

Seroconversion against *Lutzomyia longipalpis* Saliva Concurrent with the Development of Anti-*Leishmania chagasi* Delayed-Type Hypersensitivity

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Antibody responses to salivary gland sonicate (SGS) from *Lutzomyia longipalpis* were investigated using serum samples from individuals living in an area where visceral leishmaniasis is endemic. Individuals were classified into 2 groups, according to the alteration of their responses to *Leishmania chagasi* antigen over the course of 6 months. Group 1 included children who experienced anti-*L. chagasi* seroconversion from negative to positive; group 2 included children who experienced delayed-type hypersensitivity (DTH) response to *L. chagasi* antigen conversion from negative to positive. Individuals who experienced seroconversion against *L. chagasi* antigens did not have increased anti-saliva antibody response, whereas those who developed a positive anti-*L. chagasi* DTH response had increased immunoglobulin (Ig) G, IgG1 and IgE anti-SGS antibody levels. Despite wide variation, serum samples from individuals in group 2 recognized more bands in SGS than did those from individuals in group 1. This simultaneous appearance of anti-saliva humoral response and anti-*L. chagasi* cell-mediated immunity supports the hypothesis that induction of immune response against SGS can facilitate induction of a protective response against leishmaniasis.

Leishmania species are transmitted by sand flies. The saliva of these and other blood-sucking insects contains a varied repertoire of pharmacologically active molecules that are able to interfere with host hemostatic, inflammatory, and immune responses [1]. In mice, these products exacerbate infection with *L. chagasi* and may, in fact, be elemental for establishing the parasite in the vertebrate host [2]. The presence of salivary gland sonicate (SGS) changes the pattern of the anti-*L. chagasi* immune response from a protective Th1 pattern to an unresponsive Th2 pattern [3]. This effect was completely abrogated in

mice that were preexposed to salivary components or bites from uninfected sand flies [4, 5]. The protection given by immunization with SGS [2] or the 15-kDa protein from the saliva of *Phlebotomus papatasi* (SP15) [4] also is indicative of the importance of sand fly saliva on the initial steps of infection with *L. chagasi*. Of interest, even in B cell knockout mice, immunization with SP15 cDNA leads to protection, suggesting that a delayed-type hypersensitivity (DTH) response against saliva provides most, if not all, of the protective effects of this vaccine [4]. However, the importance of sand fly saliva in human *L. chagasi* infection is less clear.

We have previously shown that serum samples from children living in an area endemic for visceral leishmaniasis (VL) have anti-SGS IgG antibodies that differentially recognize salivary gland antigens [6]. Individuals with a positive anti-*L. chagasi* DTH response exhibited anti-*Lutzomyia longipalpis* saliva antibodies. A positive correlation was observed between anti-*Lu. longipalpis* saliva antibodies and anti-*L. chagasi* DTH response, but no correlation was observed between anti-saliva antibodies and anti-*L. chagasi* serologic status [6]. In the present study, we explore the change in humoral and cell-mediated anti-*L. chagasi* responses in a 6-month follow-up of individuals in an area where VL is endemic, as well as the change in anti-*Lu. longipalpis* saliva antibody responses in the same individuals.

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Informed written consent was obtained from parents or legal guardians of minors. Human experimentation guidelines from Universidade Federal do Maranhão Ethics Committee were followed and approved.

