

Antigens from *Rhipicephalus sanguineus* ticks elicit potent cell-mediated immune responses in resistant but not in susceptible animals

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Abstract

In the present study we compared the immunological reactions between *Rhipicephalus sanguineus* tick-infested susceptible (dogs and mice) and tick-resistant hosts (guinea pigs), elucidating some of the components of efficient protective responses against ticks. We found that T-cells from guinea pigs infested with adult ticks proliferate vigorously in the presence of concanavalin A (ConA), whereas ConA-induced cell proliferation of tick-infested mice and dogs was significantly decreased at 43.1 and 94.0%, respectively, compared to non-infested controls. Moreover, cells from mice and dogs submitted to one or three successive infestations did not exhibit a T-cell proliferative response to tick antigens, whilst cells from thrice tick-infested guinea pigs, when cultured with either a tick extract or tick saliva, displayed a significant increase in cell proliferation. Also, we evaluated the response of tick-infested mice to a cutaneous hypersensitivity test induced by a tick extract. Tick-infested mice developed a significant immediate reaction, whereby a 29.9% increase in the footpad thickness was observed. No delayed-type hypersensitivity (DTH) reaction was detected. Finally, the differential cell count at the tick attachment site in repeatedly infested mice exhibited a 6.6- and 4.1-fold increase in the percentage of eosinophils and neutrophils, respectively, compared to non-infested animals, while a decrease of 77.0–40.9 in the percentage of mononuclear cells was observed. The results of the cutaneous hypersensitivity test and the cellular counts at the tick feeding site for mice support the view that tick-infested mice develop an immune response to *R. sanguineus* ticks very similar to dogs, the

Abbreviations: ConA, concanavalin A; DTH, delayed-type hypersensitivity; UAE, unfed adult tick extract; UNE, unfed nymphal extract; Inf., infestation; S.D., standard deviation

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natural host of this species of tick, but very different from guinea pigs (resistant host), which develop a DTH reaction in addition to a basophil and mononuclear cell infiltration at the tick-attachment site. In conclusion, saliva introduced during tick infestations reduces the ability of a susceptible animal host to respond to tick antigens that could stimulate a protective immune response. As a consequence, the animals present a lack of DTH response and disturbed cellular migration to tick feeding site, which can represent a deficient response against ticks.

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1. Introduction

Host immunization against ticks is being investigated as a promising substitute to the expensive and laborious acaricide treatments that are currently the sole alternative for tick control. Towards this end, knowledge about naturally acquired immunity is essential. Even though mechanisms of acquired immunity against ticks are still poorly understood, it has been established that during a blood meal ticks stimulate both regulatory and effector pathways of the host immune response, involving antibodies, complement, cytokines, antigen-presenting cells, and lymphocytes (Wikel et al., 1994; Wikel, 1996). Mice can be used as a model to study *Rhipicephalus sanguineus* tick–host interactions since, similarly to dogs, they do not develop resistance to repeated tick infestations and many more immunological reagents are available (Ferreira and Silva, 1998). In contrast to mice and dogs, guinea pigs develop an outstanding immunological response to re-infestations with *R. sanguineus* ticks, which leads to resistance (Garin and Grabarev, 1972; Szabó et al., 1995b). Such a dichotomy in the host immune response to ticks provides helpful conditions to evaluate protective and non-protective mechanisms against ticks. For this purpose, a comparative study of the immunological and inflammatory reactions in tick-infested susceptible or resistant hosts is desirable.

Previous work has shown that the local inflammatory reaction at the tick feeding sites differs between dogs and guinea pigs. The inflammatory skin reaction in dogs, which are naturally susceptible to ticks, shows a prominent infiltration of neutrophils throughout infestations (Theis and Budwiser, 1974; Szabó and Bechara, 1999), while in guinea pigs the cellular infiltrate is characterized by an increase in the numbers of mononuclear cells, basophils and eosinophils (Brown and Askenase, 1981; Szabó and Bechara, 1999). In addition, repeatedly tick-infested guinea pigs (resistant to *R. sanguineus* ticks) develop both, an immediate and a strong cutaneous delayed-type hypersensitivity (DTH) reaction to tick antigens. Conversely, dogs, develop a much more intense immediate but not a cutaneous DTH reaction after repeated infestations with ticks or injection with tick antigens (Szabó et al., 1995a).

