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Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis

ALON WARBURG[1,3], ELVIRA SARAIVA[2], GREGORY C. LANZARO[1], RICHARD G. TITUS[4] AND FRANKLIN NEVA[2]

[1]*Laboratory of Malaria Research and* [2]*Laboratory of Parasitic Diseases, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Building 4, Room 126, Bethesda, Maryland 20892, U.S.A.*
[3]*Department of Parasitology, Hebrew University-Hadassah Medical School, Ein Kerem, Jerusalem 91010, Israel*
[4]*Department of Tropical Public Health, Harvard School of Public Heath, 665 Huntington Av, Boston, Massachusetts 02115, U.S.A.*

[Plate 1]

SUMMARY

Leishmania donovani chagasi parasites, transmitted by sandflies of the *Lutzomyia longipalpis* species complex, normally cause visceral leishmaniasis. However, in Central America infections frequently result in cutaneous disease. We undertook experiments to investigate the possible influence of sandfly saliva on the course of infection. Erythemas caused by feeding sandflies correlated well with the levels of the erythema-inducing peptide, maxadilan, in their saliva. Saliva of Brazilian flies was the most potent, that of Colombian flies less so, and Costa Rican saliva had very little maxadilan and lacked activity. Nucleotide sequence differences in the maxadilan gene of the three species were detected by 'single strand conformational polymorphism' electrophoresis. *Leishmania* infections proliferated fastest when coinjected with the saliva of Costa Rican flies. Brazilian flies had less influence, and Colombian flies only a slight effect. Thus Costa Rican *Lutzomyia longipalpis*, vectors of non-ulcerative cutaneous disease, have very low vasodilatory activity and very little maxadilan, but their saliva strongly enhances cutaneous proliferation of *Leishmania* infections. Conversely, flies from Colombia and Brazil, vectors of visceral disease, have more maxadilan, but exacerbate cutaneous infections to a lesser degree. These coincidental observations suggest that species of *Lutzomyia longipalpis* differ in their propensity to modulate the pathology of the disease they transmit.

1. INTRODUCTION

Human visceral leishmaniasis caused by *Leishmania donovani chagasi* is widely distributed over much of South and Central America (Grimaldi 1989). There are an estimated 1.6 million people at risk of infection with this potentially fatal disease, and 16 000 clinical cases annually (Ashford *et al.* 1992). The most important vector of *Leishmania d. chagasi* is the Phlebotomine sandfly *Lutzomyia longipalpis* (Corredor *et al.* 1989; Lainson *et al.* 1985; LePont & Desjeux 1985; Young & Lawyer 1985; Zeledon 1985). Dense populations of this sandfly are frequently associated with human habitation in many of the drier regions of Latin America, from Mexico to northern Argentina. *Lutzomyia longipalpis* is commonly attracted to domestic animals but feeds also on humans (Quinnell *et al.* 1992). About ten years ago it was suggested that *Lutzomyia longipalpis* may be a species complex (Ward *et al.* 1983), and later work suggested that reproductive isolation may be mediated by distinct male-produced sex pheromones (Hamilton & Ward 1991). Recently, we undertook isozyme analysis and cross-mating studies among three populations, and demonstrated conclusively that *Lutzomyia longipalpis* from Costa Rica, Colombia and Brazil are three separate species (Lanzaro *et al.* 1993). Thus *Lutzomyia longipalpis* is a complex of species, each of which may be capable of transmitting *Leishmania d. chagasi* to humans.

Although infections with *Leishmania d. chagasi* are most frequently associated with visceral leishmaniasis, the parasite has also been implicated in causing a non-ulcerative form of cutaneous leishmaniasis. Such atypical cutaneous manifestations were reported from Central America: one focus in northern Costa Rica (Zeledon *et al.* 1989) and another on an island off the southern coast of Honduras (Ponce *et al.* 1991). In both, *Lutzomyia longipalpis* was the predominant sandfly species, and parasite isolates proved indistinguishable from reference strains of *Leishmania d. chagasi* obtained from patients with visceral disease.

As they feed, blood-sucking arthropods inject saliva into the vertebrate host which facilitates the location of suitable vessels in the skin and prevents haemostasis (Ribeiro 1987; Ribeiro *et al.* 1989*a*). In addition, the saliva of *Lutzomyia longipalpis* contains an extremely potent vasodilator that presumably increases blood flow

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to the bite site and produces a long-lasting erythema (Ribeiro *et al.* 1989*b*). The erythema-inducing peptide from *Lutzomyia longipalpis* (Brazil) saliva has now been cloned, characterized and named maxadilan (Lerner *et al.* 1991, 1992). Interestingly, the saliva of *Lutzomyia longipalpis* (Brazil) has also been shown to enhance the development of cutaneous leishmaniasis lesions (Titus & Ribeiro 1988; Samuelson *et al.* 1991). The component in the saliva responsible for this enhancement has not been identified.

Here we show that Costa Rican *Lutzomyia longipalpis*, collected in a focus of *Leishmania d. chagasi* where only non-ulcerative cutaneous sores occur (Zeledon *et al.* 1989), have virtually no vasodilatory activity on human skin and very little maxadilan in their saliva. Despite this, saliva from these flies strongly enhances cutaneous proliferation of *Leishmania* infections. However, the saliva of flies from foci in Colombia and Brazil, where visceral disease is exclusive, both have significantly more vasodilatory activity in human skin but are less potent than Costa Rican flies at exacerbating cutaneous infections. These coincidental observations suggest that different sibling species of *Leishmania longipalpis* may differ in their propensity to modulate the long-term pathology of the disease they transmit.

2. MATERIALS AND METHODS

(*a*) *Sandflies*

The three *Lutzomyia longipalpis* colonies utilized here were previously characterized as separate species (Lanzaro *et al.* 1993). The colony from Brazil originated in Lapinha caves, Minas Gerais (date unknown). The Colombian colony was from Melgar, Tolima department (collected by R.B. Tesh in 1989). The Costa Rican colony was started with flies we collected near Liberia, Guanacaste province in 1991. Sandflies were maintained as described by Modi and Tesh (1983).

(*b*) *Bioassay for vasodilatory activity*

Two experimental groups of 20–30 female sandflies, 3–5 days old, were allowed to feed simultaneously on the underside of the forearms of a volunteer. The erythemas at the bite sites were measured 12 hours later by using a measuring magnifier.

(c) *Densitometry of maxadilan immunoblots*

Salivary glands from females 3–5 days old were dissected in phosphate buffered saline (PBS). Glands were pooled in groups of 20 per vial and stored frozen. Samples were lysed in sodium dodecyl sulphate (SDS) sample buffer, electrophoresed on 10% polyacrylamide gels (PAGE) (Bio Rad mini system), transferred to nitrocellulose filters and blotted with anti-maxadilan rabbit serum (1:200 dilution of antiserum raised against recombinant maxadilan from Brazilian flies; Lerner *et al.* 1991) and anti-rabbit alkaline phosphatase (Promega). Western blots were photographed and the maxadilan bands were compared by densitometry of photographic negatives.

(*d*) *Genetic polymorphisms in maxadilan genes*

Genomic sandfly DNA was extracted from individual flies by using the method of Ashburner (1989) and resuspended in 40 μl of dH_2O; 10% (4 μl) of this volume was used in a 100 μl polymerase chain reaction (PCR) containing 0.2 mM deoxyribonucleoside triphosphates (NTPs), 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 g l^{-1} gelatin and 100 pmol of each primer.

Four primers were used in PCR reactions. Two were those previously used by Lerner & Shoemaker (1992): Primer A was a 17-mer immediately 3′ to a TATAA signal preceding the putative exon (5′ GAGGCTATTTTGTGCTG 3). The second primer, 3′UT, was an 18-mer, 71 base pairs 3′ to the exon (5′ TTTCGGAAAATTCGTTAC 3′). Two new primers were synthesized based on the published maxadilan sequence (Lerner & Shoemaker 1992). Primer A2 was a 17-mer (5′ CTTGCCGTGTTTGCCTT 3′) and primer 3′UT2 was an 18-mer (5′ CTTTTT-CTTCTGCTTCAT 3′. These four primers were used in PCR (Perkin-Elmer 9600) reactions in all possible combinations with DNA samples from two individual female flies of each species.

