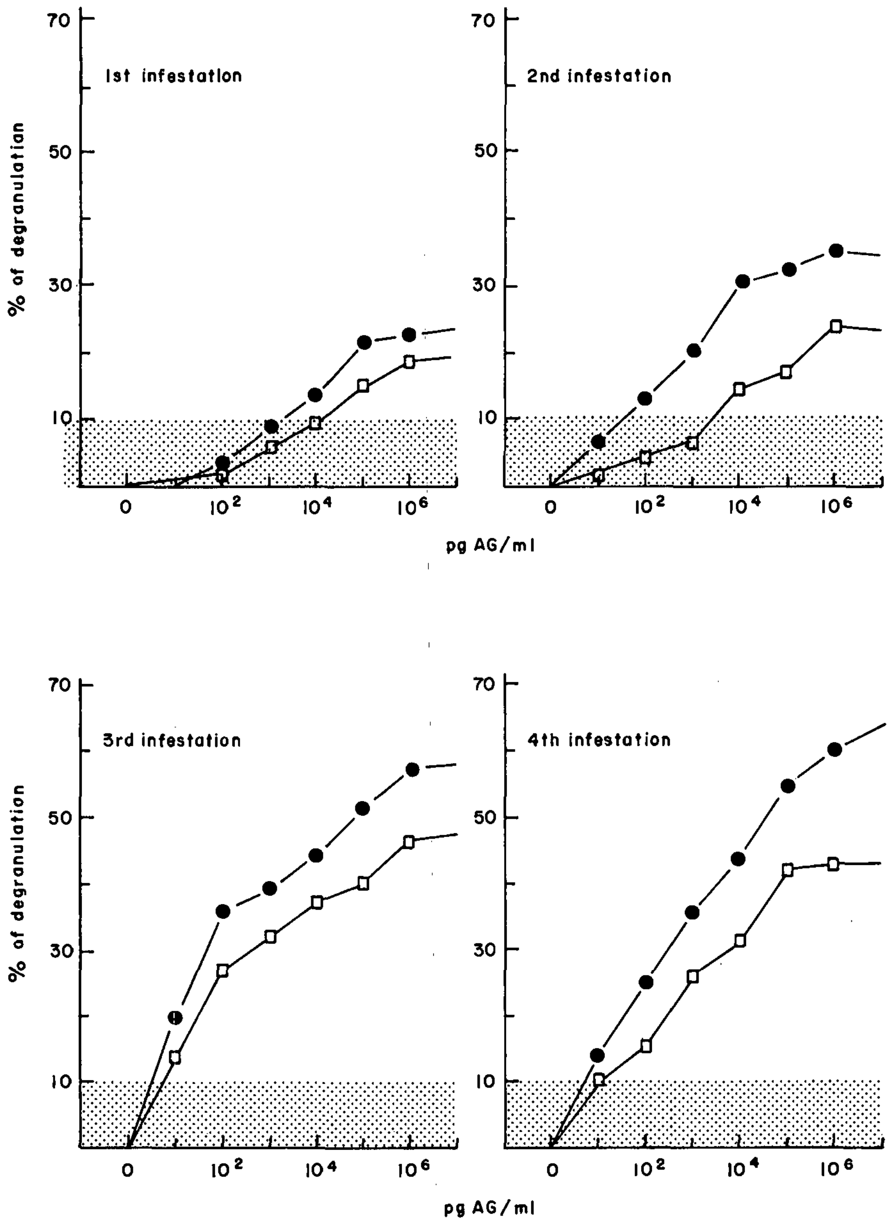

Figure 1. Progressive sensitization of circulating basophils (presence of a blocking factor). Stippled area: non-specific reactions (●——● without plasma; □——□ with plasma).

Discussion

The results of the present study on the effects of immunity on the weights of engorged *I. ricinus* ticks, on the duration of feeding, and on egg laying confirm and enlarge our earlier observations (Bowessidjaou *et al.* 1977). It is interesting to note the clear decrease of the

Table 4. Results of degranulation tests (10–15 successive infestations (a: without plasma; b: with plasma). The means are related to four rabbits

Concentration of antigen (pg/ml)	a		b	
	10^6	10^7	10^6	10^7
Before the infestation	69·0±9·8	74·8±7·9	50·0±8·2	56·0±4·6
After the infestation	75·0±5·7	79·8±7·0	49·5±3·3	52·4±4·3

rate of egg laying (0·52 to 0·29) and the prolongation of the periods of preoviposition and embryogenesis (Table 1).

