ANTIHEMOSTATIC, ANTIINFLAMMATORY, AND IMMUNOSUPPRESSIVE PROPERTIES OF THE SALIVA OF A TICK, *IXODES DAMMINI*

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Hematophagous organisms must overcome host hemostasis in order to locate blood and maintain its flow during ingestion (1, 2). Platelet aggregation provides the main hemostatic obstacle because duration of bleeding of lacerated small vessels depends mainly upon platelet function (3, 4). Important stimuli inducing platelet aggregation include ADP (released by injured cells), collagen fibrils (exposed in subendothelial tissues), thrombin (produced after activation of the coagulation cascade) and PAF[1] (platelet-aggregating factor, released by leukocytes). Activated platelets may release thromboxane A_2 (a potent vasoconstrictor and platelet-aggregating stimulus), ADP, and serotonin (which further enhance platelet aggregation and vessel constriction) (5). In addition, platelet-derived factors will also contribute to thrombin formation and clotting, thus providing rigidity to the platelet plug (4). Thus, one can anticipate that any antihemostatic properties of blood-feeding arthropods will focus mainly on platelet aggregation and, secondarily, on vascular contraction or coagulation.

Hard ticks feed solely on blood, each engorgement generally extending over at least several days, thereby providing ample time for inflammation to promote hemostasis at the feeding site, while increasing the tick's need to antagonize this process. Leukocyte-derived products, particularly PAF, would enhance hemostasis at the point of tick attachment. Immune mechanisms may further reduce feeding success by enhancing inflammatory reactions (6, 7). Interestingly, saliva of some ticks includes an anticoagulant, an antihistamine, and prostaglandins(PG); these compounds may facilitate feeding (8). Other enzymes, including esterases and glycosidases have been identified, but their functions remain unknown (8). Antiplatelet activity, however, has not been described.

Their prolonged period of contact with a narrow range of hosts suggests that hard ticks may possess effective antihemostatic mechanisms peculiarly adapted to interfere with the inflammatory mechanisms of particular hosts. Accordingly, we sought to describe the salivary armamentarium of such a tick, and to ascribe

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[1] *Abbreviations used in this paper:* IL-2, interleukin 2; mAb, monoclonal antibody, PAF, platelet-aggregating factor; PG, prostaglandin; RIA, radioimmunoassay.

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function to its various components. In particular, we attempted to identify platelet antiaggregating activity, as well as antiinflammatory components in saliva harvested from *Ixodes dammini*.

Materials and Methods

Materials. PG were kindly provided by Dr. J. E. Pike (Upjohn Co. Kalamazoo, MI). Glucose and inorganic salts used were American Chemical Society–standard. Other reagents were obtained from Sigma Chemical Co., St. Louis, MO. Anti-Thy-1 monoclonal antibody (mAb) was kindly provided by Dr. Ethan Shevach (Laboratory of Immunology, National Institutes of Health (9).

Source of Ticks. Adult *I. dammini* ticks were collected by flagging on Great Island, in West Yarmouth on Cape Cod in southern Massachusetts, during the spring of 1984.

Harvest of Saliva. Pilocarpine was used to induce ticks to salivate (10, 11). Ticks were permitted to engorge for 4–5 d on the ear of a rabbit, after which they were removed by traction. Ticks weighed 25–175 mg. To collect saliva, 1 μl of a 5% (wt/vol) pilocarpine hydrochloride solution in 0.7 M NaCl was injected through glass micropipettes near the corner of the scutum while the ticks were restrained on glass slides by adhesive tape. Saliva was collected in glass tubes throughout 2 h postinjection at 35°C in a humid chamber. 1–25 mg of saliva were collected per tick, and samples were stored at −27°C. Collections <3 mg were pooled until ≥ 3 mg was obtained. <5% of samples were small enough to require such pooling.

Platelet Aggregation. Platelet aggregation was monitored in an aggregometer equipped with a 0.1 ml cuvette (12, 13) using human citrated platelet-rich plasma (0.38% final citrate). Five or more saliva samples were pooled for each experiment.

