

# 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- $\gamma$  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- $\gamma$  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- $\gamma$  mRNA expression was detected. Draining lymph node cells of infested BALB/c produced higher level of IL-4 than IFN- $\gamma$  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- $\gamma$ , Th2*

## INTRODUCTION

In recent years, several studies have shown that helper T cell clones of CD4<sup>+</sup> 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- $\gamma$  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- $\gamma$  reciprocally regulate each other, with IFN- $\gamma$  inhibiting Th2 lymphocytes, IL-4 and IL-10 downregulating Th1 cells (6). Priming of naive CD4<sup>+</sup> 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- $\gamma$  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- $\gamma$  mRNA in draining lymph node cells using a competitive quantitative reverse transcription-polymerase

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- $\gamma$  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  $5 \times 10^5$  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  $\mu$ l sterile distilled water (RNAase free). Two  $\mu$ l containing 0.1–0.5  $\mu$ g 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- $\gamma$  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- $\gamma$ : 5'-CATTGAAAGCCTAGAAAAGTCTG-3' (sense) and 5'-CTCATGAATGCATCCTTTTTTCG-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- $\gamma$  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- $\gamma$ ) to the relative concentration of HPRT was then calculated. Results were expressed as the fold of increase in IL-4 or IFN- $\gamma$  mRNA expression in mice infested with nymphal *I. ricinus* ticks compared to non-infested mice. The formula: IL-4 or IFN- $\gamma$   $t_x$ /HPRT  $t_x$ : IL-4 or IFN- $\gamma$   $t_0$ /HPRT  $t_0$ , which was used to calculate the fold of increase in IL-4 or IFN- $\gamma$  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 ImmunoStar 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<sup>6</sup> 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<sub>2</sub>, with or without 40 µl/well of dialysed and filtrated chromatographic fractions of SGE, 1 µCi/well of methyl [<sup>3</sup>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<sup>6</sup> 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  $\mu$ l/well) at 37°C in saturated atmosphere with 5% CO<sub>2</sub>. The volume applied for each fraction is kept constant at 40  $\mu$ l/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- $\gamma$  determination. ELISA cytokine tests were performed as previously reported (19). Dilutions of rIL-4 (12.5–400 U/ml) or rIFN- $\gamma$  (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- $\gamma$ mRNA during a primary infestation**

BALB/c were infested with 40 larvae, 15 nymphs or 1 pair of *I. ricinus* adults. A suspension of  $5 \times 10^5$  cells prepared from the brachial and axillary draining lymph nodes was used for RNA extraction. IL-4 and IFN- $\gamma$  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- $\gamma$  mRNA concentrations were, respectively, IL-4 mRNA (75 pg/ $\mu$ l) > IFN- $\gamma$  mRNA (6 pg/ $\mu$ l), IL-4 mRNA (100 pg/ $\mu$ l) > IFN- $\gamma$  mRNA (4 pg/ $\mu$ l) and IL-4 mRNA (100 pg/ $\mu$ l) > IFN- $\gamma$  mRNA (4 pg/ $\mu$ l). In all cases, we observed a higher dose of IL-4 mRNA expression compared to IFN- $\gamma$  mRNA. The concentrations of IL-4 and IFN- $\gamma$  mRNA in the lymph node cells of non-infested mice were equally low.

### **Kinetic of IL-4 and IFN- $\gamma$ 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- $\gamma$  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- $\gamma$  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- $\gamma$ 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- $\gamma$  in the supernatant of the same cell culture. Cells incubated without any antigen did not produce IL-4 or IFN- $\gamma$ .

