Saliva of the Lyme Disease Vector, Ixodes dammini, Blocks Cell Activation by a Nonprostaglandin E₂-dependent Mechanism

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Summary

Tick-borne pathogens would appear to be vulnerable to vertebrate host immune responses during the protracted duration of feeding required by their vectors. However, tick salivary components deposited during feeding may inhibit hemostasis and induce immunosuppression. The mode of action and the nature of immunosuppressive salivary components remains poorly described. We determined that saliva from the main vector of the agent of Lyme disease, Ixodes dammini, profoundly inhibited splenic T cell proliferation in response to stimulation with concanavalin A or phytohemagglutin, in a dose-dependent manner. In addition, interleukin 2 secretion by the T cells was markedly diminished by saliva. Tick saliva also profoundly suppressed nitric oxide production by macrophages stimulated with lipopolysaccharide. Finally, we analyzed the molecular basis for the immunosuppressive effects of saliva and discovered that the molecule in saliva responsible for our observations was not PGE₂, as hypothesized by others, but rather, was a protein of 5,000 mol wt or higher.

In contrast to most hematophagous arthropods which feed rapidly, ixodid ticks require days to weeks to feed to repletion (1). This fact would appear to offer at least two obstacles to successful blood feeding for ticks. First, ticks must maintain blood flow and prevent blood clotting over this extended period. Ticks may circumvent these problems via pharmacologically active components in their saliva which could aid in blood feeding (2, 3, and reviewed in 4). For example, the saliva of Ixodes dammini, the tick vector of the agent of Lyme disease, contains apyrase, PGE₂, and prostacyclin which may prevent hemostasis and inflammation such that blood flow is enhanced (2–4). Second, because of the long period of time the tick remains attached to the host, certain hosts (especially unnatural ones [5]) are successful in mounting a nonspecific (neutrophils surrounding the mouthparts of the feeding tick [6]) and even a specific (T and B cell [5, 7–9]) anti-tick immune response. In fact, this immune response can be directed against components of the salivary gland itself (7, 10). This anti-tick response can decrease the tick's blood-feeding success and ultimately cause rejection of the tick. Therefore, in order to maintain feeding success, hard ticks would appear to require antiinflammatory and immunosuppressive elements in their salivary armamentarium, in addition to antihemostatic and vasodilatory activities.

The pathogens transmitted by ticks are similarly exposed to host inflammatory and immune responses at their site of inoculation. The dense cellular infiltrate that can characterize the feeding cavity surrounding a tick's mouthparts would appear to serve as an effective barrier to the entry of pathogens. Yet these pathogens still manage to infect the host and, therefore, the pathogens may also exploit the same salivary pharmacological and immunosuppressive activities that ensure successful tick feeding. Indeed, I. dammini saliva inhibits phagocytosis of the Lyme disease spirochete in vitro (11) which may enhance infectivity of the spirochete for the host. In addition, antibody responses to the Lyme disease spirochete occur rapidly when animals are infected with the spirochete by needle inoculation but these responses are greatly delayed or are nonexistent in animals infected with similar numbers of spirochetes delivered by infected ticks (12, 13). This suggests that tick saliva modifies the host-specific T and B cell response to the Lyme spirochete. However, the immunological mechanisms that underlie these observations remain poorly understood. It has been suggested by several authors (2, 3, 14–16) that the PGE₂ present in tick saliva mediates immunosuppression. Indeed, PGE₂ has effects on the immune system at several levels including the ability to downregulate the functions of T and B cells (17).

In an effort to explain how ticks might inhibit the development of effective host immunity to themselves and to the pathogens they transmit, we have used a T cell mitogen-driven response to screen for immunosuppressive effects of I. dammini saliva. If tick saliva were immunosuppressive, it should act in a nonspecific fashion and suppress even a T cell mito-

genic response. We report here that *I. dammini* saliva profoundly inhibited the mitogenic response of normal murine spleen cells (SC)[1] to the T cell mitogens Con A and PHA both with respect to T cell proliferation and IL-2 secretion. In addition, saliva prevented nitric oxide (NO) production by macrophages activated with LPS. Finally, the immunosuppressive effect of saliva was not due to PGE$_2$, but instead, was mediated by a protein of 5,000 mol wt or higher.

Materials and Methods

Animals. Adult female C57BL/6 and C3H/H mice were obtained from Taconic Farms (Germantown, NY). Adult female New Zealand white rabbits were purchased from Millbrook Farms (Wilmington, MA).

Reagents and Chemicals. Con A was purchased from Miles Laboratories, Inc. (Naperville, IL), PHA from Pharmacia (PHA-P; Uppsala, Sweden), and LPS (W *Escherichia coli* 055:B5) from Difco Laboratories (Detroit, MI). [^{3}H]methylthymidine ([^{3}H]TdR), 5 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). PGE$_2$ (P-6532), pilocarpine (P-6503), trypsin (T-2271), and soy bean trypsin inhibitor (SBTI; T-6522) were purchased from Sigma Chemical Co. (St. Louis, MO).

Ticks. Unfed adult female *I. dammini* ticks were collected by dragging vegetation on Great Island (West Yarmouth, MA) during the fall seasons of 1991 and 1992. To collect saliva, ticks were allowed to feed for 4–5 d on the ears of rabbits. Engorging ticks were removed, washed, and 1 μl of 5% (wt/vol) solution of pilocarpine in absolute methanol was applied to their dorsum. A finely drawn capillary tube was fitted over the mouthparts of each tick, which were allowed to salivate over the course of 3 to 5 h within a humidified chamber held at 37°C, as previously described (18). Saliva was pooled, filter sterilized through a 0.22 μm syringe filter (8110; Costar Corp., Cambridge, MA), and stored at −70°C until used. Each batch of saliva comprised material harvested from 20 to 30 ticks.