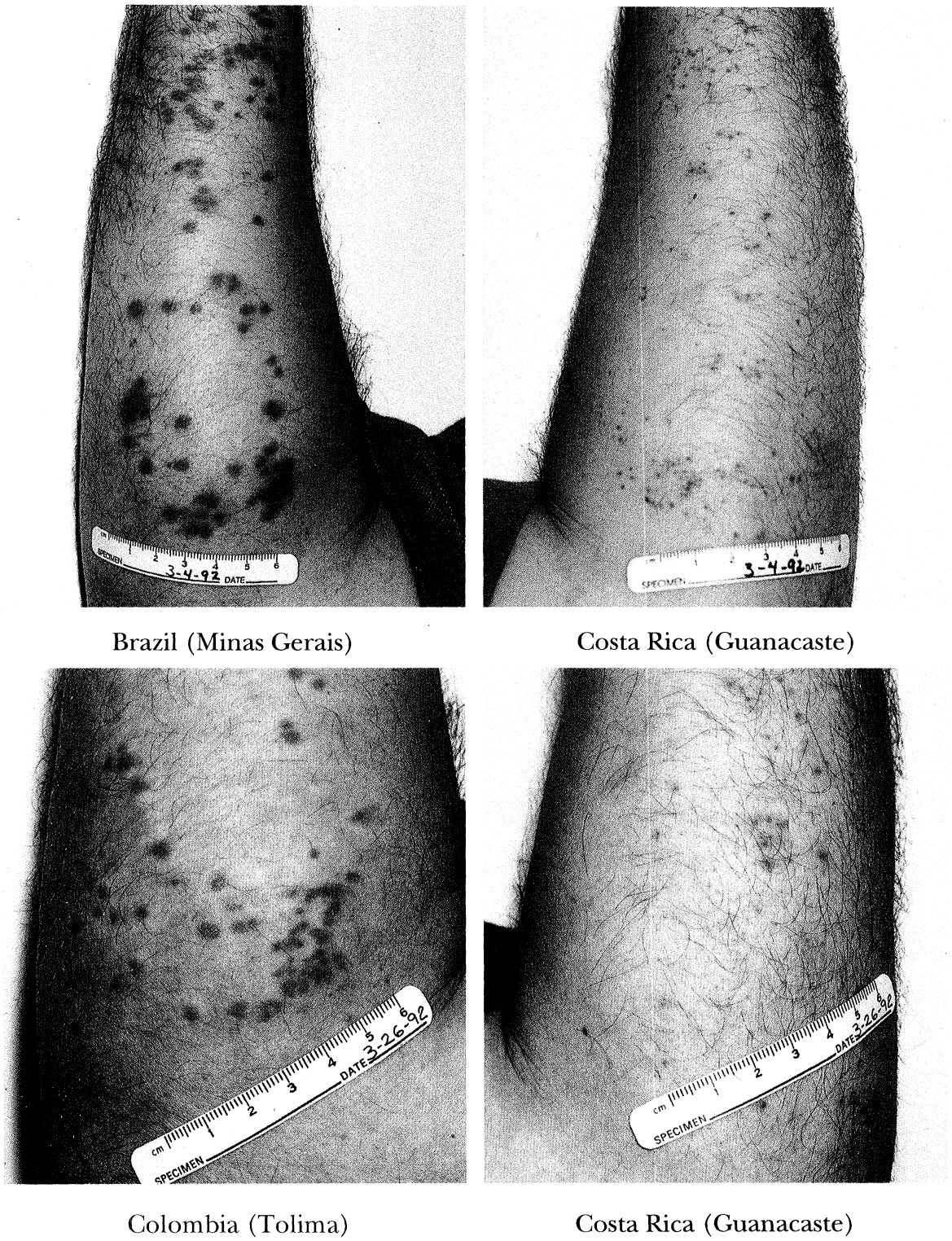
The 'Touchdown' PCR protocol (Don *et al.* 1991) was adopted to reduce artefact bands resulting from spurious priming. Under this protocol, the first cycle was run at an annealing temperature of 60°C for 30 s, extension at 72°C for 30 s and denaturation at 94C for 20 s. Thereafter, the annealing temperature was decreased by 0.5°C each cycle, so that after 21 cycles it was 50°C. The extension and denaturation conditions were the same throughout. This was immediately followed by an additional 10 cycles with annealing at 50°C for 30 s, extension at 74°C for 30 s and denaturation at 94°C for 20 s.

The electrophoretic mobility of a single-stranded DNA molecule in a native gel depends critically on the secondary structure it assumes; even a single base substitution in 900 is detectable on single strand conformation polymorphism (SSCP) gels (Orita *et al.* 1989). For SSCP analysis, genomic DNA isolated from 12 females (3 of each species) was amplified by using PCR as described above with the exception 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 onto the gel; 0.5X 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.

(e) *Effect of saliva on cutaneous lesions*

Leishmania major (LV 39) stationary phase promastigotes were mixed with salivary gland lysates in PBS + 0.1% bovine serum albumin (BSA) and injected subcutaneously into foot pads of CBA/T6

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



Brazil (Minas Gerais) Costa Rica (Guanacaste)

Colombia (Tolima) Costa Rica (Guanacaste)

Figure 1. Bite site erythemas caused by the saliva of *Lutzomyia longipalpis* spp. Viewed 12 hours after feeding. Comparisons between Brazil and Costa Rica (top) and Colombia and Costa Rica (bottom).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

mice (10^5 stationary-phase promastigotes with 0.5 of a gland per foot). Infection was monitored by measuring the increase in foot pad thickness compared with the contralateral uninfected foot using a vernier calliper (Titus & Ribeiro 1988).

The *Leishmania donovani chagasi* used in this study, MHOM/BR/00/1669, was kindly provided by M. Wilson (University of Iowa). The strain was originally isolated via bone marrow aspirate from a visceral leishmaniasis case in the state of Ceara, Brazil. Promastigotes were grown at 26°C in M-199 medium supplemented with 20% foetal bovine serum (FBS), 20 mM L-glutamine, 40 mM Hepes, 100 μM adenine, 5×10^{-4}% hemin and 10^{-4}% biotin. Parasites were harvested at the stationary phase (days 67), washed three times, resuspended in DMEM at 5×10^8 ml^{-1} and mixed with 100 g ml^{-1} peanut agglutinin (Vector Labs, Burlingham, California). After 15 min incubation at room temperature, the suspension was centrifuged at 150 *g* for 5 min. The non-agglutinated metacyclic promastigotes were washed twice and diluted into PBS for injection (see figure legends for details). BALB/c mice were inoculated with 20 μl PBS containing 10^3 metacyclic promastigotes and the equivalent of 0.5 gland each. Hamsters were inoculated with 20 μl PBS containing 10^4 metacyclic promastigotes and the equivalent of two salivary glands. Lesion growth was monitored as above.

3. RESULTS

(*a*) *Vasodilatory effect of saliva*

Flies (20–30) of the two species to be compared were allowed to feed simultaneously on the arms of a volunteer. The Costa Rican species was compared to the other two in three experiments each (figure 1). The diameter of erythemas was measured and the cumulative results were summarized (table 1). Clearly, bites of Costa Rican flies left lighter-coloured marks which were also far smaller than those of either Colombia or Brazil. Of these last two groups, Brazilian flies produced a more intensely red mark which was also somewhat larger than that of Colombian flies (figure 1; table 1). The variability in erythema diameter within each experimental group was due, in part, to the fact that sandflies tend to probe for short periods both before and after feeding. These shorter probes resulted in smaller, less intensely red erythemas.