Smooth Muscle Bioassay. Superfused rat stomach strips were prepared as previously described (14), but using aerated Tyrode's solution (15). In some experiments, laminar flow superfusion was used (14). Antagonists added to the perfusion fluid included: methysergide maleate (2×10^{-7} g/ml), mepyramine hydrochloride (10^{-7} g/ml), phenoxybenzamine hydrochloride (10^{-7} g/ml), propanolol hydrochloride (2×10^{-6} g/ml), hyoscine hydrobromide (10^{-7} g/ml), and indomethacin (2×10^{-6} g/ml). Superfusion was done either at 2.0 ml/min or 0.15 ml/min (laminar flow superfusion mode) at 30°C, because we observed maximum sensitivity of preparations at this temperature. The perfusion liquid, delivered through a microperpex peristaltic pump (LKB Instruments, Inc., Gaithersburg, MD), was interrupted for 10 s when standards or individual saliva samples were added. Such samples (≤5 μl) were added in 0.1-ml aliquots dissolved in Tyrode's solution. Isometric contractions were recorded using a Harvard isotonic/isometric transducer. Guinea pig ileum preparations were prepared using a 5 ml bath (15).

Clotting Assays. Recalcification time was determined by incubating 50 μl of citrated human plasma with 0.15 M NaCl in 1 × 7 cm glass tubes (16). After 1 min at 37°C, 50 μl of $CaCl_2$ were added, and tubes then inspected for clotting every 10 s. Tick saliva (2.5 μl from individual samples) was added with the NaCl solution, when appropriate. To determine prothrombin time (17), human citrated plasma, in 50-μl samples, was incubated for 1 min at 37°C, and 100 μl of a thromboplastin (Sigma Chemical Co.) -$CaCl_2$ (12.5 mM) mixture was added to the plasma. Clotting was followed by shaking the tube continuously on the surface of a water bath. Tick saliva (2.5 μl) or 0.15 M NaCl solutions were added to the plasma, when appropriate.

Apyrase Determination. Apyrase activity was identified by measuring orthophosphate release (18) from ATP, ADP, or AMP in a reaction medium containing 50 mM Tris HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM nucleotide, and 1 μl of tick saliva, in a final volume of 0.1 ml. Reactions proceeded for 30 min at 37°C, and were stopped by the addition of acid molybdate reagent. 1 U of enzymatic activity represents the amount of enzyme that produces orthophosphate at 1 μmol/min at 37°C, at the specified reaction mixture.

PGE_2 Radioimmunoassay (RIA). RIA for PGE_2 was performed as described previously (19).

Production and Assay of T Cell Hybridomas. The T hybridomas (E8.A1) used in these experiments were produced by fusing sheep insulin–primed lymph node cells with the BW 5147 AKR thymoma (20). This cell line is an autoreactive I-A^k-specific T cell. To assay activity, T hybridoma cells (10^5 cells/well) were cultured with anti-Thy-1 (1:1000 dilution) in complete medium (RPMI 1640 containing 8% fetal calf serum, 300 μg/ml penicillin, 50 μM 2-mercaptoethanol, and 10 mM Hepes buffer). Cells were cultured both in the presence and absence of tick saliva. Samples of saliva, ranging from 6 to 20 μl were diluted to 0.2 ml, and 1 μl of this diluted saliva was added to each well, to a 200 μl total volume per well. After incubating for 24 h at 37°C, supernatants were collected and assayed for interleukin (IL-2) content in a secondary culture using IL-2–dependent T cell line HT-2 (4×10^3 cells/100 μl/well). HT-2 cells were cultured for 24 h at 37°C in the presence of 60 μl primary culture supernatants. 1 μC of [^{3}H]thymidine (6.8 Ci/nmol; Amersham Corp., Arlington Heights, IL) was added to each well, and cells were harvested after 16–24 h incubation on a miniMASH II (M. A. Bioproducts, Walkersville, MD). The degree of stimulation was measured by the amount of radioactivity retained by the glass fiber filter, as determined by liquid scintilation counting.

Plasma Bradykinin Generation. Plasma bradykinin was generated by adding 25 μl of a 4% (wt/vol) kaolin suspension in 0.15 M NaCl to 100 μl of human citrated, platelet-poor plasma (21). After 1 min at 37°C, the mixture was added to the guinea pig ileum preparation.

