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Modulation de la réponse immunitaire de souris BALB/c infestées par la tique *Ixodes ricinus*, importance de la salive pour l'induction d'une réponse Th2 *in vitro*

Par

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La Faculté des sciences de l'Université de Neuchâtel sur le rapport des membres du jury,

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La liste des publications

Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. (*Parasitology Research* in press)

Th2 polarization of the immune response of BALB/c mice to *Ixodes ricinus* instars, importance of several antigens in activation of specific Th2 subpopulations. *Parasite Immunol*, **23**:61-69.

Induction of Th2 cell differentiation in primary immune response *in vitro* and *in vivo*: splenic dendritic cells incubated with *Ixodes ricinus* tick saliva prime naive CD4+ T cells to secrete IL-4. (submitted in *Immunology*)

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Résultat n°1

Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice. (*Parasitology Research,* in press)

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Immunosuppressive effects of *Ixodes ricinus* tick saliva or salivary gland extracts on innate and acquired immune response of BALB/c mice.

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Running title : Immunosuppressive activities of tick antigens

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ABSTRACT

Saliva and salivary gland extract (SGE) of Ixodes ricinus ticks have suppressive effects on the innate immune response of BALB/c mice. Tick saliva prevents hemolysis of sheep red blood cells (SRBC) by the human alternative pathway of complement. The adaptive immune response is also modulated by tick antigens (saliva or SGE). When stimulated *in vitro* with increasing doses of tick antigens, the proliferation and IL-4 production of draining lymph node T cells of mice infested with nymphal ticks increase, peak and then decrease. These results indicate that immunostimulative and immunosuppressive molecules have competing effects in tick saliva or in SGE. I. ricinus saliva inhibits in a dose dependent manner splenic T cell proliferation in response to concanavalin A (Con A). Tick SGE or saliva injected intraperitoneally into BALB/c mice simultaneously with SRBC systemically immunosuppress the anti-SRBC response as shown *in vitro* by the reduced responsiveness of sensitized splenic T cells to restimulation with SRBC. In brief, some components of SGE or tick saliva reduce the responsiveness of draining lymph node T cells and of sensitized splenic T cells *in vitro*. The responsiveness of naive splenic T cells to Con A stimulation *in vitro* is also decreased by tick saliva. Modulation of host responses by tick antigens may facilitate tick feeding, transmission and the propagation of pathogens.

Introduction

Ixodid ticks attach to their hosts and feed for several days during which time they may transmit various pathogens. Infested animals are immunologically tolerant or acquire resistance against ticks. One would expect that tick saliva is produced to aid feeding, as well as for the transmission and propagation of tick-borne pathogens. The few pharmacological properties of saliva molecules that have been described are related to evasive mechanisms which facilitate feeding and pathogens transmission (Ribeiro et al. 1985; Titus and Ribeiro 1990). Depending on the tick-host association, innate and adaptive immune responses have different influences on tick feeding and pathogens transmission. Females BALB/c mice repeatedly infested with pathogen-free Ixodes scapularis are tolerant to tick feeding but resistant to the subsequent tick transmission of B. burgdorferi (Wikel et al. 1997). The partial resistance of wild white-footed mice (Peromyscus leucopus) to repeatedly feeding ticks did not prevent the transmission of Borrelia burgdorferi (Richter et al. 1998). In our model I. ricinus nymphs modulate the anti-tick immune response of BALB/c mice which failed to acquire resistance against ticks (Mbow et al. 1994a). We have recently shown that some chromatographic fractions of salivary gland extract (SGE) have either stimulative or suppressive activities on the responsiveness of draining lymph node cells in BALB/c mice infested with nymphal I. ricinus (Mejri et al. 2001). Saliva molecules would have competing activities during infestation acting on several levels of the immune response. Ticks attach to their hosts in varying densities in natural conditions. The amount of salivary secretion injected into the skin could influence tick feeding as well as pathogens transmission. The balance between the immunostimulative and immunosuppressive effects of tick saliva and of SGE molecules has never been addressed.

The purpose of this work is to make an *in vitro* analyse of the effect of different concentrations of I. ricinus saliva and of SGE (tick antigens) on the non specific innate or specific acquired immunological responses of BALB/c mice. The non specific effects of tick

antigens were determined using the human alternative complement pathway and the proliferation of naive spleen cells stimulated with concanavalin A (Con-A). The specific effects of tick antigens were studied on lymphocyte proliferation and the secretion of IL-4 by lymph node cells. Low concentrations were generally immunostimulative whereas high concentrations were immunosuppressive. The influence of tick antigens was also estimated using the systemic immune response against SRBC.

Materials and methods

Animals

Eight to 12 weeks old BALB/c female mice and male rabbits (New Zealand) weighing an average of 3 kg were purchased from IFFA-CREDO (Arbresle, France) and from Elevage des Dombes (Romans, France) respectively. Ticks were reared in our laboratory as previously described (Graf 1978).

Infestations

Mice were infested with 15 I. ricinus nymphs each. These were placed into a small plastic capsule glued to shaved skin at the site drained by the brachial and axillary lymph nodes of the host shoulder using a mixture of one part beeswax and four parts colophonium (Mbow et al. 1994b). Each experiment was done using a group of five mice. To prepare tick antigens, adult female *I. ricinus* were applied to a rabbit's ears and allowed to feed for 5 days. They were contained in a nylon bag stuck to the ear with an adhesive band. A collar was placed around the rabbit's neck to prevent grooming.

Saliva preparation

Partially engorged female ticks were removed. Saliva was collected in a glass capillary tube fitted over the mouth-parts of the tick. From 0.3 to 0.5 µl of saliva was collected per tick after 10-30 min. The saliva from 80 partially fed ticks was pooled, sterilized through a 0.22 µm filter and stored at –20 °C until used.

Salivary gland extracts

An other group of partially fed I. ricinus females which had been attached to a rabbit's ears for 5 days was used to prepare SGE as previously described (Rutti and Brossard. 1989). Eighty pairs of the salivary glands were dissected out and homogenised in 1 ml of ice cold extraction buffer consisting of 50 mM phosphate-buffered saline (PBS) pH 7.4 supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM ethylene diaminetetraacetic acid (EDTA). The homogenate was centrifuged at 16 000 g for 30 min at 4 °C. The

supernatant was dialysed overnight in 10 mM (PBS) pH 7.4 in a cellulose ester membrane tube with a molecular weight cut off of 100 Da (Spectrum, Socochim, Switzerland). Dialysate was sterilised through a 0.22 μm filter and stored at –20 °C until used. The protein concentration of the saliva and the SGE was determined using a BCA protein Assay Kit (Pierce, Socochim, Switzerland).

Hemolysis test

The hemolysis test was performed according to a modified method of Ribeiro (1987) using human serum, sheep red blood cells (SRBC) (BioMérieux, Switzerland) and tick saliva. Briefly, 400 μl of SRBC at 50% (v/v) were washed twice and resuspended in 1.5 ml Veronal buffer (VBS) pH 7.35 supplemented with 0.1% BSA, 2 mM $MgCl_2$ and 5 mM EGTA (Sigma, Switzerland). SRBC at 6% were then incubated with 10 μl rabbit anti-SRBC Ab (Nordic, Netherlands) for 30 min at 37 °C. Serial dilution of the saliva protein (15, 30, and 45 μg/ml) plus 10 μl of human serum were added to the VBS-BSA-Mg^{2+} buffer followed by 30 μl erythrocytes giving a final volume of 0.1 ml. The mixture was then incubated for 1h at 37 °C. The optical density of the supernatant was measured at 405 nm with a spectrophotometer (Dynatech, Switzerland).

Proliferation of draining lymph node cells restimulated with tick antigens

Mice were killed 9 days after infestation and axillary and brachial lymph nodes were removed. A total of 10^6 lymph node cells per well were cultivated in 200 μl culture medium containing RPMI-1640 (Gibco, Basel, Switzerland), supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamin, 1 mM sodium pyruvate, 1 mM non-essential aminoacids (Sigma, St Louis, Mo), 0.05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin (Gibco) and 25μg/ml Fungizone (Gibco). Cells were stimulated for 96 h during incubation at 37 °C in a 5% CO_2 saturated atmosphere, with serial dilutions of respectively dialysed and filtered SGE (1.56, 3.12, 6.25, 12.5, 25, 50, and 100 μg/ml) or saliva (1.09, 2.18, 4.37, 8.75 and 17.5 μg/ml). At 18-24 h before harvesting, they were pulsed with 1 μCi/well of methyl [^{3}H] thymidine (specific

activity 25 Ci/mmol) (Amersham, UK). Methyl [^{3}H] thymidine incorporation was determined using a liquid scintillation counter (MR-300 DPM, Kontron, Switzerland).

Quantification of interleukin-4

Similarly to the cell cultures described above, culture supernatants collected 96h after stimulation with serial dilutions of SGE or saliva were used for IL-4 quantification using an enzyme-linked immunosorbent assay (ELISA) (Ganapamo et al. 1995). Dilutions of rIL-4 ranging from 12.5 to 400 U/ml (Pharmingen, Germany) were used to construct a standard curve. The assay for IL-4 has a sensitivity of 10 U/ml.

Con A stimulation of spleen cells

Spleen cell suspension was obtained by teasing the spleen from naive BALB/c mice with the large striated end of forceps and red blood cells were removed by incubation in hypotonic lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0,1 mM EDTA). Spleen cells were plated into 96-well culture plates (Falcon) at 10^6 cells per well in RPMI-1640 supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamin, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 0.05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin and 25 µg/ml Fungizone. The cells were then incubated with or without different protein concentrations of saliva (0.6, 1.2, 6 and 30 µg/ml) for 2h at 37°C and 5% CO_2 before being stimulated with con A (1µg/ml) in a total volume of 200 µl according to the modified procedure of Urioste et al (1994). Cells were pulsed 24 h later for 18-24h with 1µCi/well of methyl [^{3}H] thymidine. The degree of proliferation was determined by scintillation counting.

***In vitro* restimulation of SRBC-sensitized spleen T cells**

SRBC were washed twice in 50 mM PBS containing 0.1% BSA before intraperitoneal injection into three BALB/c mice. The mice were injected with 10^7 SRBC, a mixture of 10^7 SRBC and 25 µg of SGE or 5 µg of saliva respectively in 2 ml total volume of PBS pH 7.3. After 1 week, the spleens of the three treated mice were removed and 7 x 10^5 spleen cells/well were plated onto 96 well plates. Sensitized splenic T cells from each mouse were then restimulated *in vitro* with the following numbers of SRBC (5 x 10^4, 10^5, 5 x 10^5 and 10^6)

according to the method of Titus (1998). Cells were incubated for 96h at 37 °C in a 5% CO_2 saturated atmosphere. One μCi/well of methyl [^{3}H] thymidine was added 18-24 h before harvesting the cells. Methyl [^{3}H] thymidine incorporation was determined by liquid scintillation counting.

Results

Anti-complement activity of tick saliva

To examine whether the human alternative pathway of complement was affected by tick saliva, we performed a hemolysis test using different protein concentrations of saliva. The full activity of the human alternative complement pathway, corresponding to 0% of the SRBC hemolysis inhibition, was obtained in the absence of tick saliva in the culture medium. The presence of tick saliva inhibited the SRBC hemolysis in a dose-dependent manner (fig. 1). Anti-complement activity increased with increasing concentration of saliva. The optimum of anti-complement activity was reached with saliva protein concentration of 30 µg/ml. This inhibite about 70% of the SRBC lysis.

In vitro proliferation of tick-sensitized lymph node cells

To study the influence of tick saliva and SGE on the adaptive immune response, we stimulated tick-specific lymph node cells *in vitro* with increasing doses of tick antigens. The proliferation of primed lymph node cells was influenced by the concentration of tick saliva and SGE applied in the cell cultures. This proliferation increased with the protein concentrations of the saliva and SGE ranged from 1.09 to 2.18 µg/ml and from 1.56 to 12.5 µg/ml respectively (Figs. 2a, 3). With higher protein concentrations of saliva and of SGE, ranging from 2.18 to 17.5 µg/ml and from 12.5 to 100 µg/ml respectively, the proliferation of primed T cells decreased. Low doses of tick antigens were immunostimulative whereas higher doses were immunosuppressive.

In vitro IL-4 production

IL-4 secretion also depended on the protein concentrations of the tick saliva as well as of the SGE used to restimulate primed lymph node cells *in vitro*. Increasing protein concentrations of saliva (1.09-4.37 µg/ml) and SGE (1.56-25 µg/ml) induced increased production of IL-4 which reached a maximum at a concentration of 4.37 µg/ml for saliva and 25 µg/ml for SGE

(Figs. 2b, 3). Thereafter increasing protein concentrations of saliva (4.37-17.5 µg/ml) and of SGE (25-100 µg/ml) triggered the decrease of IL-4 production by primed T cells.

***I. ricinus* saliva inhibits splenic T cell proliferation to Con-A**

To show the non-specific immunosuppressive activity of tick saliva, naive spleen cells were preincubated *in vitro* for 2h with different protein concentrations of I. ricinus saliva ranging between 0.6 and 30 µg/ml. Thereafter, they were stimulated *in vitro* with Con-A. A high reduction (>90%) of cell proliferation in response to Con-A was observed with 30 µg/ml of saliva proteins (fig. 4). The inhibitory effect of tick saliva was dose-dependent. It diminished with decreasing saliva concentrations but was still evident at a concentration of 0.6 µg/ml of salivary proteins. Spleen cells untreated with saliva and stimulated with Con-A acted as controls.

Systemic immunosuppressive effect of tick antigens

To investigate the systemic immunosuppressive activity of tick antigens we injected a mixture of saliva or SGE and 10^7 SRBC intraperitoneally. At 1 week post injection, mice elicited an anti-SRBC response as shown by the proliferation of splenic T cells stimulated with SRBC *in vitro*. Spleen cells from the control mouse, which was only injected with 10^7 SRBC, showed a high proliferative response of T cells when restimulated *in vitro* with 0.5×10^5-10×10^5 SRBC, whereas splenic T cells from the mice injected with a mixture of saliva or SGE and 10^7 SRBC showed a reduction in proliferation when they were restimulated *in vitro* with SRBC (fig. 5). The impairment in proliferation of splenic T cells after stimulation with SRBC was more pronounced when mice were treated with SGE than with saliva.

