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Identification of an IL-2 Binding Protein in the Saliva of the Lyme Disease Vector Tick, Ixodes scapularis

R. Dean Gillespie,2* Marc C. Dolan, † Joseph Piesman, † and Richard G. Titus*

A potent inhibitor of mitogen-stimulated T cell proliferation exists in the saliva of several species of hard ticks, including the Lyme disease vector tick, Ixodes scapularis. Our characterization of this phenomenon has led to the identification of a possible mechanism for the T cell inhibitory activity of I. scapularis saliva. The T cell inhibitor can overcome stimulation of mouse spleen cells with anti-CD3 mAb; however, a direct and avid interaction with T cells does not appear to be necessary. Tick saliva inhibits a mouse IL-2 capture ELISA, suggesting that a soluble IL-2 binding factor is present in the saliva. This hypothesis was verified by using a direct binding assay in which plate-immobilized tick saliva was shown to bind both mouse and human IL-2. Elimination of the IL-2 binding capacity of saliva in the in vitro assays by trypsin digestion demonstrated that the IL-2 binding factor is a protein. These experiments comprise the first demonstration of the existence of such a secreted IL-2 binding protein from any parasite or pathogen. This arthropod salivary IL-2 binding capacity provides a simple mechanism for the suppression of T cell proliferation as well as for the activity of other immune effector cells that are responsive to IL-2 stimulation. Relevance of the tick T cell inhibitory activity to the human immune system is demonstrated by the ability of tick saliva to inhibit proliferation of human T cells and CTLL-2 cells grown in the presence of human IL-2.


Interactions between blood-feeding arthropods and their hosts may have profound implications for the transmission of vector-borne pathogens (1–4). Of all the blood-feeding vector arthropods, hard ticks, by virtue of their protracted feeding period, represent an extreme example of evasion of their host’s hemostatic defenses and immune response (5–8). Ticks are obligate blood-feeding ectoparasites, with many species remaining attached for periods of 3–15 days (9, 10). Many tick species are important vectors of disease worldwide. Ixodes scapularis is the main North American vector of Lyme disease, now the most prevalent vector-borne disease reported in the United States (11), as well as human granulocytic ehrlichiosis and babesiosis (12–15). Because ticks deliver the molecules used to confound host defenses in their saliva, this secretion is an important feeding and vector competence factor for these ectoparasites (9).

Several pharmacological activities have been characterized in tick saliva with important roles in blood feeding, including vasodilators, anticoagulants, and anesthetics (5, 6, 8, 9, 16). This body of observations makes it clear that ticks have evolved a remarkable array of biochemical weapons to keep the host at bay and assure their own success as a species. The protective functions of vector saliva may also have the important, though presumably inadvertent, effect of potentiating the transmission of various pathogens to hosts through the alteration of the immunologic microenvironment at the feeding site (1, 2) or the lymph nodes draining the site of attachment (17–20).

Many examples of immunomodulatory functions of tick saliva have been described in various tick species that are disease vectors to humans and livestock (reviewed in Ref. 7 and 21). Much attention has been given to the nature of the immune response to ticks as well as to the effects of tick infestations on the host’s immune competence (7, 9, 21). Tick infestation of mice has been shown to result in significant modulations of cytokine production, with a general pattern of the inhibition of proinflammatory and Th1 cytokines accompanied by enhancement of Th2 cytokines (19, 22, 23).

It has been observed that the saliva or salivary gland extracts (SGE)3 of several tick species, including I. scapularis (16, 24), profoundly inhibit the proliferative response of mitogen-stimulated T lymphocytes. This phenomenon has been demonstrated with mouse T cells by using Con A (16, 23–28), PHA (24), and anti-CD3 (20). This effect has also been demonstrated on bovine lymphocytes with Con A stimulation (29, 30). This putative immunomodulatory phenomenon is particularly intriguing because of the central role played by T cells in the orchestration of the acquired immune response (31). Determining the mechanism of this inhibitory effect of saliva may have important ramifications regarding the inability of the host to mount effective immune responses to ticks and the pathogens they transmit.

