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Infiltration of $CD4^+$ $CD8^+$ T cells, and expression of ICAM-1, Ia antigens, IL-1α and TNF-α in the skin lesion of BALB/c mice undergoing repeated infestations with nymphal *Ixodes ricinus* ticks

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SUMMARY

The skin cellular immune response of BALB/c mice was examined during three successive infestations with nymphal *Ixodes ricinus* ticks. An immunohistochemical analysis of skin cryostat sections 72 hr post-tick attachment revealed that $CD4^+$ T cells outnumbered $CD8^+$ T cells in all infestations. The $CD4^+$: $CD8^+$ T-cell ratio was 2·2 : 1 in the primary infestation, then increased to 3·2 : 1 and 4·7 : 1 in the secondary and tertiary infestations. No B lymphocytes (CD45R) were detected in the skin of control and infested mice. A positive staining of intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells, dendritic cells and some other mononuclear cells was observed in the dermis. Also, a strong positive staining of Ia antigens on dendritic cells and infiltrated mononuclear cells was noted. The staining pattern was more intense and positive cells increased in number in the skin of re-infested mice compared to the primary infestation. In addition, cells such as epidermal keratinocytes, dermal dendritic cells and infiltrated mononuclear cells positive for the 'pro-inflammatory' cytokines interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) were localized in the skin of infested mice, as detected at the mRNA level by *in situ* hybridization and at protein level by immunostaining with antibodies. These results suggest that an antigen was presented to infiltrating T lymphocytes which then became activated. This event may explain the cutaneous delayed-type hypersensitivity previously described in tick-infested BALB/c mice. Importantly, this cutaneous reaction was not sufficient to protect the mouse against tick re-infestation. Furthermore, ICAM-1 could mediate, at least in part, the extravasation of inflammatory cells into the skin of infested mice.

INTRODUCTION

Several studies have been reported about the inflammatory response in hosts infested with ticks.[1,2] BALB/c mice repeatedly infested with larval *Dermacentor andersoni* acquired resistance and their skin showed an infiltration of mast cells, neutrophils, eosinophils and mononuclear cells.[1] It was suggested that mast cells and eosinophils may participate in the acquisition of resistance to ticks in BALB/c mice. On the other hand, a local activation of complement has been suggested in resistant guinea-pigs infested with *D. andersoni* larvae.[3] Tick antigens injected into the skin via the saliva during feeding were shown to be trapped by Langerhans' cells, and presented to T lymphocytes in the draining lymph nodes.[4] BALB/c mice infested with nymphal *Ixodes ricinus* ticks failed to acquire resistance, despite the appearance of a marked cellular infiltrate in the skin, including neutrophils, eosinophils, basophils and mononuclear cells (monocytes and lymphocytes).[5] In addition, an increase in number of locally degranulating mast cells was observed.[5] The skin of infested mice became sensitized to tick antigens (immediate and delayed-type). Little importance was given to the infiltrating mononuclear cells in the skin of murine hosts infested with ticks. Therefore, attempts were made to characterize lymphocyte phenotypes infiltrating the skin of infested BALB/c mice using antibodies to mouse cell-surface markers CD4, CD8 and CD45R (mouse B lymphocytes).

Intercellular adhesion molecule-1 (ICAM-1) can mediate, at least in part, the adhesion of leucocytes to the vessel wall, and extravasation.[6] Its expression has been shown to be up-regulated by cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α),[7] which possess many other biological properties including the regulation of the pathological process in the host response to various stimuli. For example, biologically active membrane-associated IL-1 may be the form which participates in activating lymphocytes.[8] Due to the lack of information about the local immune response to ticks, we also undertook this work to examine the local production of IL-1α and TNF-α and the local expression of Ia antigens and ICAM-1 in BALB/c mice infested successively with nymphal *I. ricinus* ticks.

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MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Iffa-Credo (Arbresle, France). They were between 8 and 12 weeks old at the time of infestation.

Ticks

Ixodes ricinus nymphal ticks, originally from the pathogen-free colony of our laboratory, were reared as described previously.[9]

Infestations

Fifteen *I. ricinus* nymphs (approximately 6 weeks old) per mouse were placed within a plastic capsule glued to the skin, as previously described.[5] Three successive infestations were done and no skin site was infested more than once. Mice were given 7 days free of ticks between successive infestations. Mice prepared to be infested, but without ticks, served as controls.

