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THE SALIVARY ADENOSINE DEAMINASE ACTIVITY OF THE MOSQUITOES *CULEX QUINQUEFASCIATUS* AND *AEDES AEGYPTI*

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Summary

A cDNA coding for a protein with significant similarity to adenosine deaminase (ADA) was found while randomly sequencing a cDNA library constructed from salivary gland extracts of adult female *Culex quinquefasciatus*. Prompted by this result, we found high ADA activities in two culicine mosquitoes, *Culex quinquefasciatus* and *Aedes aegypti*, but not in the anopheline *Anopheles gambiae*. Homogenates from *Culex quinquefasciatus* also have an AMP deaminase activity that is three times greater than the ADA activity, whereas in *Aedes aegypti* the AMP deaminase activity is less than 10 % of the ADA activity. Evidence for secretion of ADA during blood feeding by *Aedes aegypti* includes the presence of ADA activity in warm solutions probed through a membrane by mosquitoes and in serotonin-induced saliva and a statistically significant reduction in the levels of the enzyme in *Aedes aegypti* following a blood meal. We could not demonstrate, however, that *C. quinquefasciatus* secrete ADA in their saliva. Male *Aedes aegypti* and *C. quinquefasciatus*, which do not feed on blood, have less than 3 % of the levels of ADA found in females. We propose that ADA activity in *A. aegypti* may help blood feeding by removing adenosine, a molecule associated with both the initiation of pain perception and the induction of mast cell degranulation in vertebrates, and by producing inosine, a molecule that potently inhibits the production of inflammatory cytokines. The role of salivary ADA in *Culex quinquefasciatus* remains unclear.

Key words: saliva, blood-feeding insect, haematophagy, adenosine deaminase, mosquito, *Culex quinquefasciatus*, *Aedes aegypti*, *Anopheles gambiae.*

Introduction

Adenosine deaminase (ADA) hydrolyzes adenosine to inosine and ammonia. Its presence in the salivary glands of a New World sand fly and tsetse flies was recently reported (Charlab et al., 2000; Li and Aksoy, 2000) following the finding of salivary cDNA sequences with homology to mammalian and bacterial ADAs. Salivary homogenates of the sand fly *Lutzomyia longipalpis* had relatively high enzyme activities compared with other enzyme sources. Activity was significantly reduced following a blood meal, suggesting that the enzyme was secreted, in accordance with the identification of a secretory signal peptide deduced from the cDNA sequence (Charlab et al., 2000). The finding of salivary ADA in hematophagous flies is puzzling because adenosine causes vasodilation and inhibits platelet aggregation (Collis, 1989; Dionisotti et al., 1992; Chinellato et al., 1994), functions that should be of help in blood feeding (Ribeiro, 1995). Conversion of adenosine to inosine abrogates these vasodilatory and antiplatelet functions. Furthermore, the Old World sand fly *Phlebotomus papatasi* actually secretes large amounts of adenosine in its saliva (Ribeiro et al., 1999) as the main salivary vasodilator and does not have salivary ADA activity (J. M. C. Ribeiro, unpublished observations). The presence of a salivary ADA in some hematophagous insects and not in others is puzzling. A possible adaptive role for salivary ADA is to reduce local pain in the host while the insect is feeding, since adenosine A2 and A3 receptors stimulate peripheral pain receptors (Burnstock and Wood, 1996; Poon and Sawynok, 1999; Charlab et al., 2000). In addition, while adenosine induces mast cell degranulation and the release at the feeding site of histamine, a substance that causes itching and burning, inosine is more than 100 times less effective in producing this effect (Tilley et al., 2000). It has also been shown that inosine formed by ADA inhibits the production of inflammatory cytokines (Hasko et al., 2000).

Whether salivary ADA is present in other hematophagous arthropods is not known. In this report, we show that the full-length clone of a cDNA from the salivary glands of the mosquito *Culex quinquefasciatus* has high homology to vertebrate and invertebrate ADA and identify large amounts of this activity in the salivary glands of adult females of both *C. quinquefasciatus* and *Aedes aegypti* but not *Anopheles gambiae*. Only *Aedes aegypti* appear to secrete the enzyme.

Materials and methods

Reagents

Organic reagents were obtained from Sigma Chemical Corporation (St Louis, MO, USA). All water used was of 18 MΩ quality and was produced using a MilliQ apparatus from Millipore (Bedford, MA, USA).

Mosquitoes

Insects were reared in the Medical Entomology Section of the Laboratory of Parasitic Diseases of the National Institute of Allergy and Infectious Diseases under the expert supervision of Mr Andre Laughinghouse. Insectary rooms were kept at 26±0.5°C, with a relative humidity of 70 % and a 16 h:8 h light:dark photoperiod. The strains of mosquito used were the Liverpool/black eye strain of *Aedes aegypti*, the G3 strain of *Anopheles gambiae* and the Vero Beach strain of *Culex quinquefasciatus*. The most recent strain (*C. quinquefasciatus*) has been in continuous culture for 8 years. Adult mosquitoes used in the experiments were given no blood meals and were maintained on a diet of 10 % Karo syrup solution. Salivary glands were dissected from adult female mosquitoes aged 3–10 days that had either never been fed blood or immediately after a blood meal, as indicated in the experiments. The glands were transferred to 10 or 20 µl of Hepes saline (HS; 0.15 mol l^{-1} NaCl, 10 mmol l^{-1} Hepes, pH 7.0) in 1.5 ml polypropylene vials, usually as groups of 20 pairs of glands in 20 µl of HS or as individual glands in 10 µl of HS. In some experiments, salivary glands from male mosquitoes were dissected. Salivary glands were kept at −75 °C until needed, when they were disrupted by sonication using a Branson Sonifier 450 homogenizer (Danbury, CT, USA). Salivary homogenates were centrifuged at 10 000 ***g*** for 2 min, and the supernatants were used for experiments. To obtain dilute saliva samples from mosquitoes, a small artificial feeder containing 100 µl of HS was offered to a cage containing approximately 100 adult female mosquitoes. The feeder membrane consisted of stretched Parafilm (American National Can, Menasha, WI, USA). The feeder was warmed by continuous circulation of water at 38 °C.