Discussion

Ixodid ticks need several days to feed. During this time hosts acquire resistance or develop tolerance to the ectoparasites. In contrast to resistant hosts, for example rabbits infested with I.ricinus adults (Bowessidjaou et al. 1977), no difference was observed in the percentage of attachment or the weight of engorged larvae or nymphs of I. ricinus during reinfestations of BALB/c mice (Christe et al. 1998). In order to feed, as well as to transmit pathogens successfully, ticks must maintain blood flow and control the immune response of their host. They must develop countermeasures to antagonize the mechanisms of the innate as well as the adaptive immune response. Some bioactive molecules confer anti-hemostatic, anti-inflammatory and immunosuppressive properties to tick saliva (Ribeiro et al. 1985). The innate immunity constitutes the first line of defense against noxious or innocuous antigens. It is followed by the specific immune response (Fearon and Locksley 1996). Activation of the alternative pathway of complement is important in the innate defense and in the development of some tissue inflammation. We showed that the saliva of I. ricinus inhibits the hemolysis of SRBC by the human alternative pathway of complement. Accordingly, the saliva of Ixodes dammini (= Ixodes scapularis) also has an anti-complement activity on human serum (Ribeiro 1987). Impairment of the innate immune system, either by the inhibition of the alternative pathway of complement or by downregulation of the activities of cellular components such as NK cytotoxicity and macrophage killing of pathogens (Kopecky et al. 1999), would contribute to the reduction of skin inflammation and protection against ticks and favour the transmission and propagation of tick-borne pathogens.

BALB/c mice infested with I. ricinus ticks develop a Th2 immune response characterized by CD4+ T cells secreting IL-4, IL-5 and IL-10 (Ganapamo et al. 1995; 1996). SDS-PAGE analysis showed some similarities in polypeptide profiles between tick saliva and SGE (Mejri et al. 2001). Therefore, both of these were used to study the dose dependent influence of tick antigens on the responsiveness of tick-sensitized lymph node T cells. The immunosuppressive effects of saliva and SGE from I. ricinus were obtained with high

concentrations of tick saliva or SGE. BALB/c mice tolerated frequent feedings by pathogens-free larval or nymphal I. ricinus. The two instars seem to engorge more effectively on repeatedly exposed mice (Christe et al. 1998). In nature, I. ricinus larvae and nymphs feed abundantly on yellow-necked mice (Apodemus flavicollis) and black-striped mice (Apodemus agrarius). These mice infect more ticks with B. burgdorferi than do other rodents (Matuschka et al. 1991; 1992). Both species fully tolerate repeated experimental feedings (Dizij and Kurtenbach. 1995). The susceptibility of either laboratory or wild mice to larval or nymphal I. ricinus feeding or to pathogens transmission may be due to the prolonged and massive exposure to these ectoparasites which deposit a high amount of salivary secretions at the site of their attachments. We suggest that the immunosuppressive activities overcome the competitively immunostimulative activites in injected saliva resulting in the reduction of primed lymph node T cells responsiveness. *In vivo* effector lymphocytes formed in the draining lymph nodes of infested BALB/c mice circulate and infiltrate the skin at the site of tick attachment. Numerous T cells have been observed in skin infested with nymphal tick. CD4+T cells outnumbered CD8+T cells from a primary to a tertiary infestation (Mbow et al.1994b). These cells could be activated by locally salivary antigens to proliferate and produce higher level of IL-4 than IFN-γ. In *situ* hybridization of skin sections showed a positive signal for IFN-γ mRNA in some infiltrating mononuclear cells in the dermis near the tick hypostome and chelecerae beside a few cells positive for IL-4 mRNA (Mbow et al: 1994c). The weak activity of locally primed Th2 cells could be due to the high amounts of salivary secretion deposited at the site of attachment of nymphal ticks. This would be in agreement with our finding showing a reduced activity of primed lymph node T cells stimulated *in vitro* with higher protein concentrations of saliva or SGE.

To test whether tick saliva inhibits the development of an immune response non specifically, we stimulated naive splenic T cells *in vitro* with Con-A. This T cell mitogen mimics the action of antigens on primed T cells (Sharon 1983). We demonstrated that I. ricinus saliva inhibited the proliferation of splenic naive T cells to Con-A in a dose-dependent manner. Accordingly I.

scapularis saliva inhibited T cell proliferation to Con-A (Urioste et al. 1994). SGE from Dermacentor andersoni fed for 9 days also suppressed Con-A stimulated T cell proliferation *in vitro* (Ramachandra and Wikel 1992). Few immunosuppressive molecules have been described in the salivary glands of ixodid ticks. We previously showed that some chromatographic fractions in the SGE of I. ricinus inhibit tick-sensitized lymph node T cells proliferation (Mejri et al. 2001). Prostaglandin E2, detected in the saliva of *I. ricinus* females (unpublished result), has a suppressive effect on Th1 cytokines elaboration (Betz and Fox 1991). A recombinant protein derived from a subtractive cDNA library of the salivary gland of female I.ricinus was also found to modulate T lymphocyte and macrophage responsiveness by inducing a Th2 response and by inhibiting the production of pro-inflammatory cytokines (Leboulle et al. 2001 and unpublished results). A calreticulin protein secreted in Amblyomma americanum saliva (Jaworski et al. 1995) as well as a protein of 36 kDa isolated from salivary glands of D. andersoni ticks (Bergman et al. 2000) also have immunosuppressive properties.

The hypostome and the chelecerae of a nymphal tick penetrates deeply into the dermis causing skin damage (Mbow et al. 1994a). Tick saliva carried with the blood stream could stimulate leucocytes in the deep lymphoid organs such as the spleen. We therefore examined the effect of tick saliva and of SGE on the priming of splenic T cells to SRBC. The results showed that both of them have a systemic immunosuppressive effect on SRBC sensitized splenic T cells for at least 1 week after intraperitoneal injection. A weak effect was already evident after 4 days of culture (unpublished result). The immune response of guinea-pigs infested with D. andersoni was also suppressed systemically (Wikel 1982). The intraperitoneal injection of sand fly salivary glands lysate induced systemic immunosuppression in C57BL/6 and BALB/c mice (Titus 1998). As with sand flies and ticks, the salivary glands of other arthropods such as black flies (Cross et al. 1994a) and mosquitoes (Cross et al. 1994b) contain immunomodulatory molecules. The immunosuppressive effect of I. ricinus saliva probably influences the transmission of tick-borne pathogens. It has been reported that the immunomodulator properties of vector saliva

may be required for the successful transmission and establishment of host infection (Titus and Ribeiro 1990).

In conclusion, the impairment of the innate and acquired immune system allows BALB/c mice to tolerate tick feeding and might facilitate the transmission of tick-borne pathogens. The identification of immunomodulatory molecules in I. ricinus tick saliva and SGE requires further investigations. This could be usefull in the conception of a vaccine against tick feeding and pathogens transmission.

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Fig.1. I. ricinus salivary inhibition of SRBC hemolysis by the human alternative complement pathway.

Results are presented as a percentage of inhibition of complement activity. No inhibition of complement activity was measured when tick saliva was absent. Results are representative of three different experiments. Each value represents the mean of quadruplicate wells ± SD.

Fig.2a, b. Effect of the protein concentration of tick saliva on the activation of tick-sensitized lymph node T cells.

The activation of primed lymph node T cells incubated with increasing protein concentrations of tick saliva was assessed by the determination of the degree of proliferation **a** and the measurement of the production of IL-4 **b**. Each column represents an average of triplicate wells ± SD.

Fig.3a, b. Effect of the protein concentrations of SGE on the activation of tick-sensitized lymph node T cells.

The activation of primed lymph node T cells incubated with increasing doses of SGE was assessed by the determination of the degree of proliferation **a** and the measurement of the production of IL-4 **b**. Each value represents the mean of triplicate wells ± SD.

Fig-4-I. ricinus tick saliva inhibition of naive splenic T cells proliferation in response to Con-A. 10^6 splenic cells/well were preincubated in medium containing increasing protein concentrations of tick saliva and stimulated *in vitro* with Con-A. Results are presented as the mean of triplicate cultures ± SD.

Fig-5- Immunosuppressive effect of tick saliva and SGE on the proliferation of SRBC-sensitized splenic T cells.

7×10^5 spleen cells/well from BALB/c mice injected with SRBC (control) or SRBC and tick-antigens were stimulated *in vitro* with increasing numbers of SRBC. Degree of proliferation is expressed as the mean of triplicate wells ± SD.

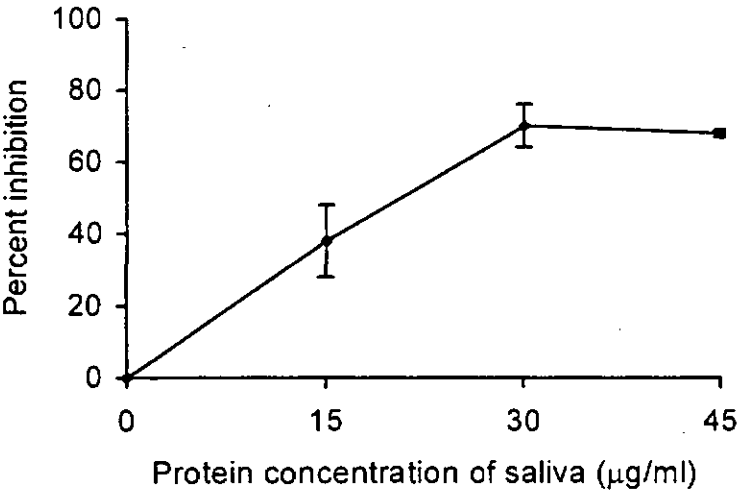
100
80
60
40
20
0
Percent inhibition
0
15
30
45
Protein concentration of saliva (µg/ml)

Fig -1-

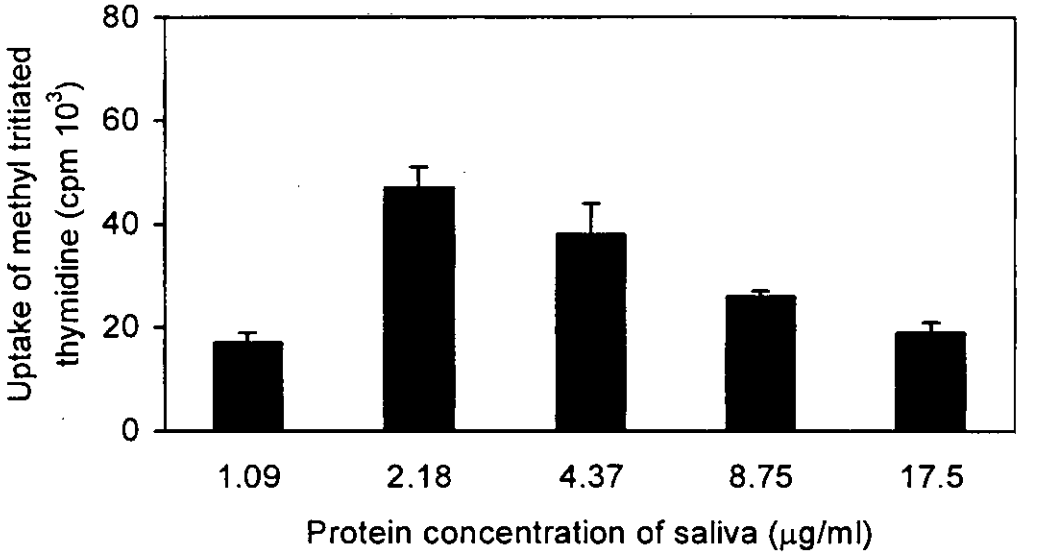
80
60
40
20
0
Uptake of methyl tritiated thymidine (cpm 10^3)
1.09
2.18
4.37
8.75
17.5
Protein concentration of saliva (µg/ml)

Fig -2a-

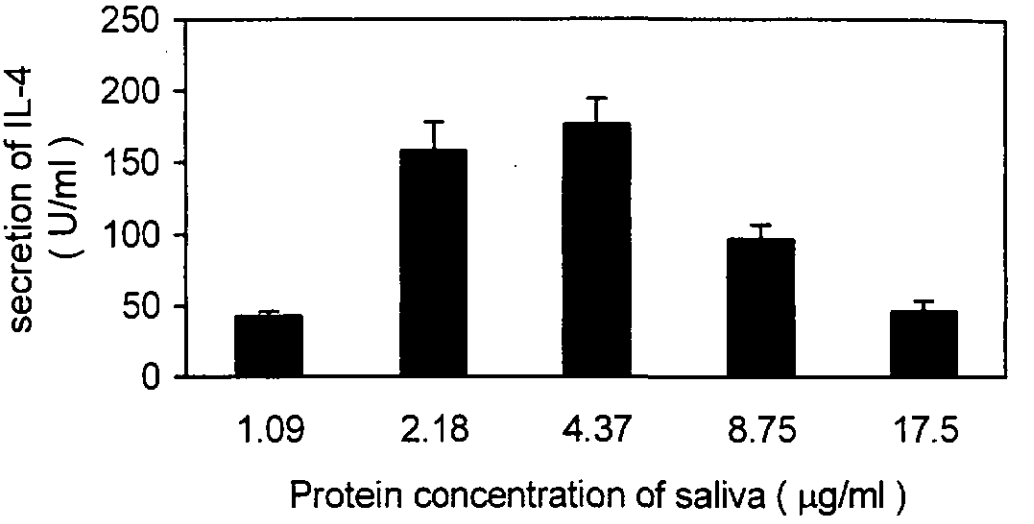
250
200
150
100
50
0
secretion of IL-4
(U/ml)
1.09
2.18
4.37
8.75
17.5
Protein concentration of saliva (μg/ml)

Fig -2b-

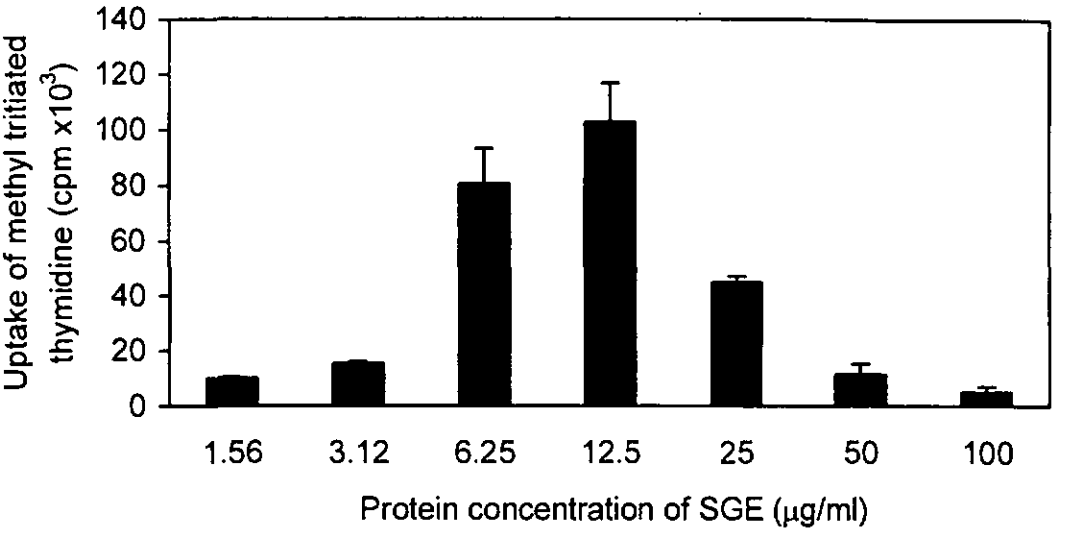
Uptake of methyl tritiated thymidine (cpm x10^3)
140
120
100
80
60
40
20
0
1.56
3.12
6.25
12.5
25
50
100
Protein concentration of SGE (µg/ml)