Salivary glands were dissected from partially fed *I. dammini* female ticks, placed in PBS (one pair of glands/100 μl) and stored at −70°C until used. At this time, the glands were disrupted by rapidly freezing-and-thawing five times using a dry ice/ethanol bath and a 37°C waterbath. The lysate was cleared of debris by centrifugation (10,000 g, 30 s), filter sterilized, and used in assays.

Con A and Phytohemagglutinin Stimulation of Spleen Cells. A suspension of SC was generated from normal C57BL/6 or C3H mice and the SC were seeded into 96-well culture plates (3596; Costar Corp.) at 2 × 10^5/ml in medium consisting of DME (19) containing 2 × 10^{-5} M 2-mercaptoethanol and 0.5% normal mouse serum. The cells were then stimulated with Con A (0.5 μg/ml) or PHA (1.0 μg/ml) in the absence or presence of tick saliva (concentrations of saliva stated in the text). 24 h later, the degree of proliferation of the SC was determined by pulsing with 1 μCi/well of [^{3}H]TdR for 18 h followed by scintillation counting.

Interleukin-2 Production by Spleen Cells and Assays for Interleukin-2. SC were seeded into 24-well culture plates (3424; Costar Corp.) at 5 × 10^6/ml in DME as described above. The cells were stimulated with Con A (0.5 μg/ml) in the presence or absence of *I. dammini* saliva for 24 h and the supernatants of the cultures were harvested and assayed for the presence of IL-2.

IL-2 was measured in supernatants by a bioassay which used the IL-2-dependent indicator cell line, CTLL-2, and by an IL-2-specific ELISA. The CTLL assay has been described in detail elsewhere (20). Briefly, CTLL cells were maintained in recombinant human IL-2 (Cetus Corp., Emeryville, CA). The effect of a sample supernatant on the proliferation of CTLL was assessed by [^{3}H]TdR incorporation followed by scintillation counting. Units/ml of IL-2 in the test supernatant were calculated from a standard curve obtained with recombinant murine IL-2 (Genzyme Corp., Cambridge, MA). Specificity was assessed using blocking anti-IL-2 mAb S4B6.

The IL-2-specific ELISA was developed using mAb's purchased from Pharmingen (San Diego, CA). A rat antimurine IL-2 mAb (rat IgG$_{2a}$; 18161D) was used to capture IL-2 and a biotinylated rat antimurine IL-2 mAb (rat IgG$_{2b}$; 18172D) was used as the detection mAb. The assay was conducted using manufacturer's directions and was developed using avidin peroxidase/TMB as the enzyme/substrate pair. Units/ml of IL-2 in test supernatants were calculated by comparison to a standard curve generated with recombinant murine IL-2.

NO Production by Macrophages. C57BL/6 mice were injected intraperitoneally with starch as described (21) and 4 d later the cells were harvested by lavaging the peritoneum with RPMI 1640 (GIBCO BRL, Gaithersburg, MD) containing 10% FCS (Hyclone, Logan, UT). The cells were plated at 10^5/0.1 ml in 96-well culture plates. After a 2-h incubation at 37°C, nonadherent cells were removed by rinsing and the adherent cells were incubated for 2 h with medium or medium containing *I. dammini* saliva. LPS was then added to a final concentration of 10 ng/ml and the levels of nitrite in the supernatants of the cultures were assessed at 24 and 72 h by the Greiss reaction as described (22). Briefly, 50-μl cell culture supernatants were mixed with 50 μl of Greiss reagent (1% sulfanilamide, 0.1% N-[1-naphthyl]ethyl-enediamine dihydrochloride [Sigma Chemical Co.] in 2.5% phosphoric acid) and incubated at room temperature for 10 min. Absorbance was measured at 570 nm. Concentration of NO$_2$ in the supernatant was determined by comparison to a standard curve generated with dilutions of NaNO$_2$.

Removing PGE$_2$ from I. dammini Saliva. To remove PGE$_2$ from *I. dammini* saliva, the saliva was centrifuged in a Centricon-3® microconcentrator (Amicon Corp., Beverly, MA). The Centricon-3 device retains 80–95% of molecules greater than 5,000 mol wt, while allowing 97% of molecules of the mol wt of PGE$_2$ (353) to pass through its membrane. 1 ml of DME containing a 1:20 dilution of saliva and 0.1% normal mouse serum was loaded into the upper chamber of the Centricon and centrifuged for 2.5 h at 5,000 g, 4°C. This reduced the volume in the upper chamber to 50 μl. The filtrate in the lower chamber was saved, 950 ml of fresh DME was added to the upper chamber and the Centricon was centrifuged again at 5,000 g for 2.5 h. In total, this process was repeated five times to assure complete removal of the PGE$_2$ from saliva. The five filtrates and the retentate were then tested for their content of PGE$_2$ (see below) and their ability to inhibit T cell proliferation (see above).

Enzyme Immunoassay for PGE$_2$. To measure PGE$_2$ concentrations in saliva and fractions thereof, a PGE$_2$ enzyme immunoassay kit (sensitivity ≤10 pg PGE$_2$/ml) was purchased from Advanced Magnetics (Cambridge, MA). The assay was performed following manufacturer's directions.