Collection of serotonin-induced saliva

To obtain saliva, female mosquitoes were lightly anesthetized by exposure to 0 °C for 1 min. After their wings and legs had been removed, 0.1 µl of a 1 mmol l^{-1} serotonin solution in HS was injected into the coelomic cavity using a heat-pulled glass capillary micropipette operated by mouth pressure (Ribeiro et al., 1984a). After the injection, the feeding fascicle was exposed from within the sheath and inserted into a 2 cm piece of polyethylene tubing (PE-10, inner diameter 0.28 mm, outer diameter 0.61 mm; Clay Adams, Parsippany, NJ, USA) containing a column of mineral oil approximately 0.5 cm long. This operation was carried out under a stereomicroscope using fine-tipped tweezers. After 10 min, to allow salivation into the oil at room temperature, the mouthparts were removed from the tubing. A no. 30 needle was inserted into the other end of the tubing, and this was used to blow the oil+saliva into 20 µl of HS, pH 7.2, with the help of a 20 µl Hamilton (Reno, NV, USA) syringe with a Luer-lock fitting. Ten samples were accumulated, each in 20 µl of HS. The samples were then centrifuged at 10 000 ***g*** for 5 min; the aqueous phase was removed for analysis.

Protein determination

Protein concentration was assessed by the Bradford method (Bradford, 1976) using a kit from Bio-Rad (Hercules, CA, USA).

Salivary gland cDNA library construction

mRNA was isolated from 80 pairs of salivary glands dissected from 0- to 2-day-old female *C. quinquefasciatus* using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA) as described previously (Charlab et al., 2000). A polymerase chain reaction (PCR)-based cDNA library was subsequently prepared according to the instructions for the SMART PCR cDNA Library Construction Kit (Protocol PT3000-1, Version PR92334, Clontech, Palo Alto, CA, USA). Briefly, the *C. quinquefasciatus* salivary gland poly(A)$^+$ RNA was reverse-transcribed to cDNA for 1 h at 42 °C using Superscript RNase H reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, USA), the SMART III oligonucleotide and a modified oligo(dT) primer (CDS III/3′ PCR primer) (Clontech). The combined use of the SMART III oligonucleotide and the CDS III/3′ primer allows the incorporation of asymmetrical *Sfi*I restriction enzyme sites at both the 5′ and 3′ ends of the cDNA.

Second-strand synthesis was performed through a PCR-based protocol using the 5′ PCR primer and the CDSIII/3′ PCR primer as sense and antisense primers, respectively. Conditions for PCR were: 95 °C for 1 min, 20 cycles of 95 °C for 15 s and 68 °C for 6 min. After proteinase K treatment for 20 min at 45 °C, double-stranded cDNA was digested with *Sfi*I for 2 h at 50 °C and size-fractionated using CHROMA SPIN-400 columns supplied by the manufacturer. The fraction profile was checked by electrophoresis on a 1.1 % agarose/ethidium bromide gel. Peak fractions were visualized under ultraviolet illumination, and the first two fractions containing cDNA were pooled, precipitated and resuspended in 7 µl of deionized water. The *Sfi*IB-digested, size-fractionated and concentrated cDNA was then ligated into the *Sfi*I-digested Lambda TriplEx2 vector (Clontech). The ligation reaction was packed using Gigapack Gold III packaging extract from Stratagene/Biocrest (Cedar Creek, TN, USA) following the supplier's recommended protocol. The library was plated by infecting log-phase *Escherichia coli* XL1-Blue (Clontech). The percentage of recombinants was determined by PCR with vector primers flanking the inserted cDNA and visualization on a 1.1 % agarose gel with ethidium bromide.

Mass sequencing of C. quinquefasciatus *salivary gland cDNA library*

The *C. quinquefasciatus* cDNA library was plated at a density of approximately 150 plaques per plate (150 mm Petri

dish). The plaques were picked at random and transferred to 96-well polypropylene plates containing 50–75 µl of deionized water per well. The plates were covered and placed on a gyratory shaker for 1 h at room temperature (25 °C). Of the phage sample, 5 µl was used as a template to amplify and sequence the cDNA according to Valenzuela et al. (Valenzuela et al., 2000). Briefly, the primers for this reaction were sequences from the triplEX2 vector, PT2F1 (5′-AAGTACTCTAGCAATTGTGAGC-3′), which is positioned upstream of the cDNA of interest (5′ end), and PT2R1 (5′-CTCTTCGCTATTACGCCAGCTG-3′), which is positioned downstream of the cDNA of interest (3′ end). High-fidelity platinum *Taq* polymerase (Gibco-BRL, Gaithersburg, MD, USA) was used for these reactions. Amplification conditions were as follows: one hold at 75 °C for 3 min, one hold at 94 °C for 3 min and 34 cycles at 94 °C for 30 s, 49 °C for 30 s and 72 °C for 80 s. Amplified products were visualized on a 1.1 % agarose gel with ethidium bromide. The concentration of double-stranded cDNA was measured using Hoechst dye 33258 on a Flurolite 1000 plate fluorimeter (Dynatech Laboratories, Chantilly, VA, USA).

To remove the primers from the PCR reaction products, PCR samples (3–4 µl) containing between 100 and 200 ng of DNA were treated with Exonuclease I (0.5 units μl^{-1}) and shrimp alkaline phosphatase (0.1 units μl^{-1}) for 15 min at 37 °C and 15 min at 80 °C on a 96-well PCR plate.

This mixture was used as a template for a cycle-sequencing reaction using the DTCS labeling kit (Beckman Coulter Inc., Fullerton, CA, USA). The primer used for sequencing (PT2F3) is upstream of the inserted cDNA and downstream of the primer PT2F1. The sequencing reaction was performed in a Gene Amp PCR system 9700 from PE Applied Biosystems (Foster City, CA, USA). Conditions were 75 °C for 2 min, 94 °C for 4 min and 30 cycles at 96 °C for 20 s, 50 °C for 20 s and 60 °C for 4 min.

To clean up the samples after cycle sequencing, they were subjected to a step using the multiscreen 96-well plate-cleaning system (Millipore); this plate was prepared by adding a fixed amount (manufacturer's specification) of Sephadex-50 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 300 µl of deionized water. After 1 h of incubation at room temperature, the water was removed from the multiscreen plate by centrifugation at 750 ***g*** for 5 min. After partially drying the Sephadex in the multiscreen plate, the whole cycle-sequencing reaction was added to the center of each well and centrifuged at 750 ***g*** for 5 min. The clean sample was collected on a sequencing microtiter plate (Beckman Coulter, Inc.). The plate was dried using a SpeedVac SC 110 with a microtiter plate holder (Savant Instruments Inc., Holbrook, NY, USA). The dried samples were immediately resuspended in 25 µl of deionized ultraPure formamide (J. T. Baker; Phillipsburg, NJ, USA), and one drop of mineral oil was added to the top of each sample. Samples were either sequenced immediately on a CEQ 2000 DNA sequencing instrument (Beckman Coulter Inc.) or stored at −30 °C for several days.