Previous work with I. scapularis saliva has shown that this phenomenon is mediated by a protein(s) due to its ability to trypsin treatment. This inhibitory effect was not dependent on mouse haplotype or the T cell mitogenic lectin used to drive proliferation (24). It was also shown that the high levels of PGE2 in tick saliva cannot account for the degree of inhibition of mouse T cell proliferation caused by saliva (24). In contrast, evidence that PGE2 in Borrelia microplus saliva is the mediator of this effect on bovine lymphocytes does exist (29). Furthermore, it was demonstrated

*Department of Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523; and † United States Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Center for Infectious Diseases, Division of Vector-Borne Infectious Diseases, Fort Collins, CO 80522

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2Address correspondence and reprint requests to Dr. R. Dean Gillespie, Department of Pathology, Colorado State University, Fort Collins, CO 80523. E-mail address: dgillesp@lamar.colostate.edu

3Abbreviations used in this paper: SGE, salivary gland extracts; IL-2BP, IL-2 binding protein; TIP, T cell inhibitory protein; SBTI, soybean trypsin inhibitor; SC, spleen cells; rm, recombinant mouse; rh, recombinant human; bio, biotinylated; IGBP, Ig binding proteins; DTH, delayed-type hypersensitivity.
with ELISA and bioassays that treatment of spleen cells (SC) with tick saliva resulted in a decrease in IL-2 production (24). This observation has also been made by others working with different tick species (20, 23, 27, 30).

Our present work was undertaken to determine the mechanism by which tick saliva nonspecifically inhibits T cell proliferation. We report here on the identification of a proteinaceous tick salivary factor that binds murine and human IL-2. Furthermore, we demonstrate that tick saliva inhibits mitogen-driven human T cell proliferation.

Materials and Methods

Animals

Adult C57BL/6 mice, >6 wk old, were obtained from National Cancer Institute (Bethesda, MD). Female New Zealand White rabbits, 5–7 wk old, were obtained from Western Oregon Rabbit (Philomath, OR).

Human PBMC preparations

Blood was obtained from healthy human donors at the Student Health Center of Colorado State University (Fort Collins, CO). PBMC were isolated from heparinized venous blood by separation on a Ficoll-Hypaque gradient (32). The cells were washed and resuspended at 1 × 10^6/ml in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 10% heat-inactivated AB human serum (Pel-Freez Biologicals, New York, NY).

Reagents and chemicals

The anti-CD3 mAb-producing clone 145-2C11 (CRL-1975) (33) was obtained from American Type Culture Collection (Manassas, VA). [3H]Tdr (5 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Pilocarpine (P-6503), PHA-L (leucoagglutinin; L-2769), trypsin (T-2271), and soybean trypsin inhibitor (SBTI; T-6522) were obtained from Sigma (St. Louis, MO). Recombinant mouse (rm)IL-2 (212-12), recombinant human (rh)IL-2 (200-02), and rmIFN-γ (315-05) were obtained from PeproTech (Rocky Hill, NJ).

Ticks and saliva collection

All experiments were done with saliva from field-isolated ticks. Unfed adult female ticks were collected by flagging vegetation at a site in Bridgeport, CT (34) during the fall (September- November) in 1996–1999. For saliva collection, ticks were allowed to feed on the ears of rabbits for 5–7 days. Near-replete and replete ticks were removed and immobilized, a finely drawn capillary tube was fitted over their mouthparts, and 2–5 μl of 5% pilocarpine in methanol was applied topically to their dorsa (35). Saliva was collected over 1–2 h in a 37°C environment, pooled, and stored at −70°C until used. For cellular assays, saliva was sterilized with a 0.22-μm syringe filter before use. Except when indicated, the results depicted in the figures are from independent batches of saliva; however, each experiment described has been replicated with different batches of saliva.

Proliferation assays

Mouse SC were prepared as described previously at 5 × 10^5 cells/ml in supplemented DMEM (24) and seeded at 5 × 10^3 well. Cultures were maintained at 37°C, 5% CO2 during the assays. The cells were stimulated with an anti-CD3 mAb culture supernatant at 1:500 in the presence of saliva (in triplicate) as described in Fig. 6. After 24 h, the cultures were pulsed with 1 μCi/well of [3H]Tdr for 18 h followed by harvesting and scintillation counting to determine the proliferative response.

Human PBMC were plated at 1 × 10^6 cells/ml in 200 μl of medium (see above for preparation details). Saliva was added to triplicate wells as indicated in Results and Fig. 1 and immediately stimulated with 2 μg/ml PHA. The cultures were maintained at 37°C, 5% CO2, during the assay; at 24 h, the wells were pulsed with 1 μCi/well of [3H]Tdr for 18 h before harvesting and scintillation counting.