Fig -3a-

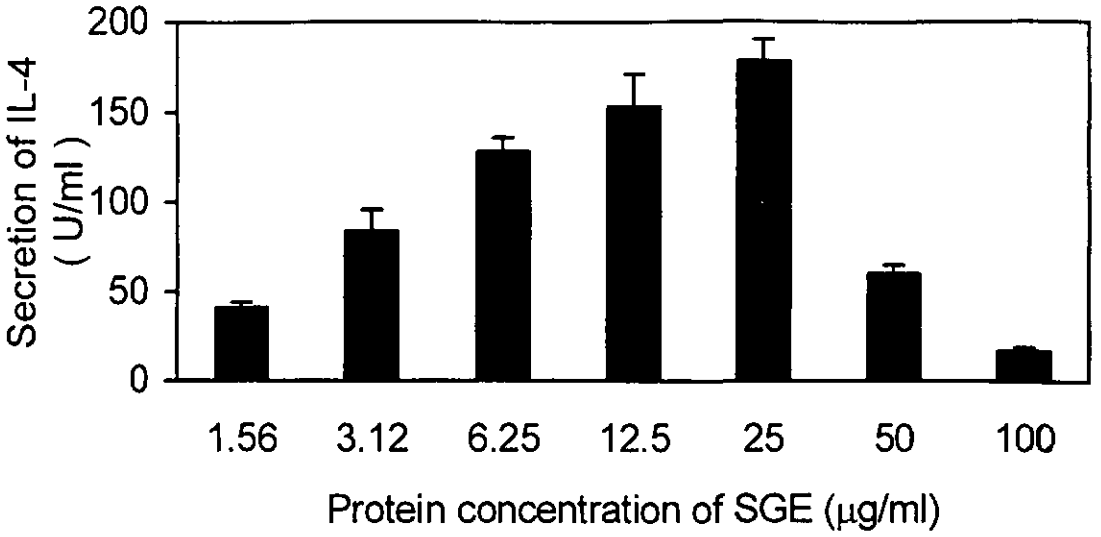
200
150
100
50
0
Secretion of IL-4
(U/ml)
1.56
3.12
6.25
12.5
25
50
100
Protein concentration of SGE (μg/ml)

Fig -3b-

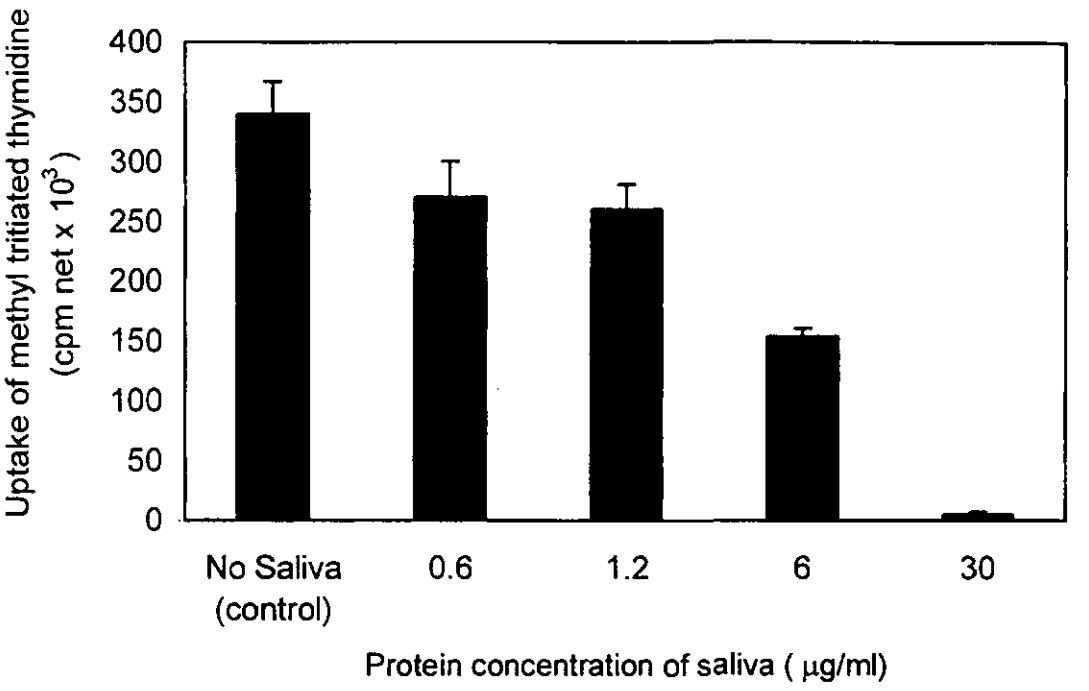
Uptake of methyl tritiated thymidine (cpm net x 10^3)
400
350
300
250
200
150
100
50
0
No Saliva (control)
0.6
1.2
6
30
Protein concentration of saliva (µg/ml)

Fig -4-

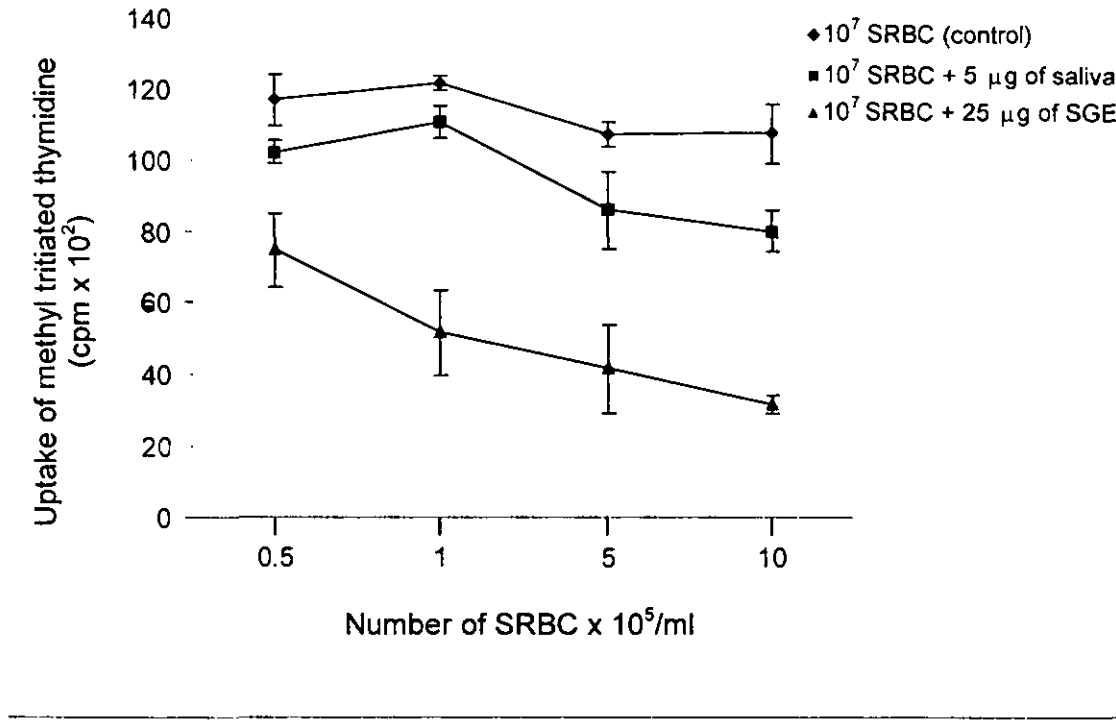
140
120
100
80
60
40
20
0
Uptake of methyl tritiated thymidine (cpm x 10^2)
0.5
1
5
10
Number of SRBC x 10^5/ml
◆10^7 SRBC (control)
■10^7 SRBC + 5 µg of saliva
▲10^7 SRBC + 25 µg of SGE

Fig-5-

Résultat n°2

Th2 polarization of the immune response of BALB/c mice to *Ixodes ricinus* instars, importance of several antigens in activation of specific Th2 subpopulations. *Parasite Immunol*, **23**:61-69

Parasite Immunology, 2001: 23: 61–69

Th2 polarization of the immune response of BALB/c mice to *Ixodes ricinus* instars, importance of several antigens in activation of specific Th2 subpopulations

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SUMMARY

BALB/c mice were infested with Ixodes ricinus *larvae, nymphs or adults. Expression of IL-4 and IFN-γ mRNA in axillary and brachial draining lymph node cells were measured by competitive quantitative reverse transcription-polymerase chain reaction 9 days after the beginning of primary-infestation. IL-4 mRNA was always higher than that of IFN-γ mRNA for all tick instars. Moreover, IL-4 mRNA expression progressively increased during nymphal primary-infestation with a high burst of expression 7 days after the beginning of infestation. No evolution of IFN-γ mRNA expression was detected. Draining lymph node cells of infested BALB/c produced higher level of IL-4 than IFN-γ following* in vitro *restimulation with adult tick saliva, salivary gland extract (SGE) or with five selected different chromatographic fractions of SGE. Anti-tick IgG1 antibodies but no IgG2a were detected in BALB/c pluri-infested with* I. ricinus *nymphs, which confirmed the Th2 polarization of the immune response.*

Keywords Ixodes ricinus, *tick instars, BALB/c, antigens, saliva, cytokines, IL-4, IFN-γ, Th2*

INTRODUCTION

In recent years, several studies have shown that helper T cell clones of $CD4^+$ phenotype can be separated into two subsets designated Th1 and Th2. These subsets can be distinguished on the basis of their pattern of cytokine secretion following stimulation with mitogens or antigens (1). Th1 cells produce IL2, IFN-γ and lymphotoxin and promote cell mediated immune responses which are important for the destruction of intracellular pathogens, such as some bacteria or protozoa (2). Th2 cells secrete IL-4, IL-5, IL10 and IL-13. IL-4 and IL-13 are effective in providing help for the expression of IgE and IgG1 in mice (3). Th2 cells are predominant in helminth infections or atopic diseases (4,5). It has also been reported that IL-4 and IFN-γ reciprocally regulate each other, with IFN-γ inhibiting Th2 lymphocytes, IL-4 and IL-10 downregulating Th1 cells (6). Priming of naive $CD4^+$ T cells in presence of IL-4 causes the development of Th2 effector cells while IL-12 yields Th1 effector cells (7,8). Other factors are implicated directly or indirectly in the orientation of the immune response such as the type of antigen presenting cells (APC) and the molecular environment of the immune induction site. In our model, it has been previously demonstrated that mice of different haplotypes develop Th2 immune responses after larval or nymphal *Ixodes ricinus* tick infestations (9). Pluri-infested mice produced high levels of IgE and draining lymph node cells from these animals produced high level of IL-4 and low level of IFN-γ after being stimulated *in vitro* with ConA. Moreover, when ticks are infected with *Borrelia burgdorferi*, the antispirochete immune response is also biased toward Th2 (10).

To characterize the primary immune response of primary-infested mice with *I. ricinus* tick, we have measured IL-4 and IFN-γ mRNA in draining lymph node cells using a competitive quantitative reverse transcription-polymerase

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chain reaction (RT-PCR) (11). After secondary *in vitro* stimulation of T cells with tick antigens, enzyme-linked immunosorbent assay (ELISA) was used to measure IL-4 and IFN-γ proteins. Despite cytokines being consumed in the culture medium, this test is sensitive enough to detect the effects of tick saliva, salivary gland extract (SGE) and SGE chromatographic fractions on cytokine production. Furthermore, specific IgG1 and IgG2a antitick antibodies, characteristic of Th2 or Th1 responses, have been analysed in mice infested four times with nymphal ticks.

Briefly, the purpose of this work was to establish the polarization of the immune response after exposure of BALB/c mice to adult and immature tick *I. ricinus* instars, and to follow *in vivo* the cytokine profile of draining lymph node cells in primary-infestation and *in vitro*, after restimulation of draining lymph node cells with different tick antigens.

MATERIALS AND METHODS

Animals

Eight to 12-week-old BALB/c female mice and male rabbits (New Zealand), weighing an average of 3 kg, were purchased, respectively, from IFFA-CREDO (Arbresle, France) and from Elevage des Dombes (Romans, France). *I. ricinus* larval, nymphal and adult ticks were reared in our laboratory as previously described (12).

Infestations

Mice were infested with 40 *I. ricinus* larvae, 15 nymphs or one pair of adult ticks. They were placed in a small plastic capsule (15 mm in diameter) glued onto the shoulders of the mice with a mixture of one part beeswax and four parts colophonium, at the site drained by brachial and axillary lymph nodes (13). Each experiment was done on a group of four mice. To detect antitick antibodies, four successive nymphal infestations were interspaced by 14 days. Mice flanks were alternated during these repeated infestations. To prepare tick antigens, adults *I. ricinus* were applied and allowed to feed for 5 days on rabbit's ears. They were contained by a nylon bag. An Elizabethan collar prevented the host from grooming.

RNA extraction

Total RNA was extracted from 5×10^5 axillary and brachial draining lymph node cells of mice infested with tick larvae, nymphs or adults using the tripure isolation kit (Boehringer Mannheim, Germany). The addition of chloroform to the solution before centrifugation allowed the formation of three phases. Total RNA in the upper phase was then precipitated with cold isopropanol (molecular grade). The pellet washed twice in 75% ethanol was then dissolved in 20 μl sterile distilled water (RNAase free). Two μl containing 0·1–0·5 μg of total RNA were used as template for the reverse transcriptase reaction.