Tissue preparation

Skin biopsies, including attached ticks, were taken 72 hr post-tick attachment and embedded in Optimal Cutting Temperature compound (OCT; Bayer-Pharma, Zürich, Switzerland), then frozen in isopentane chilled in liquid nitrogen and stored at −70°. Cryostat sections (5 μm thick) were placed on poly-L-lysine-coated slides[10] and allowed to dry in air. Sections were fixed in acetone for 10 min at 4°, air-dried and hydrated in Tris-buffered saline (100 mM Tris–HCl, 150 mM NaCl, pH 7·6) for 2 min before immunostaining.

Antibodies

The following monoclonal rat IgG antibodies were used: anti-L3T4/CD4 (IgG2a), anti-Ia^d (IgG2a) (Boehringer-Mannheim, Rotkreuz, Switzerland), anti-CD8-biotin (IgG2a) (Gibco, Basel, Switzerland), anti-CD45R (IgG2a) for murine B cells (Pharmingen, Lugano, Switzerland) and anti-ICAM-1 (IgG2a) (British Bio-technology Products, Abingdon, U.K.).

Polyclonal rabbit anti-mouse IL-1-α and TNF-α were purchased from Genzyme (Boston, MA).

Immunohistochemistry

For anti-L3T4/CD4, CD45R, Ia^d and ICAM-1 antibodies, an indirect immunoperoxidase technique was used. After inhibiting the endogenous peroxidase activity with 0·1 M sodium azide and 0·04% hydrogen peroxide,[11] sections were incubated for 30 min at room temperature (21°) with primary antibodies diluted 1/100 in 100 mM Tris–HCl, 150 mM NaCl (TBS), pH 7·6, containing 0·2% bovine serum albumin (BSA; Sigma, St Louis, MO). Control slides received the diluent alone. After three washes (5 min each) in TBS, slides were incubated for 30 min at room temperature (21°) with peroxidase-conjugated sheep anti-rat Ig (Boehringer-Mannheim), diluted 1/200 in TBS–0·2% BSA.

A biotin–streptavidin immunoperoxidase technique (Histomark kit; KPL, Gaithersburg, MD) was used for anti-CD8 biotin rat monoclonal antibodies.

Peroxidase activity was demonstrated by incubation for 5 min in diaminobenzidine/tetrahydrochloride (DAB), containing hydrogen peroxide using the KPL peroxidase detection kit.[12] Sections were then counterstained with Mayer's haematoxylin solution (Fluka, Buchs, Switzerland) and mounted with fluoromount-G (SBA, Birmingham, AL).

A biotin–streptavidin–alkaline phosphatase complex (Histomark kit; KPL) was used for the detection of IL-1-α and TNF-α. Sections were blocked with normal goat serum 20 min before immunostaining. Slides were sequentially incubated with anti-IL-1-α and anti-TNF-α, respectively diluted 1/150 and 1/400 in TBS–0·2% BSA, biotinylated goat anti-rabbit IgG and streptavidin–alkaline phosphatase. All incubations were performed for 30 min at room temperature (21°). Several washes (5 min) in TBS were done between all incubations. Phosphatase activity was revealed with fast-red development. Sections were counterstained and mounted as described above. The omission of the primary antibodies and the development of alkaline phosphatase alone yielded a negative signal.

In situ *hybridization*

Biopsy specimens were frozen as described above and the *in situ* hybridization technique was performed.[13] The ultra-pure water used for cleaning slides and for preparation of hybridization solutions was treated in 0·1% diethylpyrocarbonate (DEPC; Sigma) and allowed to stand at room temperature overnight before autoclaving, in order to remove any contaminating RNases.[14]

Probes

The cDNA for murine IL-1-α and TNF-α were synthetic oligonucleotides (30 and 28 bases, respectively) complementary to murine mRNAs cytokines. Probes were labelled at the 5′ end with digoxigenin and were purchased from British Biotechnology Products.

Hybridization

Five-micrometre thick cryostat sections were fixed in 4% paraformaldehyde for 20 min at 4°. After several washes in 0·1 M phosphate-buffered saline, pH 7·2, free amino groups were acetylated by treatment with 0·25% acetic anhydride in 0·1 M triethanolamine, pH 8. Sections were prehybridized in hybridization buffer, consisting of 2 × SSC (1 × SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7), 30% deionized formamide (Gibco), 1 × Denhardt's solution, 125 μg/ml sheared denaturated salmon sperm DNA (Gibco), for 1 hr at 37°. Slides were then drained and sections covered with 50 μl of hybridization buffer containing DNA probe at a concentration of 0·5 mg/ml. Sections were covered with siliconized coverslips, sealed with rubber cement and hybridized overnight at 37°.