Digesting I. dammini Saliva with Trypsin. *I. dammini* saliva was mixed with an equal volume of trypsin dissolved at a concentration of 1 mg/ml in 10 mM Tris-HCl buffer, pH 8.0. The mixture was incubated for 30 min at 37°C and the trypsin reaction was stopped by adding SBTI dissolved in Tris-HCl buffer so as to achieve

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a final concentration of 1 mg/ml SBTI. This mixture was then tested for its ability to inhibit Con A–driven T cell proliferation. Controls consisted of: (a) trypsin and SBTI incubated together before the addition of the saliva to determine whether trypsin or SBTI would in any way directly inhibit the actions of saliva; (b) trypsin and SBTI incubated together and then added to SC + Con A to determine whether trypsin or SBTI would in any way influence a Con A response.

Results

I. dammini Saliva Inhibits T Cell Proliferation to Con A. To test whether tick saliva has immunosuppression effects, normal SC from two mouse haplotypes, C57BL/6 (H-2^b) and C3H (H-2^k), were stimulated in vitro with Con A. As can be seen in Fig. 1, when SC were preincubated for 2 h with dilutions of *I. dammini* saliva (1:100 to 1:1,000), the response of the cells to Con A (0.5 μg/ml) was greatly diminished. When present at a 1:100 final dilution, saliva inhibited the mitogenic response by 70 to 80%. This inhibition was still evident at a 1:1,000 final dilution of *I. dammini* saliva (inhibition ∼30%). Thus, the inhibitory effects of tick saliva are dose dependent. In addition, the inhibitory effects of saliva were not restricted to one mouse haplotype; substantial inhibition of the mitogenic response was seen using both C57BL/6 and C3H SC. Finally, lysates of the salivary glands of female *I. dammini* were also found to suppress the response to Con A to an extent similar to that of saliva itself (data not shown). The experiment depicted in Fig. 1 is representative of eight similar independent experiments. Similar results were obtained with other doses of Con A (1.0, 0.25, or 0.1 μg/ml), however, the magnitude of proliferation varied with the dose of Con A.

Kinetics of the Response of Spleen Cells to Con A in the Presence of Graded Doses of I. dammini Saliva. Although *I. dammini* saliva markedly inhibited Con A mitogenesis when T cell proliferation was assessed by [³H]TdR incorporation at 24 h of culture (Fig. 1), it was possible that this inhibition was not as complete at other times of culture, or that saliva

simply shifted the kinetics of the response to a later time point. These possibilities were addressed in the experiments of Fig. 2. At 12 and 36 h of culture saliva inhibited the Con A response as much as at 24 h, and saliva did not simply delay the response to Con A since the response to Con A was subsiding at 36 h of culture whether the SC were cultured with Con A alone or Con A + saliva. In addition, Fig. 2 again shows that the inhibition of Con A mitogenesis is a dose-dependent phenomenon: a 1:50 dilution of saliva almost completely suppresses the response, a 1:100 dilution suppresses the response by ∼80%, and a 1:200 dilution suppresses 50% of the response.

Preincubation of Spleen Cells in I. dammini Saliva Is Not Required for Inhibition of T Cell Proliferation. In the experiments described above, SC were preincubated with *I. dammini* saliva for 2 h before Con A was added to the cultures. However, the experiment depicted in Fig. 3 demonstrates that preincubation was not necessary; the degree of inhibition of the Con A response (∼75%) was identical whether saliva was added 2 h in advance of Con A or at the same time as Con A.

I. dammini Saliva Also Inhibits T Cell Proliferation to Phytohemagglutinin and the Pilocarpine Used to Induce I. dammini Salivation Is Not Responsible for the Observed Immunosuppression. Since Con A is a lectin which binds α-D-mannose and α-D-glucose, it was possible that *I. dammini* saliva inhibited the Con A response due to a very large amount of one of these sugars in the saliva. To test this possibility, we stimulated SC with PHA (which binds N-acetylgalactosamine) in the presence or absence of *I. dammini* saliva. Results in Fig. 4 show that the response to PHA was also markedly inhibited by saliva; a 1:100 dilution of saliva inhibited the response to PHA (1 μg/ml) by 80%. The experiment depicted in Fig. 4 is representative of three similar independent experiments. Similar results were obtained with other doses of PHA (doses as high as 10 μg/ml), however, the magnitude of proliferation varied with the dose of PHA.

It was also possible that *I. dammini* saliva was inhibiting mitogenic responses due to a nonspecific toxic effect of saliva for the responding SC. Although this was unlikely since mi-
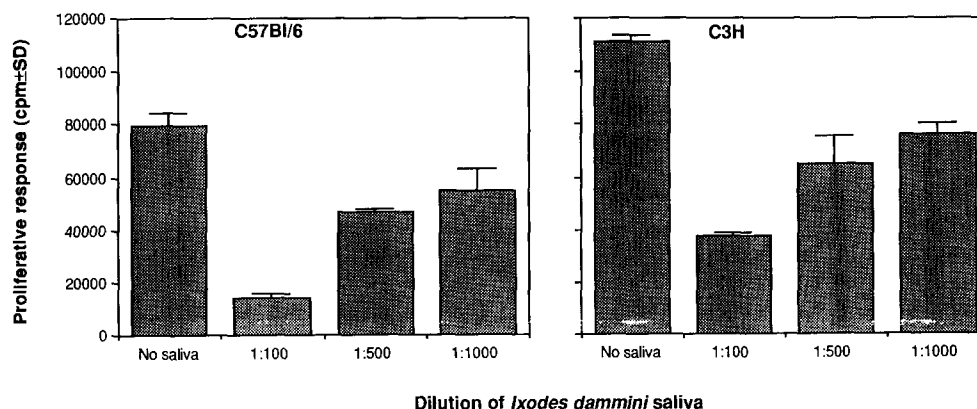