Results

Platelet Antiaggregating Activity. To detect platelet antiaggregating activity, 4 μl of tick saliva were added to 100 μl of citrated human platelet-rich plasma in an aggregometer cuvette. Platelet aggregation was triggered by adding ADP, collagen suspension, or PAF, all used in concentrations that triggered maximum platelet response. A minor and transient episode of platelet aggregation followed addition of saliva to the platelet-rich plasma (Figs. 1–3), but subsequent aggregation was aborted or delayed. We conclude that the saliva of this tick contains antiplatelet activity that is effective against the main stimuli of platelet aggregation anticipated at the tick's feeding site.

Anticoagulant Activity. Because coagulation not only consolidates the already formed platelet plug, but contributes to its formation by promoting platelet aggregation (4–5), we sought evidence of an anticoagulant in the tick saliva. First, we determined whether saliva may delay recalcification time of citrated human plasma. Samples that normally clotted in 88 ± 1 s, clotted in 131 ± 10 s when 2.5 μl of saliva were added ($\bar{x} \pm$ SE; $n = 6$; $P < 0.01$, paired t test). Addition of saliva did not affect prothrombin time. Because this assay depends on factors VII, X, and thrombin as well as fibrinogen, we conclude that saliva delays coagulation by acting on the intrinsic pathway of the coagulation cascade before factor X activation. This activity may help prevent coagulation and thrombin formation at the feeding site.

Apyrase Activity. Apyrase enzymes have recently been described in the saliva of blood-sucking bugs (26), tse-tse flies (28), and mosquitoes (29), where they account, at least in part, for the anti–platelet-aggregating properties of the saliva of these unrelated blood-feeding insects. Accordingly, we investigated whether *I. dammini* may have evolved a similar antiplatelet system. Indeed, tick saliva hydrolyzed both ATP and ADP (Table I), but not AMP (not shown), and this characterizes apyrase activity. When estimating this activity, provision was made for the presence of 2.5 ± 0.3 mM orthophosphate (n = 10) in the saliva.

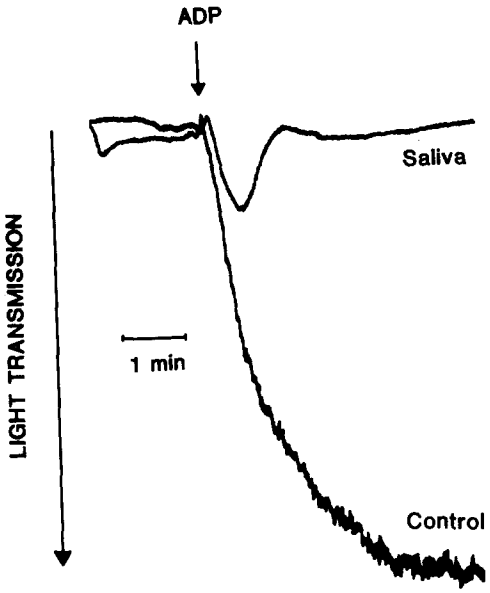

FIGURE 1. Inhibition of ADP-induced platelet aggregation by tick saliva. Platelet-rich plasma (100 μl) was preincubated for 1 min with continuous stirring. At the beginning of the tracings, saline (control) or saliva was added (4 μl). The arrow marks the addition of ADP (2 μM final concentration).

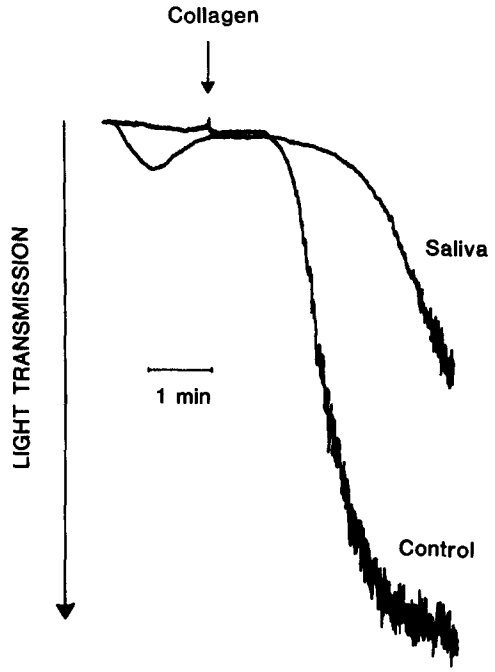

FIGURE 2. Inhibition of collagen-induced platelet aggregation by tick saliva. Other conditions as in Fig. 1.