### **SDS-PAGE analysis of SGE, saliva and chromatographic fractions**

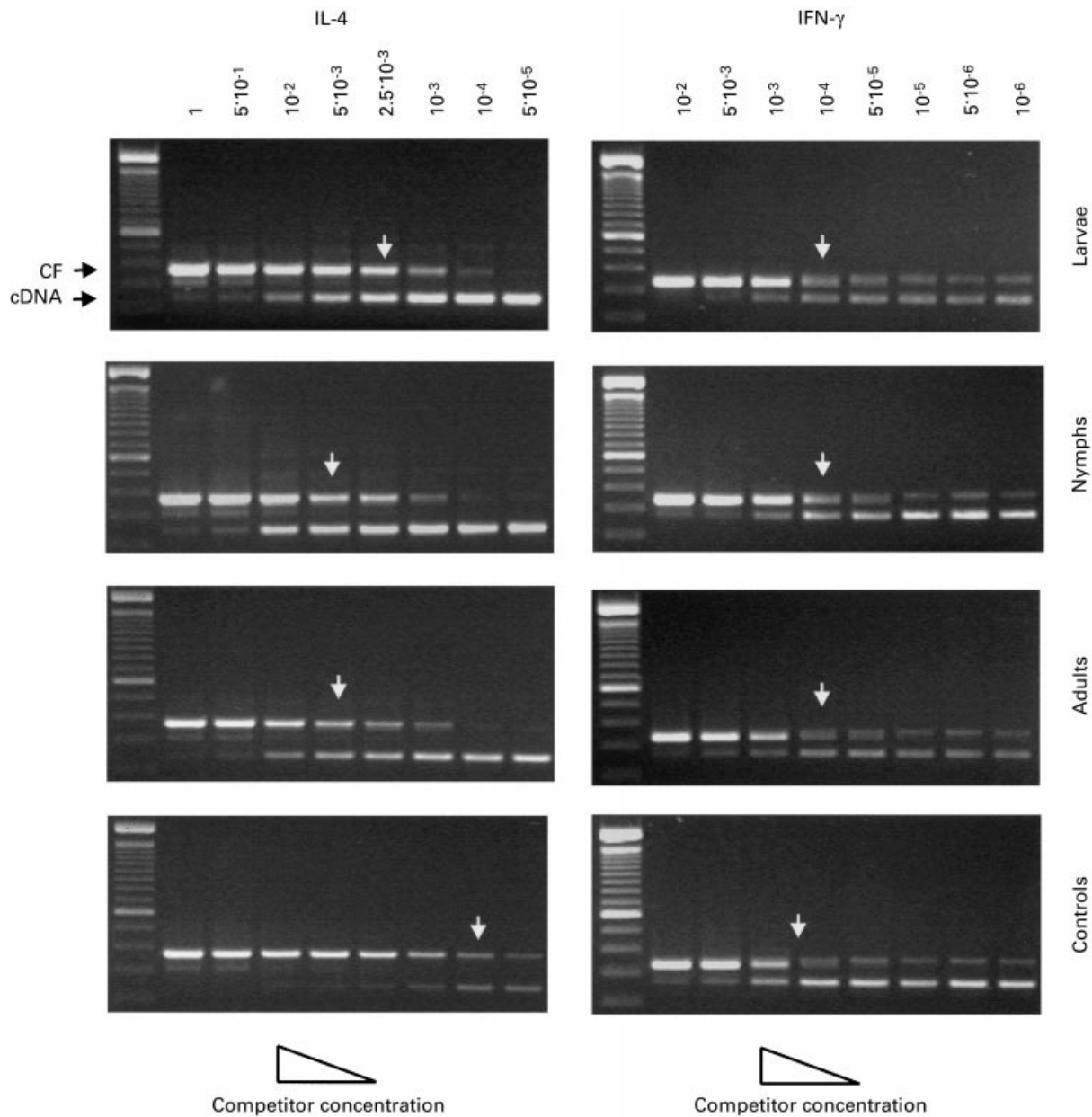
SGE, saliva or the five chromatographic fractions used in antigen-specific T cell proliferation and cytokines detection show different protein patterns (Figure 5). The pattern of SGE (lane 1) is more complex than that of saliva (lane 2) and of each chromatographic fraction (lanes 3–7). Several proteins 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

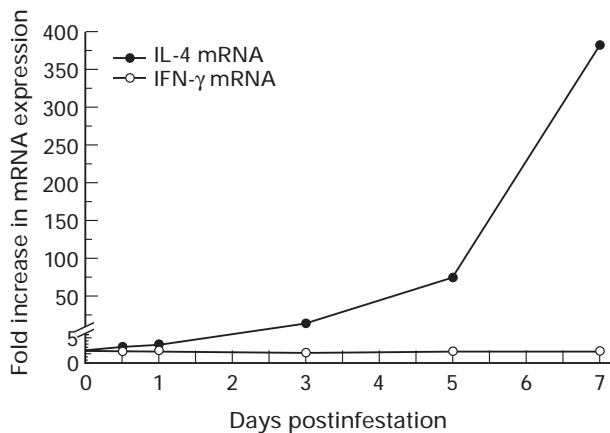
It has been reported that many factors are implicated in the regulation of Th1 and Th2 subsets in mice such as the