Brossard & Fivaz (in press) have detected the presence of basophils and their quantitative evolution in the cutaneous lesion provoked by *I. ricinus* females attached to rabbits. In the course of a primary infestation, few of these cells are present and degranulated, but they infiltrate into the tissue to a larger extent at the end of a 2nd infestation. The infiltration of basophils in the feeding lesion of ticks is not unique for the rabbit *I. ricinus* system. It is even more intense in guinea-pigs infested by *Dermacentor andersoni* (Allen 1973) or *Ixodes holocyclus* larvae (Bagnall 1975). In the latter case, degranulated cells have been observed. These leucocytes could participate in cutaneous basophil hypersensitivity phenomena (Askenase *et al.* 1978), with cells sensitized by IgE degranulating specifically on contact with allergen. From this point of view, it is interesting that specific sensitization of rabbit basophils against *I. ricinus* antigens occurs.

The results of this study seem to account for the histological observations. The number of circulating basophils increase considerably from the 1st to the 2nd infestation (103–260/mm^3, Table 3a), as did the percentage degranulation of the same cells (21·8, 23·6–34·8, 33·8 for the concentrations of antigens of 10^6 and 10^7 pg/ml respectively: Table 3a). For the subsequent infestations, we have no histological information. However the progress of basophil sensitization has been noted. At the 3rd infestation, for the same antigen concentrations, the degranulated cells represented 56·8 and 57·7% of the total number of the circulating basophils. At the 4th infestation, the maximum degranulation was equal to 59·8 and 63·8%, respectively. Thus, the basophils are sensitized progressively in the course of subsequent infestations. In order to demonstrate a further evolution of this phenomenon, four rabbits have been submitted to 10–15 infestations. At the end of the last re-infestation, the percentage of degranulated basophils was very high, reaching 75·0 and 79·8% (Table 4a).

A blocking factor, probably an IgG antibody (Feingold 1973), reduced in our *in vitro* system the basophil degranulation induced by each antigen dilution, irrespectively of the number of previous infestations. Further, the blockade reaction increased from one infestation to the next (Table 3a & b). The anti-*I. ricinus* IgG antibodies appeared in two of five cases during the 1st infestation. The titres rose gradually during the course of re-infestations (Table 2). The *in vivo* efficiency of the blocking factor seems to be good in acute anaphylaxis phenomena where antigen is scattered with the blood (Vervloet & Charpin 1980). It could be less regular in immediate allergy where antigen interacts directly with tissue mast cells. In infested rabbits, the allergen injected by the ticks reacts undoubtedly first with sensitized mast cells and basophils. Pharmacologically active substances released (e.g. histamine) could be harmful to the ticks (Brossard 1982). But these substances, in increasing vascular permeability could also facilitate access of protective elements: cells, complement and specific antibodies. The antibodies to tick salivary glands, notably the IgG demonstrated in this study, could then neutralize salivary components (e.g. enzymes) and thus impair normal food intake. Furthermore they could perhaps also react with the intestinal epithelium of the tick, thus impeding normal feeding and digestion.

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C3 Levels in the Sera of Rabbits Infested and Reinfested with *Ixodes ricinus* L. and in Midguts of Fed Ticks

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ABSTRACT

Papatheodorou, V. and Brossard, M., 1987. C3 levels in the sera of rabbits infested and reinfested with *Ixodes ricinus* L. and in midguts of fed ticks. *Exp. Appl. Acarol.*, 3: 53–59.

Serum C3 levels of rabbits infested 3 times with *Ixodes ricinus* L. females and C3 in midgut extracts of fed ticks have been measured by a single radial immunodiffusion test.

From the first tick attachment, the mean serum C3 level of hosts increased. A peak of C3 occurred 6 days after the beginning of each infestation and was highest during the third infestation (about 8 times more pronounced than the level measured before the first). After the end of all infestations of the rabbits, the serum C3 level decreased and approached normal levels.

Modifications of serum C3 levels during reinfestations influenced C3 contents in the blood meal of fed ticks. Midguts of ticks fed during the third infestation contained more C3 than midguts of ticks of the same engorged weights fed during the first or second infestations.