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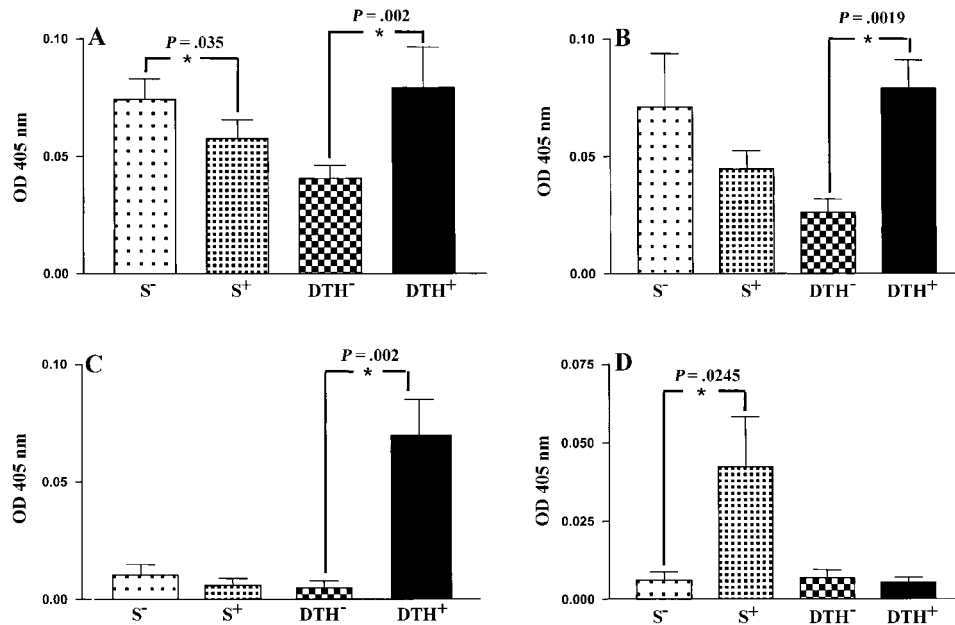


Figure 1. Antibodies against *Lutzomyia longipalpis* saliva. Human serum samples were obtained at time 0 (negative anti-*Leishmania chagasi* serologic status [S⁻] or negative delayed-type hypersensitivity [DTH] response [DTH⁻]) and 6 months later (positive anti-*L. chagasi* serologic status [S⁺] or positive anti-*L. chagasi* DTH response [DTH⁺]). ELISAs were performed with these serum samples using salivary gland sonicate of the sand fly *Lu. longipalpis*. **A**, Anti-saliva IgG levels in individuals whose anti-*L. chagasi* serologic status converted (S⁻ to S⁺) and whose anti-*L. chagasi* DTH response converted (DTH⁻ to DTH⁺). **B**, Anti-saliva IgE levels in the individuals described in panel **A**. **C**, Anti-saliva IgG1 levels in the individuals described in panel **A**. **D**, Anti-saliva IgG4 levels in the individuals described in panel **A**. The nonparametric paired Wilcoxon test was used to compare levels of anti-*Lu. longipalpis* saliva antibodies at time 0 and after 6 months. $P < .05$ was considered to be significant. OD, optical density.

Methods

Study population. Serum samples used in the present study were obtained from an epidemiologic survey of VL in children <7 years old living in a region of São Luiz, Maranhão State, in northeastern Brazil, where VL is endemic. During this prospective study, anti-*L. chagasi* DTH response and serologic tests were performed twice a year during 1997 and 1998. Only children who did not have VL, a positive serologic test result, or a DTH response at the first survey were included in the present study. None of the individuals in the data set had disease, and all had negative responses to leishmanial antigen during the preceding 6-month period. Positivity in the anti-leishmanial tests reported here indicates a recent conversion, as determined by a sensitive and specific ELISA [6] and/or DTH test [6]. To determine the cutoff value for IgG anti-*Lu. longipalpis* in ELISAs, serum samples were obtained from children in the same age range from an area where *Lu. longipalpis* is not endemic. Assuming that recent seroconversion represents infection and that a positive DTH response is a marker of protection against leishmaniasis in subclinical cases, we classified children in 2 groups, according to their anti-*L. chagasi* responses. Children in group 1 experienced serologic status conversion, from negative to positive ($n = 15$), and children in group 2 experienced DTH response conversion, from negative to positive ($n = 15$).

Anti-sand fly saliva serologic testing. Anti-sand fly saliva serologic test ELISA was performed as described elsewhere [6]. Serum IgG subclasses were determined using anti-human IgG1, IgG3, or

IgG4 alkaline-phosphatase conjugates (Sigma-Aldrich). To determine IgE levels, serum samples were previously absorbed using rheumatoid factor. Anti-human IgE (Sigma-Aldrich) was used in the ELISA.