Competitive quantitative reverse transcription-polymerase chain reaction (CQ RT-PCR)

The first-strand cDNA synthesis kit (Boehringer Mannheim) was used. The semiquantitative competitive PCR was carried out using a competitor construct (pPQRS) containing sequences for multiple cytokines including IL-4 and IFN-γ and for hypoxanthine guanine phosphoribosyl transferase gene (HPRT) (14). Primers for IL-4 were: 5′-CATCGGCATTTTGAACGAGGTCA-3′ (sense) and 5′-GCTACGGACCTAAGTAGCTATTC-3′ (antisense), for IFN-γ: 5′-CATTGAAAGCCTAGAAAGTCTG-3′ (sense) and 5′-CTCATGAATGCATCCTTTTTCG-3′ (antisense) and for HPRT: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ (sense) and 5′-GAGGGTAGGCCTATAGGCT-3′ (antisense). Sense and antisense primers were chosen on different exons separated by large intronic sequences which enables unambiguous differentiation of cDNA from contaminating genomic DNA amplification products. cDNA synthesis using RNA extracted from draining lymph node cells from mice under different infestation conditions, were used as templates. The thermal cycling conditions were: 94°C for 40 s, 60°C for 20 s, 72°C for 40 s, followed by a final incubation at 72°C for 10 min. The number of cycles varied between 33 and 36. The simultaneous amplification of the cytokine gene in the first strand cDNA reaction mixture and of an eight-fold serial dilution of competitor of known concentration allowed the determination of the level of HPRT, IL-4 or IFN-γ specific transcript. The point of equivalence in intensity between the competitor (upper band) and the cDNA (lower band) indicates the relative concentration of mRNA. The ratio of the relative concentration of the gene of interest (IL-4 or IFN-γ) to the relative concentration of HPRT was then calculated. Results were expressed as the fold of increase in IL-4 or IFN-γ mRNA expression in mice infested with nymphal *I. ricinus* ticks compared to non-infested mice. The formula: IL-4 or IFN-γ t_x/HPRT t_x: IL-4 or IFN-γ t_0/HPRT t_0, which was used to calculate the fold of increase in IL-4 or IFN-γ mRNA expression, emanated from the work of Reiner *et al.* (14) who calculated the HPRT mRNA concentration to control the varying efficiencies of the RT step among different experimental groups. The different concentrations of mRNA have been measured at t_0 and t_x which represent different times points, respectively, 0 h in

lymph node cells of non-infested mice or 12 h, 1, 3, 5, and 7 days in lymph node cells of postnymphal infested mice.

Tick antigens

To collect saliva, adult *I. ricinus* ticks were allowed to feed for 5 days on rabbit's ears. Partially engorged female ticks were removed. To activate the salivation one drop of 5% (wt/vol) solution of pilocarpine in absolute methanol was applied to their dorsum previously scratched by abrasive paper. A finely drawn capillary tube was fitted over the mouthparts of each tick which was allowed to salivate for 10–30 min (15). The volume provided by each tick was 0·5 μl in average. Saliva from 100 to 200 partially fed ticks was pooled, sterilized through a 0·22-μm filter and stored at − 20°C until use. *I. ricinus* females fed for 5 days on rabbit's ears were used to prepare SGE as previously described (16). The salivary glands were dissected and homogenized in ice cold extraction buffer consisting of 50 mM phosphate-buffered saline (PBS) pH 7·4 supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and 5 mM ethylene diaminetetraacetic acid (EDTA). The homogenate was centrifuged at 16 000 *g* for 30 min at 4°C. The supernatant was dialysed in cellulose ester membrane tube with a molecular weight cut-off of 100 Da (Spectrum, Socochim, Switzerland) overnight in 10 mM (PBS) pH 7·4. Dialysate was sterilized through a 0·22-μm filter and stored at − 20°C until use. Soluble proteins from SGE were fractionated by FPLC (Pharmacia, Switzerland). The extract was desalted on a Fast desalting Column HR 10/10 using 10 mM Tris-buffer, pH 7·5 and 50 mM NaCl as eluant. The peak containing proteins was then applied onto an anion exchange MonoQ HR 5/5 column. Bound proteins were eluted with a 50–600 mM NaCl linear gradient in 10 mM Tris-buffer pH 7·5. The main parameters were the gradient volume (20 ml), the salt concentration change/ml (25 mM/ml). The volume of each fraction was fixed at 0·5 ml. They were all dialysed against 10 mM PBS, pH 7·5 during 48 h at 4°C and stored at − 20°C until use. Protein concentration of saliva, SGE or chromatographic fractions were determined using BCA Protein Assay Kit (Pierce, Socochim, Switzerland).

SDS-page

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used with a 12% separation gel and a 6% stacking gel (17). Chromatographic fractions, saliva, and SGE from partially fed tick females were boiled for 4 min in 5% SDS buffer before loading on the gel. The proteins separated on SDS-gel were electrophoretically transferred onto a nitrocellulose sheet. The strips of different samples were rinsed in PBS and incubated in PBS-Tween 20 (0·3%) at 37°C for 30 min. Blots were then washed three times during 5 min with PBS-Tween 20 (0·3%). The strips were incubated in colloidal gold solution until optimal band visualization was obtained.

Western blot

Free binding sites on the strips were blocked by 5% (w/v) milk powder in PBS pH 7·5 during 1 h. They were then washed four times during 5 min with 1% milk powder in PBS. For the specific detection of both IgG1 or IgG2a, the strips corresponding to SGE from partially fed females were incubated with diluted pooled sera (1 : 5) from mice infested four times overnight at room temperature. The membranes were washed four times in PBS and incubated for 1 h with diluted (1 : 1000) rat antimouse IgG1 or IgG2a (Pharmingen, Germany) coupled to alkaline phosphatase. Negative controls were performed with non-infested mice sera (1 : 5). All strips were then treated with the Immun-Star chemiluminescent protein detection system (BioRad, Hercules, CA, USA) for few minutes before film exposure (BioMax, Kodak,New Haven, CT, USA).

Preparation, culture and quantification of proliferation of draining lymph node cells stimulated with tick antigens

Mice were killed 9 days after infestation and axillary and brachial lymph nodes were removed. 10^6 draining lymph node cells per well were cultivated in 200 μl total volume of complete culture medium containing RPMI-1640 (Gibco, Basel, Switzerland), supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamin, 1 mM sodium pyruvate, 1 mM nonessential aminoacids (Sigma, St Louis, MO, USA), 0·05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin (Gibco) and 25 μg/ml fungizone (Gibco). After 96 h incubation at 37°C in saturated atmosphere with 5% CO_2, with or without 40 μl/well of dialysed and filtrated chromatographic fractions of SGE, 1 μCi/well of methyl [^{3}H] thymidine (specific activity 25 Ci/mmol) (Amersham, Bucks, UK) was added 18–24 h before harvesting the cells. Tritiated thymidine incorporation was determined by liquid scintillation counting.

Quantification of IL-4 and IFN-γ in supernatants of tick antigens-stimulated draining lymph node cells

For culture supernatant collection, 10^6 draining lymph node cells in 200 μl total volume of complete culture medium were incubated with SGE (20 μg/ml), saliva (4 μg/ml) or

dialysed and filtrated chromatographic fractions of SGE (40 µl/well) at 37°C in saturated atmosphere with 5% CO_2. The volume applied for each fraction is kept constant at 40 µl/well to respect the proportionality of antigens concentrations present in the SGE cocktail. Based on recent published data (18), and preliminary assays showing that no cytokines were detected 24 or 48 h following incubation with tick antigens, supernatants of draining lymph node cells were removed after 96 h and stored at −80°C until used for IL-4 and IFN-γ determination. ELISA cytokine tests were performed as previously reported (19). Dilutions of rIL-4 (12·5–400 U/ml) or rIFN-γ (4–125 U/ml) (Pharmingen, Germany) were used as positive test controls and for the construction of standard curves.

RESULTS

In vivo expression of IL-4 and IFN-γ mRNA during a primary infestation

BALB/c were infested with 40 larvae, 15 nymphs or 1 pair of *I. ricinus* adults. A suspension of 5×10^5 cells prepared from the brachial and axillary draining lymph nodes was used for RNA extraction. IL-4 and IFN-γ mRNA levels were determined by competitive quantitative RT-PCR nine days after infestation with larvae, nymphs or adult ticks (Figure 1). The IL-4 and IFN-γ mRNA concentrations were, respectively, IL-4 mRNA (75 pg/µl) > IFN-γ mRNA (6 pg/µl), IL-4 mRNA (100 pg/µl) > IFN-γ mRNA (4 pg/µl) and IL-4 mRNA (100 pg/µl) > IFN-γ mRNA (4 pg/µl). In all cases, we observed a higher dose of IL-4 mRNA expression compared to IFN-γ mRNA. The concentrations of IL-4 and IFN-γ mRNA in the lymph node cells of non-infested mice were equally low.

Kinetic of IL-4 and IFN-γ mRNA expression

BALB/c were infested with 15 nymphs of *I. ricinus*. This was followed by the removal of draining lymph nodes after half a day or at 1, 3, 5 and 7 days post primo-infestation. A cell suspension was prepared and used for RNA extraction. The time course of IL-4 mRNA and IFN-γ expression in draining lymph node cells from infested mice is shown (Figure 2). There is a slight and regular increase of IL-4 mRNA expression during the first five days (2·5, 11·5 and 71-fold increase, respectively) followed by a high burst of expression at day 7 (> 375-fold). In contrast, IFN-γ mRNA always stay low during the corresponding days.

Specific T cell proliferation with SGE chromatographically defined fractions

Nine days after being infested with 15 nymphal *I. ricinus* ticks, cells from axillary and brachial lymph nodes draining the site of nymphs fixation respond to a wide range of chromatographic fractions of SGE. Among them fractions 10, 15, 20, 24 and 33 display a higher effect on T cell proliferation (Figure 3a), each of them corresponding to peaks in the chromatogram (Figure 3b).

In vitro IL-4 and IFN-γ production

Primed T cells of axillary and brachial lymph nodes collected from BALB/c mice infested with nymphal ticks produced high level of IL-4 (> 150 U/ml) when stimulated with either SGE or saliva and > 20 U/ml when stimulated, respectively, with fractions 10, 15, 20, 24 and 33 (Figure 4). In contrast we detected a low dose of IFN-γ in the supernatant of the same cell culture. Cells incubated without any antigen did not produce IL-4 or IFN-γ.

SDS-PAGE analysis of SGE, saliva and chromatographic fractions

SGE, saliva or the five chromatographic fractions used in antigen-specific T cell proliferation and cytokines detection show different protein patterns (Figure 5). The pattern of SGE (lane 1) is more complex than that of saliva (lane 2) and of each chromatographic fraction (lanes 3–7). Several proteins of tick saliva, SGE and chromatographic fractions comigrate, as for example proteins of 90 and 94 kDa in F20 and F24 (lanes 5 and 6) or of 65 kDa which is enriched in F33 (lane 7). F10 and F15 (lanes 3 and 4) show also some comigrating polypeptides with saliva and SGE among a relative complex pattern of proteins.

Specific IgG1 and IgG2a antibodies produced by pluri-infested BALB/c

The immunoblot analysis (Figure 6) shows two bands representing the reactivity of two SGE proteins (25 and 65 kDa) with BALB/c mice IgG1 (lane 1) infested four times with 15 nymphal *I. ricinus* (see arrows). No specific IgG2a antibodies were detected (lane 3). Controls were performed with non-infested mice sera (lanes 2 and 4) which did not show any reactivity with tick antigens.

DISCUSSION

It has been reported that many factors are implicated in the regulation of Th1 and Th2 subsets in mice such as the

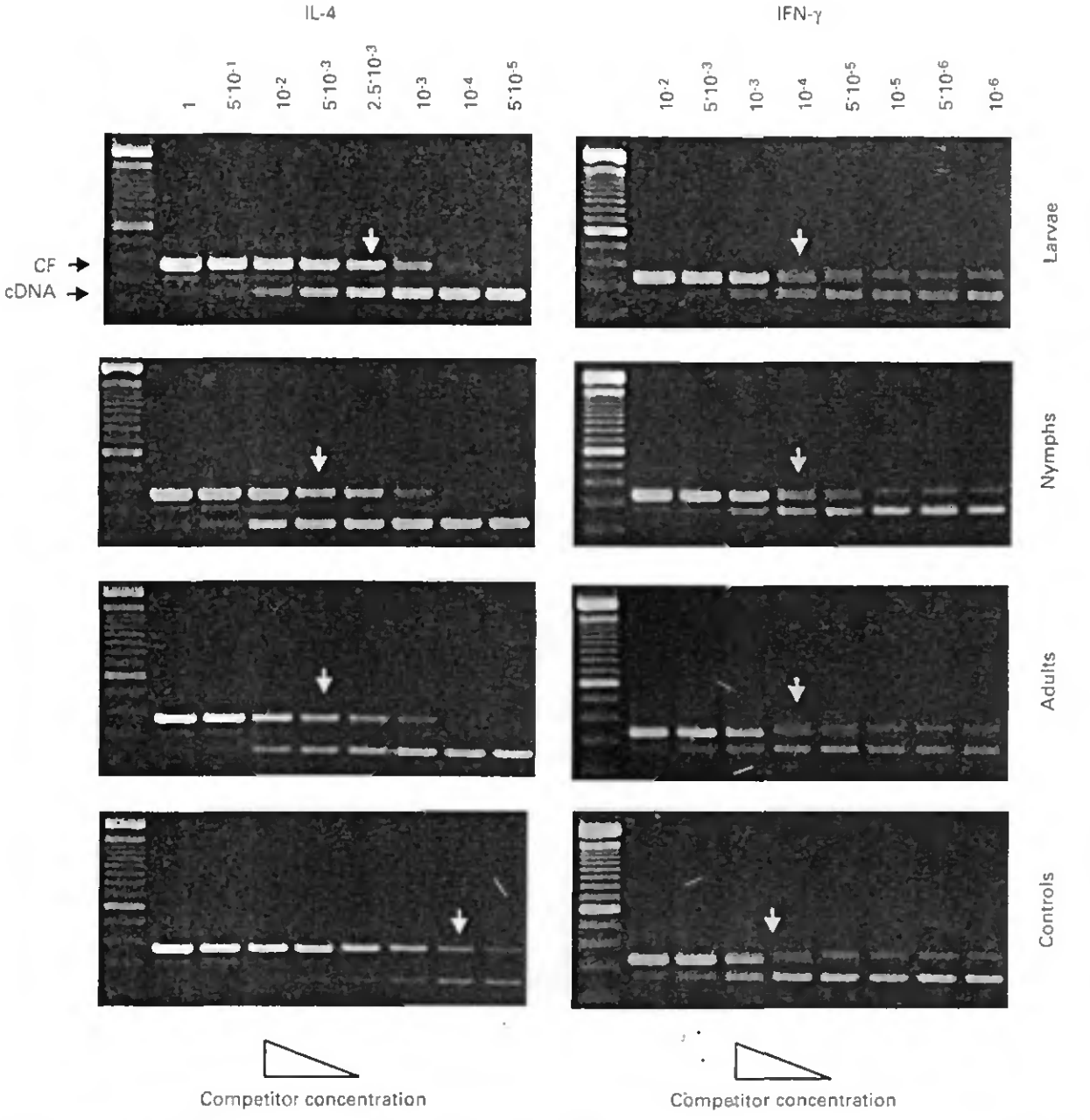

Figure 1 Determination by RT-PCR of the relative levels of IL-4 and IFN-γ mRNA. Ethidium bromide agarose stained gels of representative PCR reactions using IL-4 and IFN-γ specific primers in the presence of serial eight-fold dilutions of a competitor, 1 ng to 5×10^{-5} ng/μl for IL-4 and 10^{-2}–10^{-6} ng/μl for IFN-γ determination. Two μl of six-fold diluted cDNA (larvae) and four-fold (nymphs, adults and controls) are used as template. The point of equivalence in intensity between the competitor fragment and the cDNA indicate the relative concentration of IL-4 or IFN-γ mRNA (see arrows) in draining lymph node cells of BALB/c after a primary infestation with, respectively, larvae (75 pg/μl for IL-4 mRNA, 6 pg/μl for IFN-γ mRNA), nymphs (100 pg/μl, 4 pg/μl) or adult ticks (100 pg/μl, 4 pg/μl). As a control, levels of IL-4 and IFN-γ mRNA determined in lymph node cells from non-infested mice are quite equal (4 pg/μl).