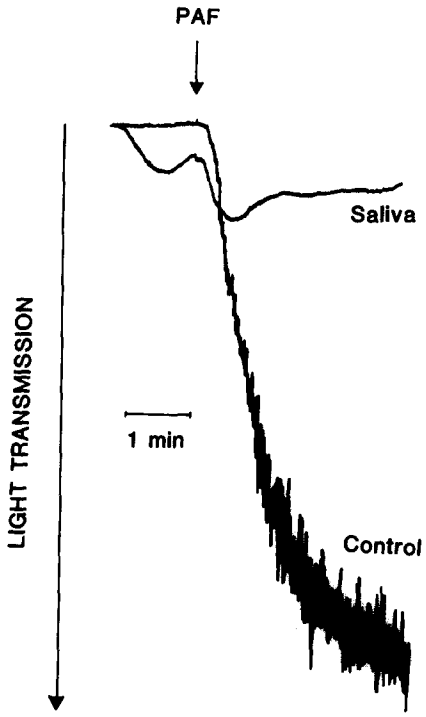

FIGURE 3. Inhibition of PAF-acether–induced platelet aggregation by tick saliva. Final concentration of PAF was 50 ng/ml. Other conditions as in Fig. 1.

TABLE I

Apyrase Activity in Samples of Saliva Individually Harvested From Adult, Female Ixodes dammini Stimulated by Pilocarpine

Sample number	Activity with substrate*	
	ATP	ADP
1	0.36	0.33
2	0.11	0.12
3	1.84	1.48
4	0.12	0.11
5	0.25	0.24
6	0.75	0.60
7	0.67	0.71
8	2.95	2.39
9	0.28	0.26
10	1.66	1.47
$\bar{x} \pm$ SE:	0.90 ± 0.30	0.77 ± 0.24

* Expressed as micromoles of orthophosphate released per minute per milliliter of tick saliva at 37°C.

Preliminary characterization of this enzyme activity in pooled saliva indicated a requirement for divalent cations, and that Mg^{++} was a better activator than Ca^{++}. Optimum pH was 8.5–9.0 both for ATP and ADP hydrolysis, and both activities were stimulated by 20 mM 2-mercaptoethanol. The ratio of ATP to ADP hydrolysis at pH 7.5 was 1.10 ± 0.04 to 1 (n = 10), demonstrating close correlation between the two hydrolytic activities, despite a broad range of activity when individual samples were compared (Table I). We conclude that tick saliva contains an enzyme with apyrase activity.

PGE_2 Characterization. Anti–platelet-aggregating activity seemed too great to be explained solely by apyrase, particularly the powerful inhibition of PAF-induced platelet aggregation (Fig. 3) (5). Substances that increase platelet cyclic AMP are good inhibitors of PAF-induced platelet aggregation (5), and among these are PG of the E series and prostacyclin (5). Evidence of salivary PG was then sought by means of a rat stomach strip bioassay. The tissue was rendered insensitive to most agonists by antagonists administered in the perfusion solution. Pilocarpine solutions, similar to that used to stimulate ticks to salivate, did not induce contractions. Saliva, on the other hand, induced contractions having an activity equivalent to 94 ± 32 ng PGE_2/ml of saliva (n = 9) (Figs. 4 and 5), thereby suggesting the presence of PG in tick saliva.

RIA of the same samples used in the bioassay, demonstrated the presence of 97 ± 36 ng PGE_2/ml of saliva. When compared individually, the bioassay and RIA results correlated closely (correlation coefficient, 0.974) (Fig. 5) and the slope of the regression line (0.88) did not differ from unity (as shown by analysis of variance). This suggests that most, if not all, rat fundus–contracting activity can be attributed to PGE_2.