Western blots. Western blots of salivary gland antigens were performed as described elsewhere [6].

Statistical analysis. The nonparametric paired Wilcoxon test was used to compare levels of anti-*Lu. longipalpis* saliva antibodies in the same children at time 0 (beginning of survey) and after 6 months. $P < .05$ was established as the significance level. GraphPad Prism Software was used to perform the statistical tests.

Results

Antibodies against *Lu. longipalpis* saliva. Individuals ($n = 15$) who experienced conversion to a positive anti-*L. chagasi* DTH response had significantly increased anti-*Lu. longipalpis* IgG (figure 1A; $P = .02$) and IgE antibody levels (figure 1B; $P = .002$). IgG1 was the principal antibody subclass involved in the increase of anti-saliva antibodies in the group who experienced anti-*L. chagasi* DTH response conversion, from negative to positive ($n = 15$) (figure 1C); no significant changes were observed in other IgG subclasses. The cutoff value for anti-*Lu. longipalpis* IgG level in ELISA was 0.045, as measured by optical density. A significant decrease in anti-saliva IgG antibody levels

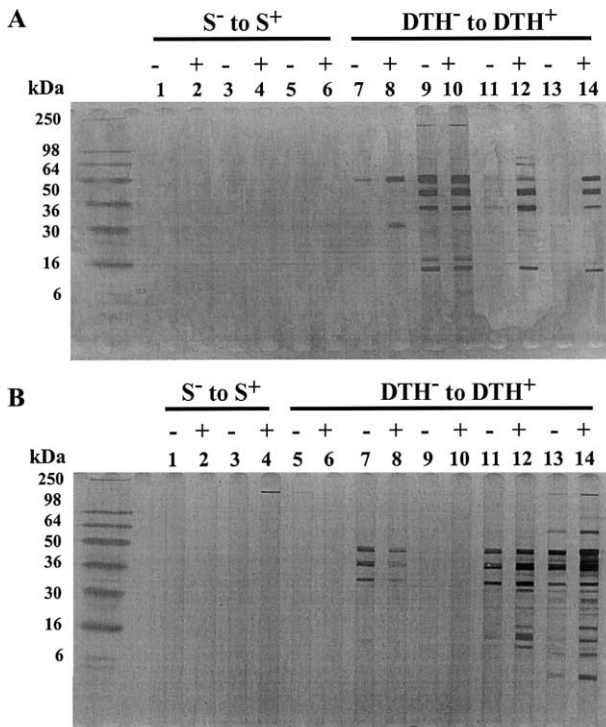


Figure 2. *Lutzomyia longipalpis* salivary proteins recognized by human serum samples of individuals whose anti-*Leishmania chagasi* serologic status converted from negative (S^-) to (S^+) or whose anti-*L. chagasi* delayed-type hypersensitivity (DTH) response converted from negative (DTH^-) to positive (DTH^+). *A* and *B*, Western blot of *Lu. longipalpis* salivary proteins reacted to human serum samples of individuals whose anti-*L. chagasi* serologic status converted from negative (S^-) to positive (S^+) (lanes 1–6) or whose DTH response converted from negative to positive (lanes 7–14). –, Time 0; +, time 6 months.

($P = .035$) was observed in serum samples from children who experienced anti-*L. chagasi* serologic status conversion from negative to positive (group 1; figure 1*A*). No significant changes were observed in anti-saliva IgE in group 1 (figure 1*B*). Although IgG anti-saliva levels in group 1 children decreased in the 6-month period, a significant increase in IgG4 anti-saliva was observed in this group ($P = .0245$; figure 1*D*).