Post-hybridization washing was performed in decreasing concentration of SSC (4 × –2 × –0·2 × SSC) with 30% formamide at 37°. After 15 min washing at room temperature in Tris-buffered saline (0·05 M Tris–HCl, 0·15 M NaCl, 2 mM $MgCl_2$, 0·1% BSA), pH 7·6, containing 0·1% Triton X-100, sections were incubated with sheep anti-digoxigenin–alkaline phosphatase conjugate (British Biotechnology Products) diluted 1/600 in TBS. Alkaline phosphatase activity was demonstrated using the β-chloroindolyl-phosphate–nitroblue tetrazolium (BCIP–NBT) medium using the digoxigenin detection kit (British Biotechnology Products) for 3–6 hr. Slides were then counterstained with Mayer's haematoxylin solution (Fluka).

Table 1. $CD4^+$ and $CD8^+$ T cells 72 hr post-tick attachment in 1·24 mm^2 of skin of BALB/c mice infested *I. ricinus* nymphs

	T lymphocytes		
	$CD4^+$ T cells	$CD8^+$ T cells	CD4/CD8 ratio
Normal skin	4·0 ± 0·0	3·66 ± 0·01	1·09
Infestation			
1	19·16 ± 1·01[†]	8·66 ± 1·20	2·2
2	78·33 ± 3·44(†) [†]	24·66 ± 3·97(*)	3·2
3	86·00 ± 12·16(†) [†]	18·33 ± 2·67(*)	4·7

( ) Comparison between re-infestation and primary infestation for each T-cell subpopulation.

[ ] Comparison between $CD4^+$ and $CD8^+$ T cells in each infestation.

*$P < 0·05$.

†$P < 0·01$.

Means ± SE are shown.

The cell counting was carried out around the tick rostrum.

Sections incubated with RNase A (Boehringer Mannheim) (0·1 mg/ml in 2 × SSC, 10 mM $MgCl_2$) for 1 hr at 37° and the application of the hybridization solution, without probe, served as negative control. This yielded the expected negative signal.

Microscopic evaluation

Two sections per mouse, for a total of three mice per infestation with nymphs, were viewed with an Olympus Vanox-S microscope and cells counted according to phenotypic distribution. A total area of 1·24 mm^2 was examined.

Statistical analysis

A non-parametric test Mann–Whitney *U*-test was performed to analyse the data from lymphocyte counting.

RESULTS

Normal skin

In non-infested mice, dendritic cells in the epidermis (Langerhans' cells), dermal dendritic cells, and resident macrophages stained positive for Ia antigens. ICAM-1 expression was confined to vascular endothelium and to some basal epidermal keratinocytes. No CD45R B cells were present, while a few $CD4^+$ and $CD8^+$ T cells were detected in the intact dermis. Immunostaining with anti-CD45R antibodies of axillary BALB/c mouse draining lymph node cryostat sections was performed as a positive control (data not shown). Some epidermal keratinocytes stained positive for both mRNA and secreted proteins IL-1α and TNF-α (data not shown).

Tick-infested skin

In the primary infestation, $CD4^+$ T cells outnumbered $CD8^+$ T cells, with a CD4/CD8 ratio of 2·2 (Table 1). T cells preferentially infiltrated the dermis and were rarely observed in the epidermis. No CD45R B lymphocytes were detected in the skin. Epidermal Langerhans' cells, dermal dendritic cells and a few infiltrating mononuclear cells stained positive for Ia antigens, while keratinocytes remained negative. ICAM-1 staining was confined to basal epidermal keratinocytes and dermal dendritic cells. An intense staining was observed on mononuclear cells in the dermis. Epidermal keratinocytes,

