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Variation in the salivary peptide, maxadilan, from species in the *Lutzomyia longipalpis* complex

G. C. Lanzaro,[1] A. H. C. S. Lopes,[2] J. M. C. Ribeiro,[3] C. B. Shoemaker,[4] A. Warburg,[5] M. Soares[6] and R. G. Titus[7]

[1] *Department of Pathology & Center for Tropical Diseases, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555–0609, USA;* [2] *Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil, 21941–590;* [3] *Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892–0425, USA;* [4] *Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115, USA;* [5] *Department of Parasitology, Hebrew University-Hadassah Medical School, PO Box 12272, Ein Kerem, Jerusalem 91120, Israel;* [6] *Laboratorio de Patologia e Biologia Celular, Centro de Pesquisas Goncalo Moniz, Fundacao Oswaldo Cruz, Salvador-Bahia, Brazil;* [7] *Department of Pathology, College of Veterinary Medicine & Biomedical Sciences, Colorado State University, Fort Collins, CO 80523–1671, USA.*

Abstract

Maxadilan is an approximately 7 kDa peptide that occurs in the saliva of the sand fly *Lutzomyia longipalpis*. This peptide is a potent vasodilator and may also have immunomodulatory effects related to the pathogenesis of leishmanial infections. Variation in the primary DNA and inferred amino acid sequence of maxadilan is reported. Differences were found within and among natural field populations as well as among sibling species. Extensive amino acid sequence differentiation, up to 23%, was observed among maxadilan from different populations. This is a remarkable degree of polymorphism considering the small size of this peptide. The vasodilatory activity of maxadilan was equivalent among recombinant maxadilan variants. All maxadilan variants induce interleukin-6. Predicted secondary structure and hydrophobicity plots suggest that these characteristics are conserved among variant peptides. However, profiles based on the antigenic index do differ among peptides.

Keywords: maxadilan, population genetics, saliva, vasodilator.

Received 20 May 1998; accepted 19 August 1998. Correspondence: Gregory C. Lanzaro, Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas, 77555-0609, USA. E-mail: glanzaro@utmb.edu

Introduction

Saliva of the sand fly, *Lutzomyia longipalpis* produces a long lasting erythema when injected into the skin of its mammalian hosts (Ribeiro *et al.*, 1986, 1989). This erythema is the result of a potent salivary peptide known as maxadilan (Lerner *et al.*, 1991). Maxadilan presumably facilitates the formation of a small pool of blood allowing the fly to obtain its blood meal. The maxadilan gene has been cloned, sequenced, and a functional recombinant peptide produced (Lerner & Shoemaker, 1992). The availability of recombinant maxadilan led to a series of studies aimed at understanding how this peptide functions as a vasodilator (Grevlink *et al.*, 1995; Moro *et al.*, 1995, 1996; Jackson *et al.*, 1996).

In addition to having a vasodilatory activity, substances in the saliva of *Lu. longipalpis* have been shown to enhance the infectivity of *Leishmania* parasites (Samuelson *et al.*, 1991; Titus & Ribeiro, 1988; Theodos *et al.*, 1991). This enhancement may be the result of sand fly saliva-induced impairment of macrophage function. Theodos & Titus (1993) have demonstrated that the saliva of *Lu. longipalpis* inhibits the ability of macrophages to present *Leishmania major* antigens to parasite-specific T cells. Maxadilan has recently been implicated as being at least one of the specific compounds in sand fly saliva that has immunodulatory properties. Specifically, it has been demonstrated that maxadilan inhibits T-cell proliferation in murine spleen cells that have been stimulated with either concanavalin A or anti-T cell receptor antibody (Qureshi *et al.*, 1996). Moro & Lerner (1997) demonstrated that maxadilan is an agonist of the type I receptor for pituitary adenylate cyclase activiating peptide (PACAP). Soares *et al.* (1998) recently showed that maxadilan has multiple effects on murine macro-

phages; it inhibits tumour necrosis factor-α but induces interleukin-6 production by the cells. They related these activities to an interaction between maxadilan and the PACAP receptor. Both the vasodilatory and immunosuppresive activities of maxadilan probably derive from its mimicking of PACAP type neuropeptides. Maxadilan may therefore play a significant role in the pathogenesis of leishmaniasis by inhibiting the immune response at the site of parasite inoculation, thereby facilitating the establishment of infection.