Dilution of *Ixodes dammini* saliva

Figure 1. *I. dammini* saliva inhibits T cell proliferation to Con A. SC from C57BL/6 or C3H/H mice were placed into 96-well microplates (2 × 10⁵/well) and incubated for 2 h in medium alone or in medium containing the indicated final dilution of *I. dammini* saliva (e.g., 1:100, 1:500, or 1:1,000). Experimental wells were then stimulated with Con A (0.5 μg/ml); control wells received no Con A. 24 h later, the wells were pulsed with [³H]TdR. 18 h later the cultures were harvested and assessed for T cell proliferation by scintillation counting. Results are presented as the mean of triplicate cultures ± SD.
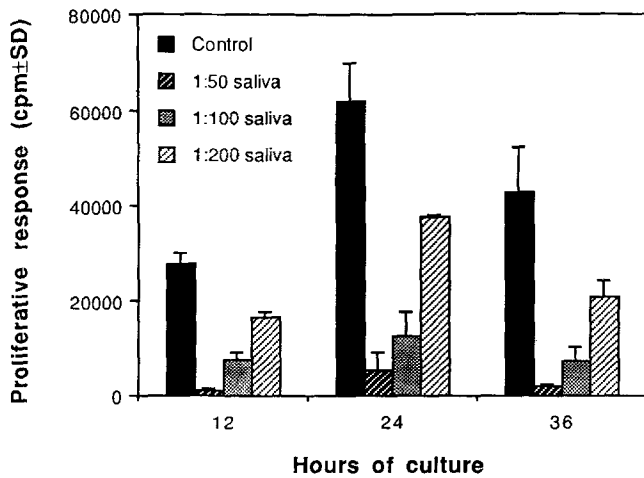
Figure 2. Kinetics of the response of spleen cells to Con A in the presence of graded doses of *I. dammini* saliva. C57BL/6 SC were preincubated in medium alone or in the indicated dilutions of *I. dammini* saliva for 2 h. Experimental wells were then stimulated with Con A (0.5 μg/ml); control wells received no Con A. At the hours of culture indicated, the cultures were pulsed with [3H]TdR. 18 h later, the cultures were harvested and T cell proliferation was assessed as in Fig. 1.

Figure 4. *I. dammini* saliva inhibits T cell proliferation to phytohemagglutinin. C57BL/6 SC were preincubated in medium alone or in a 1:100 dilution of *I. dammini* saliva for 2 h. Experimental wells were then stimulated with PHA (1.0 μg/ml); control wells received no PHA. T cell proliferation was assessed as in Fig. 1.

croscopic (inverted phase) observation of SC cultures never suggested a direct toxic effect, we routinely directly tested the viability of SC (by their ability to exclude trypan blue) at the end of culture with either Con A or Con A + saliva. Using this approach, we never detected a toxic effect of saliva for SC. For example, the C57BL/6 SC of Fig. 1 that were cultured with Con A for 24 h were 79% viable whereas the SC cultured with Con A and saliva were 85% viable. We also tested for toxic effects of tick saliva by culturing various transformed cell lines in the presence or absence for 24 h and pulsing the cells with [3H]TdR to determine whether saliva
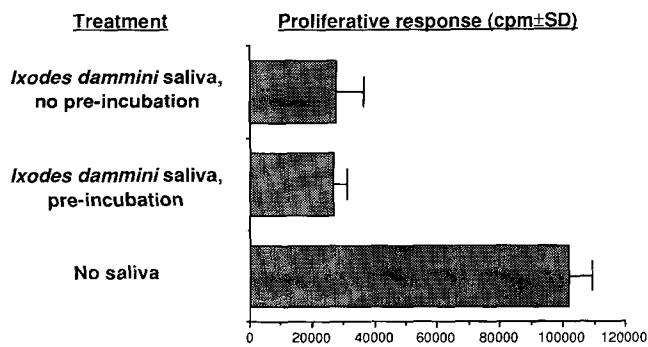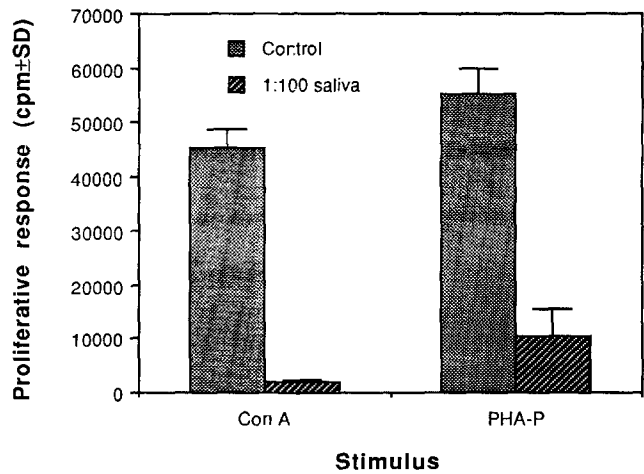

Figure 3. Pre-incubation of spleen cells in *I. dammini* saliva is not required for inhibition of T cell proliferation. C57BL/6 SC were preincubated in medium alone or in a 1:100 dilution of *I. dammini* saliva for 2 h. The SC preincubated in medium alone were then either left untreated (negative control), stimulated with Con A (0.5 μg/ml, positive control), or treated with a mixture of Con A and *I. dammini* saliva (1:100 dilution). The SC preincubated in *I. dammini* saliva were stimulated with Con A (0.5 μg/ml). At 24 h of culture, the cells were pulsed with [3H]TdR, and T cell proliferation was assessed as in Fig. 1.

altered the replicative rate of any of the cells. We found that tick saliva did not alter the degree of proliferation of any of the following transformed cell lines (American Type Culture Collection, Rockville, MD): EL4 (thymoma, ATCC no. TIB 39), WEHI 279 (B cell lymphoma, ATCC no CRL 1704), P815 (mastocytoma, ATCC no. TIB 64).

