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Identification of an *Ixodes ricinus* salivary gland fraction through its ability to stimulate CD4 T cells present in BALB/c mice lymph nodes draining the tick fixation site

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SUMMARY

BALB/c mice infested with larvae or nymphs of *Ixodes ricinus* develop in their lymph nodes a T cell-specific immune response triggered by salivary gland soluble antigens (SGA). SGA are apparently conserved in the 3 biological stages of *I. ricinus* ticks and are species specific. SGA derived from partially fed females *I. ricinus* stimulate lymph node T cells from mice infested with *I. ricinus* larvae or nymphs. In contrast, lymph node cells from mice infested with *Amblyomma hebraeum* nymphs do not respond. A chromatographic fraction enriched with a 65 kDa protein (IrSG65) isolated from salivary glands of *I. ricinus* partially fed females induces *in vitro* a specific T cell proliferation of lymph node cells from mice infested with *I. ricinus* nymphs. The depletion of $CD4^+$ T cells drastically reduces the ability of lymphocytes from infested mice to proliferate after IrSG65 stimulation.

Key words: tick, *Ixodes ricinus*, salivary gland, Th cells, immunogen.

INTRODUCTION

During tick feeding, saliva or cement components deposited in the host skin have 2 main biological functions; tick fixation and tissue digestion (enzymes) and modulation of the host immune response against tick feeding through prostaglandin secretion, anti-complement and anti-inflammatory activities (Kaufman, 1989). Some of these molecules are responsible for the host specific immune response. Langerhans cells are essential for the induction of an anti-tick immune response in guinea-pigs (Allen, Khalil & Wikel, 1979). Ultraviolet B radiation alters this type of cell (Simon *et al*. 1991) and consequently abolishes the acquisition of resistance in pluri-infested hosts (Nithiuthai & Allen, 1984). Langerhans cells initiate the recruitment process and proliferation of tick immunogen-specific T cells in the paracortical lymph node area (Nithiuthai & Allen, 1985). After antigen processing, immunogen epitopes are presented to $CD4^+$ T cells associated with MHC class II molecules (Puri & Lonai, 1980).

Anti-tick specific immune responses are generally detected by immunoblotting using tick pluri-infested animal sera (Rutti & Brossard, 1989) or by *in vitro* stimulation of host immune cells using tick antigenic extracts (Schorderet & Brossard, 1993). The specific immune response can also be detected using cutaneous assays (Mbow *et al*. 1993). Salivary gland soluble antigens obtained at different stages of *Dermacentor andersoni* development showed variable antigenic components (Gordon & Allen, 1987). *Ixodes ricinus* pluri-infested rabbits and some pluri-infested mice produce IgG antibodies reacting with tick integumental protein (Rutti & Brossard, 1989). We have previously reported that salivary gland but not integumental antigens may induce T cell proliferation *in vitro* after spleen accessory cell antigen processing (Ganapamo, Rutti & Brossard, 1995*a*). In the present work we report on the isolation, for the first time, of a salivary gland immunogen from adult *I. ricinus* tick capable of inducing a T helper cell-specific and dose-dependent proliferation in cells from draining lymph nodes from tick-infested mice. Possible roles of this immunogen and its influence on the transmission of infectious agents are discussed.

MATERIALS AND METHODS

Animals and infestations

Eight to 12-week-old female BALB/c mice were purchased from IFFA CREDO (Arbresle, France). Mice were infested with 15 nymphal ticks for both *I. ricinus* and *Amblyomma hebraeum* or 30 larvae each for *I. ricinus* (Mbow *et al*. 1994). *I. ricinus* ticks are reared in our laboratory. *A. hebraeum* nymphs were a kind gift of Ciba-Geigy, St Aubin, Switzerland.