Sand flies are generally considered to be weak fliers, so that for species such as *Lu. longipalpis* which have a very broad geographical distribution, there is a significant likelihood that local populations are genetically isolated. This results in the evolution of a significant genetic divergence between populations and may ultimately lead to the evolution of new species. The first suggestion that *Lu. longipalpis* may not represent a single species came in 1969 (Mangabeira). Ward *et al.* (1983) carried out cross-mating experiments using laboratory-reared flies. They suggested that the reduced capacity of males to inseminate females of strains bearing a different number of tergal spots indicated that *Lu. longipalpis* is a complex of at least two sibling species. Lanzaro *et al.* (1993) reported that *Lu. longipalpis* consists of at least three species. Comparisons of the composition and vasodilatory activity of the saliva between the three species were made by Warburg *et al.* (1994). They reported that the saliva of the *Lu. longipalpis* sibling species which occurs in Costa Rica has relatively little maxadilan, 10–40-fold less than in species from Brazil or Colombia. They also presented the results of single strand conformation polymorphism (SSCP) analysis which suggested the existence of species differences in the primary DNA sequence of the maxadilan gene. These observations raised several questions that we address in this study: (i) What is the extent and pattern of nucleotide differences in the maxadilan-encoding gene among different *Lu. longipalpis* species? (ii) What is the extent of amino acid differences in the maxadilan peptide in different sibling species? (iii) Do different maxadilan peptides differ in their potency as vasodilators? (iv) Do all maxadilan variants stimulate interleukin-6?

Results

Sequence variation in maxadilan gene and peptide

An ≈ 400 bp fragment of the maxadilan gene was amplified by PCR, and cloned, and a single clone was sequenced from each of two individuals from four different laboratory colonies (Fig. 1). The laboratory colony material was: (i) Brazil (BZ) established in 1972 originating from Lapinha Cave, about 60 km north-west of Belo Horizonte in Minas Gerias State, Brazil (19°03′S, 43°57′W); (ii) Marajo (MJ) from Marajó Island (0°45′S, 48°32′W), Pará State, Brazil; (iii) Colombia (CO) from El Callejón (4°11′N, 74°18′W) near Melgar in Tolima State, Colombia (established in 1989) and (iv) Costa Rica (CR) collected from Liberia (10°38′N, 85°27′W), Guanacaste Province, Costa Rica in 1991.

The 5′ end of the fragment included a sequence for 11 of the 23 amino acids making up the secretory leader and the 3′ end contained 90 bp of an untranslated sequence. The maxadilan gene contains an ≈ 90 bp intron starting at position 20 (Fig. 1). We observed a total of 69 variable sites, 32 of which were located in the coding region of the gene (Fig. 2). The between-population mean percentage nucleotide differences for 24 pair-wise comparisons (excluding comparisons to LE, which are included in Table 1 for comparison) ranged from 12.8% to 4.6% (mean = 9.1% ± 3.2%, Table 1). Within populations for four comparisons, the mean was 0.08% (± 0.06%).

The amino acid translation of that portion of the maxadilan gene represented in our clones is illustrated in Fig. 3. Differences between the amino acid sequences among the individuals studied are summarized in Table 1. On average, the percentage of amino acid differences between populations was 15.7% (± 4.2%). The largest difference was 23%,

Sample	BZ-1	BZ-2	CO-1	CO-2	CR-1	CR-2	MJ-4	MJ-5	LE
BZ-1	–	0.000	0.213	0.197	0.131	0.148	0.115	0.115	0.164
BZ-2	0.000	–	0.213	0.197	0.131	0.148	0.115	0.115	0.164
CO-1	0.131	0.131	–	0.016	0.262	0.279	0.197	0.197	0.049
CO-2	0.115	0.115	0.016	–	0.246	0.262	0.180	0.180	0.033
CR-1	0.077	0.077	0.137	0.120	–	0.016	0.197	0.197	0.213
CR-2	0.082	0.082	0.142	0.126	0.006	–	0.213	0.213	0.230
MJ-4	0.060	0.060	0.115	0.098	0.098	0.104	–	0.000	0.213
MJ-5	0.060	0.060	0.115	0.098	0.098	0.104	0.000	–	0.213
LE	0.104	0.104	0.027	0.011	0.115	0.120	0.109	0.109	–

BZ = Lapinha Cave, Brazil colony; CR = Liberia, Costa Rica colony; MJ = Marajo Island, Brazil colony; CO = El Callejon, Colombia colony; LE = recombinant maxadilan sequence reported by Lerner & Shoemaker (1992), included for comparison.

Table 1. Proportion of nucleotide (below diagonal) and amino acid (above diagonal) differences in the maxadilan gene/peptide between pairs of individual sand fly samples.