Identification and sequencing of C. quinquefasciatus *ADA*

Sequenced cDNA inserts were analysed using the BLAST programs with BLOSUM-62 matrix (http://www.ncbi.nlm.gov/BLAST/; Altschul et al., 1997). During this process, we identified a full-length cDNA with significant sequence similarity to *Lutzomyia longipalpis* ADA (GenBank AF 234182) (Charlab et al., 2000). This cDNA was then reamplified using the PT2F1 and PT2R1 primers, and the entire cDNA was fully sequenced using custom-designed primers as described above.

Enzyme assays

Adenosine deaminase activity was measured in quartz microcuvettes holding 60–100 µl samples (Starna Cells, Atascadero, CA, USA). The indicated concentrations of adenosine, AMP or adenine in 10 mmol l^{-1} Hepes plus 150 mmol l^{-1} NaCl (HS) (or buffer as otherwise indicated) were added to the cuvette, followed by saliva or salivary gland homogenate (usually equivalent to 5–10 % of one pair of glands, or approximately 50–100 ng of protein). After mixing the solution by several cycles of aspiration and deposition of the sample, the absorbance (*A*) either at 265 nm or at both 241 and 265 nm was read at 5 s intervals for the single-wavelength protocol or at 15 s intervals for the dual-wavelength protocol using a Lambda 18 spectrophotometer from Perkin Elmer (Norwalk, CT, USA). The cuvettes were thermostatted to 30 °C. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the deamination of 1 µmol of adenosine per minute (ΔA=8.6 min^{-1} ml^{-1} at 265 nm or ΔA=13.76 min^{-1} ml^{-1} when the difference in optical densities between 249 and 265 nm is used; Agarwal and Parks, 1978, and our own measurements). In some experiments, the cuvette was scanned at the indicated time intervals from 230 to 320 nm.

Chromatographic procedures

Gel permeation chromatography experiments were performed with a Superdex 75 column (3.2 mm×300 mm) isocratically perfused with 10 mmol l^{-1} Hepes, pH 7.4, 0.15 mol l^{-1} NaCl at a flow rate of 50 µl min^{-1}. A CM4100 pump and SM 4100 UV detector (ThermoSeparation Products, Riviera Beach, FL, USA) were used. Fractions were collected at 30 s intervals. Of each fraction, 5 µl was used to determine ADA activity in the assay described above, following the rate of absorbance change at 265 nm for 2 min. The column was calibrated with cytochrome *c*, myoglobin, carbonic anhydrase, ovalbumin and the monomer and dimer of bovine serum albumin.

Statistical analyses

Statistical tests were carried out using SigmaStat software (Jandel Scientific, San Raphael, CA, USA). Mann–Whitney tests were conducted in comparisons where the variances of two groups were not homogeneous; otherwise, Student's *t*-tests were performed.

Results

We randomly sequenced 600 clones from a salivary gland cDNA library obtained from female *C. quinquefasciatus*. Among the sequenced clones, we identified a cDNA with a high degree of similarity to ADA and invertebrate growth factors when the translated protein sequence was compared with the non-redundant protein database of the National Center for Biotechnology Information of the National Library of Medicine using the BlastP program (Altschul et al., 1997). This cDNA (GeneBank accession number AF298886) has the adenosine/AMP deaminase (A_deaminase) Pfam signature (Bateman et al., 2000) and a signal peptide indicative of secretion (Nielsen et al., 1997) (Fig. 1). The mature protein is predicted to have an amino acid sequence starting at the 18th amino acid residue (sequence AKLI...), an isoelectric pH of 5.78 and a relative molecular mass of 55 575.

To verify whether salivary gland homogenates of *C. quinquefasciatus* contain ADA activity, salivary homogenate equivalent to 0.2 of a pair of glands was incubated with 100 µmol l^{-1} adenosine in 100 µl of HS buffer, and the mixture was scanned at 10 min intervals from 230 to 320 nm. The spectrum shifted in a time-dependent manner from a maximum at 258 nm to one at 249 nm, consistent with the conversion of adenosine to inosine. The differential spectrum shows a maximum decrease at 265 nm and a maximum increase at 241 nm, as found for the conversion of adenosine to inosine (Agarwal and Parks, 1978) (Fig. 2).

Many ADAs can also utilise AMP as a substrate, and so we measured the ability of *C. quinquefasciatus* salivary homogenates to convert AMP to IMP. The rate of conversion of AMP by the enzyme in the homogenate is faster than that of adenosine (Fig. 3). Although part of the activity could be ascribed to the conversion of AMP to adenosine by 5′-nucleotidase, this mechanism cannot explain the increased enzyme activity with AMP. In other words, an infinite amount of 5′-nucleotidase could instantly convert all AMP to adenosine and, thus, the ADA activity would be the same for AMP and adenosine. It could not, however, convert AMP to inosine faster than adenosine is converted to inosine. We conclude, therefore, that there is AMP deaminase activity in *C. quinquefasciatus* salivary gland homogenates. In three different pools of *C. quinquefasciatus* salivary homogenates, the ratio of AMP deaminase activity to ADA activity was 3.3±0.07 (mean ± S.E.M., N=3). When adenine was used as a substrate, no reaction was observed (results not shown).

To investigate whether ADA and AMP deaminase activities co-elute following gel-permeation chromatographic procedures and to obtain an estimate of their molecular masses, we submitted 20 pairs of homogenized salivary glands from *C. quinquefasciatus* to chromatography in a Superdex 75 column. The activities of both substrates co-elute with an apparent molecular mass of 62 kDa (Fig. 4). Although this estimate is larger than the 55 kDa predicted from the translated cDNA, it is still within the margin of error of the technique. The ranges of retention times of the fraction containing the maximum activity correspond to molecular masses of 55.7–69 kDa. In addition, the mature molecule, which has several glycosylation sites, could have been post-translationally modified. In any case, these data support the hypothesis that ADA and AMP deaminase activities reside in the same molecule.