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Tick salivary gland and integumental extracts

Tegument and salivary glands were dissected out from partially fed female ticks. Antigenic extracts were prepared following the procedures previously described (Rutti & Brossard, 1989). In short, female and male *I. ricinus* adults were partially fed for 5 days on rabbit ears. Integumental antigens were extracted with a Polytron (Kinematica) grinder in ice-cold extraction buffer consisting of 50 mM phosphate-buffered saline (PBS) at pH 7·4, 5 mM phenylmethyl-sulfonyl fluoride (PMSF) and 10 mM ethylene diaminetetraacetic acid (EDTA). SGA were obtained by homogenization of dissected female tick salivary glands in the same extraction buffer. These extracts were centrifuged at 16000 ***g*** for 30 min at 4 °C. Supernatants were dialysed overnight in 25 mM PBS pH 7·4. Extracts were again centrifuged under the same conditions and the protein concentration was determined by the Coomassie blue method (Bradford, 1976). Supernatants were sterilized using a 0·2 μm Millipore filter and stored at −20 °C until use.

Fractionation of salivary gland soluble proteins

Soluble proteins extracted from 80 pairs of salivary gland were desalted on a Fast Desalting Column HR 10/10 (Pharmacia) using 10 mM Tris-buffer, pH 7·5, 50 mM NaCl as eluent. The peak containing proteins was then applied to an anion-exchange MonoQ HR 5/5 column. Bound proteins were eluted with a 0·2, 0·3, 0·4, 0·5 M NaCl step gradient of increasing concentrations. Proteins of each fraction were separated on SDS–PAGE (12 %), transferred onto nitrocellulose and stained with colloidal gold. The 65 kDa protein-containing fractions were pooled and stored at −20 °C.

Depletion of CD4$^+$ T lymphocytes of the lymph node cell population

Depletion of CD4$^+$ T cells was carried out as previously described (Ganapamo, Rutti & Brossard, 1996). A single-cell suspension of axillary and brachial lymph nodes was obtained from mice infested with nymphal ticks and killed 9 days after the infestation. Lymph node cells were incubated for 30 min at 4 °C with 0·25 μg/10^6 cells of mAb (IgG2a) rat anti-mouse CD4 receptor (Pharmingen, AMS, Lugano, Switzerland). Antibodies were diluted in phosphate-buffered saline, pH 7·4 (0·15 M NaCl, 0·01 M Na-phosphate) and supplied with 1 % foetal bovine serum (FBS). After 3 successive washes with HBSS, cells were incubated with Dynabeads M-450 incorporating sheep anti-rat IgG (Dynal, Milan Analytica, La Roche, Switzerland) diluted in PBS–1 % FBS (ratio 40:1) for 45 min at 4 °C. CD4$^+$ T lymphocytes were then depleted using a Magnetic Particle Concentrator MPC-1 (Dynal, Milan Analytica, La Roche, Switzerland). The remaining suspended cells were removed and used as the CD4$^+$ cell depleted single-cell suspension.

Assay of lymph node cell proliferation

The experimental procedures for T cell proliferation assays were as described previously (Ganapamo *et al.* 1996; Ganapamo, Rutti & Brossard, 1995*b*). To study the kinetics of antigen stimulation, mice were killed 9 days after infestation. Axillary and brachial lymph nodes were removed and a cell suspension prepared. Then 4×10^5 lymph node cells/well in 96-well flat-bottom plates (Falcon), were cultivated in complete culture medium [RPMI 1640 (Gibco), supplemented with 10 % FBS (v/v), 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids (Sigma), 0·05 mM mercaptoethanol, 100 i.u./ml pen/strep (Gibco), 0·25 μg/ml fungizone (Gibco)] at 37 °C.

For the IrSG65 T cell proliferation assays, 4×10^5 lymph node cells/well were cultivated for 96 h with or without 2 μg/well of purified IrSG65 in 100 μl of complete culture medium. Finally, 0·1 μCi/well (37·0 MBq/ml) of tritiated thymidine (Amersham Int. Amersham, UK) were added 18–24 h before harvesting the cells. The incorporation of tritiated thymidine was determined by liquid scintillation counting. Results show the mean of quadruplicate IrSG65-stimulated wells and error bars indicate the variation of individual values from the mean. Means of quadruplicate unstimulated wells were previously subtracted (cpm net).