Table 1. *Comparative size of erythemas on human skin, measured 12 hours after bites of different Lutzomyia longipalpis species*

strain	no of experiments	no of bites	size range /mm	mean diameter /mm
Costa Rica	5	222	0.1–1.5	0.3
Colombia	3	100	1.1–4.8	3.3
Brazil	3	151	2.6–5.6	4.3

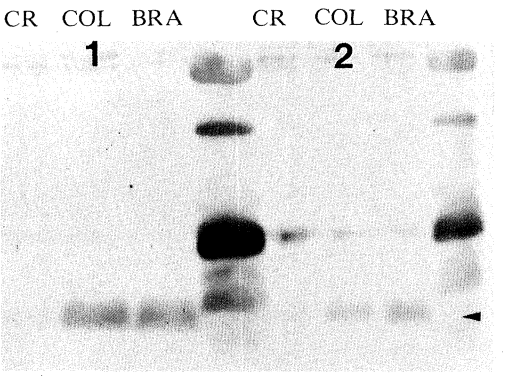


Figure 2. Western blot of salivary gland lysates of *Lutzomyia longipalpis* spp. Panel 1, 2 glands per lane; panel 2, 0.4 glands per lane. Arrow head indicates maxadilan bands. BRA = Brazil; COL = Colombia; CR.

(*b*) *Relative amounts of maxadilan in saliva*

We compared the quantities of maxadilan in portions from pools of 20 salivary glands. Western blots probed with anti-maxadilan (figure 2) showed that glands of Brazilian flies contained twice as much maxadilan as those of Colombian flies, and both these strains apparently contain 10–40 fold more than flies from Costa Rica (figure 3). However, because the antiserum used was raised against maxadilan from Brazilian flies, albeit a recombinant form, the possibility of differential affinities for the different forms of the peptide (see following sections) has to be considered. Identical gels were silver stained to verify that equivalent amounts of protein were used for each lane (data not shown). Similar comparisons were made using homogenated, individual flies. In these experiments the maxadilan bands were more diffuse, but densitometry results were similar to those in figure 3 (data not shown).

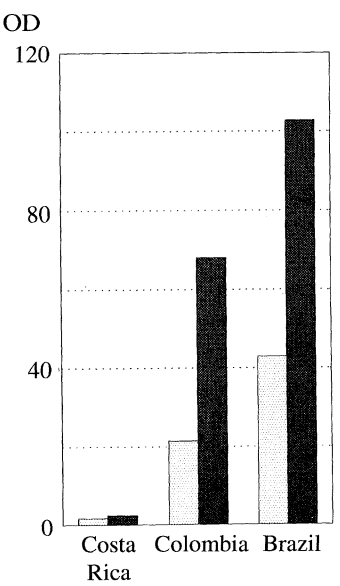


Figure 3. Densitometry of salivary gland lysates probed with anti-Maxadilan Western blot negative. Light-shaded bars, 0.4 glands; dark-shaded bars, 2 glands.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

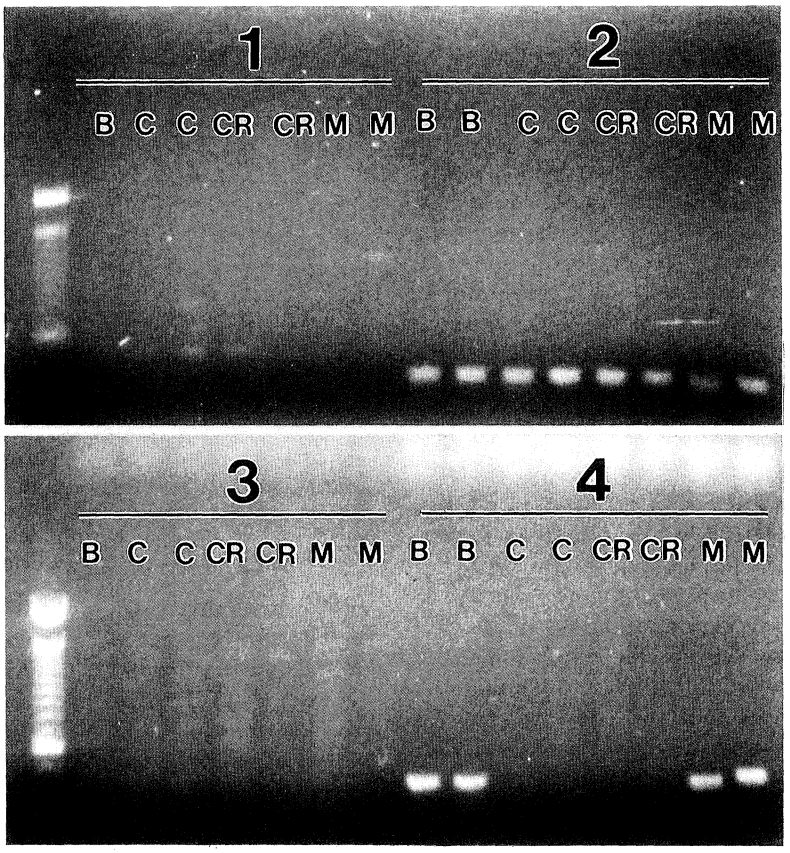


Figure 4. Development of universal PCR primers for the Maxadilan gene. 1% agarose gel. Lanes 1 and 17 = 100 b.p. ladder; panel 1 samples amplified with the primer combination A2/3′UT2; panel 2 amplified with primers A2/3′UT; panel 3 amplified with primers A/3′UT2; panel 4 amplified with primers A/3′UT. B = Brazil, C = Colombia, CR = Costa Rica, M = Marajó.

(*c*) *Genetic polymorphisms of maxadilan*

By using the primers described by Lerner & Shoemaker (1992), A and 3′UT, we were able to PCR amplify a DNA fragment from the Brazilian species and from another Brazilian colony originating in the island of Marajó (figure 4, panel 4) as well as one of three Costa Rican flies (data not shown). However, this primer combination failed to amplify anything from two other Costa Rican and all of the Colombian flies. The fragment amplified was 484 base pairs long, as predicted from the maxadilan gene sequence. Only the primer combination A2 and 3′UT successfully amplified DNA from all of the species. The DNA fragment thus produced was equal in size to the predicted maxadilan gene fragment (397 base pairs; figure 4, panel 2).

The PCR products appeared identical in all species as visualized on 1% agarose gels stained with ethidium bromide. To detect nucleotide substitutions between individual flies from the three species, we performed SSCP analysis. By using this procedure we were able to establish that there are consistent differences in the nucleotide sequence of the maxadilan gene between the sibling species, and that there are polymorphisms within the Costa Rican species (figure 5).

(*d*) *Experimental enhancement of cutaneous lesions in mice and hamsters*

Three sets of experiments were performed using different animals, different parasites cultured and purified in different ways, as well as different doses of salivary gland lysates. As they differ by more than one variable, the results are not directly comparable with each other. Figure 6 summarizes the results of experimental infections using *Leishmania major* in

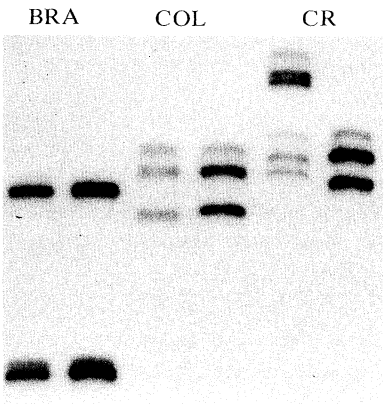


Figure 5. Single strand conformation polymorphism (SSCP) in PCR amplified DNA from the maxadilan gene. BRA = Brazil; COL = Colombia; CR = Costa Rica.

resistant (self curing) mice; there were three repeats using five mice per repeat per fly species. Measurements of infected foot pads clearly demonstrated that saliva of flies from Costa Rica was a more potent enhancer of infections than that of flies from Brazil. Saliva of Colombian flies was much less potent than either.

Essentially similar results were obtained in sensitive (BALB/c) mice and hamsters injected intradermally with metacyclic *Leishmania d. chagasi* and sandfly saliva (figures 7 and 8). Lesions appeared first in footpads into which promastigotes were coinjected with saliva from Costa Rican flies. About a week later, lesions appeared in animals injected with Brazilian saliva, and the control footpads injected with parasites alone progressed more slowly than either. Presumably because of the strain's innate susceptibility, lesions in BALB/c mice progressed so rapidly that enhancement by salivary extract, although readily observable, was not statistically significant (figure 7). Saliva from Colombian flies was not evaluated in these two experiments.