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  44 atgtggaagaaagtggtgatattcttgcttatttgggattcagcg
     M  W  K  K  V  V  I  F  L  L  I  W  D  S  A
  89 tacagtgctaaactgattagtcgccttgaccgagagtcaattctg
     Y  S  A  K  L  I  S  R  L  D  R  E  S  I  L
 134 gatgccgaaaagaagcatcgaactggtggtaacgcgtatctcacc
     D  A  E  K  K  H  R  T  G  G  N  A  Y  L  T
 179 gagagggaagcccaagcaaatgatatcgtaacgagacttcgaagt
     E  R  E  A  Q  A  N  D  I  V  T  R  L  R  S
 224 aagattctactggaaggaatcgccaactcgacaggatttgcaccg
     K  I  L  L  E  G  I  A  N  S  T  G  F  A  P
 269 gcgatgcacttttccaagcaaagccacttattgagagcagtccg
     A  M  H  F  F  Q  A  K  P  L  I  E  S  S  P
 314 atcttccgtatgttgaaagcgatgccgaaagggtcggttctacat
     I  F  R  M  L  K  A  M  P  K  G  S  V  L  H
 359 ttgcacaatacagcagctgttagttccaagtgggtcatcaaaaat
     L  H  N  T  A  A  V  S  S  K  W  V  I  K  N
 404 ttgacctaccgttccgaggcaaaactctgtgaggttaatggaaca
     L  T  Y  R  S  E  A  K  L  C  E  V  N  G  T
 449 gtgttttttacagttagacaatccaagttctgtgacagtgaacct
     V  F  F  T  V  R  Q  S  K  F  C  D  S  E  P
 494 caaaaaagcatcaccaaacttcgagcgcaaaacgcttcagcagaa
     Q  K  S  I  T  K  L  R  A  Q  N  A  S  A  E
 539 gcgttcgatctctggctggagtccttcatcaatcttaagcttcgc
     A  F  D  L  W  L  E  S  F  I  N  L  K  L  R
 584 gacccggaactgatgcacaccgacgtgaacaccgtttggaacgat
     D  P  E  L  M  H  T  D  V  N  T  V  W  N  D
 629 ttccagcagatgtttgacgcctccaaggatctcctgatgtacaaa
     F  Q  Q  M  F  D  A  S  K  D  L  L  M  Y  K
 674 ccgttcttcgaagactaccaccgtcagatgctgcgagaattttac
     P  F  F  E  D  Y  H  R  Q  M  L  R  E  F  Y
 719 gacgacaacgttcagtacattgagctacgggcgtcgctttcaaag
     D  D  N  V  Q  Y  I  E  L  R  A  S  L  S  K
 764 gtgtacgatgcgaatggaaaagattacaacgagtttgaaatcgtg
     V  Y  D  A  N  G  K  D  Y  N  E  F  E  I  V
 809 aaaataatttcggatattgtggactctttcaagaaagaccatccg
     K  I  I  S  D  I  V  D  S  F  K  K  D  H  P
 854 gacttttttggagttaagatcatttacgccaaacatagatcgatt
     D  F  F  G  V  K  I  I  Y  A  K  H  R  S  I
 899 gataacgaaactgtggaatcgttcctggagaagtttatcgcgctc
     D  N  E  T  V  E  S  F  L  E  K  F  I  A  L
 944 aatcaggaattccccgacctcgttgttggcttcgacctcgtcggc
     N  Q  E  F  P  D  L  V  V  G  F  D  L  V  G
 989 caggaggacatcaacaatccgctcatcctattcacagatcagttg
     Q  E  D  I  N  N  P  L  I  L  F  T  D  Q  L
1034 tgcaaatttgaaaagacagccccttacttttttccacgctggagag
     C  K  F  E  K  T  A  P  Y  F  F  H  A  G  E
1079 acaaacggttacggcagcgaggccgacctgaacctagtcgatgcc
     T  N  G  Y  G  S  E  A  D  L  N  L  V  D  A
1124 gtactgctcaacagtcgtcgcatcggccacgggtactcactgtac
     V  L  L  N  S  R  R  I  G  H  G  Y  S  L  Y
1169 aagcacccggtcctgtggaagatggtcaagcaaaagggtatcgcg
     K  H  P  V  L  W  K  M  V  K  Q  K  G  I  A
1214 ctggagatttgtcccctgtcgaaccaggtgctgcggctcgtgacg
     L  E  I  C  P  L  S  N  Q  V  L  R  L  V  T
1259 gacttgcgcaaccatccggcggttttctacgtgtccgagagtgtc
     D  L  R  N  H  P  A  V  F  Y  V  S  E  S  V
1304 cccatcgtgattgcacccgacgatcccggcttttgggacagtgca
     P  I  V  I  A  P  D  D  P  G  F  W  D  S  A
1349 gcggttggcttcgattattactatgcgttgatgtcgctggctccg
     A  V  G  F  D  Y  Y  Y  A  L  M  S  L  A  P
1394 cactcggctgggattggatttttgaagcagatcgtttgggattcg
     H  S  A  G  I  G  F  L  K  Q  I  V  W  D  S
1439 gtcaagtacagcacattaacagaaccagaaagaacacaatacgct
     V  K  Y  S  T  L  T  E  P  E  R  T  Q  Y  A
1484 gagttgctgcagcccaagtgggaagcattttggacttcatcatc
     E  L  L  Q  P  K  W  E  A  F  L  D  F  I  I
1529 gccagtaaagtgttgaactattaa 1552
     A  S  K  V  L  N  Y  *
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Fig. 1. Salivary *Culex quinquefasciatus* adenosine deaminase cDNA and protein sequences. The underlined sequence refers to the signal peptide identified by the program SignalP (Nielsen et al., 1997). Nucleotides are in lower case and amino acid residues in capital letters.

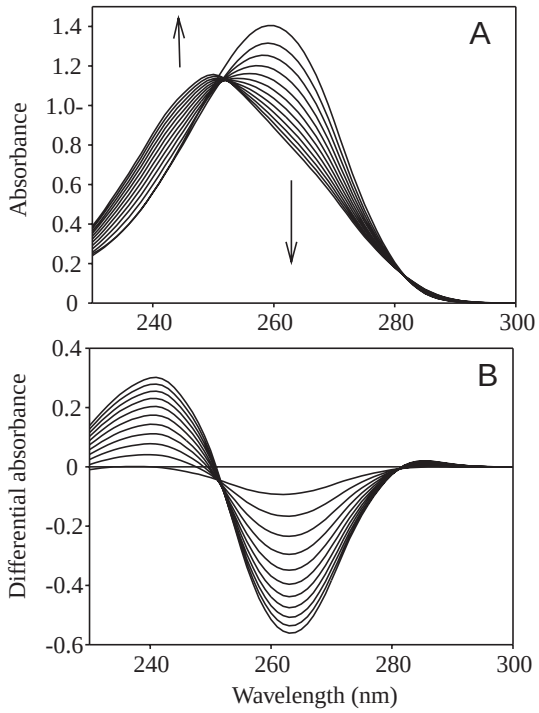

Fig. 2. Adenosine deaminase activity of salivary homogenates of adult female *Culex quinquefasciatus* mosquitoes. (A) A cuvette containing 100 μmol l^{-1} adenosine in 100 μl of HS buffer, pH 7.2, was scanned at 10 min intervals following addition of salivary homogenate equivalent to 0.2 pairs of salivary glands. The arrows indicate the direction of change of the spectrum over time. (B) Differential spectra of the data in A obtained by subtracting each scan from the scan at time zero.