CTLL-2 cell assays

CTLL-2 cells were maintained in complete RPMI 10 medium in the presence of 1 U/ml human IL-2 (36). All assays were performed 2–3 days after the last cell feeding. Cells were washed three times in sterile PBS and resedeed at 5000 cells/well in a final volume of 50 μl in the presence of human or mouse IL-2 and saliva as described in the Results and Fig. 7. The cultures were maintained at 37°C, 5% CO2, during the assay. At 18 h, the wells were pulsed with 1 μCi/well of [3H]Tdr for 8 h before harvesting and scintillation counting.

Preincubations of IL-2 with the T cell inhibitory protein (TIP)-containing fraction were done in polypolyethylene tubes for 90 min in 10 mM HEPEs (pH 7.3), 150 mM NaCl. The TIP fraction of tick saliva was obtained by gel filtration HPLC of 400 μl of saliva (360 μg protein) on a Bio-Rad Bio-Sil size exclusion 125-5 column (125-0475; Bio-Rad, Richmond, CA) that was perfused in 10 mM HEPEs (pH 7.3) and 150 mM NaCl. Parameters of the HPLC were essentially as described previously (37).

IL-2 and IFN-γ ELISAs

The IL-2-specific and IFN-γ-specific ELISAs were performed using matched sets of rat mAbs obtained from BD PharMingen (San Diego, CA), according to the manufacturer’s suggested protocol, on Nunc Pass(Sor plates (439454; Nunc, Naperville, IL). A rat anti-mouse IL-2 capture mAb (rat IgG2a; 18161D) was used to capture IL-2. Biotinylated rat anti-mouse IL-2 mAb (rat IgG2b; 18172D) was used to detect captured IL-2. A rat anti-mouse IFN-γ mAb (rat IgG1; 18181D) was used to capture IFN-γ, and the biotinylated rat anti-mouse IFN-γ mAb (rat IgG1; 18112D) was used to detect captured IFN-γ. Both assays were developed with avidin-HRP (A-3151; Sigma), and 3,3’,5,5’-tetramethylbenzidine (TMB; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used as the substrate.

Preincubation of saliva with mouse IL-2 and IFN-γ was done in 96-well polypolyethylene plates (Costar 3590; Corning Glass, Corning, NY). A serial dilution of saliva was prepared in DMEM supplemented with 5% FCS. A serial dilution of the cytokine mouse IL-2 or IFN-γ was also prepared in DMEM supplemented with 5% FCS. Equal volumes of each serially diluted reagent were combined on transverse axes, 0–1 μl saliva on one axis and 0–4 μg of cytokine on the other, and incubated for 2–4 h at 37°C. The saliva-cytokine mixtures were then transferred to the capture Ab-coated plate and incubated overnight at 4°C. The ELISA was completed using standard procedures.

A control ELISA designed to test for possible Ab-binding by plated saliva was performed as follows. Saliva (1 μl/well) was immobilized in wells as described below. Following a wash step, wells were blocked with Superblock (37353; Pierce, Rockford, IL). After another wash step, each of the Abs added to the ELISA was washed with the wells at the concentrations used in the ELISAs and incubated for 2 h at room temperature. The samples were washed again and then incubated with a 1:500 dilution of HRP-conjugated goat anti-rat IgG (H+L) (14-160-biopanhn12; Kirkegaard & Perry Laboratories) in 5% FCS/1× PBS (pH 7.4) for 45 min before a final wash and developed as described above.

Biotinylation of cytokines

rmIL-2, rhIL-2, and rmIFN-γ were biotinylated using aminohexanoyl-biotin-N-hydroxysuccinimide ester (Zymed, San Francisco, CA) and standard protocols for biotinylation of proteins in solution (38). To clear the biotinylated cytokines of excess quenched aminohexanoyl-biotin-N-hydroxysuccinimide ester and exchange buffers, the biotinylation reactions were filtered on Microcon ultrafiltration units with a molecular mass cutoff of 10 kDa that were passivated with Tween 20 following the manufactur er’s suggested procedure (Millipore, Bedford, MA). The filter was washed twice with 400 μl 1× PBS plus MgCl2 and CaCl2, and then the biotinylated cytokine was collected from the membrane in 100 μl 1× PBS plus MgCl2 and CaCl2.

The concentration of the recovered biotinylated cytokines was determined using competitive capture ELISAs with unlabeled cytokines of known concentration as standards (39). The concentration of the cytokines before dilution were: biotinylated (bio)-rmIL-2, 1.26 ng/μl; bio-rhIL-2, 0.3 ng/μl; and bio-rmIFN-γ, 1.34 ng/μl.