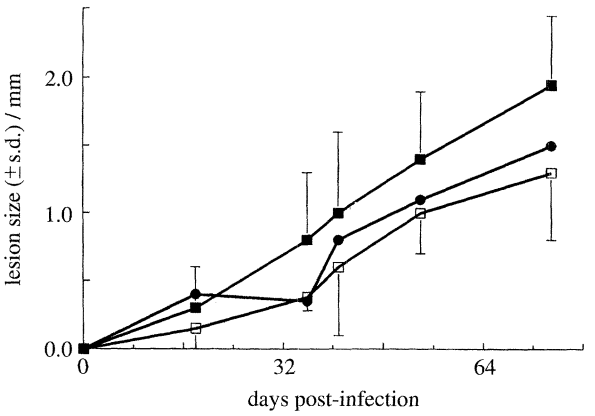


Figure 7. Influence of saliva on the development of *Leishmania donovani chagasi* infections in sensitive mice (BALB/c); 10^3 promastigotes with 1 salivary gland equivalent. Costa Rica (filled squares), Brazil (filled circles), control (without saliva; open squares).

4. DISCUSSION

There are at least two plausible conventional explanations for the different pathologies caused by *Leishmania d. chagasi* infections: the existence of *Leishmania d. chagasi* variants that differ in their virulence, and differences in the immunological make-up of the affected populations. Studies conducted thus far have failed to demonstrate any differences between parasite isolates from cutaneous and visceral cases (Ponce *et al.* 1991; Zeledon *et al.* 1989), and no comparative studies on human populations have been published to date. Thus so far the only proven difference between foci of cutaneous and visceral disease is that infections are transmitted by distinct sandfly species (Lanzaro *et al.* 1993). Whenever separate species are involved in transmission of a disease, the possibility of differential vectorial capacities has to be considered. Aside from obvious factors such as the degree of anthropophily (Quinell *et al.* 1992) and the ability to harbour and transmit a particular *Leishmania* species (Killick-Kendrick 1990), a sandfly's efficiency as a vector may also be influenced by the chemical composition of the saliva that it injects into the vertebrate host as it is feeding (Ribeiro 1987). Based on the above observations as well as results reported here, we propose a novel explanation for the different pathologies caused by *Leishmania d. chagasi*: that the vectors' saliva somehow modulates the long-term pathology of the disease. Consequently, different salivary composition of vector species predisposes the infected individual to develop either cutaneous or visceral disease. Although we could not prove this hypothesis, the experiments described here certainly demonstrate major quantitative and qualitative differences between the saliva of three *Lutzomyia longipalpis* sibling species.

The level of vasodilatory activity in the saliva of flies from Costa Rica was much lower than the other two species (figure 1; table 1), and correlated well with densitometry of Western blots of salivary gland

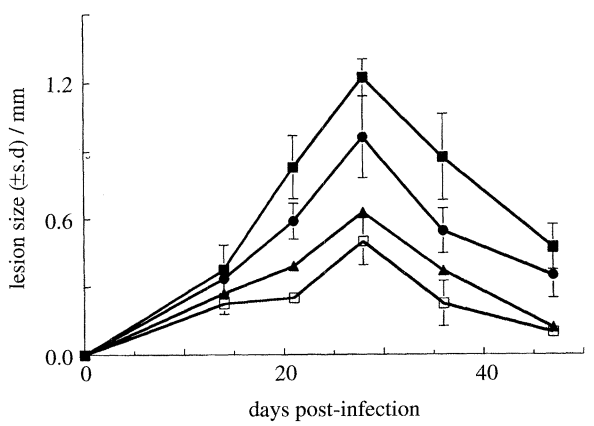


Figure 6. Influence of saliva on the development of *Leishmania major* infections in resistant mice (CBA/T6). 10^5 stationary-phase promastigotes with 0.5 salivary equivalent. Costa Rica (filled squares), Brazil (filled circles), Colombia (filled triangles), control (without saliva; open squares).

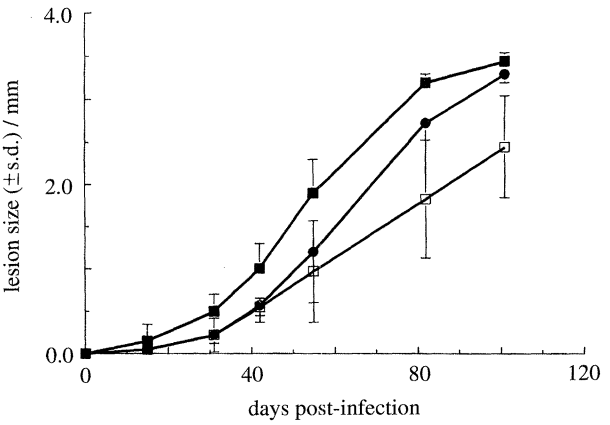


Figure 8. Influence of saliva on the development of *Leishmania donovani chagasi* infections in golden hamsters; 10^4 promastigotes with 2 salivary gland equivalent. Costa Rica (filled squares), Brazil (filed circles), control (without saliva; open squares).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

lysates. These data indicate that the lower activity was due to smaller quantities of the erythema-inducing factor, maxadilan (figures 2 and 3). However, SSCP analysis (figure 5), the variability in the PCR primer annealing regions, and preliminary DNA sequencing data (G. C. Lanzaro *et al.*, unpublished results) demonstrated significant differences in the maxadilan gene between the three sibling species. As the antiserum used for the Western blot was raised against a recombinant peptide derived from Brazilian flies, densitometry may have been affected by differential affinity for the distinct forms of maxadilan. To clarify these questions we are currently conducting studies to compare levels of maxadilan in the different flies by using antisera to conserved regions (Lanzaro *et al.*, unpublished results) and comparing the erythema-inducing properties of the different forms of recombinant maxadilan.

Measurement of footpad thickness to evaluate infection is not a very accurate method. Nevertheless, it is the best one available for comparing the progression of cutaneous leishmaniasis in rodents. When performed correctly, the method will detect even small differences. To minimize bias in our comparisons, some experiments in each set were conducted blindly.

Although it lacked vasodilatory activity, the saliva of Costa Rican flies was consistently the most potent enhancer of experimental cutaneous leishmaniasis in mice and hamsters (figures 6–8). Indeed, Costa Rican saliva enhanced infections initiated by metacyclic promastigotes, the most infective life-cycle stage of *Leishmania* (figures 7 and 8).

Recent studies have shown that *Lutzomyia longipalpis* saliva inhibits the ability of *Leishmania*-infected macrophages to present parasite-specific antigen to T cells (Theodos & Titus 1993), suggesting that this inhibition may be responsible for the enhancement of infectivity in mice. There is also evidence linking vasodilatory pharmacological activity with inhibition of macrophage function (Nong *et al.* 1989) and enhancement of cutaneous leishmaniasis in mice (Theodos *et al.* 1991). Our data suggest that the observed enhancement of infection caused by sandfly saliva does not depend on the vasodilatory activity of maxadilan.

Upon injection into the skin by the bite of an infected sandfly, *Leishmania* promastigotes invade cells of the reticulo-endothelial system. The parasites multiply intracellularly and reinvade macrophages as their host cells rupture. Therefore, if sandfly saliva somehow modulates the type of macrophages that become available at the bite site, or the behaviour of infected macrophages, it may conceivably influence the entire course of the disease. As the saliva of Costa Rican *Lutzomyia longipalpis* lacks vasodilatory activity (table 1; figures 1–3) but strongly enhances cutaneous infections (figures 6–8), we propose that promastigotes transmitted by these flies will infect mainly resident dermal macrophages and cause a rapidly proliferating cutaneous lesion. As cutaneous leishmaniasis provokes a strong cell-mediated immune response (Liew & O'Donnel 1993), visceralization may be totally prevented in cases that develop fulminating cutaneous lesions early on. However, when the same parasite is injected with saliva that contains maxadilan, a strong vasodilator, more circulating monocytes will infiltrate the bite site via blood and lymph vessels thus facilitating the early visceralization of the infection.