INTRODUCTION

Hosts infested with ticks acquire resistance which affects the ectoparasite's biology (Willadsen, 1980; Wikel, 1982). When rabbits are parasitized several times with *Ixodes ricinus* L., nutrition and egg laying of the tick are altered (Bowessidjaou et al., 1977). In these animals, specific IgG antibodies and the number of basophils increase while these leucocytes are progressively sensitized against tick salivary antigens (Brossard et al., 1982). Their skin becomes progressively more sensitive to the salivary tick antigens (immediate and delayed-type hypersensitivity; Girardin and Brossard, 1985). Skin inflammation develops at the ectoparasite attachment site (Brossard and Fivaz, 1982).

The complement system is often involved in immune and inflammatory responses (Harrewyn and Renoux, 1981) and is probably an important component of acquired resistance to ticks. In guinea pigs depleted of C3 after treatment with cobra venom factor, immunity against *Dermacentor*

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andersoni larvae is considerably diminished (Wikel and Allen, 1977).

In our study, we have considered C3, the central component of the complement system. We have measured the serological kinetics of this protein during three repeated infestations of rabbits with adult *I. ricinus*. We have also estimated the quantity of C3 ingested by ticks during their nutrition.

MATERIALS AND METHODS

Himalayan male rabbits (genotype aacHcH), weighing about 2 kg, were infested 3 times with 10 female and 10 male *I. ricinus* ticks, as described previously (Bowessidjaou et al., 1977).

Serum C3 levels of six animals (first and second infestation) or four animals (third infestation) were measured every 3 days using a single radial immunodiffusion test. In this test, 0.75 μl anti-rabbit-C3 (Cappel) per ml barbitone buffer pH 8.6 (5′5 diethyl barbituric acid $0.43 \times 10^{-2} M$, 5′5 diethyl sodium barbiturate $0.2 \times 10^{-1} M$, NaN_3 $0.15 \times 10^{-1} M$) 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. Test sera were diluted 1:6 in the same buffer and deposited into wells of 5 μl punched in the gel. Incubation was done in a humid atmosphere at room temperature. The logarithm of serum C3 level is proportional to the diameter of the precipitate. A standard curve was made using a reference serum and results expressed as percentages of this serum.

To measure midgut C3 levels, female *I. ricinus* engorged during the first, second or third infestations of rabbits were dissected. Each midgut was sonicated in 0.15 *M* PBS at pH 7.2 and 4°C. After centrifugation at 25.000 *g* for 15 min at 4°C, the supernatant was made up to 5 ml with PBS, lyophilised in aliquots of 1 ml and stored at 4°C. Midgut C3 levels were measured with the same radial immunodiffusion test. Before use, lyophilised aliquots were reconstituted with distilled water according to the respective tick weight: <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.

For each tick, results were expressed as equivalent dilutions of the standard serum. Regression lines between midgut C3 levels and tick weights were established and statistically analysed according to Hald (1952) to determine if any difference between the infestations could be demonstrated.

RESULTS

C3 level in serum of infested rabbits

Figure 1 shows the levels of serum C3 during the course of three successive infestations. The results are expressed as percentages of a reference serum (source of C3) with respect to time. From the first attachment of

