2. R. H. Barth, Jr., *Science* **149**, 882 (1965).
3. A. L. Hollander and C.-M. Yin, *Physiol. Entomol.* **7**, 163 (1982); *J. Insect Physiol.* **31**, 159 (1985); L. M. Riddiford and C. M. Williams, *Biol. Bull.* **140**, 1 (1971); M. Sasaki and L. M. Riddiford, *Physiol. Entomol.* **9**, 315 (1984).
4. H. Itagaki and W. E. Conner, *J. Insect Physiol.* **32**, 657 (1986); *ibid.* **33**, 177 (1987); M. Sasaki, L. M. Riddiford, J. W. Truman, J. K. Moore, *ibid.* **29**, 695 (1983); J. D. Tang, R. E. Charlton, R. T. Cardé, C.-M. Yin, *ibid.* **33**, 469 (1987).
5. J. N. McNeil, *Fla. Entomol.* **69**, 78 (1986); *Insect Sci. Applic.* **8**, 591 (1987).
6. J. Delisle and J. N. McNeil, *J. Insect Physiol.* **32**, 199 (1986); *Physiol. Entomol.* **12**, 157 (1987); J. J. Turgeon and J. N. McNeil, *Entomol. Exp. Appl.* **31**, 402 (1982).
7. M. A. Rankin, M. L. McAnelly, J. E. Bodenhamer, in *Insect Flight, Dispersal and Migration*, W. Danthanarayana, Ed. (Springer-Verlag, New York, 1986), pp. 27–48.
8. A. K. Raina and J. A. Klun, *Science* **225**, 531 (1984).
9. Females were reared at 25° ± 1°C, 16L:8D, 65 ± 5% relative humidity. At the time of injection, donor and recipient females were in the second to third hour of the scotophase that followed their first night of calling. Females anesthetized with CO_2 were neck-ligated in the middle of the preceding photophase with the use of cotton thread. Brain-SOG homogenates were prepared as described in (*8*). Recipient females for the photophase treatment were in the second hour of the photophase at the time of injection. The titer of the main pheromone component (Z11-16:Ac) was determined by gas-liquid chromatography of a gland extract in hexane (performed 3 hours after injection of brain-SOG homogenate), with methyl heptadecanoate used as an internal standard [see (*19*) for details on the temperature program and column used].
10. A. K. Raina and J. J. Menn, in *Pheromone Biochemistry*, G. D. Prestwich and G. J. Blomquist, Eds. (Academic Press, New York, 1987), pp. 159–174.
11. M. Cusson and J. N. McNeil, *Can. J. Zool.*, in press.
12. Rearing conditions were the same as in (*9*). Only females emerging during the photophase were used. Allatectomies were performed 1 hour after eclosion. The scales covering the head capsule and the cervical area were brushed off and the insect immobilized in plasticine at the bottom of a dish supplied with a continuous flow of CO_2. With sharp forceps and micro-scissors, a small opening was made in the head capsule near the neck membrane, and the CA was gently excised under a microscope. A few crystals of streptomycin were applied to the wound before replacing the cuticule. Sham-operated females underwent the same treatment, but the CA were left untouched. A lamp covered with Kodak Wratten filter (No. 29) was used for observations during the scotophase at 10-min intervals. To assess ovarian development, we measured the width of the penultimate basal oocyte in each ovariole of the right ovary with an ocular micrometer. Pheromone titer analysis was performed as described in (*9*).
13. One-day-old allatectomized females were injected between the fourth and fifth abdominal segment with either 5 μl of light mineral oil (Atlas), or 5 μl of light mineral oil containing 10 μg of JH II (Sigma). This dosage may appear too high (non-physiological), but the kinetics of JH release from oil carriers are such that it should result in a relatively low JH titer [L. M. Riddiford and A. M. Ajami, *J. Insect Physiol.* **19**, 749 (1973)]. We chose the C_{17}JH (JH II) since HPLC analysis indicated that it is the main JH produced by *P. unipuncta* females.
14. Larvae were immobilized with CO_2 and anchored in plasticine on the bottom of a dish, with metal restrainers. With micro-scissors, a small slit was made on the dorsum of the animal to expose the yellowish ovaries which were excised with fine forceps (or left intact in sham-operated controls). Streptomycin was applied to the wound which was then sealed with Super Glue.
15. A. L. Hollander and C.-M. Yin, *J. Insect Physiol.* **31**, 159 (1985).
16. Allatectomy was performed as described in (*12*) and allatectomized females were injected with brain-SOG homogenates from donor females similar to those described in (*9*), or saline 3 to 4 days later.
17. Calling does not appear to be influenced by PBAN as the females that we injected with brain-SOG extracts never called.
18. D. Morse and E. Meighen, *Insect Biochem.* **17**, 53 (1987).
19. J. Delisle and J. N. McNeil, *J. Insect Physiol.* **33**, 315 (1987).
20. We thank P. E. A. Teal for helpful comments on an earlier version of the manuscript. We thank S. S. Tobe for his help with the HPLC analysis. Supported by an FCAR postgraduate scholarship to M.C. and by grants from NSERC and FCAR to J.N.M.