genetic background, the form and the dose of antigen, the site of the antigens inoculation, the type of APC and environmental cytokines (20). In *Leishmania major* infection, two distinct responses occur depending of the mice genetic background (21,22). The susceptibility of BALB/c (H-2d) is consistent with $CD4^+$ Th2 type response whereas the resistance of C57BL/6 (H-2b) results in the development of a polarized $CD4^+$ Th1 response. In contrast to *Leishmania* models, mice with different haplotypes such as BALB/c or DBA (H-2d), C57/BL/6 (H-2b), C3H or CBA

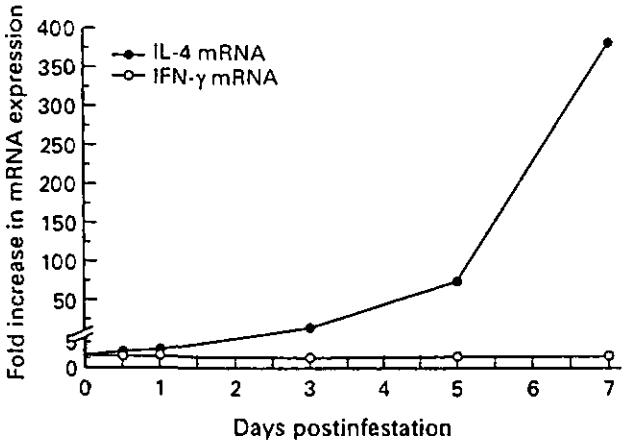

Figure 2 Kinetics of IL-4 and IFN-γ mRNA expression in draining lymph node cells during nymphal tick infestation. The curves represent the fold increase of IL-4 (full circle) or IFN-γ (empty circle) mRNA expression *in infested mice compared to non-infested ones.*

(H-2 k), SJL (H-2 s) and FVB (H-2q), infested once with 15 *I. ricinus* nymphal ticks, developed a Th2 immune response as shown after stimulation of draining lymph node cells with ConA (9). Moreover, BALB/c infested with either five or 45 nymphs of *I. ricinus* developed always a Th2 immune response. Infestations of C3H/HeN mice with *Ixodes scapularis* or *Ixodes pacificus* nymphs also polarized cytokine production towards a Th2 profile as shown after restimulation of spleen lymph node cells with ConA (23).

In our work, we have observed that adult *I. ricinus*, as well as larval or nymphal ticks, polarize the immune response of BALB/c toward Th2. Nine days after the beginning of infestation with the different tick instars, high IL-4 mRNA expression was detected in draining lymph node cells. At that time, draining lymph node cells of mice infested with nymphal ticks were successfully restimulated *in vitro* with adult tick saliva and SGE. Draining lymph node cells secrete high level of IL-4 and low level of IFN-γ in the cell culture supernatants. This suggests the presence of common immunogenic epitopes between tick instars. Following restimulation with five chromatographic fractions of SGE, draining lymph node cells show high cell proliferation (Figure 3a) and IL-4 synthesis (Figure 4). In contrast, IFN-γ always remains at base line level. The quantity of IL-4 produced upon tick antigens restimulation is correlated with T cell proliferation intensity. Nevertheless, it is not dependent of T cell proliferation as cytokine expression does not require completion of cell cycle of activated T cells (24). Saliva, SGE and five selected chromatographic fractions of *I. ricinus* SGE show different proteins pattern but contain some comigrating proteins as revealed with SDS-PAGE (Figure 5). Fraction 20 is the more efficient to stimulate proliferation of draining lymph node cells and IL-4 secretion. We hypothesize that two predominant proteins of molecular weight 90 and 94 kDa, also present in tick saliva, could be important triggering molecules. Rabbits infested with adult *I. ricinus* produced antibodies against these proteins (16).

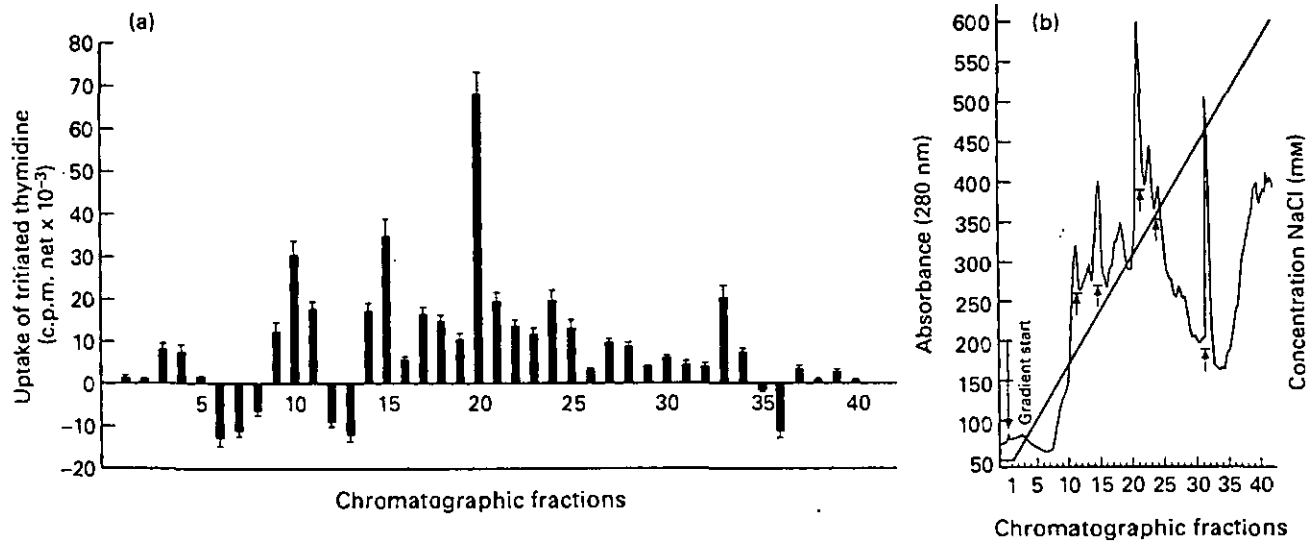

Figure 3 (a) A wide range of chromatographic fractions of salivary gland extract (SGE) induced *in vitro* lymphocyte proliferation of axillary and brachial draining lymph nodes removed from BALB/c mice infested with nymphal ticks. Results show the mean (n = 3) of stimulated wells ± SD. Incorporated radioactivity was expressed as c.p.m. from cultures with fractions minus c.p.m. from culture without additives (net c.p.m.). (b) Elution profile of SGE from anion exchange chromatography. A linear 50–600 mM NaCl gradient was used to generate fractions. Peak areas of the main stimulative fractions are indicated by arrows.

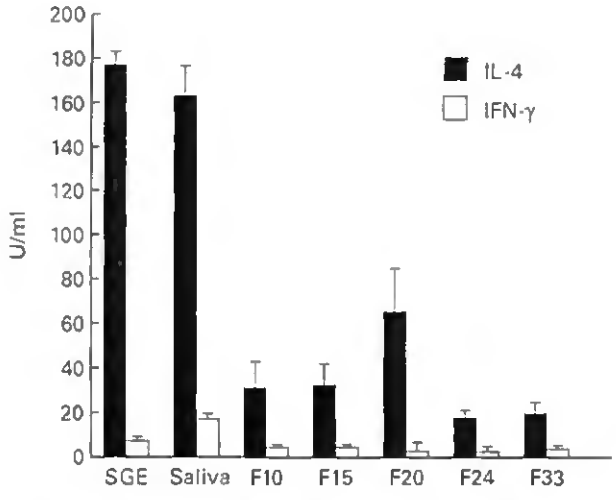

Figure 4 Measure of IL-4 (black bars) and IFN-γ (white bars) by specific ELISA. Nine days after the beginning of the first infestation, cells from axillary and brachial draining lymph nodes collected from BALB/c mice infested with nymphal ticks were stimulated *in vitro* with, respectively, SGE, saliva or chromatographic fractions 10, 15, 20, 24 and 33. Each value represents the mean of triplicate wells ± SD from a single experiment representative of three separate experiments.

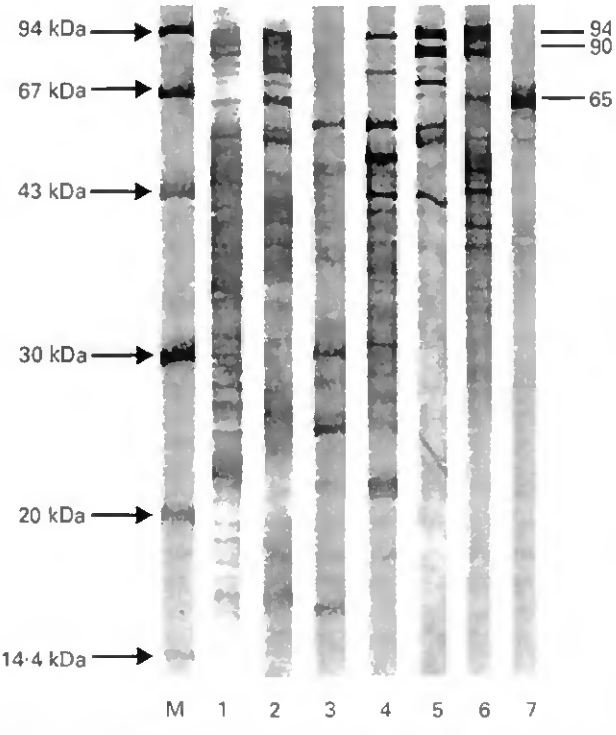

Figure 5 SDS-PAGE analysis of SGE (lane 1), saliva (lane 2) and chromatographic fractions 10, 15, 20, 24 and 33 (lanes 3–7, respectively) from ticks fed for 5 days. The strips 1–7 and molecular weight markers (M) were stained with colloidal gold.

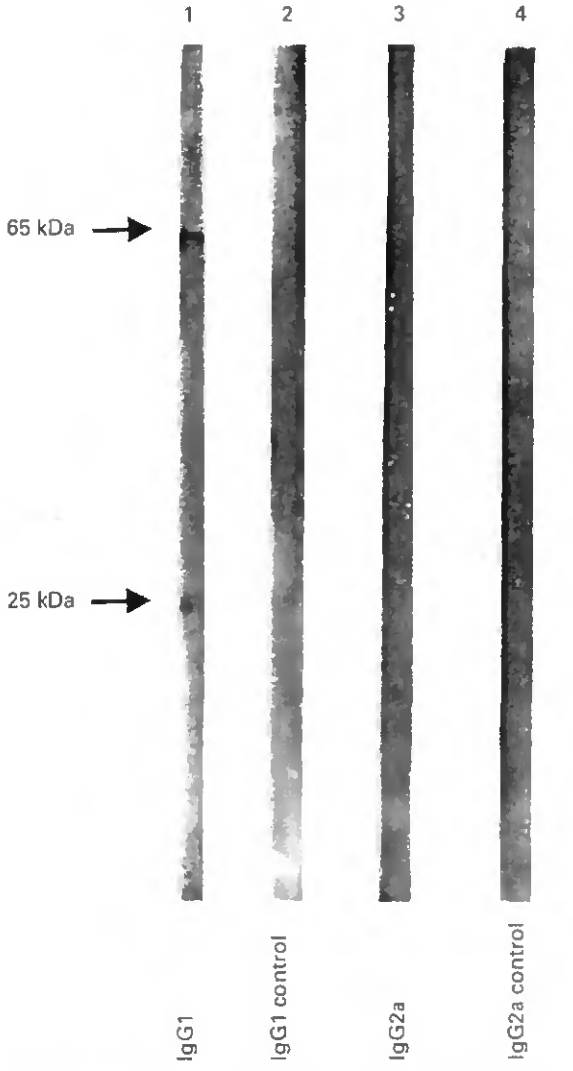

Figure 6 Immunoblot analysis of SGE. Blots were incubated with a pool of sera from four times infested BALB/c with 15 nymphs (lanes 1, 3) or as controls with a pool of non-infested mice (lanes 2, 4). This was followed by incubation with antimouse IgG1 (lanes 1, 2) or IgG2a (lanes 3, 4) coupled to alkaline phosphatase.

Fraction 33 was strongly enriched with a protein of 65 kDa, which was also detected in tick saliva. This protein was already shown to be a potent antigen in proliferation assay (25). Other tick saliva and SGE polypeptides that comigrate could also participate in the Th2 polarization of the immune response. Our results show the specificity of the response of primed draining lymph node cells to different antigens and the involvement of several subpopulations of Th2 in the antitick immune response. Tick saliva antigens are associated with Langerhans cells in the suprabasal layer of the epidermis in the skin of infested animals (26). Langerhans cells pick up and process antigens with high efficiency, and then migrate to draining lymph nodes. In contrast to other types of APC, dendritic cells are potent

activators of naive Th cells (27). The differential development of naive Th into functionally distinct effector Th cells depends upon microenvironmental factors. Only dendritic cells which have been activated by exogenous IL-12 inducing factors such as bacteria or their constituents can direct Th1 development through the release of IL-12. Increasing IFN-γ production by activated naive T cells directs their development toward the Th1 phenotype (27). PGE_2 is also an important molecule in the regulation of the immune response (28). With a colourimetric assay, PGE_2 has been detected in the saliva of 5 days engorged *I. ricinus* female ticks (0·5 μg of PGE_2/ml; unpublished results). It is also present in the saliva of other tick species such as *I. scapularis* (15). In the skin, this molecule may down regulate the expression of IL-12 by dendritic cells (28). This effect which is stable *in vitro* for at least 48 h could contribute to the development of Th2 responses in draining lymph nodes.

The *I. ricinus* rostrum penetrates deeply into the dermis (13) so that saliva molecules could be drained directly into lymph nodes. High local PGE_2 concentration should act synergistically with IL-4 on uncommitted B cells to direct isotype switching to IgE and IgG1 (29,30). The antibody isotypes produced in BALB/c pluri-infested with *I. ricinus* ticks are IgG1 (Figure 6) and IgE (31). The use of IL-4 deficient mice or the treatment of mice with anti-IL-4 monoclonal antibodies inhibited the production of IgE during successive infestations (31). IL-4 is an indispensable factor for the differential development of naive T cells into Th2 (8). A burst of IL-4 mRNA has been observed in the popliteal draining lymph node cells of BALB/c 16 h after subcutaneous injection of *L. major* promastigotes into the hind footpads (32). In this model IL-4 mRNA returned to baseline level by 48 h. For these authors, the early burst of IL-4 mRNA should play an essential role in the development of the second wave of IL-4 mRNA expressed from day 5 onwards. This new wave reflects the differentiation of parasite-specific $CD4^+$ T cells toward the Th2 functional phenotype. In tick infested BALB/c the IL-4 mRNA expression did not show an early peak but a progressive increase of IL-4 mRNA from 12 h onwards with a drastic increase at day 7 postinfestation. This observation suggests that an early burst of IL-4 secretion in draining lymph node cells is not imperious but that sufficient quantities of IL-4 are required to the development of mature Th2 cells.