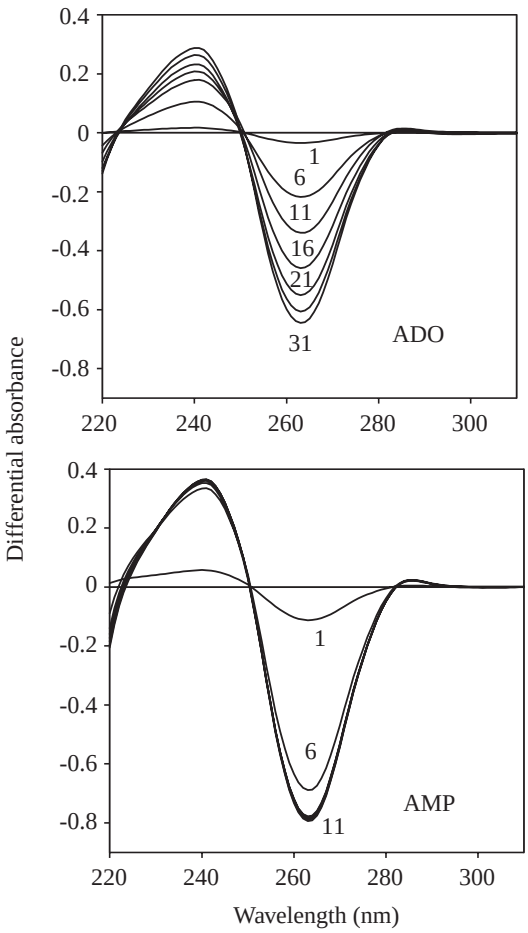

Fig. 3. Adenosine (ADO) and AMP deaminase (AMP) activities in *Culex quinquefasciatus* salivary homogenates from adult female mosquitoes. (A) Differential spectra obtained following addition of homogenate from one pair of salivary glands to 100 μmol l^{-1} adenosine in 100 μl of HS buffer, pH 7.2. (B) Differential spectra obtained following addition of homogenate from one pair of salivary glands to 100 μmol l^{-1} AMP in 100 μl of HS buffer, pH 7.2. The numbers in the figure indicate the time, in minutes, that the scan was performed following the addition of the salivary homogenate.

To determine whether other mosquitoes could have salivary ADA activity, we incubated salivary homogenates from *Aedes aegypti* and *Anopheles gambiae* with adenosine and scanned the cuvette for changes in ultraviolet absorption. Even when incubating two pairs of homogenized gland equivalents from the anopheline mosquito, no activity was found; however, incubation of salivary homogenate equivalent to 0.02 of a pair of glands from *Aedes aegypti* resulted in measurable activity (Fig. 5). *Aedes aegypti* salivary homogenates had little AMP deaminase activity. In three different pools of *Aedes aegypti* salivary homogenates, the ratio of AMP deaminase activity to adenosine activity was 0.07±0.02 (mean ± S.E.M., N=3).

To investigate whether mosquito salivary ADA could be secreted during a blood meal, we measured the enzyme activity in salivary homogenates obtained from unfed mosquitoes and from mosquitoes immediately after a blood meal. While more than 95% of *Aedes aegypti* offered a blood meal would feed readily, only 10–20% of *C. quinquefasciatus* would do so. For this reason and to obtain salivary glands from mosquitoes of the same physiological status, control (pre-blood-fed) salivary glands from *C. quinquefasciatus* were removed from mosquitoes attracted to a human hand. Because virtually all (>95%) *Aedes aegypti* were attracted to the human hand, this selection was not performed in these mosquitoes. In both mosquito species, there was a significant decrease in salivary protein content following a blood meal. Levels fell from 2.6±0.5 to 1.2±0.2 μg protein gland $pair^{-1}$ for *Aedes aegypti* (mean ± S.E.M., N=12, P=0.016, Mann–Whitney rank sum test) and from 1.00±0.36 to 0.32±0.08 μg protein gland $pair^{-1}$ (mean ± S.E.M., N=12, P=0.027, Mann–Whitney test) for *C. quinquefasciatus.* While there was a significant decrease in the amount of ADA activity in *Aedes aegypti* salivary gland homogenates before and after a blood meal (from 17.0±1.6 to 12.0 1.3 munits gland $pair^{-1}$, mean ± S.E.M., N=12, P=0.028, t-test), only a small non-significant decrease was observed in *C. quinquefasciatus* (from 2.64±0.37 to 2.53±0.34 munits gland $pair^{-1}$, mean ± S.E.M., N=12, P>0.1, t-test) (Fig. 6). Although these data suggest that the *Aedes aegypti* enzyme is secreted during a blood meal, we cannot conclude the same for the *C. quinquefasciatus* enzyme.

Unlike females, which feed on both blood and sugar, adult male mosquitoes take only sugar meals. Salivary glands of

male and female mosquitoes have similar amounts of the enzymes associated with sugar feeding (Marinotti et al., 1990), but males have much less, or none, of the enzyme activities associated with blood feeding, e.g. antiplatelet apyrase enzyme or anticlotting activities (Rossignol et al., 1984; Stark and James, 1995). Accordingly, to investigate whether mosquito salivary ADA activity is associated with blood feeding, we measured ADA activity in salivary homogenates of male *Aedes aegypti* and *C. quinquefasciatus*. Male mosquitoes had very little ADA activity, amounting to less than 2% of that of their female counterparts (Fig. 6), suggesting that female salivary ADA activity is associated with a role in blood feeding.

To obtain further evidence for secretion of salivary ADA during probing, we exposed approximately 100 female *Aedes aegypti* for 20 min to an artificial feeder containing a solution of 50 μmol l^{-1} adenosine in HS. During this period, there were always 3–5 mosquitoes probing the membrane but, perhaps because of the absence of phagostimulation, individual mosquitoes probed the membrane for only 10–30 s and then left. Nevertheless, when the solution was removed from the feeder, its spectrum had changed to one characteristic of inosine (Fig. 7A). When 5 μl of the feeder solution was added to 100 μmol l^{-1} adenosine in 95 μl of HS and the cuvette scanned at 12 min intervals (Fig. 7B,C), the resulting spectral change (Fig. 7D) demonstrated an ADA activity corresponding to 2.5 munits contained in the feeder after *Aedes aegypti* probing, or nearly 15% of the mean amount of ADA contained in one pair of female salivary glands. Thus, if each of the approximately 100 mosquitoes probed the membrane during the 20 min interval, the equivalent of 0.15% of the ADA content of one pair of glands was delivered to the feeder solution per mosquito, assuming an average of 16 munits of ADA activity per pair of female *Aedes aegypti* salivary glands (from Fig. 6).