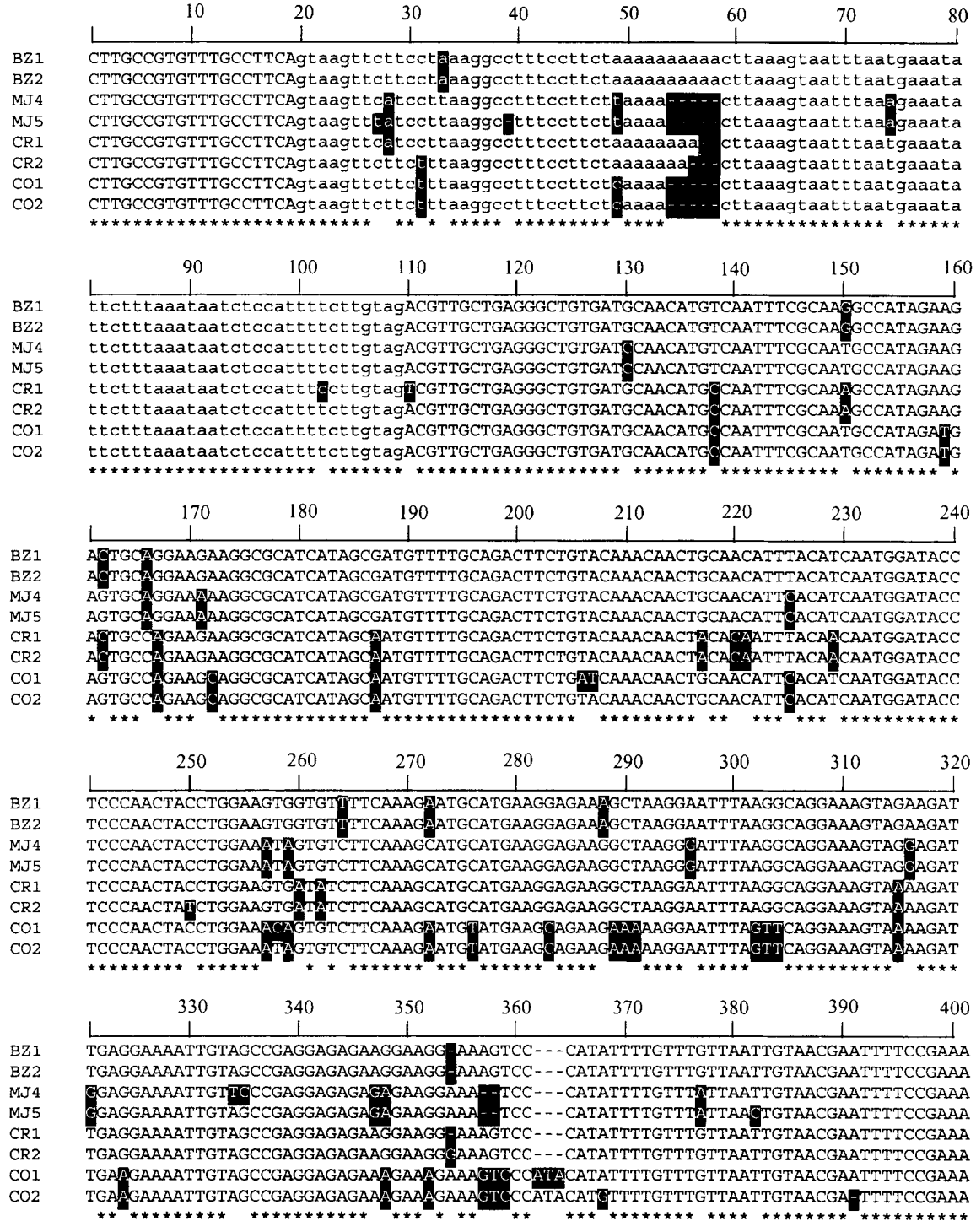

Figure 1. Multiple DNA sequence alignment for maxadilan gene for two individuals from each of four laboratory colonies. BZ = Lapinha Caves, Brazil; MJ = Marajo Island, Brazil; CR = Liberia, Costa Rica; CO = El Callejon, Colombia. Sequence in lower case indicates position of intron. Asterisks at bottom indicate 100% sequence homology at that position. Shading indicates sequences differing from the consensus.

between the Colombian and Costa Rican samples, a surprising degree of differentiation for a peptide of this size. The average difference between alleles within populations was 1% (± 1.3%). The relationship among maxadilan variants was described by calculating *p-distance*, which is simply the proportion of different amino acids between two sequences under comparison. A UPGMA dendrogram based on these

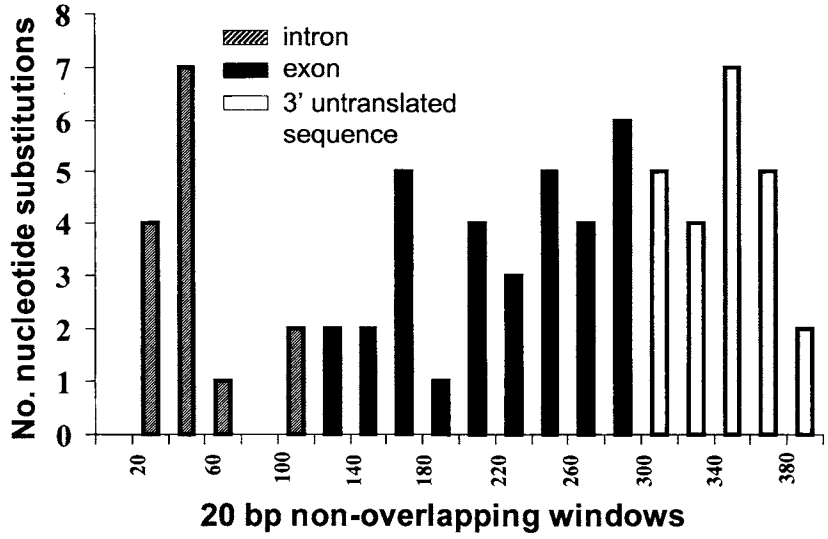

Figure 2. Distribution of variable sites within 20 base pair nonoverlapping segments of the maxadilan gene. Each division on the *x*-axis represents a 20 base pair segment of the DNA sequence. Sequence has been divided into 3 regions: (▨) intron; (■) exon and (□) 3' untranslated sequence.