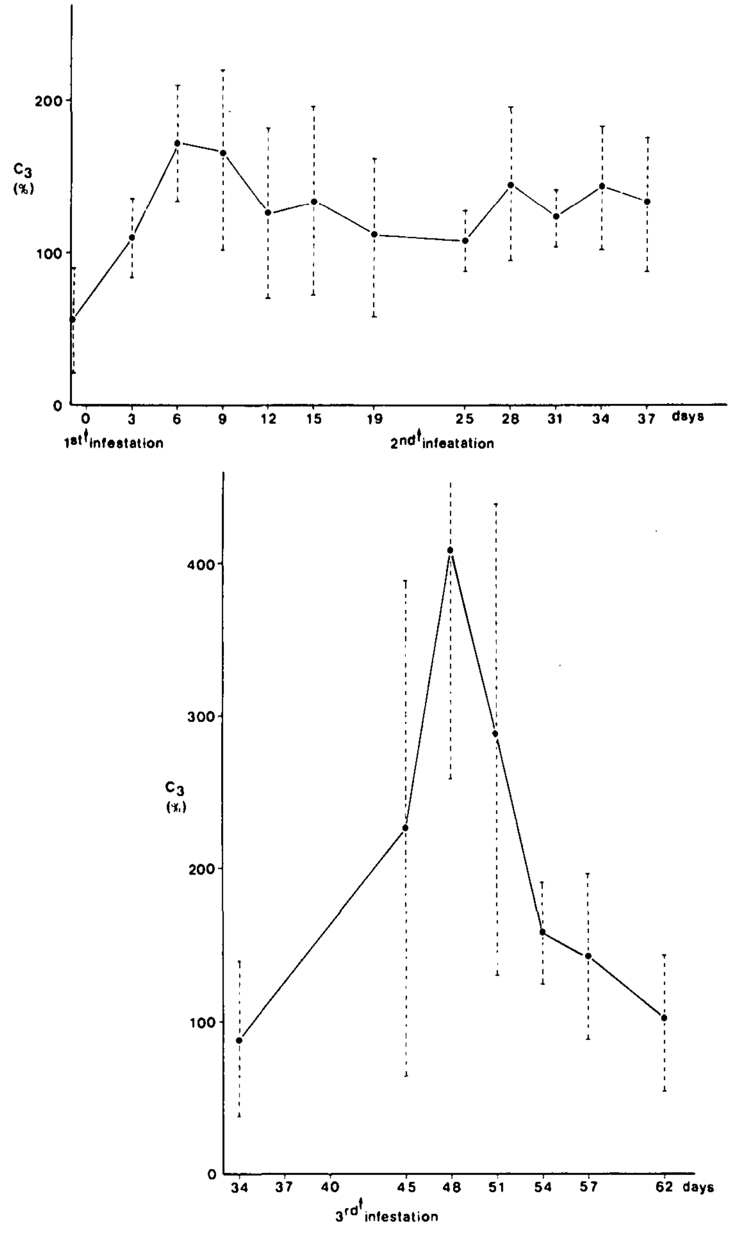

Fig. 1. Effect of *I. ricinus* infestations of rabbits on serum C3 level (mean and standard deviation). Arrows indicate beginning of first (n = 6), second (n = 6) and third (n = 4) infestations.

I. ricinus on the host (day 0), serum C3 level increased and remained higher than normal up to the last observation (day 62). A peak of C3 level was observed on day 6 of each infestation. It was most distinct and pro-