In support of this hypothesis, it has been demonstrated that artificial *Leishmania donovani* infections in hamsters can follow either a visceralizing course or a cutaneous one, depending on the way in which parasites were introduced into the animal. Progressive visceral disease, in animals inoculated intracardially, was characterized by lack of response of lymph node cells, spleen cells and peripheral blood lymphocytes to parasite antigens *in vitro*. However, animals inoculated intradermally developed cutaneous leishmaniasis with characteristic skin reactions and lymphoid cell proliferation to parasite antigens *in vitro* (Gifawesen & Farrell 1989). The difference in the mode of inoculation may be mimicked in nature by the pharmacological properties of the vectors' saliva, the early effects of which may influence the long-term pathology caused by the parasite they transmit.

Preliminary data using SSCP (figure 5) and apparent variability in the annealing regions of the PCR primer A (data not shown) support the existence of polymorphism in the maxadilan gene within the Costa Rican colony. The same species also had the highest number of isozyme allells per locus but there was no indication that the Costa Rican colony was made up of separate species (Lanzaro *et al.* 1993). Furthermore, SSCP clearly demonstrated that neither of the Costa Rican forms was the same as any of the other colonized species (figure 5). Therefore, we conclude that the observed polymorphism is intraspecific. Whereas only the non-ulcerating cutaneous pathology of *Leishmania d. chagasi* is known in Costa Rica (Zeledon *et al.* 1989), in Honduras the visceral and the cutaneous forms exist side by side (Ponce *et al.* 1991). We are planning field studies in Honduras to determine whether there are variations in salivary composition of *Lutzomyia longipalpis* or whether more than one vector species transmits *Leishmania d. chagasi* in the affected areas.

In the Old World, *Leishmania donovani* spp. normally cause visceral disease, but are not infrequently associated with cutaneous leishmaniasis. In North Africa and southern Europe, *Leishmania d. infantum*, a parasite which is arguably identical with *Leishmania d. chagasi* (Lainson *et al.* 1987), causes visceral infections in some parts but primarily cutaneous disease in other parts (Ben-Ismail *et al.* 1992; Belazzoug *et al.* 1985; Gramiccia *et al.* 1991; Rioux & Lannote 1990). Cutaneous lesions due to *Leishmania d. donovani*, the causative agent of kala-azar in Africa, have also been reported (Abdalla 1982). In the Mediterranean region a number of *Phlebotomus (Larroussius)* spp. are known to transmit *Leishmania d. infantum*, and in East Africa *P.* (*Synphlebotomus*) spp. are the probable vectors of *Leishmania d. donovani* (Killick-Kendrick 1990). Data on the saliva of local vectors and their distribution in relation to clinical manifestations and

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

parasite strains could go a long way to support or refute our hypotheses.

There are also examples of *Leishmania* species that normally cause self-curing cutaneous sores but occasionally result in visceral leishmaniasis. In Bahia state, Brazil, *Leishmania mexicana amazonensis* infections usually cause self-limiting cutaneous lesions. However, some cases develop mucocutaneous pathology, and others even visceralize to cause typical kala azar symptomatology (Barral *et al.* 1991). Different clinical manifestations of seemingly identical parasites in separate geographic regions are also known within the *Leishmania braziliensis* complex. *Leishmania b. panamensis* and *Leishmania b. braziliensis* normally produce a cutaneous sore at the site of the sandfly bite, and a certain percentage of untreated cases go on to develop secondary chronic infections in the mucosal membranes of the nose and pharynx (mucocutaneous leishmaniasis). Of the persons infected with *Leishmania braziliensis*, a larger percentage of individuals develop mucocutaneous manifestations in Brazil, Bolivia and Colombia (25–81%), whereas in countries in Central America (Costa Rica, Nicaragua and Panama) the percentages are significantly lower (2–5%) (compiled from references cited by Grimaldi *et al.* (1989)). There are two proven and 13 suspected vectors of *Leishmania b. braziliensis*, and nine suspected vectors of *Leishmania b. panamensis* in Latin America (Killick-Kendrick 1990). Although some of these differences are certainly attributable to environmental factors and parasite strains, it is not inconceivable that the different vectors also contribute to this complex epidemiological scenario.

In nature, the Costa Rican *Lutzomyia longipalpis* species transmits *Leishmania d. chagasi* that manifests itself exclusively as cutaneous non-ulcerating sores. Flies with much higher vasodilatory activity but reduced capacity to enhance cutaneous proliferation, from Brazil and Colombia, transmit an indistinguishable parasite which, nevertheless, causes visceral leishmaniasis in humans. The present report is the first to demonstrate a difference in the transmission cycle of the two forms of the disease, the vectors' saliva. Thus, some of the variability in the clinical presentations of *Leishmania d. chagasi* infections may be due to the different composition of the saliva of the sandfly that transmits them.

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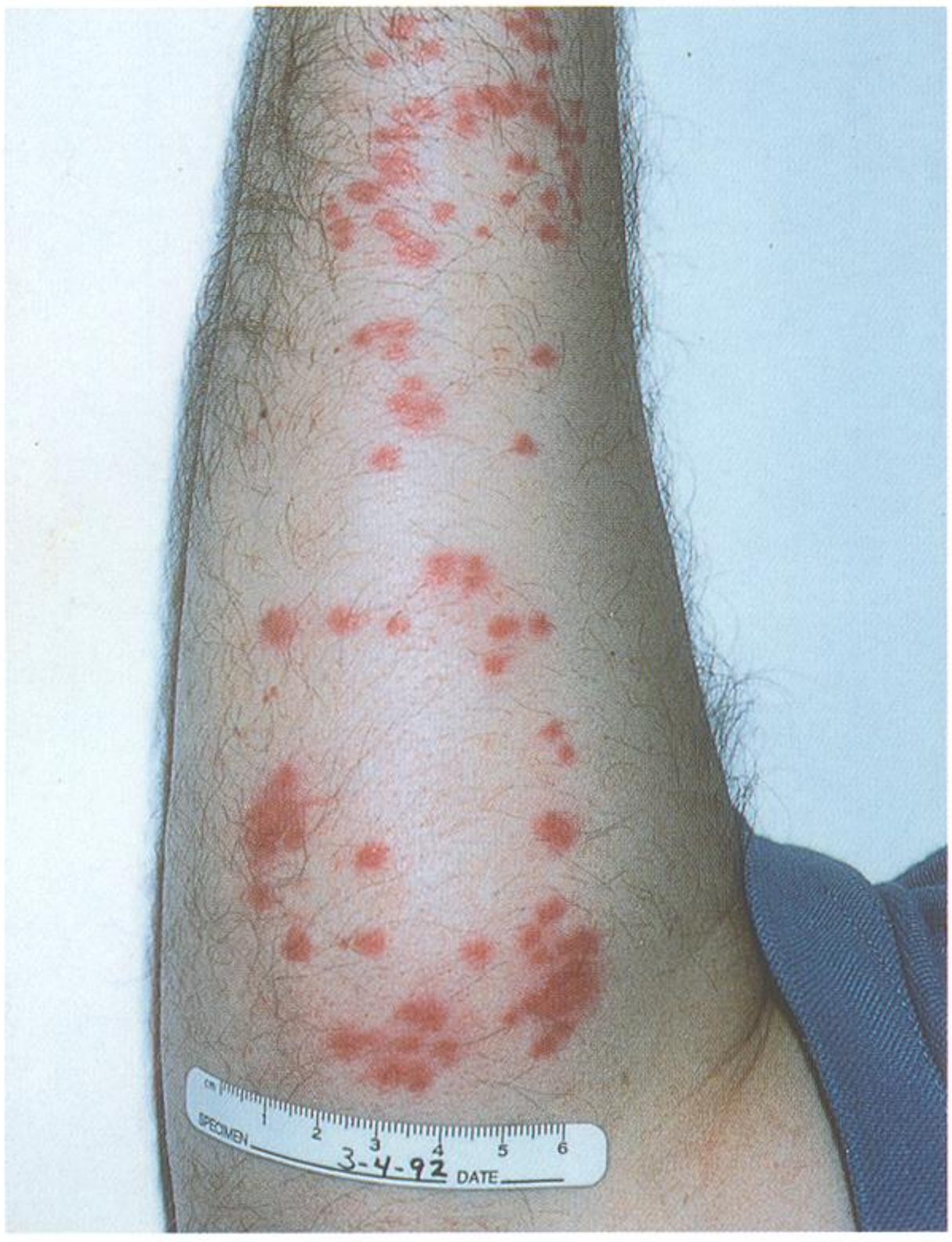