Assays with plate-bound tick saliva

A serial dilution of tick saliva was plated on Nunc Pass Sor plates in standard ELISA carbonate buffer (pH 9.6) overnight at 4°C. After washing with 1× PBS (pH 7.4), the plates were blocked with 5% BSA/0.1% Tween 20/1× PBS (pH 7.4). Following another wash step, an excess of bio-rhIL-2, -rhIL-2, or -rmIFN-γ was added to the plate for 4 h at room temperature; the plate was then washed again. A blank control of saliva-coated wells processed without a biotinylated cytokine incubation step was also run. The plate was then incubated with avidin-HRP for 30 min followed by a final wash step and development with tetramethylbenzidine reagents (Kirkegaard & Perry Laboratories). The reactions were stopped with sulfuric acid before the absorbance was read at 450 nm. Before graphing, the blank control absorbance values were subtracted from the data, and the absorbance values were normalized to account for differential levels of biotinylation of the various cytokines used. The specific activity of each
biotinylated cytokine was determined by graphical analysis of the slope of the linear data range in the quantitative competitive ELISAs, described above. Ratios of the slopes were used to derive normalization factors for the various cytokines. The normalization factors relative to the signal generated by bio-rmIL-2, which was set at a value of 1, were determined to be 1.81 for bio-rhIL-2 and 1.65 for bio-rmIFN-γ. Thus, the specific activity of the bio-rmIFN-γ and bio-rhIL-2 are nearly equivalent, whereas the bio-rmIL-2 specific activity is greater than either of the other reagents.

In the competitive binding assay, 2 μl of tick saliva was immobilized in each well of the assay plate as above. Bio-rmIL-2 was premixed with unlabeled rmIL-2 at the concentrations indicated in the Results and Fig. 4 in 5% FCS supplemented with 1× PBS (pH 7.4) in polypropylene wells. These samples were transferred to the plates with immobilized saliva and incubated for 3 h at room temperature. The assay was completed as were the noncompetitive assays. The positive control for bio-rmIFN-γ was done using the plate-bound capture Ab for the IFN-γ ELISA (described above).

**Tryptic digestion of saliva**

An aliquot of *I. scapularis* saliva was mixed with an equal volume of 1 mg/ml trypsin prepared in 10 mM Tris-HCl and 5 mM CaCl₂ (pH 8). The negative control was prepared with water in place of saliva, and the positive control was prepared with 10 mM Tris-HCl (pH 8) and 5 mM CaCl₂ without trypsin. Trypsin-only and saliva-only controls were also prepared. All samples were incubated for 24 h at 37°C and then stopped with a 2× mass excess (relative to trypsin) of 10 mg/ml SBTI prepared in dH₂O. An equivalent volume of dH₂O was added to samples that were not treated with SBTI. One trypsin-only sample was combined with the saliva-only sample to generate the “saliva with trypsin plus SBTI” control. The various treatment samples were then divided into two portions that were tested, respectively, in the IL-2 ELISA interference assay and the plate-bound saliva bio-rmIL-2 binding assays described above and in Results.

**Statistics**

The human proliferation assays were analyzed by ANOVA and the Student-Newman-Keuls method for pairwise multiple comparisons. Values of p < 0.05 were accepted as indicating a significant difference between treatments. Other experiments were run a minimum of three times, and the results of representative experiments are graphed as the mean of triplicate samples ± SD.

**Results**

The T cell proliferation inhibitory activity of *I. scapularis* saliva can be washed away after several hours of preincubation with splenocytes

The simplest apparent model for the T cell inhibitory capacity of tick saliva would be a mechanism dependent upon a direct interaction of a salivary factor(s) with T cells to mediate suppressive effects on T cell proliferation. Previous work established that preincubation of saliva with SC was not necessary to see the inhibitory effect of saliva (24). This observation was extended by testing the ability of tick saliva to mediate its effect on SC if the cells were preincubated with saliva for several hours and then washed extensively to remove saliva proteins that were not tightly interacting with cells before mitogenic stimulation. The data depicted in Fig. 1 show that the ability to inhibit T cell activity can be washed away from SC even after 2–4 h of preincubation before stimulation. From looking at the results with the unwashed saliva-treated samples, it is clear that the ability to inhibit is still present even after 4 h of preincubation. These results suggest that, although saliva must be present to mediate a suppressive effect on proliferation, a direct and avid interaction with T cells does not occur.