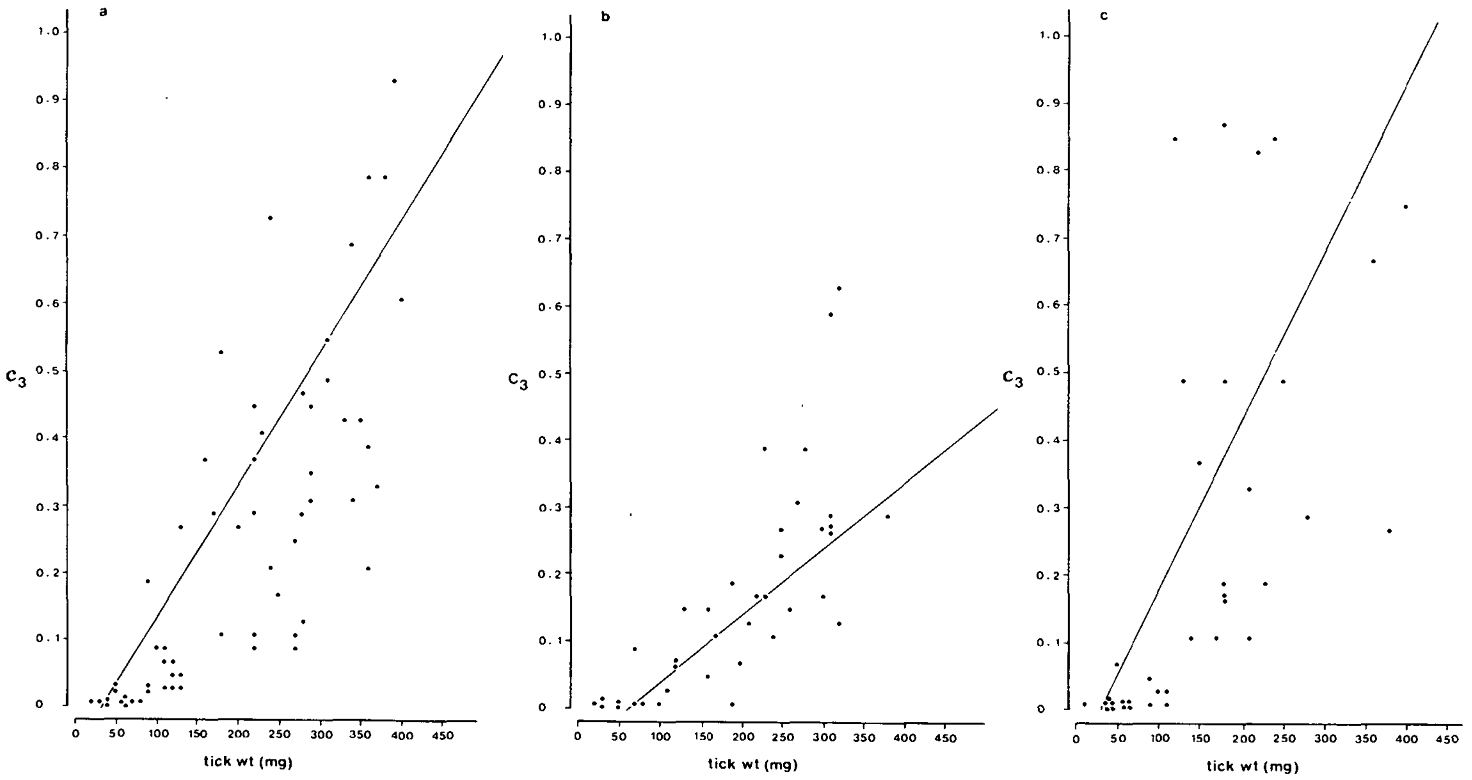

Fig. 2. Relation between C3 in the midgut of *I. ricinus* females and their engorged weights after three infestations. C3 is expressed as equivalent dilutions of the standard serum. Equations of linear regression lines: $y_1 = 0.002x - 0.070$, $r_1 = 0.78$, $df_1 = 56$; $y_2 = 0.001x - 0.063$, $r_2 = 0.77$, $df_2 = 36$; $y_3 = 0.004x - 0.223$, $r_3 = 0.61$, $df_3 = 38$. The C3 equivalents (1.96, 2.86 and 3.22) of three fed ticks (301.6, 305.6 and 325.3 mg) were too high to be represented on Fig. 2c.

nounced during the third infestation. After the end of this last infestation, serum C3 decreased and approached normal levels. High variance of the results was especially observed during the third infestation. This was due to individual, but overall to asynchronous, responses among the different animals.

C3 level in midgut extracts of ticks

After each infestation, midgut C3 levels were significantly correlated with weights of engorged ectoparasites ($P < 0.001$, $r_1 = 0.78$, $r_2 = 0.77$, $r_3 = 0.61$). Heavier ticks contained more C3 than lighter ones (Fig. 2). Regression lines differed from each other. The slope of the straight regression line for the third infestation was greater than that of the first ($P < 0.05$) and the second ($P < 0.001$), indicating that ticks of the same engorged weight ingested more C3 during the third than during the first and especially more than during the second infestation. This resulted undoubtedly from the high serological C3 level detected on day 6 of that infestation in comparison with the first and particularly the second infestation (Fig. 1).

DISCUSSION

The mouthparts of *I. ricinus* penetrate deeply into the skin of the rabbits' ear (Brossard and Fivaz, 1982). During feeding an inflammatory lesion develops in the dermis surrounding the mouthparts of the tick. Higher vertebrates respond to tissue damage and acute inflammation with an increase in serum concentration of some complement components (Weimer et al., 1964; Nishioka et al., 1976), C3 in particular (Hartweit et al., 1973).

1973).

In our study we observed that rabbit serum C3 levels increase soon after the first attachment of *I. ricinus*, to reach their maximum values during the third infestation of the rabbits. A peak level of serum C3 occurs by the end of the tick blood meal (day 6 of each infestation). A similar increase in C3 levels has been detected in other parasitic systems. It occurs in mice following intraperitoneal injection of *Trypanosoma brucei* (Shirazi et al., 1980) or percutaneous infection with cercariae of *Shistosoma mansoni* (Pepys et al., 1980). In the latter case, the increase in C3 level occurs parallel with the development of liver granuloma around the parasites' eggs.