Immunosuppressive Activity. Because PGE_2 is immunosuppressive (23), we asked whether tick saliva inhibits T lymphocyte activation. In this system, a cloned T cell hybridoma, E8.A1 was activated by the anti-Thy-1 mAb to secrete IL-2. The presence of IL-2 in conditioned media from E8.A1 cells was deter-

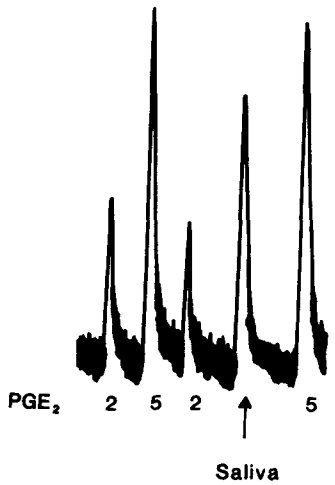

FIGURE 4. Rat stomach strip–contracting activity induced by tick saliva. PGE_2 standards of 2 and 5 ng were added as indicated. Tick saliva addition marked by arrow.

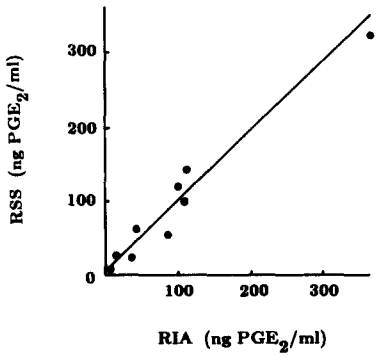

FIGURE 5. Rat stomach strip (RSS)-contracting activity and PGE_2 RIA of nine individual samples of tick saliva, both expressed in nanograms of PGE_2/ml of tick saliva. The line was obtained by linear regression.

TABLE II

Effect of Tick Saliva on the Production of IL-2 by E8.A1 T Hybridoma Cells Stimulated by Anti-Thy-1 mAb as Detected by [^{3}H]Thymidine Uptake by IL-2-dependent Cells

Sample number	Treatment	Saliva*	PGE_2‡	[^{3}H]Thymidine uptake	Inhibition
		μl/ml	*pM*	*cpm*	%
—	None	—	—	17,804	—
11	Saliva	0.57	160	798	95
12	Saliva	0.26	77	2,644	85
13	Saliva	0.23	350	2,612	85
14	Saliva	0.15	79	11,071	38

* Microliters of tick saliva per milliliter of culture during E8.A1 incubation with anti-Thy-1 mAb.

‡ Calculated concentration of PGE_2 during E8.A1 incubation, as estimated from RIA.

mined in secondary culture, by measuring uptake of [^{3}H]thymidine in an IL-2-dependent cell line. Suppression of [^{3}H]thymidine uptake in this experiment reflected inhibition of IL-2 secretion, a consequence of inhibited T cell activation. The results (Table II) show that addition of <1 μl of saliva from four individual ticks caused marked suppression of IL-2 secretion by the T cell hybridomas. Although accurate quantitative comparisons cannot be made on the basis of this data, the degree of suppression of IL-2 secretion is in the general range expected from the measured quantities of PGE_2 in these saliva specimens (38). We tentatively conclude that the suppression of IL-2 production by tick saliva can be accounted for by their content of PGE_2.

Kininase Activity. PGE_2 is hyperalgesic, increasing the sensitivity of a lesion to the pain-producing effects of bradykinin (24), which would stimulate hosts to remove ticks. PGE_2 also potentiates the edema promoted by histamine, serotonin, and bradykinin (25), a reaction that is present in lesions of hosts resistant to ticks (32). But ticks feed successfully and without causing pain, possibly because tick saliva antagonizes bradykinin. To test this hypothesis, we used a guinea pig ileum assay to determine whether saliva prevented bradykinin activity that was elicited

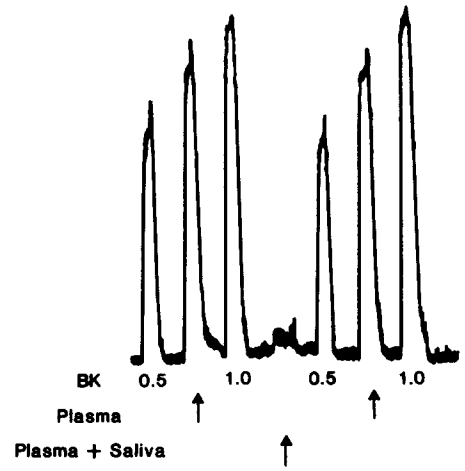

FIGURE 6. Tick saliva prevention of contractures induced in guinea pig ileum by contact-activated, human citrated plasma. Bradykinin (BK) standards were added to the organ bath to give 0.5 or 1 ng/ml of the peptide, where indicated.