*I. scapularis* saliva inhibits detection of mouse IL-2 in an ELISA

The previous experiment presented the possibility that the salivary T cell inhibitor does not interact directly with T cells. Working with this information and the results of previous studies showing decreases in the amount of IL-2 present in culture supernatants of Con A-stimulated SC that were treated with saliva (24), we hypothesized that saliva was binding IL-2. We tested this using a mouse IL-2-specific ELISA, as shown in Fig. 2A. For this experiment, tick saliva was preincubated with mouse IL-2 before being transferred to microtiter wells coated with the IL-2 capture Ab. As depicted in Fig. 2A, the detection of IL-2 in this ELISA is diminished in a dose-dependent fashion both in terms of the amount of saliva and the amount of IL-2 present. This result supports the presence of an IL-2 binding factor in tick saliva that blocks one or both of the epitopes recognized by the matched anti-IL-2 mAbs resulting in decreased IL-2 detection in the ELISA. This activity was not influenced by the glycosylation state of the mouse IL-2, because detection of both rIL-2 produced in Escherichia coli and native IL-2 from SC culture supernatants were inhibited (data not shown).

A control experiment consisting of an ELISA specific for mouse IFN-γ was run using the same batch of saliva to test the specificity of the IL-2 binding factor. This T cell cytokine was chosen as a control because its production has also been shown to be markedly decreased by in vitro-stimulated lymphocytes in the presence of SGE (19, 22, 23, 30, 40). As shown in Fig. 2B, tick saliva does not affect the detection of rmIFN-γ in a dose-dependent manner, illustrating the specificity of the IL-2 binding activity defined in Fig. 2A and also that *I. scapularis* saliva does not appear to have IFN-γ binding capacity under the conditions tested.

*I. scapularis* saliva binds both mouse and human IL-2

To directly verify the IL-2 binding capacity and specificity of the *I. scapularis* IL-2 binding activity, tick saliva was plated in microwells, and then its ability to bind bio-IL-2 was determined. Although mouse and human IL-2 sequences are 63% conserved at the amino acid level (41), they are not fully biologically interchangeable (42). Thus, the ability of saliva to bind both mouse and human IL-2 was examined. The specificity for IL-2 was again demonstrated (5). The IL-2 binding activity, tick saliva does not affect the detection of rmIL-2 in an ELISA (described above).
implies a similar binding affinity for both species of IL-2. However, the saliva does not show evidence of bio-rmIFN-γ binding, even when up to 8 μl of saliva is plated. The signal from bound bio-IL-2 titrates with the decrease in saliva plated; thus, plate-immobilized saliva retains its ability to bind the IL-2 ligand. The absence of significant binding to bio-rmIFN-γ also shows that the binding of the bio-IL-2 is not due to biotin binding activity in tick saliva. Repeating this analysis with different batches of saliva showed that the amount of IL-2 binding activity varies, but is always present. This batch-to-batch variance was also evident when the IL-2 ELISA interference assay was used (data not shown).

The binding of bio-rmIL-2 by plated saliva can be specifically competed away with rmIL-2

As a more direct means of assessing the specificity of the IL-2 binding capacity of the saliva, the direct plate-binding assay was adapted to a competitive binding format. This assay was also important as a control for nonspecific signals generated by potential protein contaminants present in the commercially obtained preparations of rmIL-2 that were biotinylated during the generation of bio-rmIL-2. The IL-2 supplier was chosen because they do not add any carrier protein to their recombinant cytokines. The rmIL-2 and rhIL-2 were HPLC-purified to >97%, and the rmIFN-γ was >95% pure. A constant amount of saliva was bound to each well and then incubated with a mixture that contained a constant amount of bio-rmIL-2 (~13 pg) and varying amounts of nonbiotinylated competitor rmIL-2. As illustrated in Fig. 4, the signal from binding of bio-rmIL-2 was competed away by addition of between 8- and 16-fold of unlabeled competitor and is essentially the same as theoretically predicted for a competitive inhibitor with equal affinity for the binding site. As a negative control, the assay was also run in parallel in wells that were not plated with saliva.

The IL-2 binding factor is a protein

Because the majority of the inhibitory effect of tick saliva on T cell proliferation has previously been shown to be due to a protein (24), we used the same approach to test whether the IL-2 binding activity was due to a protein in the saliva. Saliva was digested with trypsin for 24 h, the reaction was stopped with excess SBTI, and then tested for its ability to inhibit the IL-2 ELISA and bind bio-rmIFN-γ using the assay systems described above. As shown in Fig. 5 (groups C), trypsin treatment eliminates the IL-2 binding capacity of the saliva; therefore, the IL-2 binding factor is a protein. Control reactions showed that trypsin treated with SBTI did not affect either in vitro assay when added alone (Fig. 5, groups D) and that saliva was still capable of binding IL-2 when trypsin treated with SBTI was added to each assay system (Fig. 5, groups E).