It has been shown that lymphocytes taken from axillary and brachial lymph nodes of mice that have been infested once or thrice with ticks have reduced ability to proliferate when cultured with mitogens such as ConA or phytohemagglutinin (Wikel, 1982; Schorderet and Brossard, 1993; Ferreira and Silva, 1999). Moreover, mice re-infested with *Ixodes ricinus* and *R. sanguineus* ticks exhibit a polarized Th2-type cytokine profile, represented by

augmented IL-4, IL-10 and TGF- β (Ganapamo et al., 1995, 1996; Ferreira and Silva, 1999). However, none of these studies compared the immune response between tick-susceptible and resistant hosts.

The present study was undertaken to compare the immunological reactions between tick-infested susceptible and resistant hosts. Hence, dogs, mice and guinea pigs were infested with ticks and their cellular proliferative response to ConA and tick antigens was analyzed. Additionally, a cutaneous hypersensitivity test was employed to measure the response of tick-infested mice to a tick extract and the pattern of cellular infiltration at the tick-feeding site was determined.

2. Materials and methods

2.1. Experimental animals and tick infestations

2.1.1. Hosts

C3H/HeJ female mice (6–8 weeks of age) and albino female guinea pigs (6–12 months old) were bred and maintained under standard conditions in the animal facilities of the Department of Biochemistry and Immunology, University of São Paulo (USP), Ribeirão Preto, SP, Brazil. Mongrel dogs (1–3 years old, both sexes) were bred and maintained under standard conditions at the Department of Veterinary Pathology, University of the State of São Paulo (UNESP), Jaboticabal, SP.

2.1.2. Ticks

R. sanguineus ticks were laboratory reared as previously described (Ferreira et al., 1996). All ticks used for infestations were 1–3 months old.

2.1.3. Tick infestations

Mice, guinea pigs and dogs were infested with 2, 6 and 25 pairs of adult ticks, respectively, in plastic feeding chambers glued to the animals backs' (Britannia Adhesives P4104 Latex, UK) for the cell proliferation assays and the skin histopathological analysis. Mice were infested with 12 nymphs per mouse per tick infestation for the cutaneous hypersensitivity test. Three infestations were performed at 30-day intervals at different skin sites. Control groups for all experiments consisted of animals on whose backs empty plastic feeding chambers were glued (sham infestations).

2.2. Saliva collection and tick extract preparation

In order to obtain engorged ticks for saliva collection, dogs ($n = 10$) were infested with 60 pairs of adult *R. sanguineus* ticks contained in plastic feeding chambers fixed to their backs. The saliva collection procedure was performed using fully engorged female ticks inoculated with 10–15 μ l of a 0.2% (w/v) solution of dopamine in phosphate buffered saline, pH 7.4 (PBS) (Ferreira and Silva, 1998). Each saliva pool consisted of material harvested from 100 to 200 female ticks. Protein concentration was determined using a bicinchoninic acid solution, that binds in a concentration-dependent manner to Cu(I) produced from the

reduction of alkaline Cu(II) by proteins (Procedure TPRO-562, Sigma Chemical Co., St. Louis, MO), as previously described (Smith et al., 1985).

The unfed adult tick extract (UAE) and the unfed nymphal tick extract (UNE) were obtained as previously described (Szabó et al., 1995a).