28 July 1988; accepted 1 November 1988

A Novel Vasodilatory Peptide from the Salivary Glands of the Sand Fly *Lutzomyia longipalpis*

JOSÉ M. C. RIBEIRO,* ANDRÉ VACHEREAU, GOVIND B. MODI, ROBERT B. TESH

Salivary gland lysates of the sand fly *Lutzomyia longipalpis* contain a potent vasodilator that aids the fly to feed on the blood of its vertebrate hosts. Chromatographic analysis, antibody reactivity, and data obtained from bioassays of the salivary erythema-inducing factor indicate striking similarity with human calcitonin gene-related peptide. The erythema-inducing factor is, however, at least one order of magnitude more potent than calcitonin gene-related peptide.

IN THEIR QUEST FOR BLOOD, HEMATOPHAGOUS arthropods introduce into the skin of their hosts an array of salivary compounds that facilitate blood feeding by blocking the hemostatic reactions or by altering hemodynamics (*1*). Salivary gland homogenates of the sand fly *Lutzomyia longipalpis* contain trypsin-sensitive erythema-inducing factor (EIF) (*2*). As little as 10 ng of protein from a crude salivary lysate produced an erythema in rabbits (*2*). The EIF was differentiated from other known erythema-inducing substances such as histamine, serotonin, bradykinin, substance P, or prostaglandins on the basis of its sensitivity to trypsin, kinetics of erythema development, and absence of edema and itching. The neuropeptide CGRP (calcitonin gene-related peptide) has been characterized as the most potent and persistent vasodilator known. When injected into human skin it produces a delayed, long-lasting, erythema with a surrounding pallor (*3, 4*). In vitro, CGRP relaxes various isolated arterial preparations contracted by adrenergic agonists (*3, 5*). We investigated the extent to which the properties of EIF are similar to those described for CGRP.

We first investigated the nature of the response of human skin to the bite of a sand fly. As little as 30 s after a sand fly probed human skin, an erythema formed, reaching a maximum area at 60 min after the bite (Fig. 1). Between 30 min and 1 hour after the bite, the erythema develops pseudopod-type figures. A surrounding pallor, which contrasted with the sharp boundary of the erythema, was also observed (Fig. 1). In all four human beings tested, the erythema was still visible 24 hours after the bite, without any edema or itching.

To compare the potency of EIF with that of synthetic CGRP in inducing erythema, we injected serial dilutions of either salivary gland lysates or human α-CGRP intradermally into the back of a rabbit, starting with 100 ng of crude salivary gland protein (about 10% of one pair of glands) (*2*) or 100 ng of CGRP per injection site. The minimum dilution of the crude homogenate

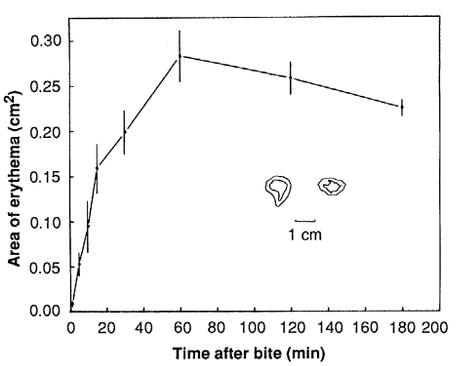

Fig. 1. Time course of the erythema induced by the bite of adult female *L. longipalpis* sand flies on humans. Flies were allowed to probe the forearm skin for 30 s. Symbols and bars represent the average and SE of four experiments. Inset: Typical contours of the erythema (inner contour) and associate pallor (outer contour) displayed by two humans 1 hour after being bitten for 30 s.