I. scapularis saliva inhibits proliferation of human T cells

Previous experimentation has established that tick saliva contains a potent T cell proliferation inhibitor by using lymphocytes from a variety of animal models including mice and cows. Due to the identification of a novel cytokine binding activity in tick saliva (described above), and because of the importance of I. scapularis as a vector of human pathogens (11, 12, 15), we tested the ability of tick saliva to inhibit human T cell proliferation. For these experiments, PBMCs were isolated from healthy human donors and
stimulated with the T cell mitogenic lectin, PHA. As is evident in Fig. 6, proliferation of T cells from all six donors was diminished from 52–88% when treated with a 1:100 dilution of saliva (p < 0.05), and the effect of the saliva could be diluted away, as the difference between each of the three treatment groups is significant (p < 0.05).

Saliva inhibits growth of an IL-2-dependent cell line

To assess the suggested mechanism of TIP as an IL-2 binding protein (IL-2BP), the effects of the saliva on the growth of the IL-2-dependent cell line CTLL-2 were tested. As CTLL-2 cells can be stimulated with both human and mouse IL-2 (36), this approach allows the hypothesis to be addressed with a bioassay system. It was found in preliminary experiments that CTLL-2 cells are very sensitive to even low concentrations of PGE2, which is present in tick saliva at relatively high levels (24, 43). Thus, to obtain TIP without contaminating PGs, the TIP-containing fraction of *I. scapularis* saliva was purified using gel filtration HPLC. All three assays for TIP (described above) showed that the TIP activity co-fractionated with an apparent mass of ~60 kDa (data not shown). As shown in Fig. 7A, proliferation of CTLL-2 cells grown in the presence of 10 pg of human or mouse IL-2 are inhibited in a dose-dependent manner by TIP.

If the inhibition of CTLL-2 proliferation is indeed the result of IL-2BP neutralization of growth factor activity of IL-2, it should be possible to negate this effect by adding extra IL-2 to the assay. This experiment was done by preincubating IL-2 and TIP before addition to the CTLL-2 cells. Results for the mouse and human IL-2 experiments are shown in Fig. 7B, where this prediction is fulfilled.

Discussion

The activation of T cells plays an important role in the orchestration of both humoral and cellular immune responses. Although a large body of evidence supports the existence of nonspecific T cell inhibitory activities in the saliva of several species of ixodid ticks (16, 20, 23–29), with one exception (29), definitive experiments aimed at elucidating the mechanism of T cell proliferation inhibitory factors have been lacking. Toward this end, Urioste et al. (24) demonstrated that the major T cell inhibitory activity in *I. scapularis* saliva is proteinaceous due to its inactivation by trypsin treatment. Bergman et al. have also recently identified a 36-kDa protein in tick SGE of *Dermacentor andersoni* that inhibits T cell proliferation (26, 28).

One of the hallmarks of T cell activation is the production of IL-2, which serves as an autocrine growth factor (44). Thus, it is not surprising that many studies have also identified a decrease in IL-2 production by lymphocytes exposed to tick SGE or saliva in vitro (16, 23, 24, 27) or in ex vivo-restimulated lymphocytes from tick-infested animals (20, 22). The full importance of this observation is brought into perspective when it is considered that IL-2 also acts as a paracrine growth factor and signaling molecule to activated T cells and other immune effector cells that express IL-2 receptors on their surface, including B cells, NK cells, CTLLs, lymphokine-activated killers, monocytes, and macrophages (44–46). Thus, the nonspecific effect of tick saliva on T cell activation has potentially far-reaching consequences in the generation of anti-tick immunity and pathogen transmission. Of interest in this regard, it has been observed that the functions of NK cells (47, 48) and macrophages (24, 27) are also inhibited by tick saliva.