Complement is certainly an important component of acquired resistance to ticks (Wikel and Allen, 1977). At the attachment site of *Dermacentor andersoni* larvae on resistant guinea pigs, salivary gland antigens, complement and IgG were deposited at the dermo-epidermal junction (Allen et al., 1979). Complement was also shown to be present in epidermal vesicles beneath attachment sites. These authors suggest that antigen-antibody reaction and complement activation may initiate and take part in the local inflammatory lesion.

In female *I. ricinus* fed on resistant rabbits (third infestation), Papatheodorou (1985) has shown that haemoglobin digestion was altered. In this period, ticks take in more C3 and anti-tick salivary gland antibodies than those fed during first and second infestations. In Western blot, antigens are recognized in tick extracts of midguts and salivary glands (B. Rutti and M. Brossard, unpublished results). We can hypothesize that in vivo an immunological reaction on midgut epithelium could perturb the normal physiology of that tissue.

We have shown that infestations of rabbits with *I. ricinus* provoke an increase in the quantity of serum C3. The amplitude of this phenomenon influences the C3 content of the tick blood meal.

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Mots-clés : Tique. *Ixodes ricinus.* Lapin. Immunité. Digestion de l'hémoglobine.

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

M. BROSSARD, V. PAPATHEODOROU

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;*

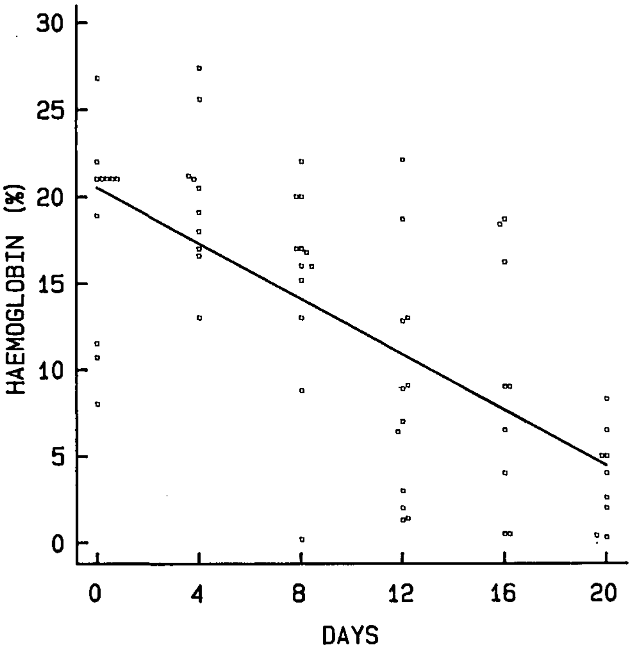

FIG. 1. — Relation between haemoglobin concentration of tick midgut and time after drop off (during 20 days) in a first infestation. Haemoglobin is expressed in mg per 100 mg of fed ticks. $y = -0.80x + 20.44$ ($r_1^2 = 46.14$ %, $n_1 = 63$, $p < 0.001$).