Components of saliva recognized by IgG. We evaluated by Western blot the number and pattern of *Lu. longipalpis* salivary proteins recognized by the serum samples of individuals who experienced anti-*L. chagasi* serologic status or anti-*L. chagasi* DTH response conversion from negative to positive. Of 7 randomly selected serum samples from individuals who experienced anti-*L. chagasi* serologic status conversion, samples from 2 individuals poorly recognized 2 different salivary proteins of 33 and 200 kDa, respectively (data not shown; figure 2*B*, lane 4). The remaining serum samples did not recognize any salivary protein at any time point.

Conversely, of 13 randomly selected serum samples of individuals who experienced anti-*L. chagasi* DTH response con-

version, 12 recognized a variety of salivary proteins with various intensities. Figure 2*A* and 2*B* show the diversity of salivary antigens recognized by these serum samples (lanes 7–14). In addition, serum samples from 6 individuals who experienced anti-*L. chagasi* DTH response conversion from negative to positive showed an increase in the number and/or intensity of salivary proteins recognition, when time points 0 (negative) and 6 months (positive) were compared (figure 2*A*, lanes 7 [negative] and 8 [positive], 11 [negative] and 12 [positive], and 13 [negative] and 14 [positive]; figure 2*B*, lanes 11 [negative] and 12 [positive], 13 [negative] and 14 [positive]; data not shown). Some individuals in this group did not show any change from time 0 to 6 months (figure 2*A*, lanes 9 [negative] and 10 [positive]; figure 2*B*, lanes 7 [negative] and 8 [positive]) or did not recognize any salivary protein (figure 2*B*, lanes 9 [negative] and 10 [positive]).

The serum samples of the individuals who experienced anti-*L. chagasi* DTH response conversion recognized a total of 16 different salivary proteins; however, the frequency of recognition varied among these individuals (figure 3). A salivary protein of 45 kDa was recognized by 12 serum samples, followed by proteins of 44, 43, and 35 kDa recognized by 8 serum samples each, a protein of 17 kDa recognized by 6 serum samples, and a protein of 16 kDa recognized by 5 serum samples. Other salivary proteins were recognized as well but with less frequency (≤ 3 serum samples).

Discussion

The present report indicates that children who experience anti-*L. chagasi* DTH response conversion also have an increase in anti-sand fly saliva antibodies, as evidenced by ELISA and Western blot results. We have previously shown a correlation between anti-saliva antibody titers and anti-*L. chagasi* DTH response [6], and the present results expand those findings, showing that the development of anti-parasite DTH coincides temporally with the development of anti-*Lu. longipalpis* saliva antibodies.

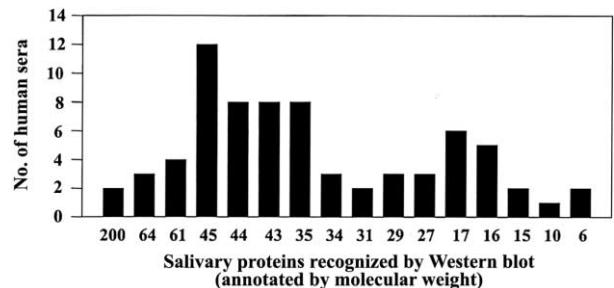


Figure 3. Frequency of salivary proteins recognized by serum samples of 13 individuals whose anti-*Leishmania chagasi* delayed-type hypersensitivity (DTH) response converted from negative to positive. X-axis, Different *Lutzomyia longipalpis* salivary proteins (labeled by the approximate molecular weight) recognized by Western blot analysis. Y-axis, No. of human serum samples recognizing a particular salivary protein.