RESULTS

Tick antigen stimulation of lymph node cells in vitro *from mice infested with ticks*

Nine days after tick infestation, lymph node cells recovered from *I. ricinus*-infested mice proliferated in the presence of SGA (Fig. 1B) and gradually incorporated tritiated thymidine from 24 h to the optimal level at 96 h. Salivary gland immunogenicity was specific to *I. ricinus* tick species and was conserved during all stages of development (Fig. 1B). Lymph node cells from mice infested with *A. hebraeum* nymphs do not proliferate when stimulated with *I. ricinus* salivary gland extracts. In contrast, lymph node cells from mice infested with nymphal or larval ticks do not respond *in vitro* to integumental antigen (Fig. 1A).

Purification of a tick salivary gland antigen (IrSG65) by anion-exchange chromatography

SGA from partially fed (5 days) females of *I. ricinus* analysed by SDS–PAGE show a complex pattern of proteins (Fig. 2A). As indicated by anion-exchange chromatography most of them are unbound (Fig.

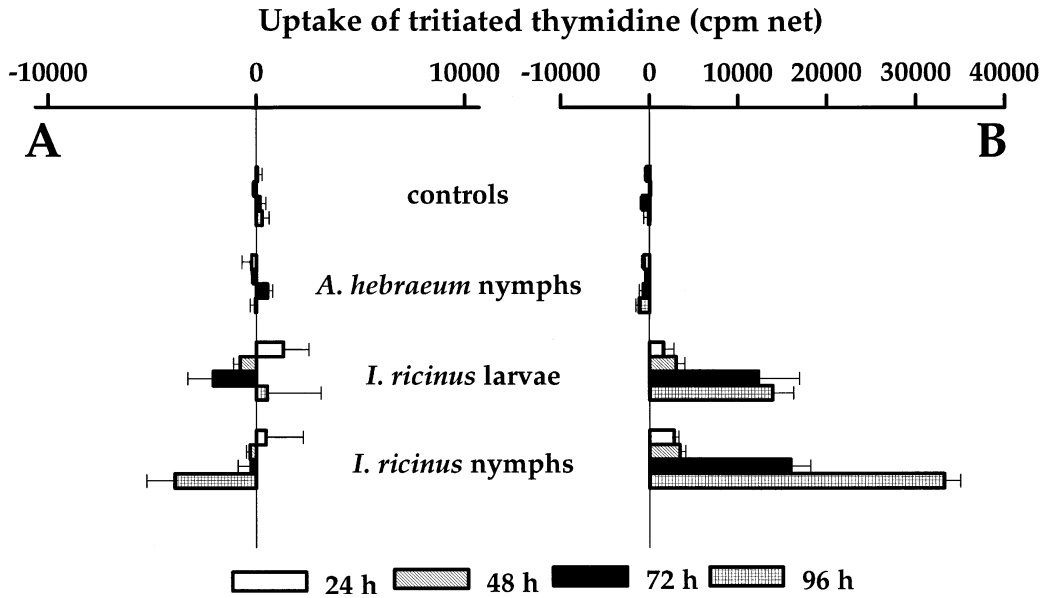

Fig. 1. Kinetics of the response of lymph node cells to salivary gland antigens (B), and integumental antigens from *Ixodes ricinus* tick (A). Results show the mean ($n = 4$) of antigen (2 μg/well) stimulated wells containing 4×10^5 cells/well of lymph node cells from mice infested with larvae and nymphs *I. ricinus* and nymphs *Amblyomma hebraeum*. Error bars indicate the variation of individual values from the mean. Means of quadruplicate unstimulated wells were previously subtracted (cpm net).