In conclusion, tick saliva molecules could have different functions in relation with the Th2 polarization of the immune response. Some of them could act already at the site of tick attachment. Dendritic cells migrating from the skin to draining lymph nodes mature, present some specific antigens to naive T cells and prime them to generate effector Th2 cells.

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Résultat n°3

Induction of Th2 cell differentiation in primary immune response *in vitro* and *in vivo*: splenic dendritic cells incubated with *Ixodes ricinus* tick saliva prime naive CD4+ T cells to secrete IL-4.
(Submitted in *Immunology*)

Induction of Th2 cell differentiation in primary immune response *in vitro* and *in vivo*: splenic dendritic cells incubated with *Ixodes ricinus* tick saliva prime naive CD4+ T cells to secrete IL-4

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Running title : CD4+ T cells primed into Th2 *in vitro* or *in vivo*.

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Summary

Dendritic cells (DCs) are the most potent antigen presenting cells (APC) in priming naive T cells. In this report we showed that DCs incubated overnight with *Ixodes ricinus* tick saliva prime CD4+ T cells to secrete IL-4 and IL-10 *in vitro*. Tick saliva pulsed DCs injected subcutaneously into BALB/c mice elicit also a Th2 immune response. This finding brings out the importance of the molecular nature of tick saliva for the induction of the DCs type that generate either *in vitro* or *in vivo* a Th2 immune response. Early during the interaction of tick saliva pulsed DCs with bulk CD4+ T cells, the presence of IL-4 in the cell culture environment is of great importance to initiate the Th2 differentiation of CD4+ T cells. This is monitored indirectly by neutralizing IL-4 with specific monoclonal antibodies which blocked the differentiation of CD4+ T cells into Th2. This finding suggests that among bulk CD4+ T cells used in the culture certain populations such as memory Th2 cells, NK 1.1^+/ NK 1.1^- CD4+ T cells, conventional CD4+αβ T cells themselves are a potential source of IL-4. CD4+ T cells, differentiated into Th2 cells following stimulation *in vitro* with tick saliva pulsed DCs, proliferate only when IL-1β was added early in the culture. The early presence of IL-1β is required for the proliferation but not for the differentiation. Our priming *in vitro* system was restricted to major factors implicated in the anti-tick immune response such as naive CD4+ T cells, APCs and tick saliva. It provides the possibility to delimit further the antigenic molecule(s) and type of CD4+ T cells involved in the Th2 polarization of the immune response and to manipulate this response.

Key words: *Ixodes ricinus* saliva, cellular activation, priming *in vitro*, priming *in vivo*, dendritic cells, Th1/Th2, IL-1β, IL-4, IL-10.

Introduction

Cytokines produced by CD4+ T cells polarize the immune response *in vivo* or *in vitro* in response to antigen challenge. Th1 cells are characterized by the predominance of IFN-γ production while Th2 cells produce predominantly IL-4 (1). This dichotomy in T helper activity is reported to be associated to susceptibility or resistance to pathogens. A well studied example is experimental murine cutaneous leishmaniasis in which selective activation of Th1 cells is associated with resolution of the disease whereas selective activation of Th2 cells is associated with disease progression (2). Ectoparasites such as tick or sand fly inject pathogens into skin along with saliva. The old world sand fly *Phlebotomus papatasi* salivary gland lysate exacerbated lesion size and enhanced *Leishmania major* burden in disease-resistant CBA mice. This observation was correlated with inhibition of the production of Th1 cytokines and enhancement of Th2 ones (3). Likewise BALB/c mice infested by *Borrelia burgdorferi* infected nymphal *I. ricinus* ticks enhance Th2 immune response by reducing anti-borrelial IgG2a antibody production (4). These examples revealed the promoting effect of ectoparasite saliva toward a Th2 immune response. Pathogen free tick instars salivary secretions elicit a Th2 immune response in infested BALB/c mice (5). Many factors had been reported to be implicated directly or indirectly and may act independently or together to polarize the immune response *in vivo* (6). In our model of BALB/c mice infested with nymphal ticks, neither the genetic background nor the antigen doses are implicated in the orientation of the immune response (7). Other factors such as the molecular nature of tick saliva and the environment at the immune induction site may selectively differentiate naive CD4+ T cells into Th1 or Th2 cells. The activation of CD4+ T cells occurs often in secondary lymphoid organs and requires the involvement of antigen presenting cells. Regarding their wide distribution in the body of the host and their efficiency as antigen presenting cells (APCs) dendritic cells (DCs) are the most important cells implicated in the activation of CD4+ T cells (8). In peripheral tissues such as skin DCs trap antigens. They migrate to draining lymph nodes where they present antigen to T cells (9). The original

microenvironment of DCs including cytokines and antigens may influence their antigen presentation properties and the induction of naive CD4+ T cells to differentiate along either a Th1 or Th2 pathway.

An *in vitro* priming system has been set up to reproduce the leukocyte reaction *in vivo* and bring out the importance of the molecular nature of tick saliva for the polarization of the immune response. DCs pulsed *in vitro* with tick saliva induced the Th2 differentiation of naive CD4+ T cells. We also showed the ability of DCs pulsed *in vitro* with tick saliva and injected subcutaneously to BALB/c to generate a Th2 immune response *in vivo*. To investigate whether endogenous IL-4 is present early in the environment of the culture and if its presence is indispensable to direct the immune response toward Th2, we added neutralizing anti-IL-4 monoclonal antibodies to the primary *in vitro* (PIV) culture. Results showed that this neutralization inhibits the differentiation of CD4+T cells into Th2. Moreover we demonstrated that the early presence of IL-1β is indispensable as costimulator for the proliferation but not for the differentiation of Th2 cells. In this study we showed that the nature of tick saliva influenced DCs to promote Th2 immune response *in vivo*. Pulsed DCs synergize *in vitro* with induced endogenous IL-4 to prime naive CD4+ T cells to secrete IL-4 and IL-10.

MATERIALS AND METHODS

Animals

Male rabbits (New Zealand) weighing an average 3 kg and BALB/c female mice 8 to 12 weeks old were purchased from Elevage des Dombes (Romans, France) and IFFA-CREDO (Arbresle, France) respectively. *I. ricinus* adult female ticks were reared in our laboratory as previously described (10).

Tick saliva preparation

To collect saliva, adult *I. ricinus* ticks were allowed to feed for 5 days and 12 hours on rabbit's ears. Partially engorged female ticks were removed and fixed by their dorsum on a sticky paper. To enhance the salivation, 5 µl of dopamine solution 0.2% (w/v) (Fluka, Buchs, Switzerland) in 50 mM phosphate-buffered saline (PBS) pH 7.4 were injected into the hemocoele of the tick using a 0.3 x 13 mm needle (Becton Dickinson, Switzerland) (11). A finely drawn capillary tube of 20 µl of countenance was fitted over the mouthparts of each tick which was allowed to salivate for 20 to 40 min. The volume provided by each tick was 3 µl in average. Saliva from 100 partially fed ticks was pooled and stored at –20 °C until use. Protein concentration of saliva was determined using BCA Protein Assay Kit (Pierce, Socochim, Switzerland).

Purification of cell populations

Dendritic cells separation

A cellular suspension was obtained by teasing BALB/c mice spleens with a large striated forceps. The red blood cells were removed by incubation in ACK hypotonic lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1 mM Na_2EDTA) during 5 to 10 s. Briefly, for DCs enrichment, 5 x 10^7 cells / spleen were washed and suspended in the isolation buffer containing PBS 50 mM, pH 7.4 supplemented with 5% FCS, 5% NRS (normal rat serum) and 1 mM EDTA. DCs were then separated by a negative selection system, using the StemSep biotinylated antibody cocktail (StemCell, Basel, Switzerland) in combination with StemSep magnetic colloid, according to the manufacturer's instructions. Intact DCs which flow through the column were washed and

suspended in culture medium containing RPMI-1640 (Gibco, Basel, Switzerland), supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamin, 1 mM sodium pyruvate, 1 mM non-essential aminoacids (Sigma, St Louis, MO, USA), 0.05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin (Gibco) and 25 μg/ml fungizone (Gibco).

$CD4^+$ T cells separation

Red blood cells depleted spleen cell suspension prepared from BALB/c mice was washed twice in HBSS and suspended in the isolation buffer without EDTA. $CD4^+$ T cells were then separated using the StemSep biotinylated antibody cocktail (StemCell, Basel, Switzerland) for $CD4^+$ T cells in combination with StemSep magnetic colloid, according to the manufacturer's instructions. Highly purified $CD4^+$ T cells collected in the column flowthrough were suspended in complete RPMI-1640.

$CD4^+$ T cells priming *in vitro*

5×10^5 purified splenic DCs / well were pulsed or not (control) *in vitro* with 15 μl of *I. ricinus* tick saliva (1 mg/ml) in 500 μl of complete RPMI-1640 during 24 hours. Thereafter 5×10^6 purified $CD4^+$ T cells / well were added in 500 μl of complete RPMI-1640 alone or modified medium with adjunction of 10 ng/well of IL-1β or 50 μg of neutralizing anti-IL-4 monoclonal antibodies (Becton Dickinson, Switzerland). IL-1β and anti-IL-4 were added at the beginning of cultures. CD4+ T cells primed in absence or in presence of IL-1β were designated respectively pCD4+ T and $p_{IL\text{-}1\beta}$ CD4+ T. In all cultures cells were incubated in 1 ml total volume of complete RPMI-1640 in 24-well plates (Falcon, Becton Dickinson, Switzerland) during 7 days at 37 °C in saturated atmosphere and 5% CO_2.

Competitive PCR analysis of cytokine messenger RNA production

RNA extraction

Total RNA was extracted from 5×10^5 $CD4^+$ T cells primary stimulated *in vitro* with tick saliva pulsed DCs or DCs alone either in presence or absence of anti-IL-4. The prescript isolation kit

(Gentra Systems, Minneapolis USA) was used according to manufacturer's instructions. Briefly following CD4+ T cell lysis step a protein-DNA precipitation solution was added to the cell lysate. After centrifugation the supernatant containing the RNA was removed from the precipitated protein-DNA pellet. Total RNA was then precipitated with cold pure isopropanol. The pellet was washed in 70% ethanol, air dried, suspended in 20 µl of water treated with diethyl pyrocarbonate (DEPC) 1% (Sigma, Switzerland) and stored at -80°C until use. 1 µl containing 0.1 to 0.5 µg of total RNA were used as template for the reverse transcriptase reaction.

cDNA synthesis

Total RNA was heated to 57 °C for 10 min, cooled on ice for 5 min and used as template for the cDNA synthesis. 1 µg of total RNA contained in 5 µl of diethylpyrocarbonate-treated H_2O was added to the master mix (kit Omniscript Qiagen) including 2 µl of reverse transcription buffer (100 mM tris, 500mM KCl, pH 8.3), 2 µl of mixed dNTP, 2 µl of oligo $(dT)_{15}$, 1 µl of omniscript reverse transcriptase and 8 µl of sterile H_2O. The reaction mixture was incubated 60 min at 37 °C, heated to 93 °C for 5 min and chilled on ice for 5 min. The cDNA contained in 20 µl total volume was stored at –20 °C until use.

Competitive PCR

The first-strand cDNA synthesis kit (HotStarTaq, Qiagen) was used in this competitive PCR. The semi-quantitative competitive PCR was carried out using a competitor construct (pPQRS) containing sequences for multiple cytokines including IL-4, IL-10, IFN-γ and IL-2. Primers for IL-4 were: 5'–CATCGGCATTTTGAACGAGGTCA-3' (sense) and

5'–GCTACGGACCTAAGTAGCTATTC-3' (antisense),

for IL-10: 5'–CCAGTTTTACCTGGTAGAAGTGATG-3' (sense) and

5'-AACTCAGACGACCTGAGGTCCTGGATCTGT–3' (antisense),

for IFN-γ: 5'–CATTGAAAGCCTAGAAAGTCTG-3' (sense)

and5'–CTCATGAATGCATCCTTTTTCG-3'(antisense)and

for IL-2: 5' – TCCACTTCAAGCTCTACAG - 3' (sense) and 5' – CCGTACAAGACCTAAACTGAG - 3' (antisense). Sense and antisense primers were chosen on different exons separated by large intronic sequences which enables unambiguous differentiation of cDNA from contaminating genomic DNA amplification products as described by Reiner et al (12). 40 cycles were performed in this PCR. The thermal cycling conditions were: 94 °C for 40 s, 60 °C for 20 s, 72 °C for 40s, followed by a final incubation at 72 °C for 10 min. The simultaneous amplification of the cytokine gene in the first strand cDNA and of a serial dilution of competitor of known concentration allowed the determination of the level of IL-4, IL-10, IFN-γ and IL-2 specific transcripts. The point of equivalence in intensity between the competitor (upper band) and the cDNA (lower band) indicates the relative concentration of mRNA.

Secondary stimulation of primed CD4+ T cells *in vitro*

2 x 10^4 purified splenic DCs / well were pulsed or not with 4 µl of tick saliva overnight *in vitro* in 100 µl of complete RPMI-1640 and cultivated with 2 x 10^5 of homologous $p_{IL\text{-}1\beta}$ CD4+ T cells or p CD4+ T cells *in vitro* / well in the presence or absence of 2 ng / well of IL-1β. Cells were incubated in 96-well plates (Falcon, Becton Dickinson, Switzerland) in 200 µl total volume of complete RPMI-1640 during 96 h at 37 °C and saturated atmosphere and 5% CO_2. Cultures were pulsed each with 1 µCi/well of methyl [^{3}H] thymidine (specific activity 25 Ci/mmol) (Amersham, Bucks,UK) 24 h before harvesting the cells. Tritiated thymidine incorporation was determined by liquid scintillation counting.

Sub-cutaneous injection of tick saliva pulsed DCs

2 x 10^5 purified splenic DCs were pulsed or not with 15 µg of tick saliva *in vitro* overnight in 500 µl of complete RPMI-1640. Cells washed and suspended in 1 ml of PBS (50 mM, pH 7.4) were then injected intradermally at the level of the shoulder in one flank of BALB/c mice. Nine days after cells inoculation mice were killed and the axillary and brachial draining lymph nodes were removed. The draining lymph node cell suspensions were prepared and used in secondary

stimulation *in vitro* with tick saliva. Results were assessed by the quantification of T cells proliferation and ELISA cytokine assay.