When the same experiment was attempted with *C. quinquefasciatus*, they did not attempt to probe the membrane of the feeder. To entice these mosquitoes to probe the membrane, a human finger was placed on the surface of the net covering the mosquito cage. While the mosquito was attempting to probe the finger, the finger was gently withdrawn from the cage surface and replaced by the feeder. In this manner, three mosquitoes were enticed to probe the membrane for periods of 15–30 s each. The ADA activity measured in the feeder amounted to less than 0.1% of the ADA content of one pair of glands (results not shown), indicating that insignificant amounts of ADA were lost from *C. quinquefasciatus* while probing the membrane feeder.

As an independent test for possible ADA secretion in *C. quinquefasciatus* and *Aedes aegypti* saliva, we measured ADA and AMP deaminase activity in individual samples of female mosquito saliva. While both activities were readily detected in *Aedes aegypti* saliva, with higher values for ADA than for AMP deaminase, ADA and AMP deaminase activities for *C.*

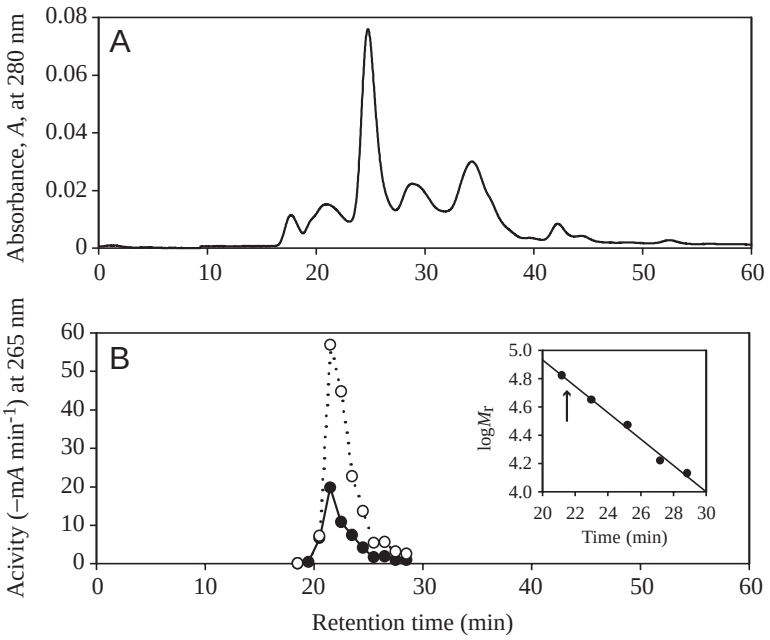

Fig. 4. Gel permeation chromatography of 20 pairs of homogenized salivary glands from adult female *Culex quinquefasciatus*. (A) Absorbance at 280 nm of the column eluate. (B) Adenosine deaminase (ADA) (●) and AMP deaminase (○) activities. The inset indicates the elution of molecular mass (M_r) standard markers; the arrow points to the elution time of the ADA/AMP deaminase activities.

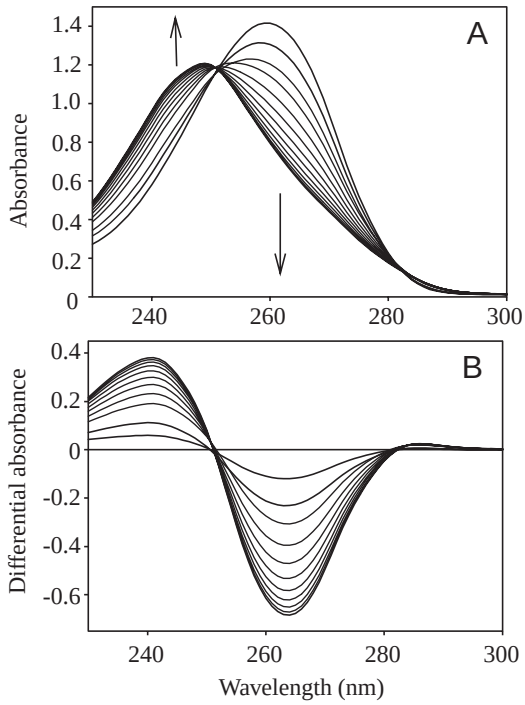

Fig. 5. Adenosine deaminase (ADA) activity in salivary homogenates of adult female *Aedes aegypti*. (A) A cuvette containing 100 μmol l^{-1} adenosine in 100 μl of HS buffer, pH 7.2, was scanned at 6 min intervals following addition of salivary homogenate equivalent to 0.02 of a pair of salivary glands. The arrows indicate the direction of change of the spectra over time. (B) Differential spectra for the data in A.

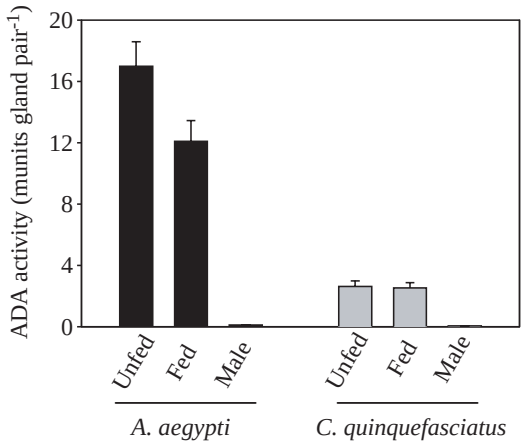

Fig. 6. Salivary adenosine deaminase (ADA) activity in blood-fed and unfed female mosquitoes and in male mosquitoes. The columns and error bars represent the mean + S.E.M. of 12 determinations in individual pairs of homogenized salivary glands from *Aedes aegypti* and *Culex quinquefasciatus*.

quinquefasciatus were at the detection limit of the assay, and were not significantly different from zero (Fig. 8). The ADA values for *Aedes aegypti* corresponded to the equivalent of 2.3% of the content of one pair of glands, on average, per collected saliva sample. These results indicated that *Aedes aegypti* salivary ADA and AMP deaminase are secreted, and failed to reveal secretion from *C. quinquefasciatus* salivary glands.