2.3. *In vitro* cell proliferation assay

T-cell proliferation assays were performed with 30 female C3H/HeJ mice and 24 guinea pigs submitted to none, one or three infestations (equally divided groups). Additionally, 12 dogs were used for this assay, 4 for each group (control, once and thrice tick infested). Animals were killed 6 days after the beginning of each infestation. Axillary and/or brachial lymph nodes (tick-chamber draining lymph nodes) were removed and the cells were obtained by teasing the organs in Hanks' medium. Cells from each experimental group were centrifuged for 10 min at $150 \times g$, washed three times and diluted in RPMI-1640 (Flow Laboratories, McLean, VA) supplemented with heat inactivated 5–10% fetal calf serum (Gibco-BRL Life Technologies, Grand Island, NY), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. Triplicate cultures were made in 96-well flat bottom culture plates (Corning, New York, NY) at 5×10^5 cells per well and stimulated with ConA (2 μ g/ml) (Sigma), UAE (10 μ g/ml) or tick saliva (35 μ g/ml). The ConA and antigen doses were established based on preliminary experiments using naïve mice, guinea pigs and dogs. ConA was tested at 1, 2 and 5 μ g/ml; UAE at 1, 10 and 40 μ g/ml and saliva at 1, 10, 35 and 70 μ g/ml. Cultures were maintained at 37 °C in a humidified 5% CO₂ incubator. After 64 h (mice and guinea pigs) and 85 h (dogs) the cultures were pulsed with methyl-³H-thymidine (Amersham Life Science, Buckinghamshire, UK) at a concentration of 0.5 μ Ci per well. Eight to 10 h later, the amount of label incorporated into cells was determined using a cell harvester (Cambridge Technology Inc., Watertown, MA) and a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA). The incubation period used for the dog cell cultures (85 h) was based on other work (Letwin and Quimby, 1987; Greeley et al., 1996). Mean counts per minute (cpm $\pm$ S.D.) were determined and the stimulation index was calculated for each experimental group as the ratio of counts in the ConA or antigen (UAE or saliva)-stimulated cultures over the basal counts (cells in medium only).

2.4. *Cutaneous hypersensitivity test*

On the sixth day of the third infestation with tick nymphs, female C3H/HeJ mice ($n = 6$) were injected subcutaneously with 10 μ g of UNE or PBS into the left and right footpad, respectively. The thickness of both feet was measured three times at various intervals post-inoculation (10 min, 1, 2, 6, 24, 48 and 72 h) with a Mitutoyo dial-thickness gauge that employs a uniform pressure of 85 gm, and the results were expressed as mean percent changes in foot thickness in relation to pre-inoculation values. The final UNE-induced increase in foot thickness was calculated by subtracting values measured in the right footpad from those of the left footpad. The control group consisted of female C3H/HeJ mice ($n = 6$) which were submitted to sham tick infestations.

2.5. Histopathology of tick attachment site

Histopathological analysis of the tick attachment site was performed on mouse skin samples. For this purpose, mice were infested with two couples of adult *R. sanguineus* ticks and killed on the sixth day of tick infestation. Skin samples were taken from the back of mice suffering first ($n = 9$) and third infestations ($n = 8$), and from non-infested mice (sham tick-infested mice) ($n = 7$) and immediately immersed in fixative (buffered formalin, pH 7.0).

Skin samples were kept in the fixative for 24 h, embedded in paraffin and processed according to routine histological techniques. Each sample was serially sectioned at a thickness of 4 μm , and stained with hematoxylin–eosin (HE) and May–Grünwald–Giemsa. Skin sections were analyzed under light microscopy. General features were evaluated on HE stained sections. Total and differential cell counts were made on sections stained with May–Grünwald–Giemsa on oil immersion fields (objective 100 $\times$). For this purpose, cells from the dermis were counted below the cement cone in intensely cell infiltrated areas. Counting fields of 0.026 mm² were delimited with the aid of a micrometer (Olympus, Tokyo). Four fields of one section per skin sample were analyzed. Only sections displaying the central tick attachment lesion were considered. This was indicated by the presence of a bell shaped mass of cement cone on the skin surface (produced by the tick to achieve a stronger attachment).

2.6. Statistical analysis

Data were analyzed using the Tukey–Kramer test for multiple comparisons.