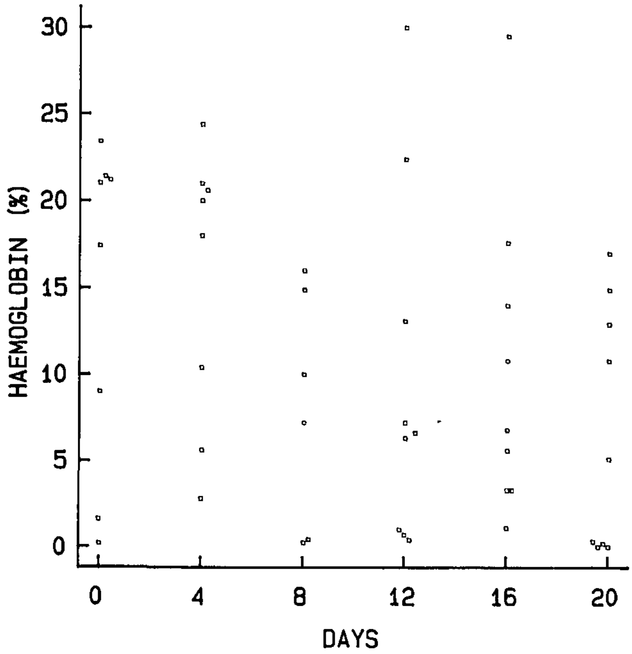

FIG. 2. — Relation between haemoglobin concentration of tick midgut and time after drop off (during 20 days) in a third infestation. $y = -0.36x + 14.86$ ($r_3^2 = 7.15$ %, $n_3 = 49$, $p > 0.05$).

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*; * $p < 0.001$.

($r_3^2 = 7.15$ %, $n_3 = 49$, $p > 0.05$). 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*). 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;

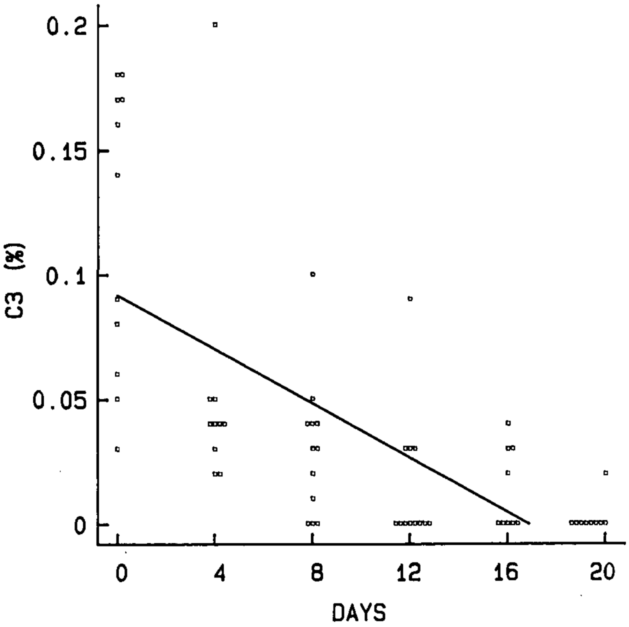

FIG. 3. — Relation between C_3 concentration of tick midgut and time after drop off (during 20 days) in a first infestation. $y = -0.005x + 0.091$ ($r_1^2 = 43.18$ %, $n_1 = 63$, $p < 0.001$).

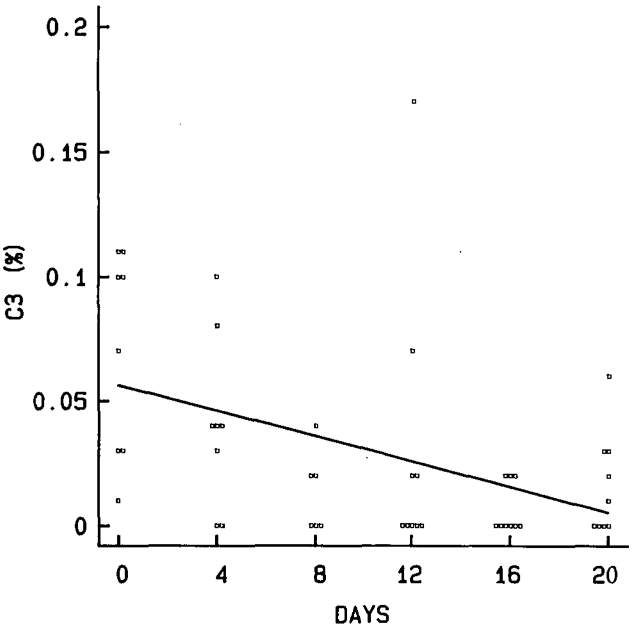

FIG. 4. — Relation between C_3 concentration of tick midgut and time after drop off (during 20 days) in a third infestation. $y = -0.002x + 0.056$ ($r_3^2 = 20.51$ %, $n_3 = 49$, $p < 0.01$).

($r_3^2 = 20.51$ %, $n_3 = 49$, $p < 0.01$ 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	$H = -0.80x + 20.44$	$r_1^2 = 45.26$ %	$n_1 = 63$
3	$H = 1.64y + 0.03z + 1.04$	$r_2^3 = 60.99$ %	$n_3 = 49$

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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