J. M. C. Ribeiro and A. Vachereau, Department of Tropical Public Health, Harvard School of Public Health, Boston, MA 02115.
G. B. Modi and R. B. Tesh, Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06510.

*To whom correspondence should be addressed.

causing a visible erythema 1 hour after injection was 8 ± 2 ng of protein, compared to 18 ± 3 ng of pure CGRP (mean ± SE; $n = 5$ in five different rabbits). Therefore, it appeared that the salivary gland lysates were more potent than synthetic CGRP in inducing skin erythema.

To determine whether the erythema could be mediated by the local release of prostaglandins, we injected intradermally 1 μg of indomethacin 15 min before the injection and again at the time of injection of 0.1 μg of salivary protein. The areas of erythema produced in the presence of indomethacin (0.777 ± 0.149 cm^2) did not differ significantly from that of controls injected with salivary gland lysates 10 cm away from the indomethacin-treated site (0.738 ± 0.194 cm^2, mean ± SE of three rabbits).

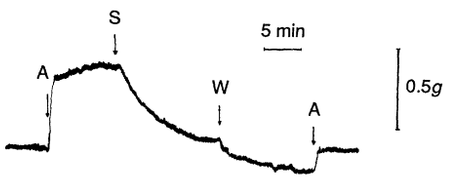

Fig. 2. Relaxation of a constricted rabbit aortic ring by *L. longipalpis* salivary gland lysates (*9*). A, addition of adrenalin, 200 ng total; S, addition of salivary gland lysates from three sand flies; W, washing of the preparation. Thoracic aortas were obtained from adult New Zealand rabbits. Four-millimeter-wide rings were suspended on a 3-ml bath containing Tyrode's solution bubbled with 95% O_2 and 5% CO_2 and kept at 37°C under initial tension of 1*g*.

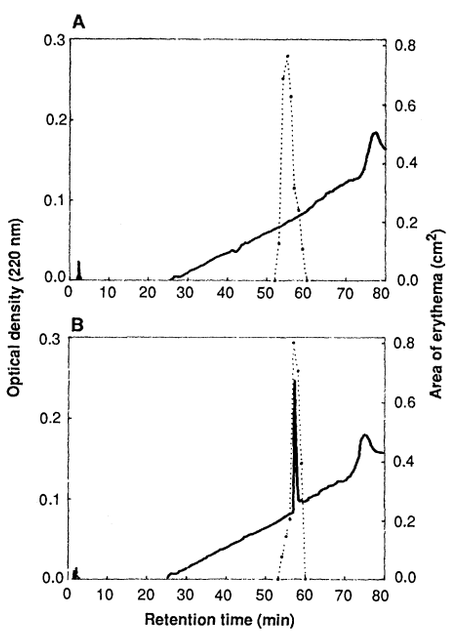

Fig. 3. Reversed-phase HPLC of (**A**) crude salivary protein (6 μg), containing EIF, and (**B**) synthetic human α-CGRP (6 μg) (Peninsula Labs). Continuous line, absorption at 220 nm; and dotted line, area of erythema. An ODS-5 column (Bio-Rad) was equilibrated with 20% acetonitrile and 0.1% trifluoroacetic acid in H_2O at a flow rate of 1 ml/hour. After sample injection, this solution flowed for 20 min when a linear gradient started attaining 60% of acetonitrile at 80 min after the injection. Fractions were collected every minute. After evaporating the solvent, samples were diluted at 50 μl with phosphate-buffered saline containing bovine serum albumin (1 mg/ml) and bioassayed for EIF on the rabbit skin (*2*).