3. Results

3.1. Tick-infested resistant and susceptible animals displayed different lymphoproliferative responses to ConA and to *R. sanguineus* antigens

Guinea pigs, mice and dogs were repeatedly tick-infested in order to obtain immune cells and compare their response to ConA and tick antigens. Firstly we determined the best concentration of ConA and tick antigens, which yielded the highest T-cell proliferation response in cells from control or experimental animals. Our results showed that the ConA concentration that better stimulated cell proliferation in controls of all animal species tested was 2 $\mu\text{g/ml}$. Testing the different doses of tick antigens we also observed that the concentrations that induced higher proliferation of resistant guinea pigs cells were 10 and 35 $\mu\text{g/ml}$ of saliva and UAE, respectively. Higher concentrations did not improve cell proliferation rate in guinea pigs, neither induced any significant increase in cell proliferation of tick-infested mice and dogs.

Lymph node cells harvested from once and thrice tick-infested guinea pigs cultured with ConA exhibited a stimulation index similar to control animals (non-infested) (Fig. 1A). On the other hand, cell proliferation of thrice tick-infested mice and dogs was significantly decreased in 43.1% ($P < 0.01$) and 94.0% ($P < 0.001$), respectively, when compared

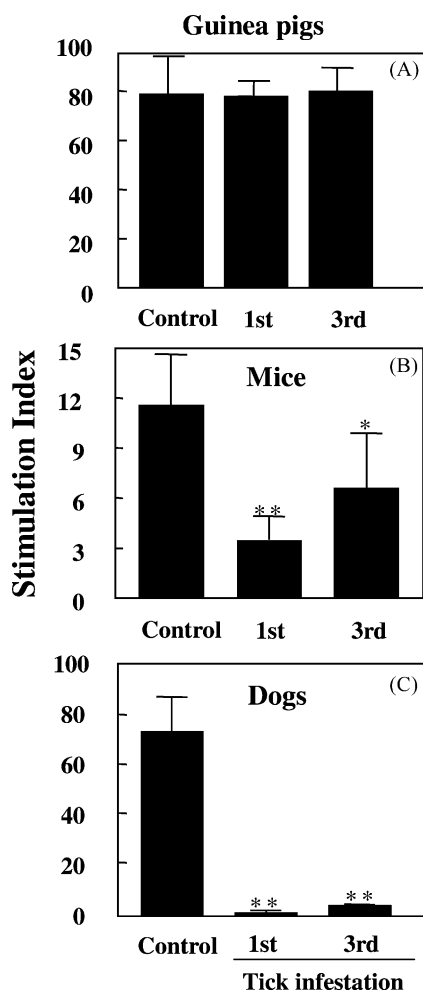


Fig. 1. Effect of *R. sanguineus* tick infestations on guinea pig, mouse and dog lymphocyte proliferation induced by ConA. Lymph node cells (5×10^5 cells per well) from control and tick-infested (once and thrice) guinea pigs (A), mice (B) and dogs (C) were incubated with ConA ($2 \mu\text{g/ml}$) and assayed for cell proliferation by ^3H -thymidine incorporation. Mean counts per minute were determined and the stimulation index was calculated for each experimental group as the ratio of counts in the ConA-stimulated cultures over the basal counts (cells in medium only). Results are expressed as stimulation index $\pm$ S.D. ** $P < 0.001$ and * $P < 0.01$ compared to control animals.

to controls (Fig. 1B and C). Additionally, lymphocytes harvested from lymph nodes of thrice tick-infested guinea pigs recognized tick antigens in the UAE and in tick saliva and proliferated significantly more than the controls (stimulation index of 14.8 ± 4.7 ($P < 0.001$) and 17.3 ± 8.7 ($P < 0.001$), respectively) (Fig. 2A). Conversely, cells from tick-infested mice and dogs, even after repeated infestations, did not significantly recognize tick antigens in UAE or in tick saliva (Fig. 2B and C). Similar results were obtained when BALB/c or