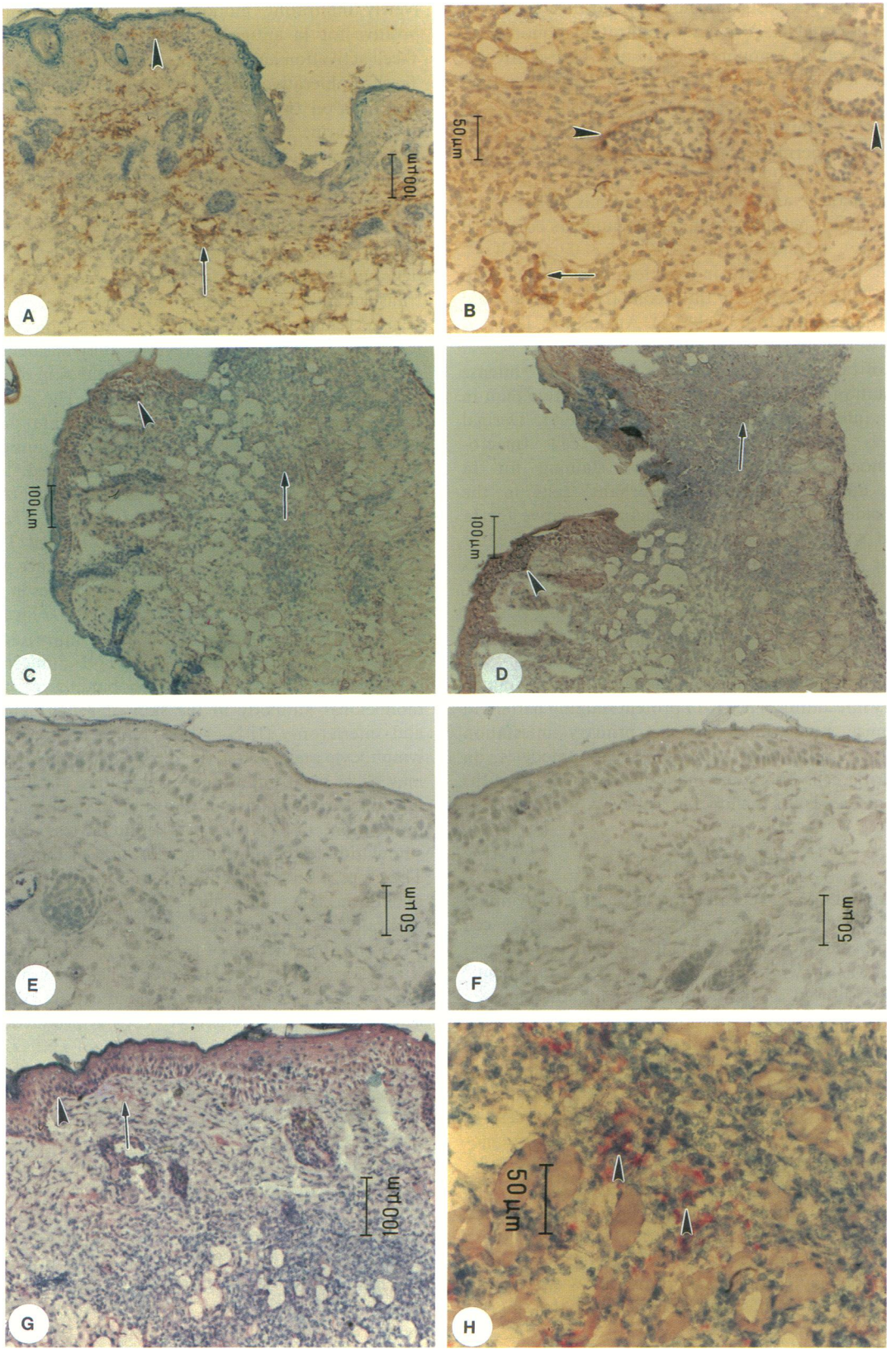
Table 2. Summary of results obtained by immunostaining with antibodies or using *in situ* hybridization on skin biopsies taken 72 hr post-tick attachment on BALB/c mice

	Immunostaining				*In situ* hybridization	
	Ia	ICAM-1	IL-1α	TNF-α	IL-1α	TNF-α
Control mice	+*, LC, DC, MC	±, VE, KC	±, KC	±, KC	±, KC	±, KC
Infestation 1	+, LC, DC, MC	±, KC, VE, MC, DC	+, KC, DC	+, KC, DC	+, KC, MC	+, KC, MC
Infestation 2	+ +, LC, DC, MC	+ +, KC, VE, MC, DC	+, KC, DC	+ +, KC, DC, MC	+, KC, MC	+ +, KC, MC
Infestation 3	+ +, LC, DC, MC	+ +, KC, VE, MC, DC	+, KC, DC	+ +, KC, DC, MC	+, KC, MC	+ +, KC, MC

*Intensity of coloured end product : ±, weak; +, light; + +, strong.