The vasodilatory activity of saliva of the sand fly was characterized on a rabbit aortic ring preparation constricted with adrenalin (*3*). Aortic relaxation of more than 50% was achieved when three pairs of glands were added to the 2.5-ml chamber (58 ± 8%, mean ± SE; $n = 4$). In all cases there was a 15- to 30-s delay between addition of the salivary homogenate and the beginning of the aortic relaxation. After the preparation was washed, further addition of adrenalin did not restore the level of contraction before treatment, indicating that the activity persisted (Fig. 2).

Reversed-phase high-performance liquid chromatography (HPLC) of sand fly salivary gland lysates indicated that the EIF peak eluted 2 min earlier than CGRP in an acetonitrile-H_2O-trifluoroacetic acid gradient (Fig. 3). In this experiment, fractions of similarly potent doses of salivary gland lysates and CGRP (6 μg of crude salivary protein and 6 μg of synthetic human CGRP) were compared for their erythema-inducing ability in the rabbit skin and the absorbance of the column effluent was monitored at 220 nm. Although the CGRP peak was detectable by ultraviolet (UV) spectrometry, no absorbance peak was evident in the fractions containing EIF, indicating that EIF was a minor component of the salivary gland total protein. The molecular weight estimate of EIF was 3900, based on its elution pattern in a molecular sieving column (*6*). The retention time in the column coincided with that of synthetic CGRP (*7*).

Radioimmunoassay of salivary gland lysates for CGRP, performed with a polyclonal rabbit antibody to human CGRP and radioactive human CGRP tracer (*8*), indicated partial displacement of the tracer from the antibody when 1 μg of salivary protein was used per assay (17.5 ± 3.8% displacement, mean ± SE; $n = 4$; $P < 0.05$ for paired t test). Salivary gland homogenates of *L. longipalpis* thus contain one or more substances that share partial immunologic identity with human CGRP.

These results indicate that salivary gland lysates of the sand fly *L. longipalpis* contain pharmacologically active properties that are similar to those displayed by CGRP. Indeed, the time course, shape, and indomethacin insensitivity of the erythema (Fig. 1) are remarkably similar to those described for CGRP, which were, heretofore, distinct characteristics of this neuropeptide (*3, 4*). Moreover, salivary gland homogenates were able to relax constricted aortic rings (*9*) (Fig. 2), a property not unique to but displayed by CGRP (*3, 5*). From a pharmacological standpoint, the only substance known to produce the same effects as EIF is CGRP.

Furthermore, EIF elutes as a single peak from a molecular sieving column, with a retention time identical to that of CGRP. Also, a single peak elution for EIF is obtained in reversed-phase HPLC. The 2-min difference in retention time between EIF and CGRP by reversed-phase chromatography represents less than a 2% difference in the acetonitrile gradient position and indicates EIF is similar to CGRP, but is possibly a less hydrophobic molecule than CGRP. Furthermore, 17.5% cross-reactivity was found when one pair of salivary glands was radioimmunoassayed with a specific rabbit antibody to human CGRP. These data support the hypothesis that the salivary glands of this sand fly contain a peptide of the CGRP family.

When equal amounts of crude salivary protein and CGRP were injected into rabbit skin, they elicited equivalent degrees of erythema. Evidence that EIF is more potent than CGRP comes from the results obtained by reversed-phase HPLC in which equal amounts of salivary gland protein and CGRP were chromatographed, resulting in the lack of EIF UV absorbance detection as compared to a conspicuous CGRP absorbance peak. Thus, it is likely that the sand fly CGRP-like substance has a different, more active structure than CGRP. In any event, if we conservatively assume that EIF is no more than 10% of the total salivary gland contents, and that the UV detection difference between EIF and CGRP is only a tenfold difference, then EIF could be at least ten times as potent as CGRP and thus would represent the most potent substance known that induces erythema.