**Figure 1** Determination by RT-PCR of the relative levels of IL-4 and IFN- $\gamma$  mRNA. Ethidium bromide agarose stained gels of representative PCR reactions using IL-4 and IFN- $\gamma$  specific primers in the presence of serial eight-fold dilutions of a competitor, 1 ng to  $5 \times 10^{-5}$  ng/ $\mu$ l for IL-4 and  $10^{-2}$ – $10^{-6}$  ng/ $\mu$ l for IFN- $\gamma$  determination. Two  $\mu$ l 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- $\gamma$  mRNA (see arrows) in draining lymph node cells of BALB/c after a primary infestation with, respectively, larvae (75 pg/ $\mu$ l for IL-4 mRNA, 6 pg/ $\mu$ l for IFN- $\gamma$  mRNA), nymphs (100 pg/ $\mu$ l, 4 pg/ $\mu$ l) or adult ticks (100 pg/ $\mu$ l, 4 pg/ $\mu$ l). As a control, levels of IL-4 and IFN- $\gamma$  mRNA determined in lymph node cells from non-infested mice are quite equal (4 pg/ $\mu$ l).

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

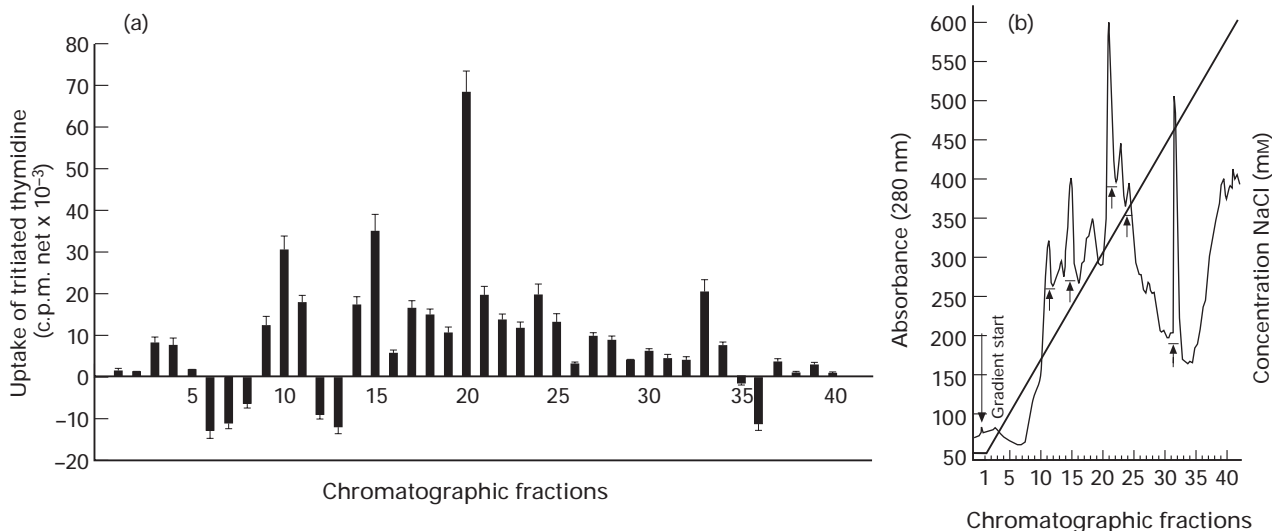
(H-2d) is consistent with CD4<sup>+</sup> Th2 type response whereas the resistance of C57BL/6 (H-2b) results in the development of a polarized CD4<sup>+</sup> Th1 response. In contrast to *Leishmania* models, mice with different haplotypes such as BALB/c or DBA (H-2d), C57/BL/6 (H-2b), C3H or CBA



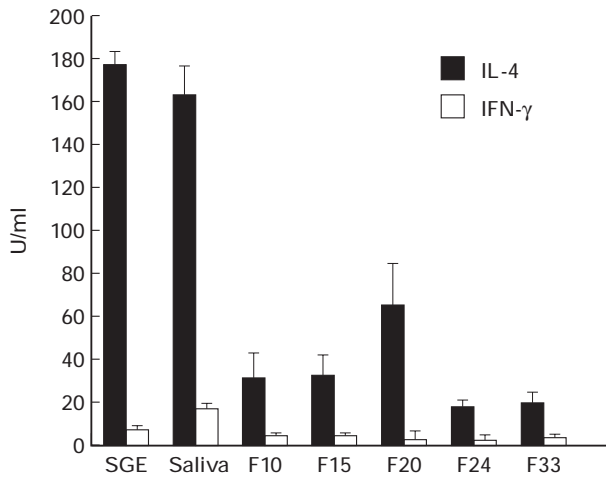
**Figure 2** Kinetics of IL-4 and IFN- $\gamma$  mRNA expression in draining lymph node cells during nymphal tick infestation. The curves represent the fold increase of IL-4 (full circle) or IFN- $\gamma$  (empty circle) mRNA expression in infested mice compared to non-infested ones.