Finally, salivation of *I. dammini* was induced by placing 1 μl of a 5% (wt/vol) pilocarpine solution in methanol on the dorsum (see Materials and Methods). Since it was possible that some of this pilocarpine contaminated the induced saliva and might be involved in the suppression of T cell mitogenesis, we tested the ability of pilocarpine to inhibit a Con A response. Pilocarpine in methanol (5%, wt/vol), methanol alone or saliva were added to SC at dilutions of 1:100 or greater, and the SC were stimulated with Con A. Results in Fig. 5 show that while saliva profoundly inhibited the mitogenic response, neither pilocarpine in methanol nor methanol alone had any effect on the response to Con A.

Taken together, these results suggest that *I. dammini* saliva inhibited T cell responses in a consistent, nonartifactual manner either via an effect on the T cells themselves and/or via inhibition of cytokines produced by the cells.

I. dammini Saliva Inhibits IL-2 Secretion by T Cells. Since IL-2 is the principal autocrine growth factor for T cells, *I. dammini* saliva could be inhibiting T cell replication through its ability to inhibit IL-2 production by the cells. To test this, we stimulated SC with either Con A alone or Con A + *I. dammini* saliva and harvested the supernatants of the cultures 24 h later. To assess the levels of IL-2 in the test supernatants, we first used the IL-2-dependent cell line, CTLL-2. Results in Table 1 show that saliva markedly inhibited IL-2 production (77.4%). Since this degree of suppression of IL-2 production is approximately equal to the degree of suppression of T cell proliferation seen in Fig. 1–5, it is likely that *I. dammini* saliva is inhibiting T cell proliferation through its ability to inhibit IL-2 production by the cells.
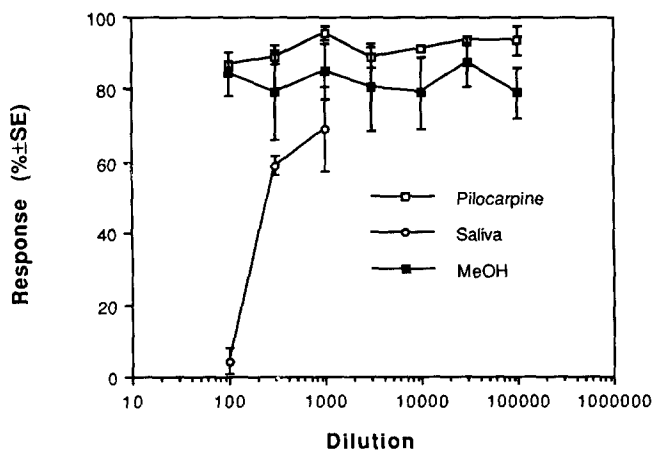
Figure 5. Pilocarpine/methanol used to induce *I. dammini* salivation does not inhibit T cell proliferation. C57BL/6 SC were preincubated 2 h in either medium alone, methanol at the indicated final dilutions, pilocarpine (5% wt/vol in methanol) at the indicated final dilutions, or *I. dammini* saliva at the indicated final dilutions. The cells were then stimulated with Con A (0.5 μg/ml) and T cell proliferation was assessed as in Fig. 1. The figure depicts the results of three replicate experiments, thus, the data were normalized for comparison. Actual proliferative responses ranged between 4 × 10⁴ and 8 × 10⁴ cpm.

Although the results obtained in the CTLL-2 assay system showed that *I. dammini* saliva greatly reduced the levels of biologically active IL-2 secreted by T cells, this result could be due to one of two different mechanisms which the CTLL-2 assay cannot distinguish between. Either: (*a*) saliva contains an antagonist for IL-2 such that although IL-2 is secreted by T cells, its activity is neutralized by saliva; or (*b*) saliva truly prevents the secretion of IL-2 by T cells. To distinguish between these two possibilities and to confirm the results

Table 1. *I. dammini Saliva Inhibits IL-2 Secretion by T Cells*

	Units IL-2/ml detected by	
Cell stimulus	CTLL bioassay	IL-2–specific ELISA
Con A	31.0	24.6
Con A + *I. dammini* saliva	7.0	4.9

Normal C57BL/6 SC were placed into 24-well macroplates (5 × 10⁶/well) and incubated for 2 h in medium alone or in medium containing a 1:100 final dilution of *I. dammini* saliva. Experimental wells were then stimulated with Con A (0.5 μg/ml); control wells received no Con A. 24 h later, the supernatant of the cultures was harvested and assayed for IL-2 by the CTLL bioassay or an IL-2–specific ELISA as described in the Materials and Methods. In the CTLL bioassay, an IL-2–specific neutralizing mAb was used to confirm that the proliferation of the CTLL cells was due to genuine IL-2. Control values (SC not stimulated with Con A) have been subtracted from the figures in the Table; control values were 1 U IL-2/ml in the CTLL assay and 0.1 U IL-2/ml in the ELISA assay.

we obtained in the CTLL-2 assay, we used an IL-2–specific ELISA. Results with this assay (81.1% suppression) were almost identical to those obtained with the CTLL-2 assay (Table 1), which indicated that saliva inhibits T cell proliferation by inhibiting IL-2 secretion by the cells.