Finally, to exclude the possibility that the presence of salivary ADA in *C. quinquefasciatus* was due to contamination of the salivary homogenates by fat body cells, fat body from *C. quinquefasciatus* was removed from adult female mosquitoes and homogenized as for the salivary glands. Using the same protein content as that of one whole salivary gland in our ADA assay, no ADA activity was found in three independent fat body homogenates (results not shown).

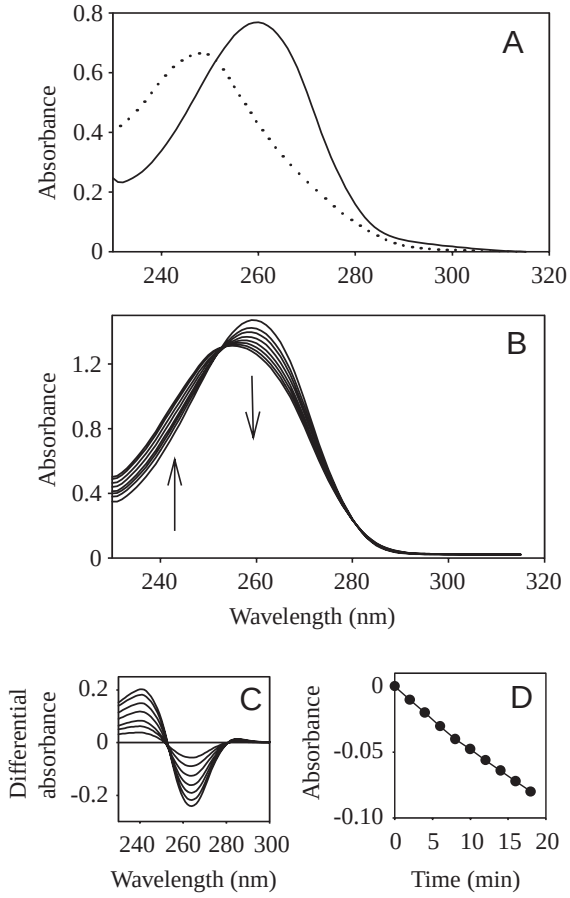

Fig. 7. Adenosine deaminase (ADA) activity left by adult female *Aedes aegypti* while probing an artificial feeder containing 50 μmol l^{-1} adenosine in HS. (A) Continuous line, spectrum of a control solution exposed in a feeder for 20 min; dotted line, spectrum of a solution after exposure to approximately 100 female mosquitoes for 20 min. (B) Mosquito-exposed feeder solution (5 μl) was diluted with 100 μmol l^{-1} adenosine in 95 μl of HS, and the resulting solution was scanned at 12 min intervals. The arrows indicate the change in the spectrum over time. (C) Differential spectra of the data in B, obtained by subtracting each scan at 12 min intervals from the scan at time zero. (D) Rate of change of absorbance at 265 nm, used to calculate the specific activity of ADA.

Discussion

The presence of salivary ADA activity in hematophagous insects is puzzling, because adenosine is a vasodilatory and anti-platelet substance (Collis, 1989; Dionisotti et al., 1992; Chinellato et al., 1994), which might be expected to increase blood availability at the feeding site. However, adenosine may be implicated in peripheral pain perception (Burnstock and Wood, 1996; Poon and Sawynok, 1999; Charlab et al., 2000), although its role in nociception is contradictory: while adenosine A2 and A3 receptors appear to be clearly nociceptive, adenosine A1 receptor stimulation appears to be anti-nociceptive (Poon and Sawynok, 1998; Poon and Sawynok, 1999). Another effect of salivary ADA may be related to a local accumulation of inosine at the feeding site. Inosine has been shown to be a potent inhibitor of the production of the inflammatory cytokines TNF-α, IL-1, IL-12, macrophage-inflammatory protein-1α and IFN-γ (Hasko et al., 2000). IL-1, in particular, is a potent peripheral nociceptive agent (Bianchi et al., 1998). However, it is doubtful whether these cytokines could be produced by the host and released during the few minutes that mosquitoes take to feed, although they may have an effect on the fate of the host immune response to salivary allergens and pathogens co-injected with the mosquito saliva. While the role of adenosine in nociception is unclear, adenosine is known to induce mast cell degranulation (Tilley et al., 2000), resulting in the release of histamine. From the point of view of the mosquito, the itching induced by histamine would have the undesirable effect of alerting the host to its presence. Although it is clear that adenosine could be beneficial to a hematophagous insect, to the point that phlebotomine sand flies produce copious amounts of the substance in their salivary glands (Ribeiro et al., 1999; Ribeiro and Modi, 2001), the presence of ADA in the saliva of

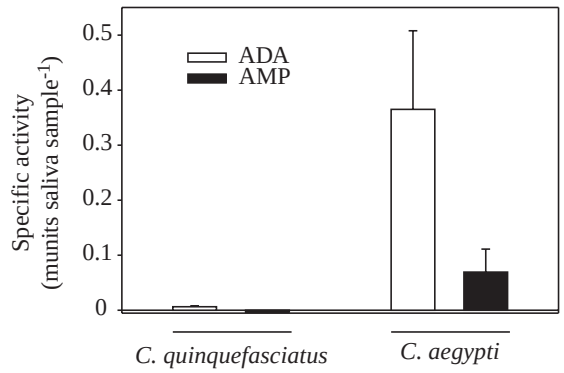

Fig. 8. Adenosine deaminase (ADA) and AMP deaminase (AMP) activities in serotonin-induced saliva from adult female *Culex quinquefasciatus* and *Aedes aegypti*. Individual samples of serotonin-induced mosquito saliva collected in mineral oil were transferred to 10 µl of Hepes saline (10 mmol l^{-1} Hepes, pH 7.2, 100 mmol l^{-1} NaCl) and centrifuged. Enzyme levels were determined by measuring the decrease in absorbance at 265 nm in microcuvettes containing 71 µl of Hepes saline, 0.1 mmol l^{-1} substrate and 4 µl of the diluted saliva sample. Each salivary sample was used for one adenosine and one AMP deaminase reaction. Values are the mean + S.E.M. of five samples from each mosquito species.

other hematophagous insects indicates that, if it has any evolutionary significance, adenosine may not be a desirable substance at the feeding site.