Cys	Asp	Ala	Thr	Cys	Gln	Phe	Arg	Asn	Ala	Ile	Glu	Asp	Cys	Gln	Lys	Lys	Ala	His	His	Ser	Asp	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	Majority
									10										20										30		
Cys	Asp	Ala	Thr	Cys	Gln	Phe	Arg	Lys	Ala	Ile	Glu	Asp	Cys	Arg	Lys	Lys	Ala	His	His	Ser	Asp	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	BZ-1
Cys	Asp	Ala	Thr	Cys	Gln	Phe	Arg	Lys	Ala	Ile	Glu	Asp	Cys	Arg	Lys	Lys	Ala	His	His	Ser	Asp	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	BZ-2
Cys	Asp	Pro	Thr	Cys	Gln	Phe	Arg	Asn	Ala	Ile	Glu	Glu	Cys	Arg	Lys	Lys	Ala	His	His	Ser	Asp	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	MJ-4
Cys	Asp	Pro	Thr	Cys	Gln	Phe	Arg	Asn	Ala	Ile	Glu	Glu	Cys	Arg	Lys	Lys	Ala	His	His	Ser	Asp	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	MJ-5
Cys	Asp	Ala	Thr	Cys	Gln	Phe	Arg	Asn	Ala	Ile	Asp	Glu	Cys	Gln	Lys	Gln	Ala	His	His	Ser	Asn	Val	Leu	Gln	Thr	Ser	Asp	Gln	Thr	Thr	CO-1
Cys	Asp	Ala	Thr	Cys	Gln	Phe	Arg	Asn	Ala	Ile	Asp	Glu	Cys	Gln	Lys	Gln	Ala	His	His	Ser	Asn	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	CO-2
Cys	Asp	Ala	Thr	Cys	Gln	Phe	Arg	Lys	Ala	Ile	Glu	Asp	Cys	Gln	Lys	Lys	Ala	His	His	Ser	Asn	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	CR-1
Cys	Asp	Ala	Thr	Cys	Gln	Phe	Arg	Lys	Ala	Ile	Glu	Asp	Cys	Gln	Lys	Lys	Ala	His	His	Ser	Asn	Val	Leu	Gln	Thr	Ser	Val	Gln	Thr	Thr	CR-2

Ala	Thr	Phe	Thr	Ser	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Ser	Ser	Val	Phe	Lys	Ala	Cys	Met	Lys	Glu	Lys	Ala	Lys	Glu	Phe	Lys	Ala	Majority
								40									50										60		
Ala	Thr	Phe	Thr	Ser	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Ser	Gly	Val	Phe	Lys	Glu	Cys	Met	Lys	Glu	Lys	Ala	Lys	Glu	Phe	Lys	Ala	BZ-1
Ala	Thr	Phe	Thr	Ser	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Ser	Gly	Val	Phe	Lys	Glu	Cys	Met	Lys	Glu	Lys	Ala	Lys	Glu	Phe	Lys	Ala	BZ-2
Ala	Thr	Phe	Thr	Ser	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Asn	Ser	Val	Phe	Lys	Ala	Cys	Met	Lys	Glu	Lys	Ala	Lys	Gly	Phe	Lys	Ala	MJ-4
Ala	Thr	Phe	Thr	Ser	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Asn	Ser	Val	Phe	Lys	Ala	Cys	Met	Lys	Glu	Lys	Ala	Lys	Gly	Phe	Lys	Ala	MJ-5
Ala	Thr	Phe	Thr	Ser	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Asn	Ser	Val	Phe	Lys	Glu	Cys	Met	Lys	Gln	Lys	Lys	Lys	Glu	Phe	Ser	Ser	CO-1
Ala	Thr	Phe	Thr	Ser	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Asn	Ser	Val	Phe	Lys	Glu	Cys	Met	Lys	Gln	Lys	Lys	Lys	Glu	Phe	Ser	Ser	CO-2
Thr	Gln	Phe	Thr	Thr	Met	Asp	Thr	Ser	Gln	Leu	Pro	Gly	Ser	Asp	Ile	Phe	Lys	Ala	Cys	Met	Lys	Glu	Lys	Ala	Lys	Glu	Phe	Lys	Ala	CR-1
Thr	Gln	Phe	Thr	Thr	Met	Asp	Thr	Ser	Gln	Leu	Ser	Gly	Ser	Asp	Ile	Phe	Lys	Ala	Cys	Met	Lys	Glu	Lys	Ala	Lys	Glu	Phe	Lys	Ala	CR-2

Figure 3. Amino acid sequence of the mature maxadilan peptide derived from the primary DNA sequence presented in Fig. 1. Shading indicates sequences differing from the consensus.

results is illustrated in Fig. 4. Maxadilan peptides from each population cluster together. In the Lapinha Cave (BZ) and Marajo Island (MJ) populations the two individuals examined had identical peptides, whereas in the El Callejon (CO) and Liberia (CR) populations the two were different.