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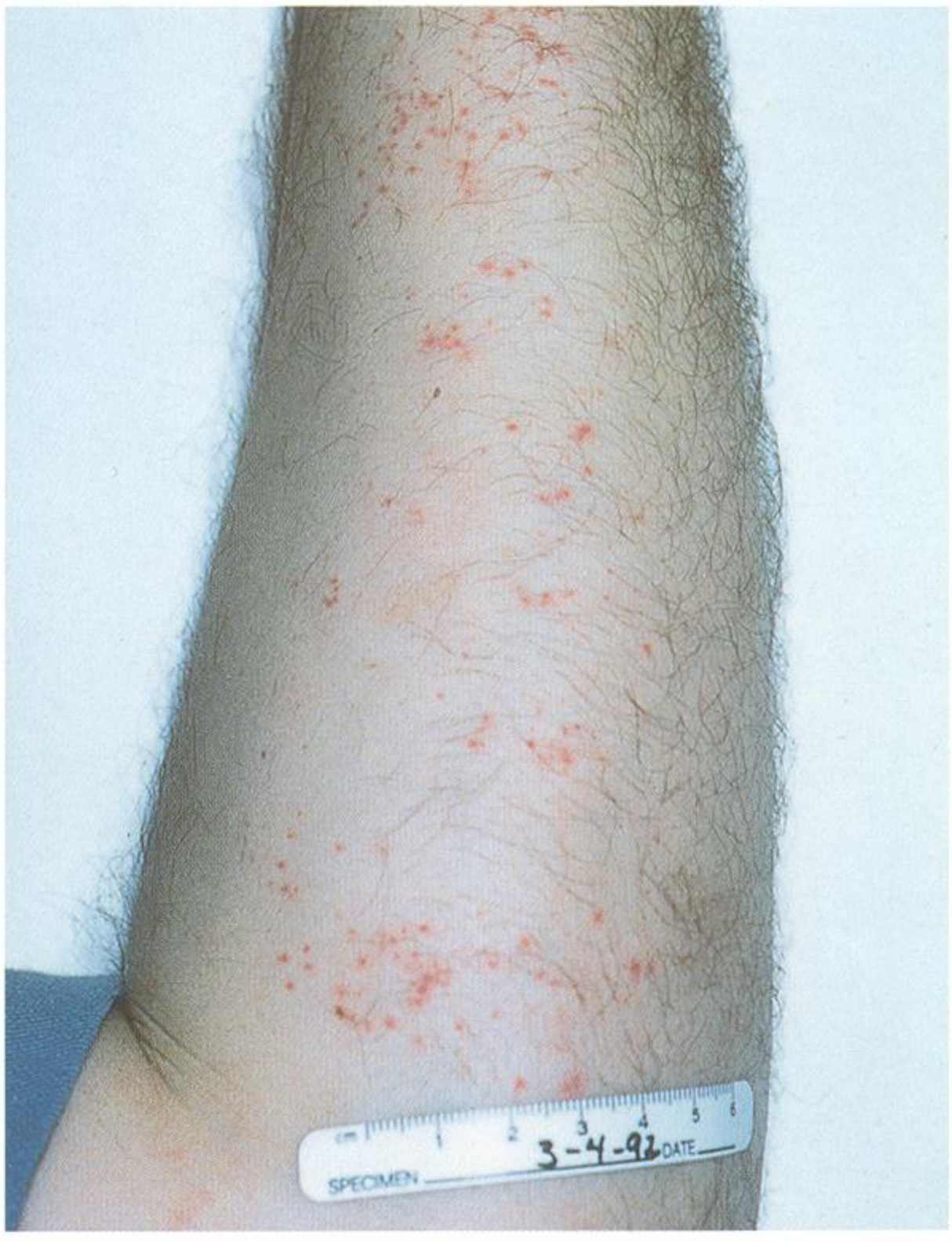

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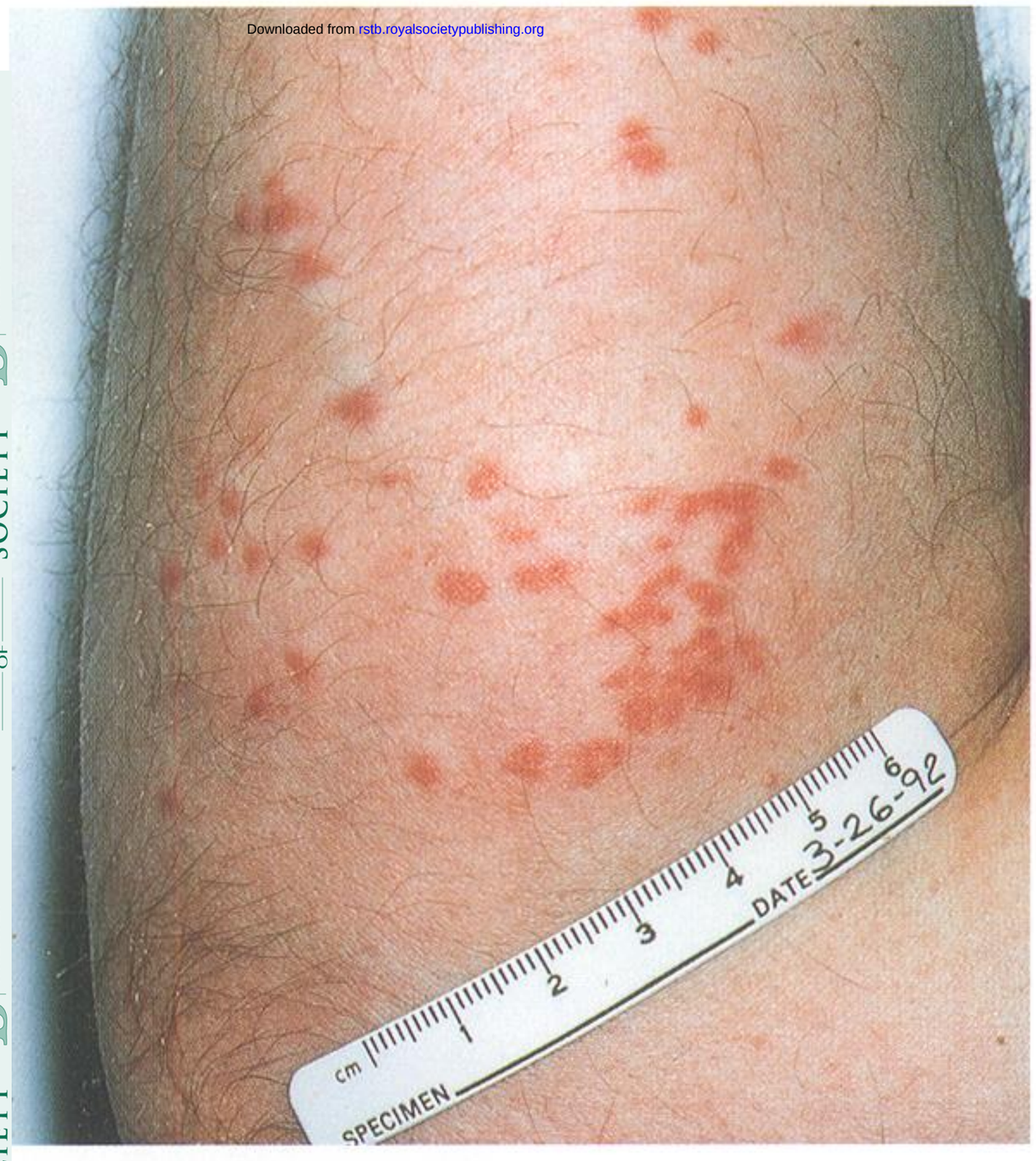