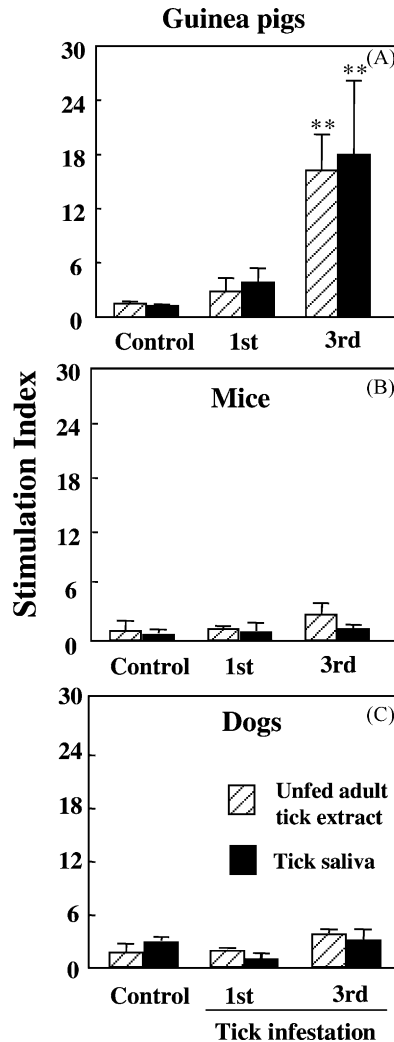


Fig. 2. Effect of *R. sanguineus* tick infestations on guinea pig, mouse and dog lymphocyte proliferation induced by UAE and saliva. Lymph node cells (5×10^5 cells per well) from control and tick-infested (once and thrice) guinea pigs (A), mice (B) and dogs (C) were incubated with UAE (10 $\mu\text{g/ml}$) or tick saliva (35 $\mu\text{g/ml}$) and assayed for cell proliferation by ^3H -thymidine incorporation. Mean counts per minute were determined and the stimulation index was calculated for each experimental group as the ratio of counts in the antigen (UAE or saliva)-stimulated cultures over the basal counts (cells in medium only). Results are expressed as stimulation index $\pm$ S.D. $^{**}P < 0.001$ compared to control animals.

C57Bl/6 mice were tested, indicating a lack of restriction by mouse genotype (data not shown).

Since cells from regional lymph nodes of dogs and mice did not proliferate in the presence of ConA and tick antigens, we asked if the pattern of response of T-cells present at distant

locations from the tick-attachment sites could be diverse. To test this hypothesis, we cultured cells from popliteal lymph nodes collected from repeatedly tick-infested mice or dogs in the presence of ConA (2 µg/ml), UAE (10 µg/ml) and saliva (35 µg/ml). ConA-stimulated non-regional lymphocytes exhibited a stimulation index similar to cells from control animals (86.5 ± 12.4 and 23.4 ± 5.8 for dogs and mice, respectively), indicating that cells from distant locations from the tick-attachment sites are responsive. Moreover, cells from the remote lymph nodes of dogs and mice did not proliferate when stimulated with tick antigens.

3.2. Tick-infested susceptible animals displayed an immediate type hypersensitivity response to tick antigens, and exhibited a cellular infiltrate dominated by neutrophils and eosinophils at the site of tick attachment

In order to generate results which could allow straight comparison between both tick-susceptible host species (mice and dogs) we evaluated the development of cutaneous reactions in mice infested with *R. sanguineus* nymphs. The cutaneous hypersensitivity response to UNE of dogs and guinea pigs infested with *R. sanguineus* nymphs was published elsewhere (Szabó et al., 1995a). Mice infested with nymphs developed a significant ($P < 0.001$) increase (29.9%) in footpad thickness 10 min after a subcutaneous inoculation of UNE. One hour later, the footpad thickness had diminished and was equal to control values and this pattern was maintained throughout 72 h. Control tick-infested mice, inoculated with PBS, did not exhibit any increase in footpad thickness throughout the observation period (Fig. 3).