Secondary structure and hydropathy for different maxadilans were compared using algorithms which predict these features from the primary peptide sequence. Mature maxadilan contains four cysteine residues at positions 1, 5, 14 and 51; these were conserved in all the sequences we examined (Fig. 3). The distribution of hydrophobic residues were likewise conserved and the predicted hydrophobicity plots (Hopp & Woods, 1981) were essentially identical. The predicted secondary structure was determined using the Chou–Fasman algorithm (Chou & Fasman, 1978). All maxadilans had similar predicted secondary structures, being characterized by the presence of two α-helices, at roughly residues 6–20 and 46–60, and a β-sheet from residues 24–36 in the Lapinha Cave and Liberia peptides, but at residues 30–36 in the El Callejon peptide. Potential antigenic determinants were identified using the antigenic index algorithm of Jameson & Wolf (1988). This algorithm generates values for surface accessibility and combines them with values obtained for regional backbone flexibility and predicted secondary structure. The results of these analyses are used to create a linear surface contour profile of the protein. We observed differences in the antigenic index profile among the three sequences. All three maxadilans shared a peak at residues 38–41; however, the El Callejon maxadilan has a second peak at residues 26–30.

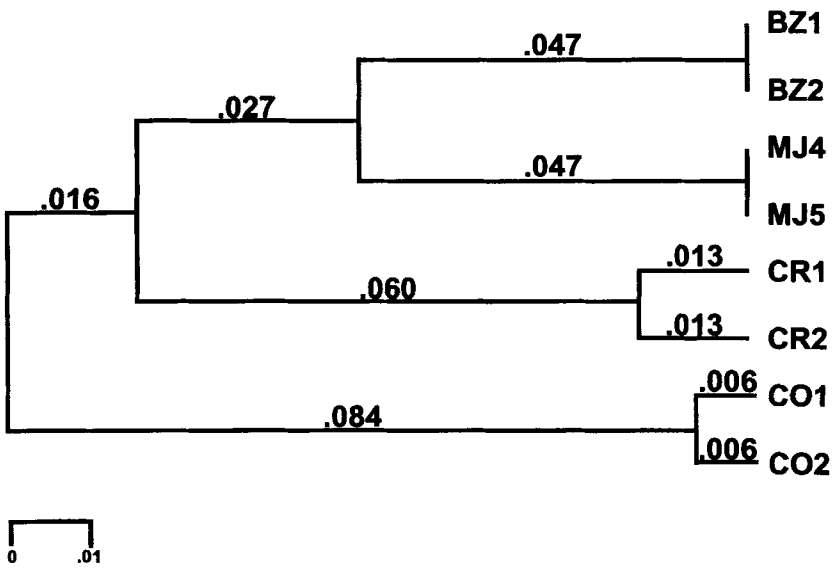

Figure 4. Phenogram based on UPGMA of *p-distance* values among maxadilan amino acid sequences. BZ = Lapinha Caves, Brazil; MJ = Marajo Island, Brazil; CR = Liberia, Costa Rica; CO = El Callejon, Colombia.

Single strand conformation polymorphism variation in natural populations

SSCP analysis was conducted on field collected samples from three Colombian populations (Bucaramanga (n = 14), Durania (n = 7) and Neiva (n = 12)) and on a population from Brasilito, Costa Rica (n = 13). We observed at least six putative maxadilan alleles, revealed as bands with distinct electrophoretic mobilities on SSCP gels (Fig. 6). Additional variants were present, but were not clearly resolved. This small survey precludes any estimate of allele frequencies in these populations. However, it does reveal that a very high degree of variation in the maxadilan encoding gene exists among individuals within natural *Lu. longipalpis* populations.

Vasodilatory activity of maxadilan variants

Recombinant maxadilan was generated from six of the eight clones developed for sequencing. The relative potencies of these recombinant maxadilans plus an earlier recombinant (Lerner & Shoemaker, 1992) and synthetic maxadilan (prepared by the Biopolymers Laboratory, Harvard Medical School; amino acid sequence based upon the predicted sequence of mature, secreted maxadilan (Lerner & Shoemaker, 1992)) were evaluated in a rabbit aortic ring bioassay (Fig. 5). Vasodilatory potencies, expressed as the concentration of maxadilan resulting in a 50% relaxation of rabbit aortic ring smooth muscle ($k_{0.5}$), ranged from 82.4 $\pm$ 19.9 ng/mL to 18.2 $\pm$ 2.5 ng/mL, a roughly fourfold difference. A one-way analysis of variance revealed that mean $k_{0.5}$ values were significantly different (F = 4.218, P = 0.004). However, only one of 28 comparisons (paired t-test) was significantly different at the 0.01 level (Lapinha Cave vs. synthetic maxadilan). It should be noted that this difference is between an insect derived maxadilan and a synthetic one; there were no significant differences among maxadilans derived from sand flies.