LC, Langerhans' cells; DC, dermal dendritic cells; KC, keratinocytes; MC, mononuclear cells; VE, vascular endothelial cells.

Figure 1. (*opposite*) (a) Expression of Ia antigens on Langerhans' cells in the epidermis (arrowhead), dendritic cells and infiltrated mononuclear cells (arrow) in the dermis of BALB/c mice infested with *I. ricinus* ticks; tertiary infestation, 72 hr. (b) Expression of ICAM-1 in the skin of BALB/c mice infested with *I. ricinus* ticks; secondary infestation, 72 hr. Note the positive staining on vascular endothelium (arrowheads) and some infiltrated mononuclear cells in the dermis (arrow). (c) Keratinocytes (arrowhead), primarily near the tick rostrum, and some infiltrating mononuclear cells (arrow) positive for IL-1α mRNA; tertiary infestation, 72 hr. (d) Keratinocytes (arrowhead) and some infiltrating mononuclear cells (arrow) positive for TNF-α mRNA in skin biopsy of BALB/c mice infested with nymphal *I. ricinus* ticks; tertiary infestation, 72 hr. (e) and (f) Control sections treated with RNase, and hybridized with IL-1α (e) and TNF-α (f) probes. No localizing signal. (g) IL-1α in the skin of BALB/c mice infested with *I. ricinus* ticks; tertiary infestation, 72 hr. Epidermal keratinocytes (arrowhead), hair follicles and some dendritic cells (arrow) appear positive. (h) TNF-α in the skin of BALB/c mice infested with *I. ricinus* ticks; tertiary infestation, 72 hr. Note the positivity of some infiltrating mononuclear cells in the dermis (arrowheads).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primarily near the tick rostrum, showed a positive signal for IL-1α and TNF-α mRNA. Also, a few infiltrating mononuclear cells showed detectable amounts of IL-1α and TNF-α mRNA. At the protein level, TNF-α was detected on dermal dendritic cells and in the same cell types that were positive for this cytokine's mRNA, contrasting with IL-1α, whose protein was only detected in epidermal keratinocytes and dermal dendritic cells.

In the secondary and tertiary infestations, $CD4^+$ and $CD8^+$ T cells infiltrated the skin in higher numbers compared to the primary infestation ($P < 0{\cdot}01$, $P < 0{\cdot}04$). $CD4^+$ outnumbered $CD8^+$ T cells in re-infestation ($P < 0{\cdot}003$), with a CD4/CD8 ratio of 3·2 in the secondary infestation and 4·7 in the tertiary infestation (Table 1). Again, no CD45R B lymphocytes were detected in re-infested mice.

Staining of Ia and ICAM-1 antigens became more intense with re-infestation and many more cells were positive than in the primary infestation (Fig. 1a, b, respectively). Dermal dendritic cells and infiltrated mononuclear cells (macrophages, monocytes) showed a positive staining for Ia antigens, in addition to some Langerhans' cells in the epidermis. Epidermal keratinocytes remained negative for Ia antigens, but some basal keratinocytes stained positive for ICAM-1. The intensity of ICAM-1 staining on keratinocytes remained at constitutive levels observed in normal skin.

Mononuclear cells infiltrating the dermis showing positive signals for IL-1α and TNF-α mRNA (Fig. 1c, d, respectively) became more numerous than in primary infestation. The staining pattern in the epidermis was identical to that obtained in the primary infestation. The IL-1α protein staining pattern in re-infested mice was similar to the primary infestation (Fig. 1g). TNF-α protein was detected on keratinocytes, on dermal dendritic cells and some mononuclear cells (Fig. 1h) (a summary of the results is shown in Table 2).

DISCUSSION

BALB/c mice undergoing repeated infestations with nymphal *I. ricinus* ticks fail to acquire resistance.[5] Inflammatory cell infiltration into the dermis of nymphal *I. ricinus* tick-infested BALB/c mice was shown to peak 72 hr post-tick attachment.[5] The epidermis showed a slight infiltration of inflammatory cells.[5] During the three successive infestations, 72 hr post-tick attachment, $CD4^+$ T cells outnumbered $CD8^+$ T cells, while no B cells were detected in the skin of susceptible BALB/c mice. Differences in number and phenotypes of lymphocytes subpopulations recruited in the skin have been suggested to determine the outcome of challenge with metacyclic trypanosomes.[15] A marked infiltration of $CD4^+$ T-helper cells, $T19^+$ ($CD4^-$, $CD8^-$) T cells and $CD45R^+$ (B cells) was shown in the skin of sheep infected with the larvae of *Lucilia cuprina* during both primary and secondary infestations compared with control sites.[16] The presence of B cells in the skin could represent an important event because it may allow a protective humoral response to be effected at the level of the skin, as previously suggested.[15]