Secondary stimulation of draining lymph node T cells with tick saliva *in vitro*

***In vitro* proliferation**

10^6 draining lymph node cells / well prepared from mice injected with DCs pulsed or not with tick saliva were stimulated with 2, 4 and 8 µg of tick saliva. Cells were cultivated in 200 µl total volume of complete RPMI-1640 during 96 h at 37 °C and saturated atmosphere and 5% CO_2. 24h before harvesting cells, 1 µCi/well of methyl [^{3}H] thymidine was added. Methyl tritiated thymidine incorporation was determined by liquid scintillation counting.

***In vitro* IL-4 and IFN-γ production**

10^6 draining lymph node cells / well prepared from mice injected with pulsed DCs or DCs alone were stimulated with 4 µg of tick saliva. Culture supernatants were collected and used for IL-4 and IFN-γ quantification using an Enzyme-linked immunosorbent assay (ELISA) as previously reported (13). Dilutions of rIL-4 (12.5-400 U/ml) or rIFN-γ (4-125 U/ml) (Pharmingen, Germany) were used for construction of standard curves.

Results

Expression of type 1 (IFN-γ, IL-2) and type 2 (IL-4 and IL-10) cytokines mRNA during a primary stimulation of CD4+ T cells

5 x 10^5 naive splenic CD4+ T cells primary stimulated *in vitro* in neutral condition with tick saliva pulsed DCs or DCs alone were collected for mRNA analysis. IL-4, IL-10, IFN-γ and IL-2 mRNA levels were determined by competitive quantitative RT-PCR at the 7^{th} day of culture. We have compared cytokine mRNA concentrations of the Th1 and Th2 phenotype (Fig.1a). Splenic CD4+ T cells primed *in vitro* with saliva pulsed DCs expressed more IL-4 mRNA (100 pg/μl) than IFN-γ mRNA (20 pg/μl) and IL-2 mRNA (5 pg/μl). These cells expressed also more IL-10 mRNA (100 pg/μl) than naive CD4+ T cells (80 pg/μl) stimulated with DCs alone considered as control. These results indicate that tick saliva pulsed DCs induce the differentiation of naive CD4+ T cells into Th2 in PIV culture. In control culture naive splenic CD4+ T cells didn't undergo any differentiation. The high levels of IL-4, IFN-γ and IL-2 mRNA are certainly related to a mixture of memory Th1 and Th2 cells present in the cell culture among selected splenic CD4+ T cells. Neither IL-4 nor IL-10 mRNA were detected in CD4+ T cells primary stimulated *in vitro* with tick saliva pulsed DCs in the presence of neutralizing anti-IL-4 monoclonal antibodies (Fig.1b). lower amount of IFN-γ mRNA (5 pg/μl) was expressed by CD4+ T cells when culture was treated with anti-IL-4 compared with that of untreated culture (20 pg/μl). *In vitro* Th2 differentiation of CD4+ T cells didn't occur after treatment with neutralizing anti-IL-4. Nevertheless this treatment didn't lead to the development of Th1 cells.

In vitro activation of primed lymph node T cells

Nine days after subcutaneous injection of tick saliva pulsed DCs into BALB/c mice, we observed *in situ* an increase of the size of axillary and brachial lymph nodes draining the site of inoculation. In contrast the size of lymph nodes of the non-inoculated side didn't change. In secondary stimulation *in vitro*, draining lymph node T cells primed *in vivo* with tick saliva pulsed

DCs showed a higher degree of proliferation when stimulated with 2 to 8 μg/ml of protein saliva (>90 $x10^3$ cpm) than T cells primed *in vivo* with only DCs (< 25 $x10^3$ cpm) (Fig.2). The maximum of proliferation (145 x 10^3 cpm) was obtained when primed T cells were stimulated with 4 μg/ml of protein saliva.

Draining lymph node T cells from mice injected with tick saliva pulsed DCs produced higher level of IL-4 than IFN-γ when stimulated *in vitro* with 4 μg/ml of protein saliva. In the same conditions T cells primed *in vivo* with only DCs produced low amount of IL-4 and IFN-γ (Fig.3). This result indicates that BALB/c mice inoculated with tick saliva pulsed DCs developped a Th2 immune response.

Effect of IL-1β addition during the primary culture on stimulated CD4+ T cells proliferation *in vitro*.

CD4+ T cells primed *in vitro* with tick saliva pulsed DCs in presence or in absence of IL-1β were stimulated *in vitro* with tick saliva pulsed DCs or naive DCs (Fig.4). Results showed that CD4+ T cells primed in presence of IL-1β displayed a higher proliferation when secondary stimulated with tick saliva pulsed DCs than with unpulsed DCs (control). Syngenic mature DCs that didn't bear salivary antigens trigger a lower degree of proliferation of primed CD4+ T cells. CD4+ T cells primed in absence of IL-1β didn't display a significant proliferation when secondary stimulated either with tick saliva pulsed DCs or with unpulsed DCs (control).

Effect of IL-1β addition during the primary or secondary *in vitro* stimulation on stimulated CD4+ T cells proliferation.

CD4+ T cells were primed *in vitro* with tick saliva pulsed DCs in presence or in absence of IL-1β. CD4+ T cells primed in absence of IL-1β didn't proliferate when stimulated *in vitro* by freshly tick saliva pulsed DCs in the presence or absence of IL-1β. While CD4+ T cells primed in presence of IL-1β displayed a same high degree of proliferation whether IL-1β was added or not to the culture medium in the secondary stimulation *in vitro* (Fig.5). Results show that only CD4+ T cells

primed *in vitro* in presence of IL-1β proliferate in secondary stimulation . This finding indicates that the presence of IL-1β is required early during primary stimulation but not during secondary stimulation to generate proliferation of Th2 cells primed *in vitro*.

Discussion

The intracellular tick-borne pathogens elicit a protective Th1 immune response in different mice strains. The intraperitoneal injection of the agent of *human granulocytic ehrlichiosis* bacteria into mice induces the differentiation of IFN-γ secreting Th1 cells (14). IFN-γ secreting CD4+ T cells provide protective immunity to BALB/c mice against challenge infection with *Babesia microti* protozoan (15). *I. scapularis* infected with *Borrelia burgdorferi* induce a Th2 immune response in Lyme disease susceptible C3H/HeJ mice (16). Exogenous delivery of the Th1 cytokines, IFN-γ and IL-2, given at the time of tick feeding, suppresses spirochetes transmission by *I. scapularis* in mice (17). Th1 subset seems to be indispensable for clearing these tick-borne bacteria or protozoan pathogens, in the opposite Th2 subset appears to be detrimental. Christe *et al* (4) showed that BALB/c mice developed a mixed Th1/Th2 response after syringe inoculation of *B. burgdorferi* and a Th2 immune response against the pathogen after tick inoculation. These results bring out the importance of *I. ricinus* tick saliva in the Th2 polarization of the immune response and in the establishment of infections. Pathogen-free Ixodid vectors such as *I. scapularis*, *I. pacificus* (18), or *I. ricinus* skewed the immune response of mice toward Th2 (5). To show the ability of *I. ricinus* tick saliva to influence the differentiation of IL-4 secreting naive CD4+ T cells and the development of Th2 immune response, we developed a PIV system in which tick-saliva-sensitized Th2 cells are derived by coculturing bulk naive CD4+ T cells with tick saliva pulsed DCs. In this PIV approach DCs which are distributed as sentinels throughout the body were used as APCs. These cells are specialized APCs required for the priming and activation of CD4+ T cells (9). They are the most potent APCs in priming *Leishmania major*-specific T cells, followed closely by B cells and finally by macrophages. *L. major*-specific Th1 secretion of cytokines which were generated in PIV system with all APCs was not greatly altered after depletion of DCs. Thus DCs alone do not appear to particularly favor the activation of either specific Th1 or Th2 T cells (19). By the only use of DCs pulsed with tick saliva in coculture with purified CD4+ T cells, we revealed in neutral conditions the importance of tick saliva in the

selective differentiation of Th2 subpopulation. IL-4 promotes differentiation of the TH2 pathway and downregulates IFN-γ production (20). This chain of regulatory events might require a period of 7 days in *in vitro* priming cultures as described by Macatonia et al (21) and by Shankar and Titus (19).

DCs pulsed overnight with tick saliva and injected subcutaneously to BALB/c mice induce also the development of a Th2 immune response. These experiments confirm the importance of the nature of *I. ricinus* saliva molecules which may signal DCs to drive development of tick antigens specific Th2 cells. Among molecules detected in *I. ricinus* saliva prostaglandin E2 impaired the production of IL-12 by maturing DCs (22). Accordingly DCs promote Th2 immune response. A recent study showed that DCs maturing in presence of LPS promote a Th1 response while phosphorylcholine-containing glycoprotein, ES 62, secreted by the filarial nematode *Acanthocheilonema viteae* induces the maturation of DCs with the capacity to induce Th2 response (23).

Another potential factor that mediates naive CD4+ T cells differentiation into Th2 cells in our PIV system may be endogenous IL-4. In many works the initial content of IL-4 in the medium was shown to be a dominant controller of Th2 differentiation. Experimental modification of the initial cytokine environment affects either T helper proliferation or cytokine production. The addition of exogenous IL-4 to *in vitro* culture of naive CD4+ T cells in the presence of mitogen (anti-CD3) (6) or alloantigen (24) directs the naive CD4+ T cells to develop into Th2 effector cells which on restimulation produce high levels of IL-4, IL-5 and IL-10 as well as IL-13 and GM-CSF. In our work we showed for the first time that DCs pulsed overnight with tick saliva were able to trigger *in vitro* the differentiation of syngenic naive CD4+ T cells into Th2 cells without addition of IL-4. These cells expressed higher level of IL-4 than IFN-γ and IL-2 mRNA. The downregulation of IL-2 mRNA expression by IL-4 was more pronounced than that of IFN-γ. Primed CD4+ T cells expressed also IL-10 mRNA which may synergyze with IL-4 to inhibit IL-2 and IFN-γ mRNA

expression. In accordance with our observations IL-10 inhibits the production of IFN-γ by CD4+ T cells stimulated *in vitro* by allogenic DCs (25). It suppressed also the production of IL-2 by CD4+T cells inducing the inhibition of their proliferation (26).

The early treatment of PIV culture with anti-IL-4 provides a useful tool to determine whether endogenous IL-4 was early secreted in sufficient quantity to initiate the anti-tick Th2 polarization of the immune response. Indeed neutralizing anti-IL-4 monoclonal antibodies applied in the culture inhibit the development of IL-4 secreting Th2 cells. Subsequently no more expression of IFN-γ mRNA by CD4+ T cells occurred. In contrast the addition of anti-IL-4 in order to neutralize any endogenous IL-4 during primary culture of T cells from TCR transgenic mice specific for ovalbumine (OVA) with various types of APC and OVA resulted in the appearance of T cells that produced IFN-γ and IL-2 upon stimulation (27). Our experiments suggest that IL-4 dependent signal pathway is indispensable to bias strongly the cytokine secretion pattern toward Th2 phenotype. However in our PIV assay the neutralization of the endogenous IL-4 didn't lead to the development of IFN-γ secreting Th1 cells. The endogenous IL-4 may be secreted by minor population of splenic CD4+ T cells present in the culture such as memory Th2 cells, NK 1.1^+ / NK 1.1^- CD4+ T cells or naive αβ CD4+ T cells themselves. The composition of salivary secretion is complex (5). Memory Th2 cells could cross-react with some salivary antigens and provide IL-4 necessary to trigger IL-4 expression by naive Th cells. NK 1.1^+ and NK 1.1^- CD4+ splenic T cells may also produce enough IL-4 following stimulation by salivary antigens. NK 1.1^+ CD4+ T cells are responsible for the rapid (30 to 90 min) and transient IL-4 production after *in vivo* challenge with anti-CD3 or staphylococcal enterotoxin B (28). NK 1.1^- CD4+ T cells express a high level of IL-4 transcripts 90 min after intravenous injection of *L. major* (29). The early inducing IL-4 may be produced by naive αβ CD4+ T cells themselves. Small percentage of transgenic αβ CD4+ T cells from IL-4Rα$^{-/-}$ mice can acquire IL-4-producing capacity when primed in the absence of IFN-γ and IL-12. This finding demonstrates that there is an IL-4-independent

pathway of IL-4 secretion by conventional αβ CD4+ T cells. The amount of IL-4 secreted by these cells may be sufficient to cause a certain degree of polarization (30). This endogenous IL-4 could be sufficient to initiate the differentiation of naive CD4+ T cells into Th2.

Tick saliva specific Th2 differentiated *in vitro* didn't display any proliferation neither in a primary stimulation nor in a secondary *in vitro* stimulation with tick saliva pulsed DCs, but strongly proliferate when IL-1 was added as costimulator in the PIV culture (Fig.4). In accordance with our finding, Chang *et al* (31) showed that the optimal proliferation of Th2 clones in response to IL-4 required the addition of IL-1. In contrast Th1 clones secrete IL-2 and proliferate without any requirement for IL-1. Exogenous IL-1 is needed because IL-4 secreted by Th2 cells inhibits the transcription and secretion of IL-1 (32). In a TCR transgenic T cell *in vitro* priming system, neither IL-1 receptor antagonist (IL-1 Ra) nor antibodies to IL-1 diminished priming for IL-4 production mediated by antigen, APC and IL-4 (27; 33). If IL-1β is not required for the differentiation of murine CD4+ T cells into Th2 clones, it is for the proliferation of some Th2 clones in response to IL-4. Proliferative responses and lymphokine production by Th2 cells have distinct signaling requirements. Moreover we showed that IL-1β is not required in secondary stimulation of differentiated Th2 cells neither when CD4+ T cells were primed in presence of IL-1β nor in its absence (Fig.5). This suggested that proliferation of differentiated Th2 cells require an early IL-1β-dependent signaling pathway during primary *in vitro* culture only.

Our PIV system is useful to analyze further the salivary antigens and the cells implicated in the anti-tick Th2 response. Neutralization of Th2 triggering factors could invert the response and confer resistance against tick and tick borne pathogens.

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Fig. 1a) Determination by RT-PCR of the relative levels of IL-4, IL-10, IFN-γ and IL-2 mRNA expressed by CD4+ T cells primed *in vitro* in neutral condition. **b)** Determination by RT-PCR of the relative levels of IL-4, IL-10 and IFN-γ mRNA expressed by CD4+ T cells primed *in vitro* in presence of anti-IL-4.

Ethidium bromide agarose stained gels of representative PCR reactions using IL-4,IL-10, IFN-γ and IL-2 specific primers in the presence of indicated serial dilutions of a competitor, for IL-4, IL-10, IFN-γ and IL-2 mRNA. Two μl of two-fold diluted cDNA are used as template. The point of equivalence in intensity between the competitor fragment and the cDNA indicates the relative concentration of IL-4, IL-10, IFN-γ and IL-2 mRNA (see arrows) in CD4+ T cells primed *in vitro* without or in presence of anti-IL-4.