Interleukin-6 stimulatory activity of maxadilan variants

The ability of maxadilan variants to induce interleukin-6 production by macrophages was also tested using methods described elsewhere (Soares *et al.*, 1998). Results are shown in Table 2. First, it should be noted that similar to our experience in Soares *et al.* (1998), the level of interleukin-6 produced from experiment to experiment varied widely. This is possibly a reflection of the degree to which thioglychollate (used to elicit the macrophages) stimulated the cells from experiment-to-experiment. All maxadilan variants appeared to stimulate interleukin-6 by murine macrophages. For example, in experiment 1, maxadilan BZ-1 stimulated interleukin-6 slightly more efficiently than maxadilan CR-1 (Table 2). However, in experiment 2, CR-1 stimulated slightly more efficiently. Likewise, in experiment 2, maxadilan CR-2 elicited slightly more interleukin-6 than BZ-1, but in experiment 3 the pattern was

Table 2. Relative ability of maxadilan variants to induce interleukin-6 production by murine macrophages.

Experiment no.	Interleukin-6 production (pg/mL) induced by:					
	BZ-1	CR-1	CR-2	MJ-4	MJ-5	CO-1
1	810	390	–	–	–	625
2	70	152	84	–	130	–
3	7954	1182	3180	3048	3436	–
4	576	160	–	–	–	566

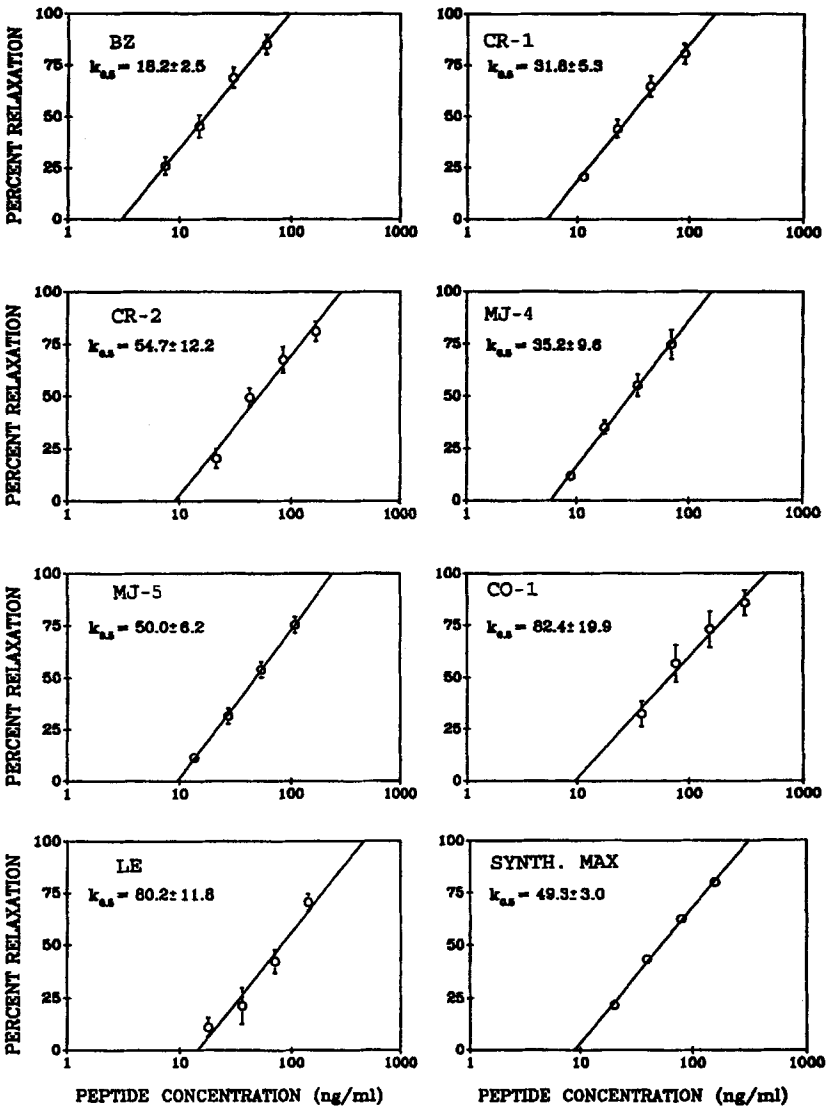

Figure 5. Cumulative dose response curves for recombinant and synthetic maxadilan in a rabbit aortic ring assay. $K_{0.5}$ values were calculated as the value giving 50% relaxation derived from regression lines where the percentage relaxation was regressed against the logarithm of the peptide concentration. BZ = Lapinha Cave, Brazil colony; CR = Liberia, Costa Rica colony; MJ = Marajo Island, Brazil colony; CO = El Callejon, Colombia colony; LE = recombinant maxadilan of Lerner & Shoemaker, 1992; SYNTH. MAX = synthetic maxadilan.