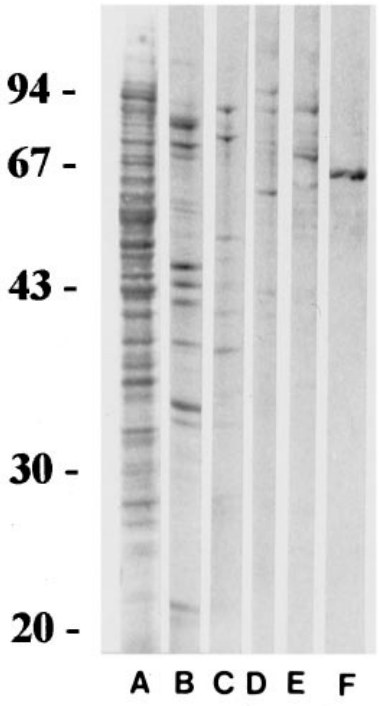

Fig. 2. Fractionation of salivary gland soluble proteins. Salivary gland soluble proteins from partially fed female *Ixodes ricinus* (A), unbound proteins on anion-exchange Mono Q column (B) or proteins eluted with 0·2, 0·3, 0·4 or 0·5 M NaCl (C, D, E, F, respectively) were separated on SDS–PAGE 12%, transferred onto nitrocellulose and stained with colloidal gold. Molecular weight markers (M_r) were: phosphorylase b (94 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa).

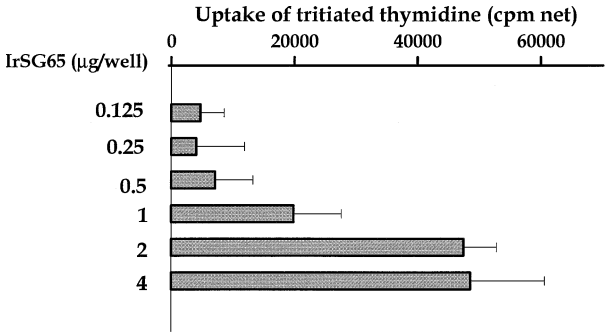

Fig. 3. Antigen dose-dependent response of lymph node cells from infested mice to *in vitro* stimulation by IrSG65. Cells were removed 9 days after infestation. Results show the mean ($n = 4$) of IrSG65 stimulated wells (4×10^5 cells/well). Error bars indicate the variation of individual values from the mean. Means of quadruplicate unstimulated wells are previously subtracted (cpm net).

2B) or eluted with 0·2, 0·3, 0·4 M sodium chloride (Fig. 2C–E). One fraction enriched with a 65 kDa protein bound more tightly to the column. This protein which eluted with 0·5 M NaCl (Fig. 2F) reacted with sera from rabbits repeatedly infested with *I. ricinus* adults (Rutti *et al.* unpublished observations).

Regionalization and CD4⁺-specific proliferation of lymph node cell response to IrSG65 in vitro *stimulation*

The antigen concentration effect on the lymph node cell responses after IrSG65 stimulation *in vitro* is shown in Fig. 3. Purified IrSG65 induced a T cell