I. dammini Saliva Inhibits NO Production by Macrophages. Since *I. dammini* saliva profoundly inhibited T cell activation, we wished to determine whether it would inhibit activation of a different cell type. Macrophages were pre-incubated with saliva for 2 h and stimulated with LPS to induce NO production. NO production was monitored by measuring the levels of nitrite released into the supernatants of the cultures. Saliva dramatically inhibited NO production by macrophages (Fig. 6). When present at a 1:100 dilution, saliva completely inhibited NO production (both at 24 and 72 h poststimulation with LPS); when present at a 1:1,000 dilution, it inhibited NO production by ~50%. Thus, saliva can inhibit the functions of T cells, which are cells that are central in the development of specific immune responses; and saliva can inhibit the functions of macrophages, which are cells that play important roles in both specific and nonspecific defense mechanisms of the host.

Ability of Prostaglandin E₂ to Mimic the Effects of I. dammini Saliva. Next we attempted to determine the mechanism by which saliva inhibits cell activation. We first tested whether PGE₂ might be responsible for the inhibition by determining whether PGE₂ could substitute for saliva in the Con A mitogenic response assay system. Tick saliva was assayed for the level of PGE₂ present using the enzyme immunoassay for PGE₂ described in Materials and Methods and was found
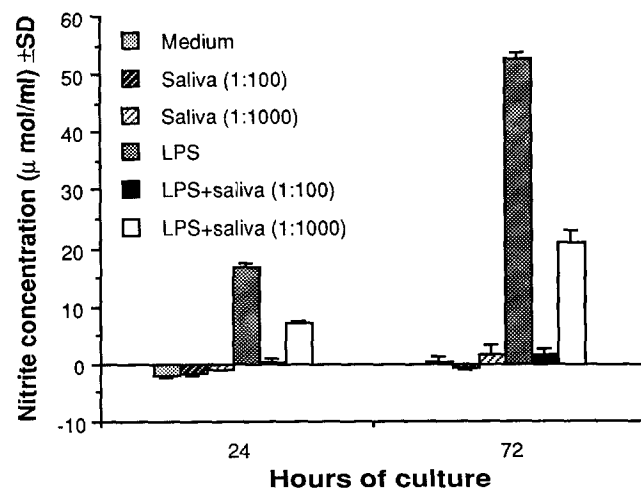

Figure 6. *I. dammini* saliva inhibits nitric oxide production by macrophages. C57BL/6 macrophages were generated as described in Materials and Methods and were preincubated with the indicated dilutions of *I. dammini* saliva for 2 h. Control cells were incubated in medium alone. After 2 h, medium ± LPS (10 ng/ml) was added to the wells. Aliquots (50 μl) of the culture supernatants were measured for their concentration of nitrite at 24 and 72 h after stimulation with LPS using techniques described in Materials and Methods. Results are presented as the mean of triplicate cultures ± SD.

to contain 2.2 μg PGE$_2$/ml. SC were stimulated with Con A in the presence or absence of dilutions of tick saliva or synthetic PGE$_2$ and the ability of each to inhibit T cell proliferation was determined. Results in Fig. 7 are plotted such that the amount of tick saliva present is given in terms of the amount of salivary PGE$_2$ present and not in terms of the dilution of whole saliva. What is clear from Fig. 7 is that the PGE$_2$ present in tick saliva cannot solely account for its immunosuppressive effects; saliva is approximately 1,000-fold more potent than synthetic PGE$_2$. In addition, tick saliva exerts its immunosuppressive effects over a narrower range of dilutions than PGE$_2$; indeed, PGE$_2$ did not completely inhibit T cell proliferation even when present at 1 μg/ml.

PGE$_2$ in I. dammini Saliva Plays a Minor Role in Its Immunosuppressive Effects. To confirm the results of Fig. 7, we removed PGE$_2$ from saliva to determine whether this diminished its capacity to inhibit T cell proliferation. 1 ml of a 1:20 dilution of I. dammini saliva was loaded into the upper chamber of a Centricon-3® microconcentrator (see Materials and Methods for details of use). The 1:20 dilution of saliva contained 108 ng PGE$_2$/ml. The microconcentrator was centrifuged until 50 μl remained in the upper chamber, the filtrate was saved, the fluid in the upper chamber was replenished and this process was repeated for a total of five centrifugations. This process completely removed the PGE$_2$ from tick saliva. Filtrate 1 contained 100.0 ng PGE$_2$/ml; filtrate 2 = 9.3 ng/ml; filtrate 3 = 3.3 ng/ml; filtrate 4 = 0.8 ng/ml; filtrate 5 = 0.1 ng/ml; and the retentate contained 0.03 ng PGE$_2$/ml.

All the filtrates and the retentate were then tested for their ability to inhibit T cell proliferation compared to that of whole saliva. Filtrates 2–5 were unable to inhibit T cell proliferation, but filtrate 1 had some capacity to inhibit proliferation

and the retentate was as effective as whole saliva in its ability to inhibit T cell proliferation (Fig. 8). Interestingly, the first filtrate which contained almost all the PGE$_2$ present in tick saliva suppressed T cell proliferation in a manner almost identical to synthetic PGE$_2$. The concentrations of salivary PGE$_2$ are given for three filtrate 1 data points in Fig. 8. When one compares Figs. 7 and 8, it can be seen that when present at 50 ng/ml, both synthetic and salivary PGE$_2$ suppressed the Con A response by approximately 30%.

Taken together, these results suggest that PGE$_2$ plays a minor role in the immunosuppressive effects of tick saliva and that a molecule of >5,000 mol wt (the cut-off of the Centricon-3® microconcentrator is 5,000–10,000 mol wt) is largely responsible for the immunosuppression.