In the search for a mechanism of the T cell inhibitory action of tick saliva, we hypothesized that the salivary factor(s) responsible for inhibiting T cell proliferation interacted directly with T cells. The ability to wash away saliva without residual inhibition of proliferation demonstrated that this hypothesis was probably incorrect. Although the results do not eliminate the possibility that T
cell inhibitors interact directly with T cells, they indicate that any interactions are likely to be weak or to occur after T cell activation. Furthermore, this experiment also illustrates the temporal requirement for the presence of saliva during and after mitogenic stimulation of SC to mediate its effect on the in vitro proliferation of T cells. This result led us to test whether tick saliva was directly interfering with IL-2 activity using an IL-2-specific ELISA. The results of this analysis were in keeping with the observation that the presence of saliva was necessary for the inhibited proliferation to be evident. T cells produce IL-2 when they are stimulated, so the IL-2BP cannot function until after T cell activation. Previous studies have also shown that IFN-γ production is decreased by tick SGE exposure (23, 30, 40) or tick infestations (19, 22). Although our analysis shows that *I. scapularis* saliva does not appear to bind IFN-γ under the tested conditions, it is known that IL-2 induces T cells and NK cells to produce IFN-γ (49). Thus, it is possible that the IL-2BP is responsible for several identified effects of tick saliva on host immune effector cells; however, clarifying this will require further studies that address causal linkages between the observed phenomena.

The identification of Ig binding proteins (IGBPs) in the saliva of several species of ixodid ticks (50, 51) raises the caveat that the inhibition of the IL-2 ELISA is due to IGBP (though this activity has not been reported in *I. scapularis*). The lack of IFN-γ ELISA inhibition by the saliva dispels this concern. However, because the isotypes of the matched pairs of rat IgG anti-cytokine mAbs used in the ELISAs were not all the same, the ability of plate-bound *I. scapularis* saliva to bind each of the mAbs used was tested (see Materials and Methods). This experiment showed that there was not significant binding of any of the rat mAbs by plate-bound saliva (data not shown). Thus, IGBP activity is not a concern in the interpretation of the results of the experiments depicted in Fig. 2.

Most previous analyses of the immunomodulatory effects of tick saliva have focused on animal models, leaving the relevance to humans unaddressed. It has been suggested that ectoparasites are closely adapted to their hosts (5) so that they can counteract their hosts’ defenses. This idea has also been used to support observations that more robust resistance to tick feeding has typically been associated with unnatural tick-host associations (5, 21). In one report, Fuchsberger et al. demonstrated that Rhipicephalus appendiculatus SGE inhibits expression of several cytokine mRNAs in LPS-stimulated human PBMCs (40). Although they were not able to detect IL-2 mRNA, they did recognize that SGE effects on cytokine expression levels were dependent on stimulation of the PBMCs. Due to the significance of *I. scapularis* as a vector for several emerging human diseases, we included experiments that showed that tick saliva binds not only to mouse IL-2, but also to human IL-2 with similar affinities. Interspecies cross-reactivity of the IL-2BP points to the likelihood that the binding site on IL-2 is a conserved region or feature of the molecule. Furthermore, we used PBMCs from human donors to show that *I. scapularis* saliva acts as an inhibitor of human T cell proliferation. This finding was further supported by the use of a CTLL-2 cell bioassay to show that binding of IL-2 and neutralization of its growth factor activity appears to be the mechanism responsible for inhibition of both human and mouse T cell proliferation. These experiments support the idea that tick saliva may play a role in the transmission of
diseases to humans and that the same phenomena identified in animal models are likely to occur in humans exposed to tick bites. The studies reported here were done with saliva from adult ticks, whereas, in the field, nymphal ticks are the primary threat for disease transmission to humans. Studies of saliva and SGE of nymphs are exceedingly challenging due to the difficulty of obtaining sufficient amounts of material for detailed analysis. However, we have done preliminary experiments that verified that T cell proliferation is inhibited by nymph SGE (R. D. Gillespie, unpublished data).

Support for the proposed IL-2 binding mechanism of inhibited T cell proliferation is provided indirectly by other studies. Zeidner et al. demonstrated that the passive administration of IL-2 to mice during challenge feeding by *I. scapularis* nymphs infected with the Lyme disease spirochete, *Borrelia burgdorferi*, resulted in 50% protection from infection (52). In studies of the effects of protective rhIL-2 administration to rabbits during infestation with *Ixodes ricinus* adults, the European vector of Lyme disease spirochetes, it was found that the rabbits were more resistant to subsequent tick infestations and developed dramatically stronger delayed-type hypersensitivity (DTH) reactions against SGE (53). Furthermore, the development of DTH to SGE occurred in the IL-2-treated animals after only one infestation, compared with the multiple infestations usually necessary for DTH development to SGE of *I. ricinus* (54). The importance of IL-2 to this phenomenon was established by showing that cyclosporin A treatment resulted in attenuation of the DTH response to SGE in rabbits (54). In a similar study, it was shown that acquired resistance to *I. ricinus* by the bank vole (a natural host for the ectoparasite) could be partially disrupted by cyclosporin A treatment (55).