(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).

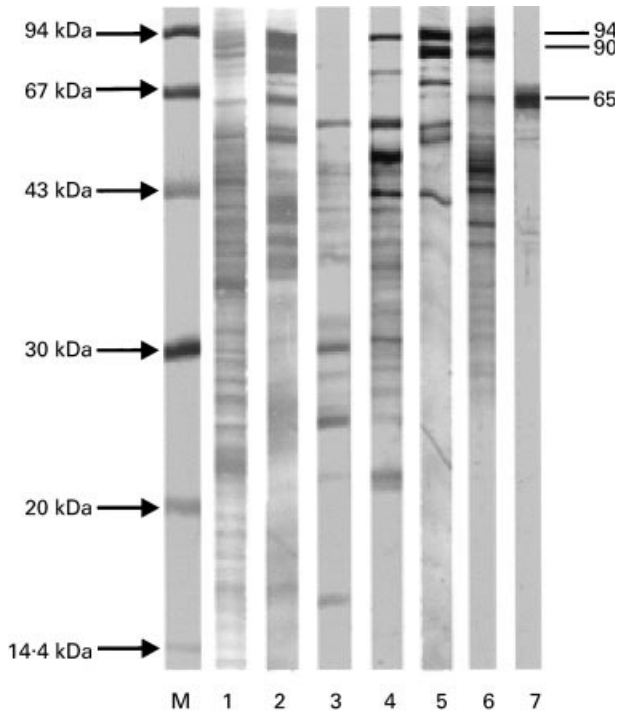
In our work, we have observed that adult *I. ricinus*, as well as larval or nymphal ticks, polarize the immune response of BALB/c toward Th2. Nine days after the beginning of infestation with the different tick instars, high IL-4 mRNA expression was detected in draining lymph node cells. At that time, 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- $\gamma$  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- $\gamma$  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).



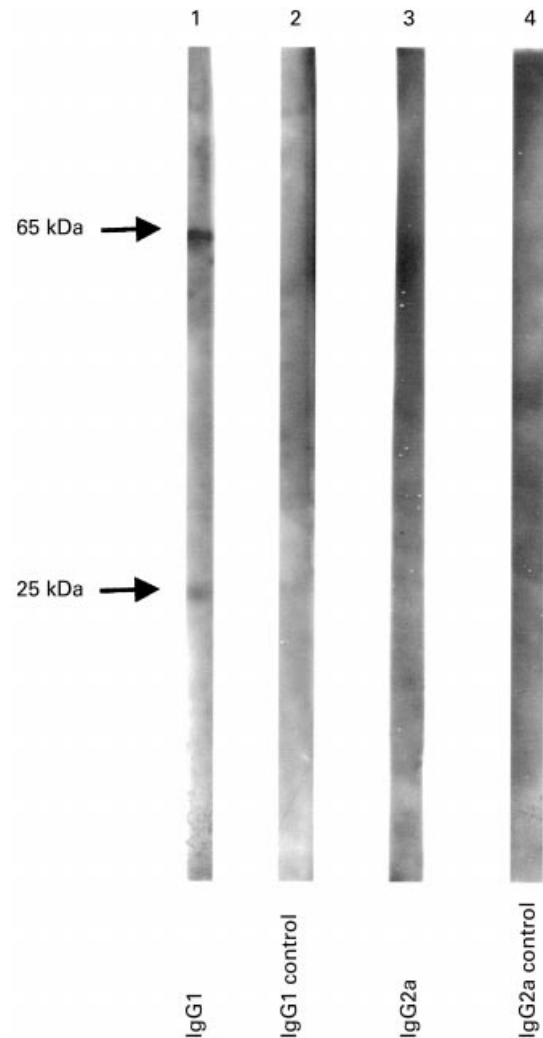
**Figure 3** (a) A wide range of chromatographic fractions of salivary gland extract (SGE) induced *in vitro* lymphocyte proliferation of axillary and brachial draining lymph nodes removed from BALB/c mice infested with nymphal ticks. Results show the mean ( $n = 3$ ) of stimulated wells  $\pm$  SD. 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- $\gamma$  (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  $\pm$  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- $\gamma$  production by activated naive T cells directs their development toward the Th1 phenotype (27). PGE<sub>2</sub> is also an important molecule in the regulation of the immune response (28). With a colourimetric assay, PGE<sub>2</sub> has been detected in the saliva of 5 days engorged *I. ricinus* female ticks (0.5  $\mu$ g of PGE<sub>2</sub>/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<sub>2</sub> 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<sup>+</sup> 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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