The Molecule in I. dammini Saliva Responsible for Immunosuppression Is a Protein. Since the factor in tick saliva responsible for immunosuppression was not PGE$_2$, we tested whether the factor was a protein. I. dammini saliva was digested with trypsin, the reaction was stopped with SBTI as described in Materials and Methods and the product tested for its ability to inhibit T cell proliferation. As can be seen in Fig. 9 (group D), trypsin digestion destroyed the ability of saliva to inhibit T cell proliferation. Importantly, this was not due to the presence of trypsin and SBTI in the cultures since control cultures showed that: (a) I. dammini saliva was capable of suppressing a Con A–driven response when it was added after the SBTI (Fig. 9, group E); and (b) neither trypsin nor SBTI affected the response of SC to Con A (group C).

Discussion

A seminal report by Trager (5) demonstrated that the guinea pig, which is not a natural host for *Dermacenter variabilis*, mounts a very efficient immune response to the tick. In ad-
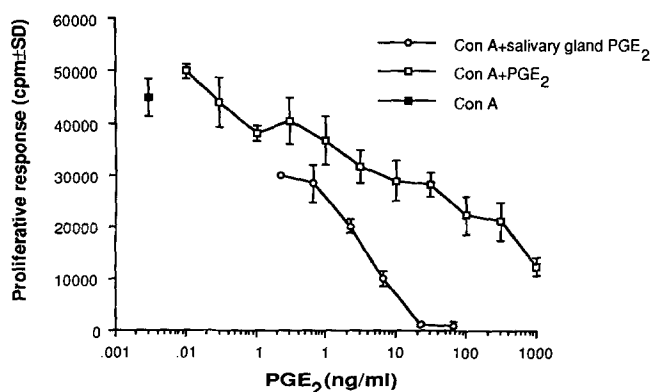

Figure 7. Ability of prostaglandin E$_2$ to mimic the effects of I. dammini saliva. C57BL/6 SC were preincubated 2 h in either PGE$_2$ at the concentrations indicated or I. dammini tick saliva diluted so as to achieve the indicated concentrations of salivary PGE$_2$. The I. dammini tick saliva used in these experiments contained 2.2 μg PGE$_2$/ml (techniques used to determine PGE$_2$ concentration given in Materials and Methods). The cells were then stimulated with Con A (0.5 μg/ml) and T cell proliferation was assessed as in Fig. 1. The figure is representative of two independent experiments.

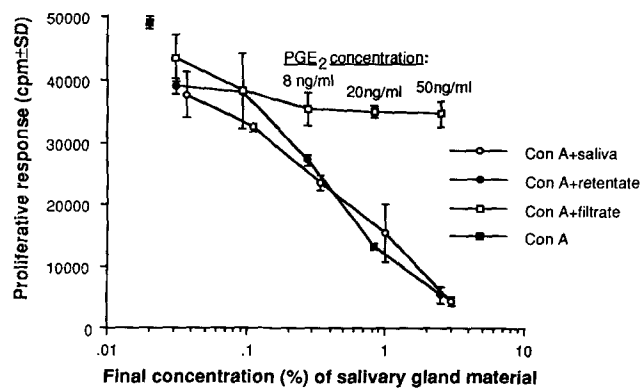**Figure 8.** PGE$_2$ in *I. dammini* saliva plays a minor role in its immunosuppressive effects. PGE$_2$ was removed from *I. dammini* saliva by repeatedly centrifuging saliva in a Centricon-3® microconcentrator as described in Materials and Methods. The figure depicts the ability of the retentate (i.e., molecules in tick saliva that are >5,000 mol wt) and of the filtrate (i.e., molecules in saliva that are <5,000 mol wt) to inhibit Con A–driven proliferation compared to the inhibition seen with unfractionated saliva. Since the filtrate contained virtually all of the PGE$_2$ present in *I. dammini* saliva, the concentrations of salivary PGE$_2$ present in the assay are indicated above the symbols for the dilutions of filtrate. The figure is representative of three independent experiments.
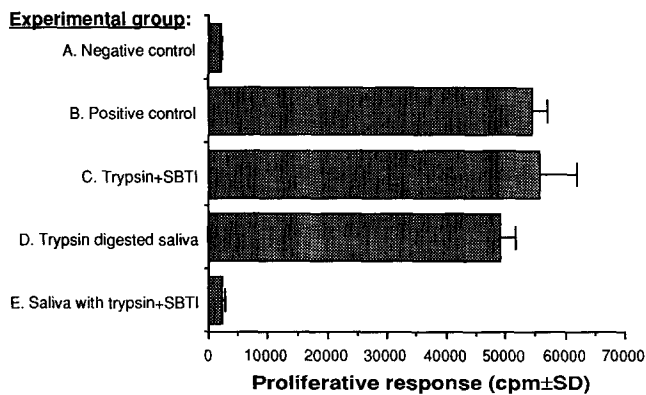
Figure 9. The molecule in *I. dammini* saliva responsible for immunosuppression is a protein. *I. dammini* was digested with trypsin and the reaction was stopped by the addition of SBTI (see Materials and Methods). To test whether this digested tick saliva was still capable of mediating immunosuppression, a 1:100 final dilution of the digest was incubated with SC for 2 h and Con A (0.5 μg/ml) was added to the cultures (group D). Controls consisted of SC to which was added trypsin that had already been blocked by SBTI followed by the addition of saliva; these cultures determined whether trypsin + SBTI was able to inhibit the immunosuppressive effect of saliva via a mechanism other than proteolysis (group E). The remaining control consisted of SC to which was added trypsin that had already been blocked by SBTI; these cultures determined whether trypsin + SBTI in any way modified the response of SC to Con A (group C).

dition, cattle and laboratory animals may develop a potent anti-tick response characterized by inflammatory cells (e.g., neutrophils, mast cells, basophils, eosinophils) present at the tick feeding site and the development of specific cell-mediated and humoral immunity (6, 7, 23). This anti-tick response may decrease the tick's blood-feeding success and ultimately cause rejection of the tick. In contrast to the guinea pig, Trager (5) found that such anti-tick immunity developed poorly if at all in the white-footed mouse, *Peromyscus leucopus*, which is a natural host for *D. variabilis*. These observations were interpreted (24) to mean that either natural hosts were immunoincompetent or that in natural hosts, the tick was able to evade the host's immune response. However, the mechanism by which the tick could evade the host immune response was not clear.