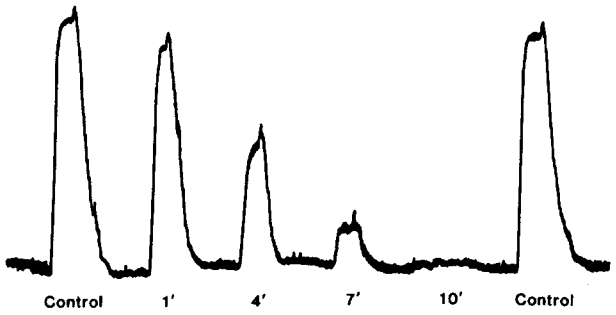

FIGURE 7. Kininase activity of tick saliva. 50 μl of a 1:200 dilution of tick saliva were mixed with 50 μl of bradykinin (100 μg/ml) in Tyrode's solution and incubated at 37°C. At the indicated time intervals in minutes, 10-μl aliquots were added to the guinea pig ileum preparation.

by contact activation of human plasma (21). In effect, 1 μl of saliva prevented contractions brought on by the addition of 0.1 ml of citrated plasma plus kaolin to the preparation (Fig. 6). This effect is consistent with that of a kininase, because, in another demonstration, bradykinin incubated in Tyrode's solution became progressively inactivated in the presence of saliva (Fig. 7). Such activity was destroyed by heat (100°C for 1 min), and abolished in the presence of EDTA (3 mM), cysteine (3 mM), Zn^{++} (0.25 mM), or Co^{++} (3 mM). The presence of this kininase in tick saliva resolves the paradox presented by the otherwise hyperalgesia- and edema-promoting activities of PGE_2.

Discussion

Saliva ejected by adult *I. dammini* contains antihemostatic, antiinflammatory, and immunosuppressive components, properties that appear to facilitate blood-feeding success of this tick during its prolonged period of host attachment. This

combination of pharmacologically active attributes of saliva in ticks are described for the first time.

Platelet antiaggregating activity has previously been described for blood-sucking bugs (27), tsetse flies (28), and mosquitoes (2, 29) and is here reported for the first time in the saliva of a tick. Indeed, *I. dammini* saliva inhibits platelet aggregation induced by ADP, collagen, or PAF (Figs. 1–3), thus counteracting the main expected stimuli of platelet aggregation at the tick's feeding site. Thrombin formation, a similarly anticipated factor, is prevented by a salivary anticoagulant. *I. dammini*, thus, is well equipped to prevent host hemostasis.

The crucial role of ADP in aggregating vertebrate platelets (5, 30, 31), and a common requirement for blocking host hemostasis, must have promoted the apparently convergent development of salivary apyrase enzymes in such distantly related arthropods as the mosquito *Aedes aegypti* (29), the blood-sucking bug *Rhodnius prolixus* (26), the tse-tse fly *Glossina tachinoides* (28), and the tick *I. dammini*. The observed apyrase activity may account, at least in part, for the inhibitory effect of saliva on platelet aggregation. Because platelet aggregation is redundantly stimulated (4, 5), effective prevention of hemostasis requires a redundant pharmacological cocktail. PGE_2 (22), and possibly other as yet unidentified components, may contribute to the inhibitory effect of saliva on platelet aggregation.

Salivary apyrase may promote other effects important to a tick's successful feeding. In addition to inhibiting hemostasis by degrading ADP, salivary apyrase may prevent those inflammatory processes stimulated by ATP (34), including mast cell degranulation (35), and aggregation of neutrophils (36). These processes are associated with release of prohemostatic compounds such as thromboxane, PAF, and vasoactive amines (33). Apyrase converts ATP to AMP, which is pharmacologically inactive or even inhibitory to purinergic P_2 receptors (37). Thus, apyrase contributes to antiinflammatory as well as antihemostatic activity.