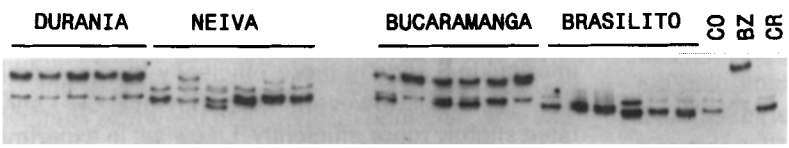

Figure 6. SSCP analysis of variability in the maxadilan gene in four field populations of *Lu. longipalpis*. Durania, Neiva and Bucaramanga = collection sites in Colombia. Brasilito = collection site in Costa Rica. CO = El Callejon colony; BZ = Lapinha Cave, Brazil colony; CR = Liberia, Costa Rica colony.

reversed. Therefore, on balance, we can simply state that all maxadilans appeared to stimulate interleukin-6.

Finally, it should be noted that the maxadilans used in the experiments described in Table 2 were produced as recombinant proteins in *Escherichia coli*. However, the observations of Table 2 were not due to the contamination of *E. coli* lipopolysaccharide since a mutated recombinant form of maxadilan that lacks vasodilatory activity, but which is produced in *E. coli* in a fashion identical to the functional maxadilan, did not induce interleukin-6 (Soares *et al.*, 1998).

Discussion

Warburg *et al.* (1994) described variation in the saliva among *Lu. longipalpis* sibling species. They showed that the erythema produced by the bite of Colombian and Brazilian *Lu. longipalpis* species were greater than 10-fold larger in diameter than those produced by

the bite of the Costa Rican species. They showed, furthermore, that this was correlated to the relative amounts of maxadilan contained in the salivary glands, as detected on Western blots probed with antimaxadilan. Brazilian and Colombian flies had 10–40-fold more maxadilan in their saliva than Costa Rican flies. Finally, Warburg *et al.* (1994) reported the results of an SSCP analysis on maxadilan encoding genes from the different species, which established that the primary DNA sequence for the maxadilan gene in each species was different. In this report we describe differences in the maxadilan encoding gene and maxadilan peptide among geographical strains and sibling species of *Lu. longipalpis*. We found a substantial variation in the maxadilan encoding gene, with as high as 12.8% nucleotide differences between sequences (Fig. 1 and Table 1). We found fixed differences distinguishing the four populations and polymorphisms within two of the populations (El Callejon and Liberia, Figs 1 and 4). Two individuals from each of four laboratory colony populations were studied, each originated from a different location, with the closest still separated by over 1000 km.

It is highly unlikely that the observed sequence variation is an artefact of cloning or a polymerase error. The observed patterns of variation are not random, as would be expected if these were artefactual. For example, the two BZ samples are identical, as are the two MJ samples. Furthermore, many of the substitutions and indels, which differentiate populations, are shared by individuals from the same population (Figs 1 and 4).

Over 45% (32/68) of the variable sites occurred within the coding region of the gene (Fig. 2). These result in a remarkable degree of polymorphism in the amino acid sequence of the maxadilan peptide. Each population had a unique peptide (Fig. 3), with populations differing in amino acid sequence by as much as 23% (Table 1). Predicted secondary structure and hydrophobicity plots suggest that, although there are numerous amino acid substitutions, these do not substantially affect structural features in the mature peptide. Bioassays to determine vasodilator function likewise showed no biologically significant differences in this activity amongst sand fly derived peptides (Fig. 5). This result suggests that differences in the erythema-producing capabilities of the saliva of South American (Brazil and Colombia) and Central American (Costa Rica) *Lu. longipalpis* are in the quantity of maxadilan present in the saliva and are not related to differences in the peptide itself. The Jameson & Wolf (1988) antigenic index algorithm was applied to maxadilan sequences to suggest whether differences in antigenicity might exist. We found that the profile of the El Callejon sequence was different, having more putative antigenic regions than the others we tested. This suggests that differences in the amino acid sequence of maxadilan which do not affect vasodilatory function may otherwise affect antigenicity.

Our survey of DNA sequence variation in the maxadilan-encoding gene was restricted to a study of two individuals from each of four laboratory colonies. This modest sample revealed that polymorphisms exist in at least two of these colonies (El Callejon and Liberia). A survey of variation in the maxadilan gene among individuals taken from four sites in nature was conducted. Three sites in Colombia and one in Costa Rica were included. It was hoped that an SSCP analysis would allow us to identify genotypes, but the banding patterns were complex, and since the survey was carried out on individuals collected from the field it was not possible to determine the inheritance patterns of band phenotypes (Fig. 6). Nevertheless, the SSCP banding patterns revealed substantial levels of polymorphism, both within and between populations.

In conclusion, we found a high degree of divergence in the salivary peptide maxadilan. The reported difference in the erythema-producing qualities of the saliva of different sibling species of *Lu. longipalpis* is not due to differences in the vasodilatory potency of different maxadilans, but rather appears to be due to differences in the amount of maxadilan in the saliva. Amino acid substitutions among different maxadilans do not appear to affect the peptide's secondary structure, hydrophobicity plot, or vasodilatory function. All maxadilans stimulate interleukin-6 production. The antigenic index does appear to be affected by amino acid substitutions. This suggests the possibility that polymorphism in maxadilan peptides modifies the antigenicity of the molecule, while conserving its vasodilator function. The significance, if any, of the antigenic variability of maxadilan is unknown. However, one might speculate that natural selection favours polymorphism in maxadilan peptides as a means of avoiding host immune responses to sand fly feeding. More work on the patterns and extent of maxadilan sequence variation in natural populations should shed light on this possibility.