Recently it was reported that *L. longipalpis* saliva enhanced *Leishmania* transmission (*10*). Because EIF has so far been found only in this sand fly (*1, 2*), we determined whether EIF and CGRP could modify macrophage function. Incubation of blood monocytes with either CGRP or salivary gland lysates prevented macrophage activation by γ-interferon, and the potency of the salivary lysate was found to be equivalent to that of CGRP (*11*). The multiple pharmacological actions of sand fly saliva therefore overlap with the known properties of CGRP. Research on the pharmacology of saliva from a bloodsucking arthropod has thus led to the finding of both a novel substance and a new function of a known agent.

REFERENCES AND NOTES

1. J. M. C. Ribeiro, *Annu. Rev. Entomol.* **32**, 463 (1987).
2. ———, P. A. Rossignol, A. Spielman, *Comp. Biochem. Physiol. A* **83**, 683 (1986).
3. S. D. Brain, T. J. Williams, J. R. Tippins, H. R. Morris, I. MacIntyre, *Nature* **313**, 54 (1985).
4. W. Piotrowski and J. C. Foreman, *Br. J. Dermatol.* **114**, 37 (1986); J. Wallengren and R. Håkanson, *Eur. J. Pharmacol.* **143**, 267 (1987); R. W. Fuller, T.-B. Conradson, C. M. S. Dixon, D. C. Crossman, P. J. Barnes, *Br. J. Pharmacol.* **92**, 781 (1987).
5. T. Shoji, H. Ishihara, T. Ishikawa, A. Saito, K. Goto, *Naunyn Schmiedeberg's Arch. Pharmakol.* **336**, 438 (1987).
6. Molecular sieving chromatography was performed on a 300 mm by 7.5 mm TSK-125 column (Bio-Rad) equilibrated with 0.1*M* sodium phosphate, *p*H 7.0. Salivary protein (5 μg) was added to the column in 50 μl at a flow rate of 0.8 ml/min. Fractions were collected every 12 s. Four microliters of each fraction were diluted to 50 μl with phosphate-buffered saline containing bovine serum albumin (1 mg/ml) and bioassayed for EIF on the rabbit skin (*2*). Molecular weight markers were immunoglobulin, ovalbumin, myoglobin, CGRP, and cyanocobalamin.
7. J. M. C. Ribeiro *et al.*, unpublished data.
8. Rabbit antibody to human CGRP (Accurate Chemical and Scientific Corporation) was used at a dilution of 1:10,000. ^{125}I-Labeled human CGRP (Amersham) was added to give 20,000 cpm per vial. The antibody was precipitated out of solution with 0.5 mg of goat antibody to rabbit immunoglobulin G beads (Bio-Rad).
9. Sand flies were reared by the procedure of G. B. Modi and R. B. Tesh [*J. Med. Entomol.* **20**, 568 (1983)]. Salivary glands of 5- to 7-day-old female flies were removed, put into a solution containing 5 m*M* tris-Cl, *p*H 7.4, and frozen at −70°C until use.
10. R. G. Titus and J. M. C. Ribeiro, *Science* **239**, 1306 (1988).
11. Y.-H. Nong, R. G. Titus, J. M. C. Ribeiro, H. G. Remold, in preparation.
12. Supported by grants AI18694-0481 and AI21049 from NIH and from the MacArthur Foundation.

20 June 1988; accepted 18 October 1988

A New Cluster of Genes Within the Human Major Histocompatibility Complex

THOMAS SPIES, GEORGE BLANCK, MAUREEN BRESNAHAN, JOHN SANDS,* JACK L. STROMINGER

A 435-kilobase (kb) DNA segment, which is centromeric to HLA-B in the human major histocompatibility complex, was isolated by chromosome walking with overlapping cosmids. Within the cloned region, the genes for the tumor necrosis factors (TNFs) α and β and HLA-B were 210 kb apart. The human homolog of a mouse gene, B*144*, was located next to TNFα. Moreover, the presence of additional genes was suggested by a large cluster of CpG islands. With cosmid probes, several distinct transcripts were detected in RNA samples from a variety of cell lines. Altogether, five novel genes were identified by isolation of corresponding complementary DNA clones. These "HLA-B–associated transcripts" (BATs) were mapped to different locations within a 160-kb region that includes the genes for TNFα and TNFβ. The presence of the genes for BAT1 to BAT5 in the vicinity of HLA-B again raises the question of which gene in this region determines susceptibility to ankylosing spondylitis.