CF = competitor fragment

Fig. 2

Proliferation of draining lymph node T cells primed *in vivo* with *I. ricinus* saliva pulsed DCs, or DCs alone.

Nine days after subcutaneous injection of tick saliva pulsed DCs, T cells from axillary and brachial draining lymph nodes were stimulated *in vitro* with increasing protein concentrations of saliva. Each value represents the mean of triplicate wells ± SD from a single experiment representative of three separate experiments.

Fig. 3

Measure of IL-4 (black bars) and IFN-γ (white bars) by specific ELISA.

Nine days after subcutaneous injection of tick saliva pulsed DCs, or DCs alone, T cells from draining lymph nodes were stimulated *in vitro* with 4 μg/ml of protein saliva. Results show the mean values recorded for triplicate wells ± SD.

Fig. 4 Effect of the addition of IL-1β during PIV stimulation on the proliferation of primed splenic CD4+ T cells following secondary stimulation with *I. ricinus* saliva pulsed or unpulsed DCs.

Splenic CD4+ T cells cultivated with ticks saliva pulsed DCs in presence or in absence of IL-1β were restimulated with *I. ricinus* saliva pulsed DCs or DCs alone. 24h before harvesting cells, 1 μCi/well of methyl [^{3}H] thymidine was added. Methyl tritiated thymidine incorporation was determined by liquid scintillation counting. Each value represents the mean of triplicate wells ± SD from a single experiment representative of three separate experiments.

$p_{IL\text{-}1\beta}$ CD4+ T cells and p CD4+ T cells = CD4+ T cells primed in presence or in absence of IL-1β respectively.

Fig. 5 Comparison between the effect of the addition of IL-1β during primary and secondary *in vitro* stimulation on restimulated splenic CD4+T cells proliferation.

Splenic CD4+ T cells primed *in vitro* with ticks saliva pulsed DCs in presence or absence of IL-1β were restimulated in the same condition. 24h before harvesting cells, 1 μCi/well of methyl [^{3}H] thymidine was added. Methyl tritiated thymidine incorporation was determined by liquid scintillation counting. Each value represents the mean of triplicate wells ± SD from a single experiment representative of three separate experiments.

$p_{IL\text{-}1\beta}$ CD4+ T cells and p CD4+ T cells = CD4+ T cells primed in presence or in absence of IL-1β, respectively.

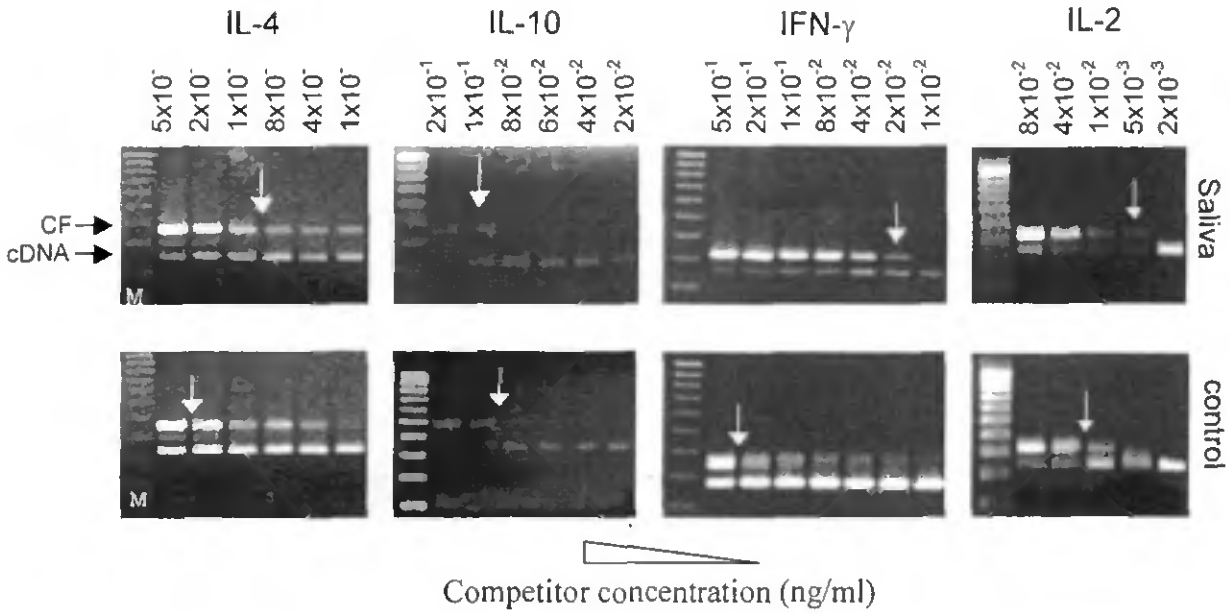
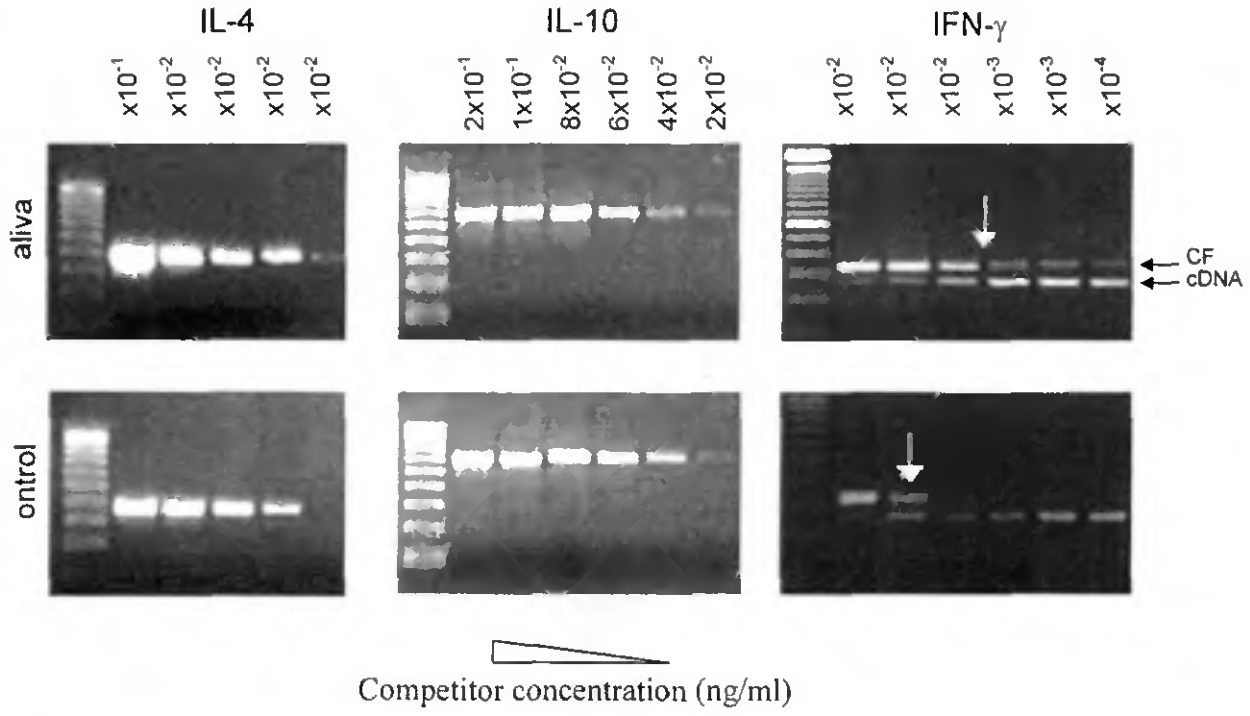

IL-4
IL-10
IFN-γ
IL-2
5x10⁻
2x10⁻
1x10⁻
8x10⁻
4x10⁻
1x10⁻
2x10⁻¹
1x10⁻¹
8x10⁻²
6x10⁻²
4x10⁻²
2x10⁻²
5x10⁻¹
2x10⁻¹
1x10⁻¹
8x10⁻²
4x10⁻²
2x10⁻²
1x10⁻²
8x10⁻²
4x10⁻²
1x10⁻²
5x10⁻³
2x10⁻³
CF
cDNA
M
Saliva
control
Competitor concentration (ng/ml)

Fig.1a


IL-4
IL-10
IFN-γ
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x10-2
x10-2
x10-2
x10-2
2x10-1
1x10-1
8x10-2
6x10-2
4x10-2
2x10-2
x10-2
x10-2
x10-2
x10-3
x10-3
x10-4
aliva
ontrol
CF
cDNA
Competitor concentration (ng/ml)

Fig.1b

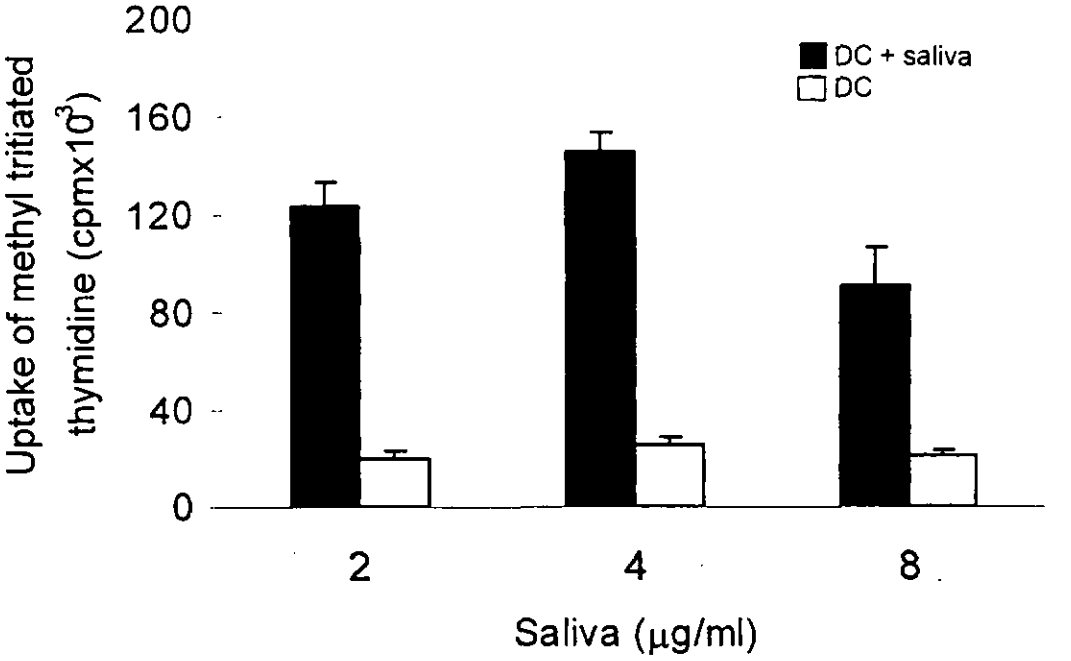
200
160
120
80
40
0
Uptake of methyl tritiated thymidine (cpmx10^3)
DC + saliva
DC
2
4
8
Saliva (μg/ml)

Fig.2

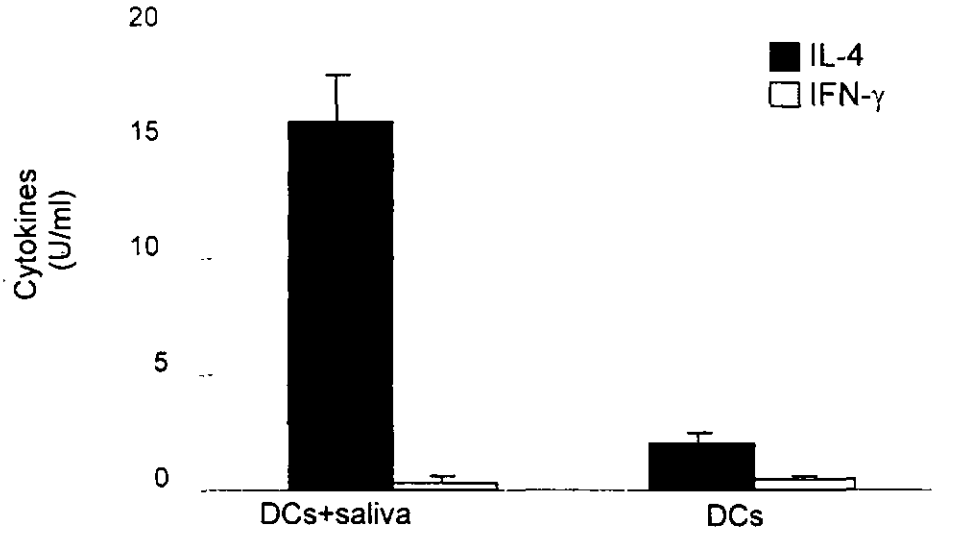

Fig.3

Primary conditions	Secondary conditions
(DCs+saliva)+CD4+T+IL-1β	(DCs+saliva)+$p_{IL\text{-}1\beta}$CD4+T
	DCs+$p_{IL\text{-}1\beta}$CD4+T
(DCs+saliva)+CD4+T	(DCs+saliva)+pCD4+T
	DCs+pCD4+T

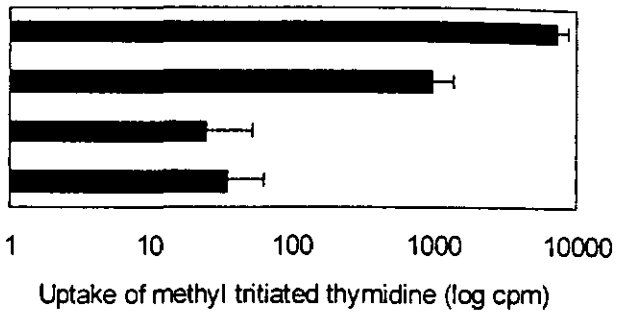

Fig.4

Primary conditions	Secondary conditions
(DCs+saliva)+CD4+T+IL-1β	(DCs+saliva)+$p_{IL-1\beta}$CD4+T+IL-1β
	(DCs+ saliva)+$p_{IL-1\beta}$CD4+T
(DCs+saliva)+CD4+T	(DCs+saliva)+pCD4+T+IL-1β
	(DCs+saliva)+pCD4+T

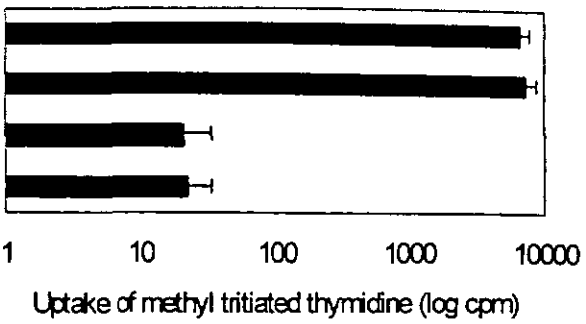

Fig.5