Experimental procedures

Cloning and sequencing maxadilan gene

Genomic DNA was isolated from individual sand flies following the protocol of Ashburner (1989). A 397-bp fragment of the maxadilan gene was amplified by PCR as described by Warburg *et al.* (1994). Briefly, 10% (4.0 μL) of an individual sand fly genomic DNA preparation was used in a 100-μL polymerase chain reaction (PCR) containing 0.2 mM dNTPs,

10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 100 pmol of each primer and 2.5 units of Taq DNA polymerase (AmpliTaq, Perkin Elmer). The primers A2 and 3'UT were used: A2 is a 17-mer, 5'-CTTGCCGTGTTTGCCTT-3' and 3'UT is an 18-mer, 71 bp 3' to the exon: 5'-TTTCGGAAAATTCGTTAC-3'. The PCR protocol involved an initial 'hot start' at 95 °C for 2 min, followed by thirty cycles at an annealing temperature of 50 °C for 30 s, extension at 72 °C for 30 s and denaturation at 94 °C for 30 s.

PCR products were cleaned using Magic Prep (Promega) columns. The 3' end of the PCR products were filled in by nick-translation using Klenow DNA polymerase I (Gibco/BRL). These were blunt-end ligated into the *Sma*I site of the phagemid vector, pBluescript SK + /– (Stratagene). The competent *E. coli* strain DH5α (Gibco/BRL) was transformed with the vector plus maxadilan insert. White colonies were selected and, following incubation, plasmid DNA was prepared using the Plasmid Midi Prep (Qiagen) following the manufacturer's protocol. The maxadilan insert was recovered by digestion of the plasmid prep DNA with *Eco*RI and *Bam*HI. A sequence analysis was completed using T7 and M13 primers and the Sequenase version 2.0 protocol (United States Biochemical). The sequence was read manually from autoradiograms.

Production and purification of recombinant maxadilan

The coding DNA of mature maxadilan was amplified by PCR and introduced into the pRSET (InVitroGen Inc.) polylinker to generate the various expression vectors. *E. coli* harbouring the plasmids were induced for expression as recommended by InVitroGen, to produce recombinant maxadilan having amino terminal fusion partners that contain an amino terminal hexahistidine to facilitate purification. The recombinant fusion proteins were purified from inclusion bodies as described by Zhong *et al.* (1995).

Vasodilation bioassay

Vasodilatory potency of recombinant peptides were assayed in the rabbit aortic ring preparation bathed with Krebs-ringer solution (NaCl 118.3 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, glucose 5.6 mM) to which 30 mM EGTA was added to chelate any contaminating heavy metals, and 0.1 mM dexamethasone was added to prevent induction of nitric oxide synthase (Moncada *et al.*, 1991). The solution was continually gassed with 96% O_2 and 4% CO_2. Aortic rings, 2–3 mm wide were stretched to 3 g with frequent readjustments for 90 min, and were contracted with 200 ng/mL noradrenaline. Isometric transducers were used to measure the ring tension. After a steady contraction was achieved, cumulative doses of the peptides were added at 10 min intervals. Total addition volume was less than 4% of the total bath volume. An initial trial was made to find the range of doses giving inhibitions between 10% and 90% of the noradrenaline induced contraction. Results are expressed as percentage relaxation of the aortic rings under the conditions described. $K_{0.5}$ values were calculated as the value giving 50% relaxation.

Interleukin-6 bioassay

The assay is based upon previously published procedures (Soares *et al.*, 1998). Briefly, thioglycollate-induced BALB/c (Charles River Laboratories, Boston, MA) peritoneal lavage cells were placed into culture and allowed to adhere for 2 h. The nonadherent cells were rinsed away, which resulted in a population of adherent cells that were > 95% Mac-1^+. These cells were then further incubated for 6 h with 10 ng/mL of each of the maxadilan variants indicated in the text. Maxadilans were quantified using the Pierce BCA reagent following the manufacturer's instructions. Control cultures did not receive maxadilan. The supernatants of the cultures were then assessed for the presence of interleukin-6 using an interleukin-6 specific ELISA.

SSCP analysis

Individual sand fly DNA isolates were amplified by PCR as described above, except that ^{32}P-deoxycytidine 5'-triphosphate (dCTP) was added to the reaction mixture. The PCR products were heated at 94 °C for 2 min. The denatured DNA was then placed directly into an ice bath for several minutes prior to loading on to the gel; 0.5 × Hydro-Link MDE gels (AT Biochem) were run at 8 W constant power for 14 h at room temperature. Gels were dried and exposed to film for 10–16 h.

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