The selective infiltration of T cells in the skin of nymphal *I. ricinus*-infested BALB/c mice may be partially mediated by adhesion molecules such as ICAM-1. Our results showed that ICAM-1 is expressed on vascular endothelium and the mononuclear cells infiltrating the skin of re-infested mice. Newly infiltrating mononuclear cells also stained strongly positive for Ia antigens. The involvement of Ia antigens in T-cell activation has been demonstrated.[17,18] In addition, a low T-cell proliferative response to antigen occurred when expression of Ia on antigen-presenting cells was reduced.[19]

In murine contact allergic dermatitis, the expression of ICAM-1 on epidermal keratinocytes precedes infiltration of the epidermis by T lymphocytes.[20] The adhesion of eosinophils to endothelial cells appeared to be favoured by the expression of adhesion molecules such as ICAM-1. Anti-ICAM-1 antibodies inhibit eosinophilia of the bronchial mucosa.[21] Mast cell degranulation also participates, *in vitro*, in the rapid and transient induction of adhesion molecules for leucocytes on nearby venular endothelium.[22,23] This event depends on the local release of TNF.[22] A previous study has demonstrated an increase of degranulated mast cells in the skin of BALB/c mice infested with nymphal *I. ricinus* ticks.[5] The co-expression of ICAM-1 and Ia antigens could facilitate adhesion and subsequent MHC class II antigen-restricted antigen presentation to T lymphocytes, as previously suggested.[24]

Both IL-1α and TNF-α were detected at mRNA and protein levels at the tick fixation site of BALB/c mice. The detection of mRNA and the secreted proteins IL-1α and TNF-α in the epidermis of non-infested mice is in accordance with previous studies showing the presence of these cytokines in unstimulated mouse epidermal cells.[25,26] IL-1α and TNF-α locally produced in the skin of infested mice may up-regulate ICAM-1 expression on the endothelial cell.[7] TNF-α injected intracutaneously was shown to increase binding of anti-ICAM-1 monoclonal antibody in the skin of *Papio anubis*.[27] TNF-α and interferon-γ (IFN-γ) may represent potent inducers of lymphocyte migration in the skin.[28] The local release of TNF-α may be of great importance in initiating and maintaining the inflammatory response in the skin. In addition, an increased production of TNF-α by keratinocytes after an initial stimulation leads to an immobilization of Langerhans' cells.[29] Therefore, a substantial number of antigen-presenting cells will remain in the skin where they can interact with specific infiltrated T lymphocytes.[30] The precise role of IL-1α and TNF-α in the outcome of the immune status of hosts infested with ticks is not known. However, it has been shown that adult *Dermacentor andersoni* salivary gland extract partially inhibits *in vitro* cytokine (IFN-γ, IL-1, TNF-α) production by mononuclear cells.[31] Due to positive staining of TNF-α and IL-1α on some mononuclear cells at the tick feeding site of non-resistant hosts, the question arises as to whether TNF-α and/or IL-1α may be detrimental to BALB/c mice in mounting an efficient resistance to tick feeding.

According to our results, it is tempting to predict a local antigen presentation to $CD4^+$ T cells, which becomes activated and may release cytokines such as IFN-γ. This event may explain the cutaneous delayed-type hypersensitivity (DTH) observed in re-infested BALB/c mice,[5] because $CD4^+$ T cells are believed to be effector cells which mediate the DTH response.[32–34] In addition, *in vivo* ICAM-1 induction accompanies T-cell-mediated hypersensitivity reactions in human skin.[35] Importantly, this DTH response is not sufficient to protect the mouse against re-infestation.

In conclusion, this study shows a selective infiltration of T cells in the skin of mice infested with nymphs of *I. ricinus* ticks, with local production of TNF-α and, to a lesser extent, IL-1α by

mononuclear cells in non-resistant BALB/c mice. This immunological response to tick feeding is also accompanied with an increase of cells expressing Ia antigens and ICAM-1, which may partially mediate the extravasation of inflammatory cells described in the skin of infested BALB/c mice.[5]

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