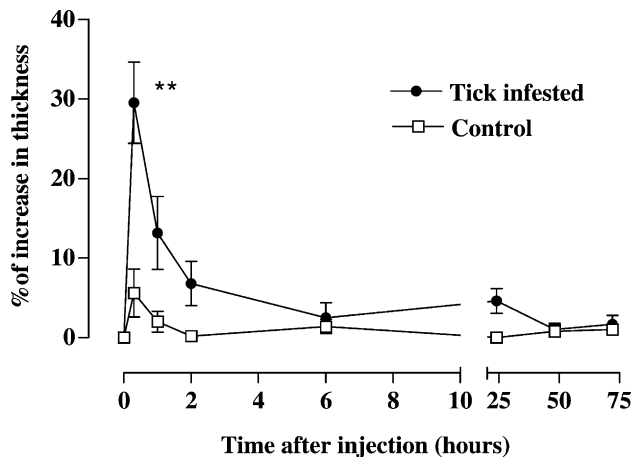


Fig. 3. Cutaneous hypersensitivity response of *R. sanguineus* tick-infested mice. C3H/HeJ mice were thrice infested with nymphs and 6 days after the third infestation were submitted to a cutaneous hypersensitivity test consisting of a subcutaneous inoculation of 10 µg of UNE in to their right footpad. The left footpad was inoculated with the same volume of dilution buffer. The thickness of both footpads was measured and the results were expressed as mean percent changes in the footpad thickness in relation to pre-inoculation values. The final increase in footpad thickness was calculated by subtracting values measured in the right footpad from those of the left footpad $\pm$ S.D.

** $P < 0.001$ compared to control animals.

Skin histopathology at the adult tick-attachment site showed that control (sham) mice displayed some skin alterations, probably because of the glue used to fix the chamber. These skin alterations included epidermal hyperplasia, spongiosis and diffuse dermal infiltration and edema. On tick-infested mice the alterations were confined to the immediate surroundings of the tick attachment site. During the first infestation (day 6), epidermal hyperplasia was slight and dermal edema and necrosis were common features, while exocytosis, microvesicles and hemorrhage were seen less frequently. The cellular infiltrate was more intense in the deeper layers of the skin, becoming diffuse in the superficial dermis. Lesions from skin samples of mice suffering the third infestation resembled those from the first infestation, but displayed a more intense epidermal hyperplasia, which also affected larger areas. Cellular infiltration in these samples was prominent and affected all skin layers including the superficial dermis.

The total and differential cell counts at the tick lesion site on mice are summarized in Table 1. Total cell numbers in skin sections from tick-infested mice were significantly higher during the tertiary infestation when compared to the controls (sham mice) ($P < 0.001$).

Differential cell counts showed that, already during the primary infestation, mice exhibited a slight (but not statistically significant) increase in the population of eosinophils and neutrophils in the skin compared to control mice. During the third infestation the proportion of granulocytes increased even more compared to sham-infested mice (increase of 6.4 and 4.1 times for eosinophils and neutrophils, respectively). A compensatory drop in the percentage of mononuclear cells at the tick-feeding site of thrice tick-infested mice (from 77.0 to 40.9) was observed (Table 1 and Fig. 4). Although not significant, the mast cell count at the tick attachment site for once or thrice tick-infested mice was reduced by 81.9 and 62.8%, respectively, compared to control mice (Table 1 and Fig. 4). Basophils were not detected throughout the study.

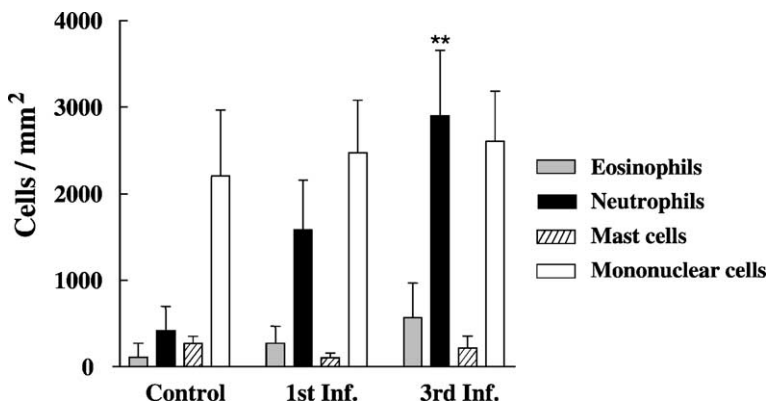


Fig. 4. Differential cell counts at *R. sanguineus* attachment sites 6 days after the beginning of each infestation in C3H/HeJ mice. Results are expressed as mean cell counts $\pm$ S.D. per mm^2 . ** $P < 0.001$ compared to control animals. Inf.—infestation.