Experiments using *I. ricinus* infestations of mice have shown that the effects of tick saliva are mediated at the attachment site and the lymph nodes draining the area (17–21), although systemic effects have also been documented with *I. scapularis* (22, 56). The IL-2 ELISA inhibition data show that 1 μl of saliva is capable of binding ~4 pg of IL-2, although we routinely measure binding capacities ranging between ~1 and 6 pg/μl in different batches of saliva using this assay. This variability is not surprising, because the ticks are not truly synchronized in their feeding/salivation state; thus, the simple and elegant mechanism of tick salivary IL-2BP, the sequestration of IL-2, has multiple potential effects on the immune system of the host. At the local level of the draining lymph node, IL-2 sequestration by IL-2BP would effect the proliferation of naïve T cells and Th1 cells, as Th2 cells are not dependent on IL-2 for expansion after Ag stimulation (57, 58). This scenario fits nicely with the generalized model of Th1 cell and inflammation suppression and Th2 cell enhancement caused by tick infestations (19–23). It is known that starvation for IL-2 in activated T cells can lead to anergy of the activated cells (59, 60); thus, IL-2BP may adversely effect the ability of a host to develop adaptive immunity to ticks and tick-borne pathogens. The full biological consequences of this activity are likely to depend on the issue of scale, both with respect to the developmental stage of the tick, i.e., larvae, nymph, or adult, and degree of infestation, e.g., a heavy infestation on a small animal vs one or a few ticks on a large animal.

It is important to note that recent studies using mice (61) and guinea pigs (62) have indicated that it is possible to generate adaptive immunity to *I. scapularis* that also results in the blocking of transmission of *B. burgdorferi*. This strategy required multiple infestations of the host with uninfected nymphal ticks before challenge with infected nymphs. However, this approach did not result in protection when using a mouse model for *I. ricinus* (63). Despite variable success, these studies provide the impetus for the pursuit of tick Ags that can protect the host from ticks and tick-borne pathogens.

The identification of a secreted IL-2 binding factor is quite novel for an ectoparasite system, and, to the best of our knowledge, the IL-2BP in *I. scapularis* saliva represents the first evidence for direct IL-2 targeting by any parasite or pathogen of mammals. Various means of subverting the immune system of hosts through their cytokines and chemokines have evolved in several classes of pathogens, although none of these directly target IL-2 (64). It has been suggested that no viral or bacterial pathogens have directly targeted IL-2 because the general immunosuppression that would ensue might give other pathogens a chance to successfully compete against them (64). Perhaps ectoparasites such as *I. scapularis* can succeed with the strategy of direct subversion of a central signaling cytokine because they do not multiply within the host. Alternatively, if the effect of tick saliva on host immune function is predominantly local rather than systemic, direct IL-2 targeting may not adversely effect the entire immune system of the host. In either case, this strategy may be important to the effectiveness of ticks as disease vectors.

The IL-2BP could be important to the potentiation of the feeding site for transmission of pathogens by aiding establishment of the infection. Indeed, the saliva of the vector should not only be considered a pharmacological milieu that allows for blood to be drawn over a protracted period and a vehicle for the transmission of pathogens, but also as an important vector competence factor (9).

Although we have functionally identified an IL-2BP in *I. scapularis* that may provide the mechanism for the inhibition of T cell proliferation by tick saliva, it will now be useful to know whether this IL-2 binding capacity is found in other tick species or other blood-feeding ectoparasites. Furthermore, is this the only example of cytokine-targeted host immunomodulation present in arthropods or other ectoparasites? This discovery can be seen as another example of how well evolved ectoparasite-host interactions appear to be. Thus, the simple and elegant mechanism of tick salivary IL-2BP, the sequestration of IL-2, has multiple potential effects on the quality and specificity of the immune response generated to other foreign Ags present during the feeding process, including those of pathogens introduced at the feeding site. Given the nature of this activity’s assault on the immune system of the host, the discovery of this mechanism also raises important issues regarding the development of vaccines against ticks, an idea that has gained much support because it holds the potential of blocking transmission of several pathogens with a single vaccination (7, 8). The IL-2BP may inhibit the effectiveness of this approach and, thus, may turn out to be an important target that must be overcome for this strategy to be successful.

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**References**