THE HUMAN MAJOR HISTOCOMPATIBILITY complex (MHC) includes numerous genes involved in immune system responses. The class I (HLA-A, -B, and -C) and class II (DR, DQ, and DP) gene families encode highly polymorphic cell surface receptors that bind peptides derived from antigen and mediate T cell activation (*1–3*). The class III genes code for components of the complement system (C4A, C4B, Bf, and C2) and steroid 21-hydroxylase (*4*). These three regions are arranged in the order class II, class III, and class I, from centromere to telomere on the short arm of chromosome 6. In addition, the genes for TNFα and TNFβ are located within the MHC (*5*). A 3500-kb molecular linkage map of the entire MHC has been established by pulsed-field gel electrophoresis (PFGE) (*6–8*). The distance between the most telomeric class II locus, DR, and the class III region is about 400 kb. The class III gene C2 is separated from the proximal class I locus, HLA-B, by 600 kb. This interval includes the closely linked genes for TNFα and TNFβ.

Population studies suggest that the MHC region between DR and HLA-B may encode important information. Some HLA-B and DR alleles together represent haplotypes that prevail through strong linkage disequilibrium (*9, 10*). Moreover, susceptibility to a number of diseases is associated with certain HLA-B and DR alleles. Among the tightest correlations is that between HLA-B27 and the rheumatoid disease ankylosing spondylitis (*11, 12*).

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.

*Present address: Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

In the present study, the possibility that unidentified genes are located in proximity to HLA-B has been explored. Linkage data generated by PFGE in combination with cosmid cloning (this report) have localized the TNFα and TNFβ gene pair about 200 kb centromeric of HLA-B (*7*). The genes for the two TNFs are contained together within 6 kb and are in the same transcriptional orientation (*13*). TNFβ is nearest to HLA-B, and the 5′ ends of these two genes are directed toward each other (*7*). The genetic organization in the mouse H-2 complex is similar, except that the TNFβ gene is only 70 kb distant from H-2D, the murine homolog of HLA-B (*14*). These facts guided a bidirectional chromosome walk between the genes for the TNFs and HLA-B.

Cosmid libraries were screened with labeled TNFα cDNA and a locus-specific genomic probe for HLA-B, and isolated clones were analyzed by restriction mapping and DNA blot hybridization. The HLA-B gene was encoded in the cosmids M20A and O32A (Fig. 1). Its position and relative orientation were determined with probes for the 5′ and 3′ ends, respectively. The cosmids M2A, M31A, O19A, O30A, and O31A contained the genes for TNFα and TNFβ (Fig. 1). The arrangement of the two genes was inferred from hybridization experiments in which the corresponding probes were used individually. The results obtained were in accord with published data (*13*). To expand the cloned TNF and HLA-B regions toward each other, overlapping series of cosmids were isolated with walking probes (wp's) that were prepared from several locations upstream from the genes for TNFβ and HLA-B, respectively (*15*). Thus, the genes for the TNFs and HLA-B were linked within a continuous stretch of cosmids in a 210-kb distance (Fig. 1). This result was corroborated by data derived from PFGE (*7, 8*). Additional cosmids were cloned downstream from TNFα (*15*). Altogether, a total of 435 kb was obtained in a contiguous cosmid cluster, currently the largest cloned segment of the human genome. Upon alignment with a PFGE linkage map, the centromeric breakpoint of the cloned region was about 200 kb from the C2 gene in the MHC class III complement gene cluster (*8*).

In the mouse H-2 complex, the gene B*144*, which is transcribed specifically in B cells and macrophages, is located 10 kb downstream from TNFα (*16*). The human B*144* homolog was found in a corresponding position within the cosmid O30A by DNA blot hybridization with the mouse cDNA probe (Fig. 1).

Within the cloned region, an accumulation of Bss HII and Sac II sites, which are relatively rare in mammalian genomes, was