Table 1
Total and differential cell counts at the tick attachment site on C3H/HeJ mice on the sixth day of tick infestation*

	Total counts	Eosinophils	Neutrophils	Mast cells	Mononuclear cells
Control	3000 ± 2074	108 ± 282 (1.6%)	420 ± 484 (10.9%)	267 ± 145 (10.5%)	2205 ± 1322 (77.0%)
First infestation	4479 ± 246 NS ^a	270 ± 342 NS (8.8%)	1582 ± 995 NS (34.3%)	101 ± 90 NS (1.9%)	2471 ± 1052 NS (54.9%)
Third infestation	6288 ± 968**	568 ± 695 NS (10.3%)	2903 ± 1305** (44.8%)	213 ± 247 NS (3.9%)	2605 ± 1006 NS (40.9%)

^a Non-significant.
* The results are expressed as means ± S.D. per mm² and mean percentage (in parenthesis).
** *P* < 0.001 compared to the means of the control group.

4. Discussion

In this work we compared the immunological reactions between tick-infested susceptible (dogs and mice) and tick-resistant hosts (guinea pigs), elucidating some of the components of efficient protective responses against ticks. We found that leucocytes from tick-infested guinea pigs proliferated in the presence of ConA and of antigens derived from UAE and tick saliva. Conversely, cells from draining lymph nodes of tick-infested mice and dogs (susceptible hosts) did not proliferate, even after testing a broad range of mitogen and antigen concentrations. This suggests a significant role for the T-cell-mediated immune response in inducing resistance to ticks. Salivary gland extracts of *Dermacentor andersoni*, *Amblyomma americanum* or *I. ricinus* ticks also stimulated in vitro proliferation of cells collected from tick-resistant hosts (Wikel et al., 1978; Wikel and Osburn, 1982; George et al., 1985; Schorderet and Brossard, 1993; Macaluso and Wikel, 2001), but not tick-susceptible hosts (Borsky et al., 1994; Schoeler et al., 2000). Taken together, these data indicates involvement of immunosuppressive mechanisms during tick infestations on susceptible hosts. It is also possible that ticks can be selectively injecting different molecules in one or another animal host species. Actually, salivary glands of *Rhipicephalus appendiculatus* ticks that were fed on cattle had reduced activity of glycoprotein secretory granules in comparison to ticks that were fed on rabbits (Walker et al., 1985). Alternatively, guinea pigs may perhaps overcome *R. sanguineus* tick immunosuppressive molecules and develop a protective immune response against ticks. In support of this, suppression of T-cell responses to mitogens is higher during a primary than in a subsequent infestation with *D. andersoni* ticks in resistant hosts (Wikel, 1982), suggesting a host ability to neutralize the immunosuppressive factors of saliva.

Impaired cellular proliferation observed in tick-infested mice could be due to several regulatory mechanisms, including the pattern of cytokine production developed (Ganapamo et al., 1995; Ferreira and Silva, 1999) and reduced secretion of IL-2 (Ribeiro et al., 1985; Urioste et al., 1994; Ferreira and Silva, 1998, 1999). Alternatively, it could be due to neutralization of the IL-2 function by an IL-2-binding protein, as described in the saliva of *I. scapularis* ticks (Gillespie et al., 2001). Reduced IL-2 function may lead to decreased T-helper and effector functions, including a reduction of DTH reactions, cytotoxic T-cell responses, as well as antibody production. Although we do not know the mechanism of action, we found at least two proteins in the saliva of *R. sanguineus* that decreased T-cell proliferation (Ferreira and Silva, 1998). Another immunosuppressive factor was identified in the salivary glands of *D. andersoni* ticks (Bergman et al., 1998).