The cardinal signs of inflammation, erythema, edema, and pain, each may affect the outcome of a tick's attempt to draw blood from its host. The erythematous reaction would help by increasing the flow of blood to the feeding site (8,32). Edema, on the other hand, reduces blood flow or induces bleb formation, as in the skin of cattle resistant to *Boophilus microplus*, but not in succeptible animals (32). Pain, which focuses attention of the host on the parasite's feeding site, would increase grooming behavior. Because inflammation both helps and hinders feeding, ticks may modulate inflammation selectively.

One salivary component, PGE_2, presents a particular problem in this regard by producing a spectrum of effects that may hinder as well as help feeding. The helpful category includes erythema (increasing the flow of blood to the feeding tick), inhibition of mast cell degranulation (which helps minimize release of platelet-aggregating, edema-promoting, and vasoconstrictive factors) (33), and immunosupression (23) (potentially preventing the production of antibodies against salivary antigens). On the other hand, PGE_2 potentiates pain produced by bradykinin (24), as well as edema caused by substrates that increase vascular permeability (25). The tick's salivary kininase may counteract these "undesirable" PGE_2 side effects by destroying bradykinin. In this manner, ticks antagonize

their host's hemostatic and inflammatory responses by the actions of several distinct salivary components.

We found that *I. dammini* saliva is immunosuppressive, as measured by inhibition of T hybridoma activation. This inhibitory activity can be explained by the PGE_2 content of saliva, although detailed quantitative studies have not yet been performed. This evidence may provide an explanation for the failure of salivary vaccines to induce host resistance to ticks (39), the poor mitogen responsiveness of T cells from tick-infected hosts (40), and the frequently described immunosuppressed state of tick-infected animals (7). Indeed, T cell activation occurs at the very beginning of the cascade of cellular events leading to antibody production, acting at the site where the antigen is deposited (41). This action of tick saliva may delay, reduce, or abolish the host's response to the tick's salivary antigens, thus reducing immune-mediated inflammatory responses at the tick's feeding site.

Tick-resistant hosts reject feeding ticks by means of immune-mediated, immediate inflammatory skin reactions (6, 7). In nature, however, ticks are not rejected. But the mechanism that permits such stable, chronic associations of ticks and their hosts have not been described. It seems likely that the pharmacological armamentarium in saliva may specifically prevent antisalivary antibody production and antagonize chemical mediators of the host's inflammatory response. Different hosts have evolved characteristic methods for mediating immune and inflammatory responses. For example, rat and mouse mast cells contain important amounts of serotonin, whereas this amine may not be detectable in other mammalian mast cells (42). Some animals release more histamine than serotonin after platelet aggregation (43). Asthma in guinea pigs is mediated mostly by histamine, whereas leukotrienes predominate in the human disease (44). Stable host associations require that the tick match its host's defenses with an appropriate array of its own, suggesting that these defenses may fit as a "lock and key." These considerations support the idea that ticks, like other parasites, evade host reactions that would cause rejection, and that this adaptation is a component of host specificity.

Finally, the salivary components injected by ticks may promote invasion of the host by tick-borne pathogens. For example, by preventing macrophage activation and neutrophil activity (45), PGE_2 would protect the pathogen during its initial phase of adaptation in the skin of a new host. *I. dammini* is the vector of human babesiosis and Lyme disease (46); coinjected saliva may facilitate transmission of these newly discovered agents of human disease.

Summary

Pilocarpine-induced saliva of the tick, *Ixodes dammini*, inhibited platelet aggregation triggered by ADP and collagen, as well as platelet-aggregation factor. In addition, we found apyrase activity (which degrades ATP and ADP to AMP and orthophosphate) and an anticoagulant. We showed the presence of prostaglandin E_2 (PGE_2) by bioassay and radioimmunoassay. This saliva inhibited interleukin 2 production by T cell hybridomas, an activity consistent with that of PGE_2. A kininase was demonstrated, and this may counteract the algesia- and edema-promoting properties of PGE_2. Together, these salivary components produce

antihemostatic, antiinflammatory, and immunosuppressive effects that may facilitate feeding, as well as transmission of tick-borne pathogens.

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