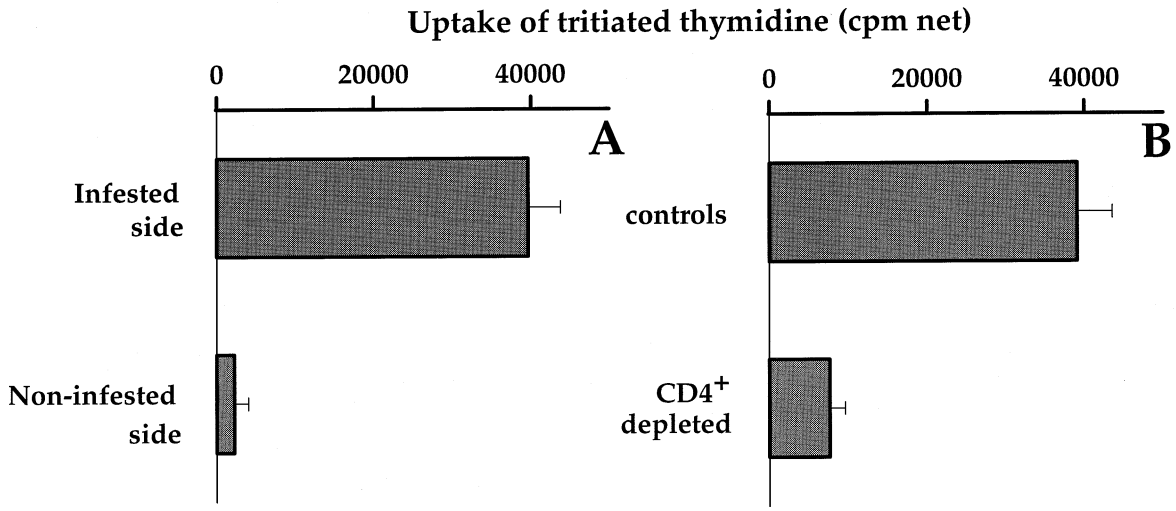

Fig. 4. Regionalization of IrSG65-specific lymphocytes and contribution of $CD4^+$ T cells to the *in vitro* response of lymph node cells from infested mice to IrSG65 stimulation. Infested and non-infested lateral lymph node cells from tick-infested mice were removed and pooled 9 days after the first infestation. (A) Regionalization of IrSG65-specific lymphocytes. (B) Contribution of $CD4^+$ lymphocytes to lymph node cell response to IrSG65. Treatment of results is the same as in Fig. 3.

antigen dose-dependent proliferation. The optimal stimulation was obtained with 2–4 μg/well of IrSG65. IrSG65 *in vitro* induces proliferation of axillary and brachial lymph node T cells which drain the tick fixation site. Lymph node cells localized opposite to the tick attachment site did not proliferate (Fig. 4A). Depletion of $CD4^+$ T cells drastically reduced the ability of lymph node cells from infested mice to proliferate after IrSG65 stimulation *in vitro*. The remaining cell proliferation may have been due to undepleted $CD4^+$ T cells or to other cell types such as B and T (CD8, CD4–$CD8^-$, $\gamma\delta$) lymphocytes (Fig. 4B).

DISCUSSION

The induction of anti-tick specific immune responses is influenced by tick saliva components and there is a Th2 polarized cytokine pattern in BALB/c mice infested with *I. ricinus* nymphs (Ganapamo *et al.* 1996, 1995*b*). Prostaglandin E_2 (PGE_2), in salivary glands of certain tick species (Inokuma, Kemp & Willadsen, 1994), may selectively orientate the immune response towards a Th2 polarized cytokine profile (Betz & Fox, 1991). The aqueous nature of tick saliva could also facilitate this response (Burstein, Shea & Abbas, 1992). The molecular events involved in the generation of an anti-tick specific immune response remain poorly understood. Tick salivary gland immunogens are processed in pH-sensitive antigen-presenting cell compartments as shown by *in vitro* inhibition with chloroquine (Ganapamo *et al.* 1995*a*) and transported to the plasma membrane as MHC-tick epitope complexes for T cell presentation (Peters *et al.* 1991). Only salivary gland and not integumental antigens can be processed and presented by mice spleen accessory cells in an *in vitro* assay (Ganapamo *et al.* 1995*a*). The different amplitude of cell responses to SGA in mice infested with nymphs or with *I. ricinus* larvae may be due to the quantity of saliva injected during the infestation. In fact, it has been reported that heavy tick burdens induce a higher specific immune response than smaller tick burdens in rabbits infested with *I. ricinus* adults (Schorderet & Brossard, 1993).

In the present study, we have demonstrated *in vitro* for the first time that $CD4^+$ T cells from tick-infested mice can be induced to proliferate by a chromatographic fraction highly enriched in a 65 kDA SGA protein (IrSG65). IrSG65 from adult *I. ricinus* salivary glands allows $CD4^+$ specific cell induction in mice infested with larvae and nymphs of this tick species. This observation seems to suggest the conservation of a common immunogenic structure between the three tick instars.