It is tempting to speculate that neutralization of sand fly salivary component(s) by antibodies or cellular response to salivary proteins allows for a more efficient mounting of an anti-*L. chagasi* cell-mediated immune response, probably developing a Th1 response against the parasite. Sand fly saliva components, such as maxadilan, are able to impair macrophage function [7], which interferes with *L. chagasi* survival and antigen presentation [8]. Recently, it was reported that this molecule exacerbated infection with *L. chagasi* to the same degree as whole saliva and that vaccinating against maxadilan protected mice against infection with *L. chagasi* [9]. The higher antibody levels observed in individuals who experienced anti-*L. chagasi* DTH response conversion suggest that mounting an anti-saliva components immune response is linked to developing cell-mediated immunity against *L. chagasi*. In our study, we did not evaluate anti-saliva cell-mediated immunity in the exposed children. Serum samples used in this study were made available from a study conducted during 1997–1998, and it was impossible to perform anti-saliva DTH tests. Even in a prospective study, performing anti-saliva DTH tests in a population is a challenging task, because of limitations of SGS availability and problems of injecting material from sand flies in children.

The Western blot analysis results reported here show that serum samples from individuals who experienced anti-*L. chagasi* serologic status conversion did not recognize any salivary protein, whereas serum samples from individuals who experienced anti-*L. chagasi* DTH response conversion recognized a number of different salivary proteins. The frequency of salivary antigens recognized by these serum samples reveals a cluster of only few proteins, including antigens with approximate molecular masses of 45, 44, 43, 35, 27, and 16 kDa, respectively (figure 3). Among these antigens, we suggest the recognition of at least 2 salivary proteins (45 and 35 kDa), which may be similar to the Yellow related protein and to the salivary apyrase from the saliva of *Lu. longipalpis*, respectively [10]. These salivary proteins (45 and 35 kDa) represent 2 of the highest frequencies of recognition by human serum samples and could be candidates to either study the exposure to sand fly bites or as vaccine candidates to control infection with *L. chagasi*. Although the molecular weight of these salivary antigens are related to the previously described *Lu. longipalpis* proteins [10], identification and characterization of all these antigens need further study. Surprisingly, only 2 serum samples recognized a protein at the range of 6 kDa, the molecular weight of maxadilan [1], suggesting that, in humans, maxadilan may not induce a strong antibody response but could be a strong inducer of cellular immunity.

Individuals who experienced anti-*L. chagasi* cell-mediated immunity conversion had increased IgG1 and IgE levels. IgG1 has been related to a human Th1 response [11], although this relationship has not been fully established. The elevation of IgE antibodies suggests the development of an immediate hypersensitivity, since IgE is considered to be a marker of a Th2 type responses [11]. It is likely that a mixed Th2 type (related to

immediate hypersensitivity) and Th1 type response (related to DTH) against saliva components coexist in individuals who recently experienced anti-*L. chagasi* DTH response conversion. In fact, this type of mixed response was reported in individuals exposed to insect bites, where the host immune response against insect saliva starts with DTH response, evolves to a predominant immediate type hypersensitivity, and finally desensitization [12]. In addition, we have observed in mice that immunization with *Lu. longipalpis* salivary genes resulted in a typical DTH and/or antibody response to *Lu. longipalpis* salivary proteins (J.G.V., unpublished results), suggesting that bites of *Lu. longipalpis* could induce Th1 and Th2 responses in humans, a phenomenon described for the bites of the sand flies from the Eastern Hemisphere [4]. Of interest, SP15, which is responsible for the DTH response in mice, is highly homologous to the SL1 protein present in the saliva of *Lu. longipalpis* [10]. Our results suggest that a mixed anti-saliva response with both Th1 and Th2 components may help in establishing an anti-immune *L. chagasi* response.

An indication of a possible down-modulation of immediate hypersensitivity came from elevation of IgG4 antibodies in individuals who experienced anti-*L. chagasi* serologic status conversion. IgG4 has been implicated as a blocking antibody in the control of allergic reactivity in human filariasis [13]. Developing allergy against insect saliva is frequently observed in populations exposed to insects [14]. The presence of an immediate hypersensitivity at the site of phlebotomine bite may create an inhospitable microenvironment, noxious to the blood feeder [15], or even impairing parasite survival. The lack of studies evaluating anti-saliva immune responses in vector-transmitted protozoan diseases may have precluded the description of immediate hypersensitivity as a protective mechanism in such conditions.

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