In accordance with the ambivalent role of adenosine in blood feeding, the data presented in this paper indicate that the presence of salivary ADA in mosquitoes is not a consistent finding, unlike the ubiquitous occurrence of diverse anticoagulants or the enzyme apyrase (Ribeiro, 1995). No ADA activity was found in *Anopheles gambiae*, but relatively high activities were found in both *Culex quinquefasciatus* and *Aedes aegypti*. The specific activity of 16 munits gland pair^{-1} for *Aedes aegypti* represents a specific activity of 5 units mg^{-1} salivary protein, while the value of 2.6 munits gland pair^{-1} for *C. quinquefasciatus* represents 2.5 units mg^{-1} salivary protein. This amount is larger than the specific activity of erythrocyte ADA (0.2 units mg^{-1} protein; Agarwal et al., 1975) and is similar to that in tissues specialized in digestion, such as the crude ADA sold by Sigma Chemical Co. obtained from calf intestinal mucosa (1–5 units mg^{-1} protein) (Sigma Chemical catalog, 2000–2001 edition). In the case of *Aedes aegypti*, salivary ADA appears to be secreted during a blood meal, as demonstrated by the significant decrease in levels in the salivary glands following a blood meal, its presence in solutions probed by hungry female mosquitoes and its presence in serotonin-induced saliva. In *C. quinquefasciatus*, no activity could be detected in serotonin-induced saliva, although very low levels of the enzyme (less than 0.03 % of the content of one pair of glands per probing mosquito) were left in feeders probed by this species. No significant decrease in enzyme levels was detected in *C. quinquefasciatus* salivary glands following a blood meal. This suggests that ADA serves a largely endogenous function in the salivary glands of *C. quinquefasciatus*.

We can only speculate about the evolutionary scenario that has led to the presence or absence of ADA in the saliva of hematophagous arthropods, but we may assume that adenosine has both positive and negative effects on blood feeding. The positive effects could be due to its actions on platelet aggregation and vasodilation. Perhaps for this reason, the sand flies *Phlebotomus papatasi* and *P. argentipes* have abundant adenosine in their salivary glands (Ribeiro et al., 1999; Ribeiro and Modi, 2001). It is possible that these phlebotomine hosts are less sensitive to the negative effects of adenosine, that they feed when their hosts are in profound sleep and unresponsive to relatively small disturbances or that sand flies have other mechanisms to counteract the negative effects of adenosine on feeding. However, both *Aedes aegypti* and *C. quinquefasciatus* have their own salivary vasodilatory molecules: the peptide sialokinin in *Aedes aegypti* (Champagne and Ribeiro, 1994) and an unknown substance in *C. quinquefasciatus* (Ribeiro, 2000). In addition, *Aedes aegypti* has large amounts of salivary apyrase, which inhibits platelet aggregation (Ribeiro et al., 1984b), while *C. quinquefasciatus* has a potent salivary phospholipase C that destroys platelet activating factor (J. M. C. Ribeiro and I. Francischetti, in preparation). Other molecules can therefore replace the positive functions of adenosine at the feeding site, and we are left only with its negative effects.

If we assume that the negative role of adenosine is related to its ability to bring the attention of the host to the feeding site, then the intensity of this negative effect would be affected by two variables: the degree to which adenosine induces pain or itching at the feeding site and the behavioral response of the host to pain and itching. The first variable can vary from species to species, e.g. the density of mast cells in the skin of the host selected for feeding by the mosquito, the number and affinity of adenosine receptors inducing cell degranulation or the density of adenosine nociceptive receptors in the skin. The second variable depends, for example, on whether the vertebrate is asleep or alert and how it reacts to the same stimulus in these two conditions. Depending on these variables, it may be of more or less selective advantage to neutralize the negative effect of adenosine on blood feeding, and more than one strategy may be employed to counteract its effects. Thus, adenosine could be destroyed *via* ADA or its effects reduced by the use of physiological inhibitors, e.g. molecules that act in an opposite way on the target cell, such as mast cell degranulation inhibitors or anesthetics.

It is interesting to note that *Aedes aegypti* is a diurnal feeder and that its main hosts are humans. Salivary homogenates from *Aedes aegypti* have the ability to prevent the release of tumor necrosis factor (TNF) by mast cells (Bissonette et al., 1993), indicating that this insect may be under pressure to avoid causing degranulation of the mast cells of the host. In contrast, *C. quinquefasciatus* is a nocturnal feeder on humans and may avoid the negative behavioral effects of adenosine by feeding when the physiological state of the host is less responsive or unresponsive to an increase in the levels of adenosine at the feeding site. Alternatively, *C. quinquefasciatus* may have additional salivary components that antagonize the nociceptive

and/or mast cell degranulating effects of adenosine. In this regard, it is interesting to note that both triatomine bugs (Ribeiro and Walker, 1994) and ticks (Paesen et al., 1999) have salivary proteins that neutralize histamine, and an anesthetic substance has recently been reported from the saliva of a triatomine bug (Dan et al., 1999). The presence of ADA in *Aedes aegypti* may represent one of several ways in which the negative effects of adenosine can be counteracted. While these considerations may help to explain why *Aedes aegypti* could benefit from secreting ADA, we have no clue as to the role of salivary ADA in *C. quinquefasciatus*, although we can speculate that it may be involved in some endogenous function, such as regulating transmitter signaling by deactivating adenosine or producing inosine or an inosine derivative as a secretory product. Such speculations suggest the hypothesis that anti-nociceptive and anti-mast cell salivary substances should be found abundantly in the salivary glands of hematophagous arthropods.

From a biochemical perspective, it is interesting that the salivary AMP deaminase activity of *C. quinquefasciatus* is approximately three times greater than that of ADA, while in *Aedes aegypti* it is one-tenth of the ADA activity. AMP deaminase and ADA are enzymes that probably evolved by gene duplications (Becerra and Lazcano, 1998). Assuming that the two activities reside in a single enzyme in mosquito salivary glands, cloning and expression of these two enzymes, one from each mosquito species, might indicate the amino acid residues that confer greater specificity towards either AMP or adenosine. Alternatively, each mosquito species may express the two enzymes (ADA and AMP deaminase) in their glands in different ratios.

The cDNA discussed in this paper may or may not be responsible for the majority of the enzyme activity seen in *C. quinquefasciatus* salivary glands. The work reported here confirms that sequencing cDNA libraries from salivary glands of hematophagous insects can yield information leading to the detection of many salivary enzymes not previously identified, such as amylases, hyaluronidases, ADA and 5′-nucleotidases (Charlab et al., 1999; Charlab et al., 2000). Coupled with appropriate biochemical and pharmacological assays, this approach lends impetus to our understanding of how insects have evolved to become one of the most successful and diverse groups of species in the animal kingdom.

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