The ability of arthropod vector to transmit pathogens may be firstly due to non-specific molecules with potent pharmacological and immunomodulating activities such as PGE_2 (Bissonnette, Rossignol & Befus, 1993). Secondly, the type of anti-ectoparasite immune response (Th1 or Th2) could potentiate the transmission of some pathogen species (Titus & Ribeiro, 1990). The specific immune response induced by the vector is essential to assure pathogen transmission and is strictly linked to the specificity of the pathogens (Titus & Ribeiro, 1988). Accordingly, co-injection of *Leishmania braziliensis braziliensis* promastigotes with sandfly salivary gland extracts enhance the skin lesion in BALB/c mice, while *Ixodes dammini* salivary gland extracts do not influence it (Samuelson *et al.* 1991). Furthermore, a salivary gland protein from *Rhipicephalus appendi-*

culatus adult tick increases the transmission of Thogoto (THO) virus to guinea-pigs (Jones & Nuttall, 1989).

In our model, draining lymph node cells produce high levels of Il-4 and low levels of IFN-γ, suggesting a Th2 polarization of cytokine pattern (Ganapamo *et al*. 1995*b*). IL-4 and IL-10 have been shown to strongly down-regulate the Th1 cell mechanisms responsible of intracellular micro-organism destruction (Fiorentino *et al*. 1991; Seder & Paul, 1994). *Babesia* and *Theileria* species are intracellular protozoa transmitted by ticks. Type 1 $CD4^+$ T cell responses are required in the induction of protective immunity against *Babesia bovis* and *Babesia bigemina* (Brown & Rice-Ficht, 1994; Brown *et al*. 1996; Rodriguez *et al*. 1996). Th1 cytokines activate $CD8^+$ cytotoxic T cells, which are necessary to confer protection against *Theileria parva* (McKeever *et al*. 1994). Th2 immune responses induced during helminth infection in mice down-regulate Th1 cytokine production and virus-specific $CD8^+$ cytotoxic T cells that are important in microorganism destruction (Le Gros & Erard, 1994). The initiation of this Th2 polarized cytokine pattern must occur before the tick transmission of pathogens (Jones & Nuttall, 1989; Reiner, 1994). In our model, the Th2 cytokine polarization is already established 2 days after the beginning of the infestation (Ganapamo *et al*. unpublished observations).

As previously proposed by other authors tick-borne viruses are able to adapt and to exploit the saliva-induced changes that occur at the skin site of tick feeding (Jones, Kaufman & Nuttall, 1992; Nuttall *et al*. 1994). The immune mechanism and especially the contribution of the different immune cell types involved in this phenomenon are not known. Recent observations show that early during primary infestation $\gamma\delta$ T cells may contribute to the development of $\alpha\beta$ T helper subsets (Ferrick *et al*. 1995). Rapid expansion of human $\gamma\delta$ T cells after *in vitro* activation by mycobacterial components as well as the early appearance of $\gamma\delta$ T cells in experimental listeriosis and tuberculosis indicates that $\gamma\delta$ T cells precede $\alpha\beta$ T cells at the site of bacterial growth (Follows *et al*. 1992). They could provide a first line of anti-bacterial defence (Kaufmann, 1993).

It appears that the ability of some immunogenic component of the tick salivary gland to trigger type 2 effector T cells could create an optimal micro-environment for the pathogens to develop, or from where they can more easily disseminate in their optimal 'niche'. We consequently hypothesize that during the first step of the tick fixation to the host, the skin $\gamma\delta$ T cells localized at the tick-fixation site may already influence the polarization of the cytokine pattern, by preferentially inducing a Th2 developing environment (IL-4, IL-10) and not Th1 generally due to IFN-γ (Skeen & Ziegler, 1995). Furthermore, IL-10 as well as some glycosylated components of tick saliva could decrease some macrophage activities (Maldonado *et al*. 1994; Appelberg, 1995) and then prevent the destruction of infectious agents in the vicinity of their entry site.

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