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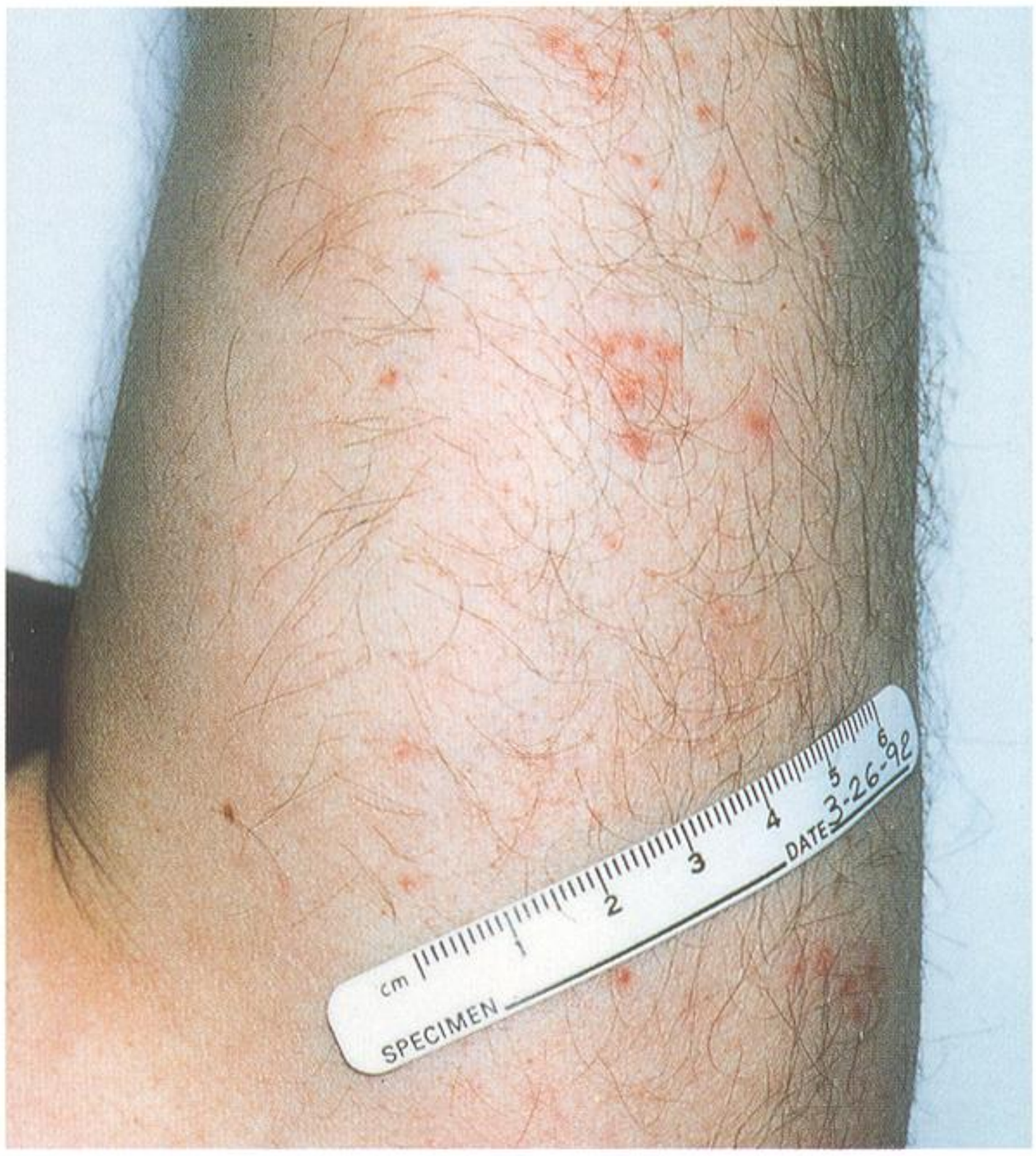
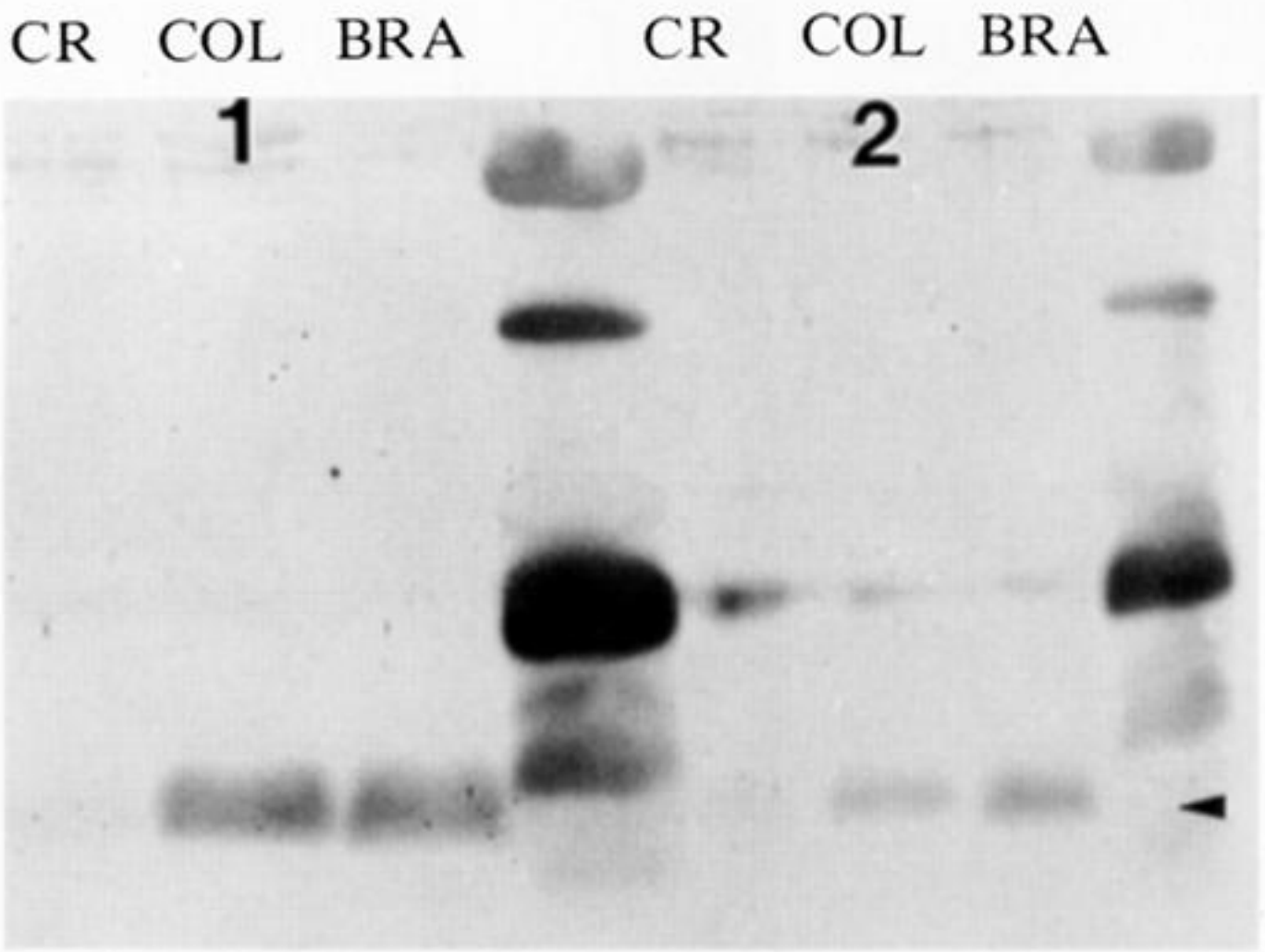
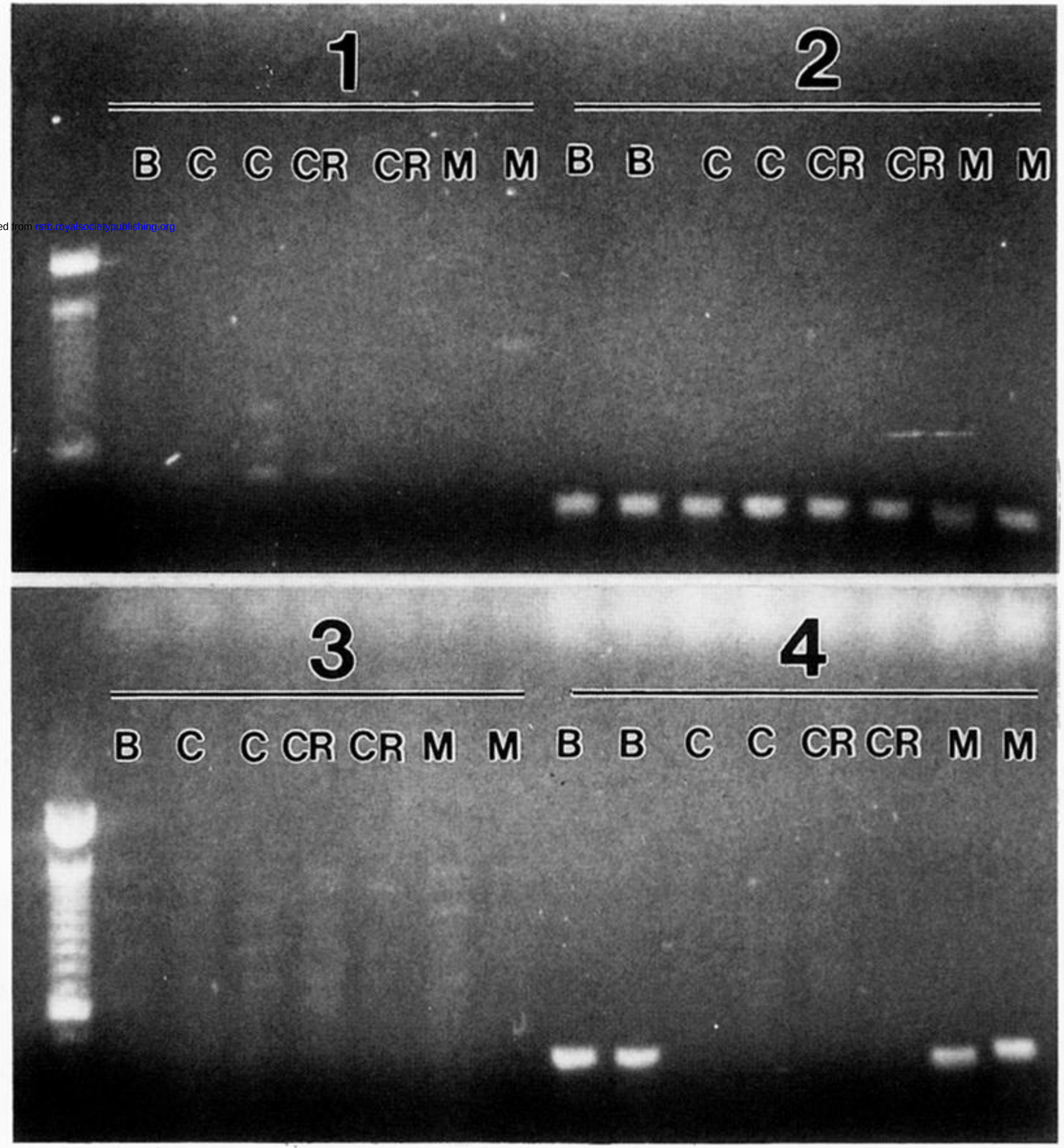