Subsequent work by Wikel (25) demonstrated that when guinea pigs were infested with *D. andersoni*, their responses to the T cell mitogens Con A and PHA were reduced. In addition, a recent report of Roehrig et al. (12), suggests that tick infestation may modify antipathogen responses as well. When hamsters were infected with *B. burgdorferi* (the causative agent of Lyme disease) delivered by syringe needle, the animals mounted an early and potent antibody response to two antigens of the spirochete, OspA and OspB. In contrast, when the hamsters were infected by the bite of infected ticks, the animals did not have a detectable antibody response to OspA and OspB. This result is identical to that seen in human beings that acquire Lyme disease by tick bite, because humans do not develop antibody to OspA and OspB until late in the course of infection. Thus, not only are ticks able to circum-

vent host immunity, but microorganisms that are transmitted by ticks may also not elicit an effective immune response.

All of the observations discussed above may be explained by the activity of a potent immunosuppressive factor in tick saliva. Many authors have speculated that this factor might be PGE_2 (2, 3, 14–16). However, PGE_2 is quite labile in vivo; one transit through the pulmonary circulation removes >95% of PGE_2 activity (26). Thus, it is unlikely that PGE_2 could have been responsible for the immunosuppression observed, especially given the work of Wikel (25) which indicated that infestation of guinea pigs with ticks had a long-lived, systemic immunosuppressive effect. We show here that PGE_2 is not the main factor responsible for the immunosuppressive effects of female *I. dammini* saliva or salivary gland lysates. Instead, suppression is primarily mediated by a protein of >5,000 mol wt (Figs. 7–9). Interestingly, this protein may be sex specific; preliminary experiments reveal that male salivary glands do not contain an immunosuppressive factor. Male *Ixodes* ticks may not be as dependent upon immunosuppressive factors of their saliva since unlike metastriate *Dermacentor* or *Amblyomma* male ticks, male *Ixodes* do not require a bloodmeal even though they do attach and a variable proportion will ingest RBC's (27; Telford, S. R., III, unpublished results). In addition, male *Ixodes* do not transmit *B. burgdorferi* or coinfecting *Babesia microti* (Telford, S. R., III, unpublished results). Finally, it should be mentioned that salivary gland proteins are also expressed in a sex-specific manner in the mosquito, *Aedes aegypti*. The salivary glands of females contain abundant amounts of a protein termed D7, but male glands contain little of this protein (28).

The specific pattern of feeding by each *I. dammini* instar may have driven this tick to develop mechanisms for preventing the development of host anti-tick immunity. Nymphal forms of *I. dammini* feed on and transmit *B. burgdorferi* to *P. leucopus* (the natural mouse host for the tick) early in the transmission season (April to July). Subsequently (July to September), larval forms of *I. dammini* feed on these infected mice and acquire the spirochete in the bloodmeal. This seasonal inversion of feeding activity then forcefully maintains the agent of Lyme disease from year to year (29). If on the other hand, host anti-tick immunity were to develop, the feeding success of the tick (especially larval forms which feed after nymphal forms) and the survival of the spirochete would both be jeopardized. Because the immunosuppressive activities within tick saliva may inhibit a host anti-tick response, the molecules responsible for such activities may have driven the coevolution of ticks as well as pathogens with their hosts.

Although Lyme disease is transmitted very efficiently by *I. dammini*, very few spirochetes appear to be delivered by the tick to the site of its bite (30, 31). The cellular infiltrate at this site (neutrophils, mast cells, eosinophils) should, but would appear not to, offer a formidable barrier for the spirochete. Therefore, it is possible that the immunosuppressive armament within *I. dammini* saliva is used by the spirochete to evade host defenses and to successfully establish infection. If tick saliva enhances the infectivity of the pathogens transmitted by ticks, sensitizing the host against ticks should alter disease transmission by ticks. In fact, this is the case since

tick-sensitized guinea pigs are refractory to tularemia (32). Thus, tick saliva may enhance the infectivity of tick-transmitted pathogens as has been demonstrated for the saliva of two other arthropod vectors: sand fly saliva enhances the infectivity of *Leishmania* (33–36) and tick saliva the infectivity of Thogoto virus (37). These observations have led to the hypothesis that an alternative method for vaccinating against the pathogens that blood-sucking arthropod vectors transmit is to vaccinate the host against the immunosuppressive factors of the vectors' saliva (4).

Taken together, the observations presented here as well as the work of others that is discussed, suggests that coevolution of ticks, tick hosts, and the pathogens that ticks transmit may have, in part, been driven by immunosuppressive factors in the tick's saliva. That is, ticks that had these immunosup-

pressive factors may have been more successful at blood feeding, and thus reproduction; and pathogens transmitted by these ticks may have been more successful at infecting the host, and thus, once again, more successful at reproduction. Because hard ticks are vectors of a variety of prevalent zoonoses in the United States (Lyme disease, Rocky Mountain spotted fever, Colorado tick fever, tularemia, babesiosis, and ehrlichiosis, all transmitted via the tick salivary gland), and because the efficiency of transmission and the course of infection may be influenced by immunosuppressive molecules present in the tick's saliva, further analysis of the pharmacologically/immunologically active molecules of vector saliva may serve to broaden our basic understanding of disease transmission and may contribute to public health worldwide.

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