Our results also showed that mice repeatedly infested with *R. sanguineus* ticks presented a clear immediate cutaneous hypersensitivity reaction to the tick extract and no detectable DTH. This pattern of response was similar to the one described for tick-infested dogs, but not for guinea pigs (resistant host), which exhibit both an immediate and a strong DTH reaction to a nymph tick extract (Szabó et al., 1995a). These data suggest that resistance to ticks is associated with a DTH reaction, which is mediated by a T helper 1-type cytokine response (Cher and Mosmann, 1987). In accordance, passive transfer of rIL-2 to rabbits during infestation with *I. ricinus* induced resistance to subsequent tick infestations and strong DTH reaction to a salivary gland extract (Schorderet and Brossard, 1994). In this context, the lack of resistance and the development of immediate-type hypersensitivity

could be due to a deviation of immunity to a Th2-type response. Indeed, a large body of data has indicated that tick-susceptible mice respond with polarization of the immune response towards Th2-lymphocytes (Ganapamo et al., 1995; Zeidner et al., 1997; Ferreira and Silva, 1999).

To further characterize reactions of mice to *R. sanguineus* ticks, we studied the cellular infiltrate at the tick-feeding site on once or thrice tick-infested mice. Neutrophils were predominant at *R. sanguineus* tick-feeding sites on mice, particularly during re-infestations, as also is observed in the tick-attachment site on dogs (Szabó and Bechara, 1999). Increased neutrophilic infiltration maybe due to molecules present in tick saliva (Berenberg et al., 1972) or to chemokines, which may be produced in the inflammatory focus (Gillitzer and Goebeler, 2001; Lira et al., 1994). However, despite the persistent presence of neutrophils, no significant gross alteration was observed at the tick-attachment site, suggesting that *R. sanguineus* tick saliva may contain anti-inflammatory molecules. Indeed, tick saliva can inhibit neutrophil function in vitro (Ribeiro et al., 1990; Inokuma et al., 1997). Therefore, saliva can modulate innate and adaptive immunity of the host ensuring successful blood-feeding. Eosinophils were also found to be enhanced at the tick-attachment site on mice. A rise in this cell population on the skin of tick-infested animals was also observed in other tick–host models, regardless of the acquisition of resistance (Mbow et al., 1994; Szabó and Bechara, 1999), suggesting that eosinophils are not involved in protective responses against ticks. Our data also revealed decrease and then a recovery of the percentage of mast cells at the tick-attachment site from the first to the third tick infestation. This phenomenon could be related to degranulation and regranulation, since degranulated mast cells cannot be seen in skin sections by routine histotechnology. Hence, we cannot associate mast cells with resistance or susceptibility to tick infestation. Overall, histopathological analysis of *R. sanguineus* tick-attachment sites on mice resembled more the kind of reaction of the susceptible natural host, the dog, than that of the guinea pig, the resistant host. Such observation strengthens the rationale of using mice as an alternative for dogs in studies of host immunity to *R. sanguineus* ticks.

Taken together, our data indicate that the host species that can develop acquired immunity against ticks are not immunosuppressed during the initial stages of tick–host interaction and, for this reason, develop a cellular immune response and protection against ticks. This possibility is compatible with the results showing that BALB/c mice infested twice with *D. andersoni* nymphs (resistant hosts) also developed a T-cell proliferative response to tick antigens and ConA (Macaluso and Wikel, 2001). On our model, *R. sanguineus* tick-infested mice and dogs presented a consistent immunosuppression during the tick fixation period, which impaired their T-cell response to tick antigens that could induce protection.

In conclusion, saliva introduced during tick infestations reduces the ability of a susceptible animal host to respond to tick antigens that could stimulate a protective immune response. As a consequence, the animals present a lack of DTH response and disturbed cellular migration to tick feeding site, which can represent a deficient response against ticks. Efforts are being employed on the identification of candidate antigens from tick saliva by fractionation and serological expression cloning of a tick salivary gland expression library. The candidate antigens will be identified based on their capacity of stimulating tick-resistant guinea pig T-cell proliferation.

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