Costa Rica (Guanacaste)

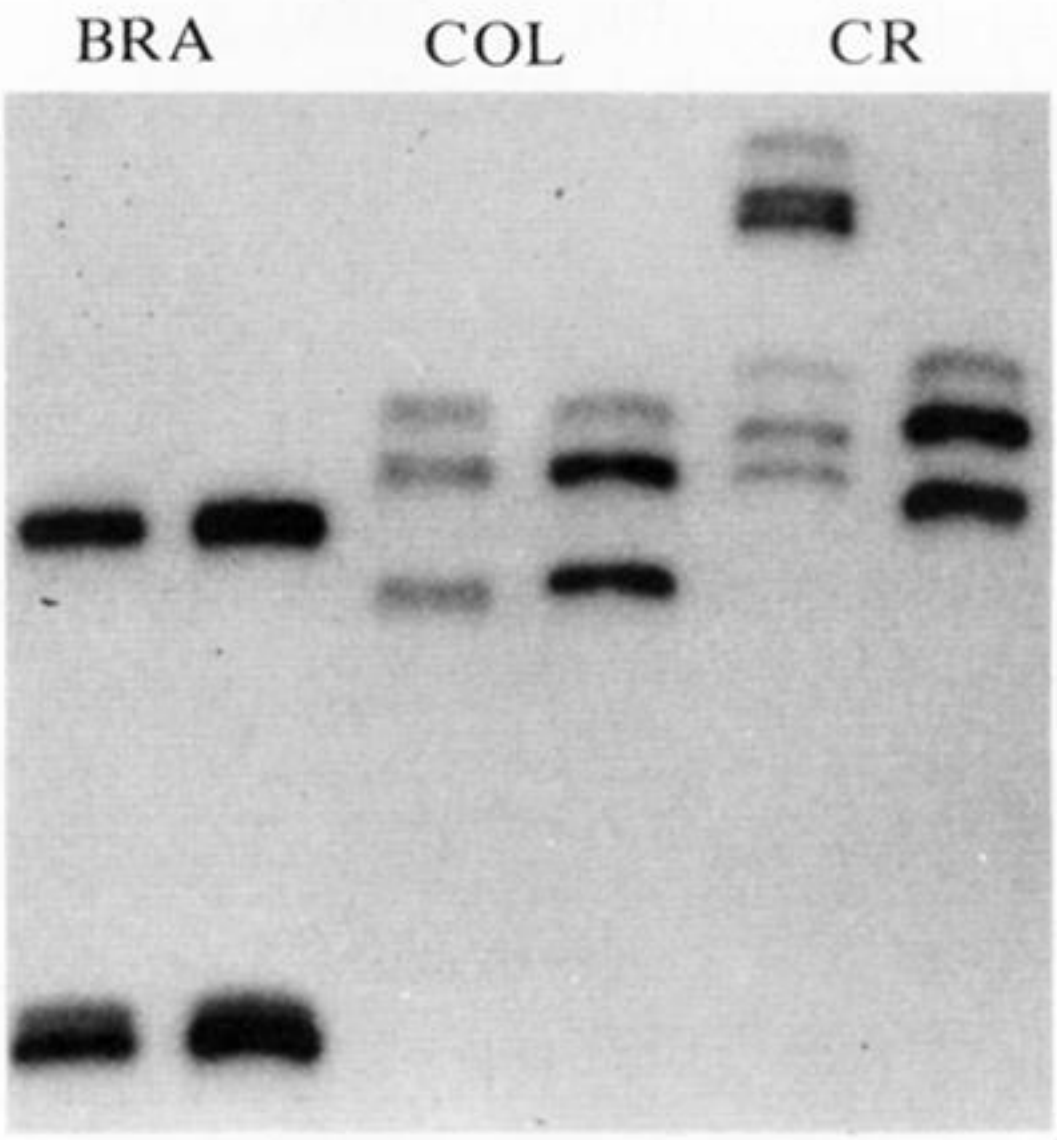
Figure 1. Bite site erythemas caused by the saliva of *Lutzomyia longipalpis* spp. Viewed 12 hours after feeding. Comparisons between Brazil and Costa Rica (top) and Colombia and Costa Rica (bottom).



gure 2. Western blot of salivary gland lysates of *Lutzomyia ngipalpis* spp. Panel 1, 2 glands per lane; panel 2, 0.4 glands er lane. Arrow head indicates maxadilan bands. RA = Brazil; COL = Colombia; CR.



igure 4. Development of universal PCR primers for the Maxadilan gene. 1% agarose gel. Lanes 1 and 7 = 100 b.p. ladder; panel 1 samples amplified with the primer combination A2/3′UT2; panel 2 amplified with rimers A2/3′UT; panel 3 amplified with primers A/3′UT2; panel 4 amplified with primers A/3′UT. B = Brazil, = Colombia, CR = Costa Rica, M = Marajó.



gure 5. Single strand conformation polymorphism (SSCP) PCR amplified DNA from the maxadilan gene. RA = Brazil; COL = Colombia; CR = Costa Rica.