LOW SERUM LEVELS OF DEHYDROEPIANDROSTERONE AND CORTISOL IN HUMAN DIFFUSE CUTANEOUS LEISHMANIASIS BY LEISHMANIA MEXICANA

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Abstract. Low levels of dehydroepiandrosterone (DHEA) and cortisol hormones produced by the suprarenal cortex have been associated with diseases involving chronic inflammation, low interferon (IFN)-γ, and high interleukin (IL)-6. Diffuse cutaneous leishmaniasis (DL), a long-lasting intracellular parasitic infectious disease, can spread unknown levels of DHEA and cortisol. Serum concentrations of both were measured in 5 patients with DL, in 15 patients with localized lesions produced by Leishmania (LL), and in 20 healthy volunteers. Leishmania mexicana mexicana was identified as the causal agent in patients with DL and LL. Hormone levels were lower in DL compared with controls and LL. Furthermore, we detected a lower percentage of IFN-γ-positive cells with higher levels of IL-6 and higher titers of anti-Leishmania antibodies in patients with DL, whereas patients with LL were similar to controls. These data suggest that patients with DL may be good candidates for DHEA and cortisol supplementation.

INTRODUCTION

Cutaneous leishmaniasis is an infectious disease caused by several species of the protozoan parasite Leishmania, including Leishmania braziliensis, L. tropica, L. major, L. aethiopica, and L. mexicana.1,2 Skin lesions are painless but can persist as long-lasting single ulcers or nodes during localized cutaneous leishmaniasis (LL). Lesions result from Leishmania parasites conveyed by the bite of infected sand flies and usually heal spontaneously or after specific treatment. However, under certain circumstances, there are two other forms of cutaneous leishmaniasis that do not spontaneously heal, even after specific treatment. During diffuse cutaneous leishmaniasis (DL), the nodes extend to exposed areas of the skin, such as the arms or face, endowing the patient with a granulomatous appearance. Mucocutaneous leishmaniasis (MCL) commonly devastates the oromucosal tissue to complete loss of cartilage and mucous membrane, leading to patient disfiguration. In these two forms, elimination of the parasite after treatment may be incomplete, which may be an explanation for lesion relapse.3 DL and MCL have been associated with a deficient immune response that is ineffective at clearing the infection; however, a specific immunologic defect has not yet been identified.4

Extensive work with L. major in murine models has revealed that Th1 cells and the Th1-associated cytokines, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, mediate the healing of lesions, whereas Th2 cells and the Th2-associated cytokine, interleukin (IL)-4, enhance the progression of leishmaniasis.5 However, the way cytokine patterns are modulated toward a Th1 or Th2 response is still a matter of discussion. These patterns are being studied in the context of antigen presentation, concentrations and kind of cytokines produced during immune cell priming, and the modulation initiated by the endocrine system.

Dehydroepiandrosterone (DHEA) plays a vital role in regulating the immune responses in some autoimmune and infectious diseases. Low levels of DHEA modify the Th1/Th2 balance, biasing the immune response toward a Th2 response, influencing the outcome of the disease. For instance, DHEA serum levels decrease during progression of HIV infection or tuberculosis, shifting Th1-associated cytokine production toward a Th2 pattern. Low DHEA serum levels are common in patients with rheumatoid arthritis (RA), who exhibit high production of antibodies, which could decrease in response to DHEA administration.10 DHEA levels are low in the elderly, and these levels are inversely associated with high IL-6 plasma levels. Furthermore, DHEA exerts a direct suppression of the human IL-6 gene promoter and inhibits in vitro IL-6 production by human peripheral blood mononuclear cells.14 DHEA also stimulates monocyte-mediated cytotoxicity, natural killer cytotoxicity, and IL-2 secretion on both human and murine T cells.16

In many aspects, DHEA has an opposite effect than cortisol. In fact, DHEA has been used to counteract the glucocorticoid side effects incurred during prolonged treatment of autoimmune diseases, such as systemic lupus erythematosus (SLE). Patients experience a diminution of symptoms when they receive DHEA supplementation.17 Glucocorticoids, including cortisol, interfere with the gene expression of some proinflammatory cytokines, through enhancement of inhibitor of nuclear factor (NF)-κB (I-κB) transcription, which prevents signaling through NF-κB.18,19 Glucocorticoids also bias the immune response of T lymphocytes toward a Th2 pattern by inhibiting the expression of signal transducer and activator of transcription (STAT)1 mRNA and enhancing the expression of Th2 cytokines through STAT3.21

It is reasonable to assume that deficient production of DHEA in patients with DL may cause them to develop extended disease. In this study, serum DHEA and cortisol were measured in patients with DL and compared with both patients with LL and healthy controls. Production of IL-6, IFN-γ, specific anti-Leishmania antibodies, and Leishmania species of the clinical isolates were also determined to identify their relationship to DHEA and cortisol serum levels under these clinical conditions.

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MATERIALS AND METHODS

Patients. At the time of this study, there were only five live patients with DL in Tabasco, a state in the southeast of Mexico. We studied all 5 patients with DL and 15 patients with LL during the course of a project approved by the Research and Ethics Committee of the participating institutions. After informed consent was obtained from patients, their clinical records were complemented by appropriate questions, physical examination, and laboratory tests. All 20 patients agreed to give 20 mL of venous blood. Blood samples were obtained at 8:00 AM in the closest health facility to their homes, where they had been diagnosed and received treatment provided by their family physicians. The diagnosis was verified by the presence of Leishmania amastigotes in touch preparation. Patients were treated with eight doses of glucantime, both intramuscularly and intralesion, every third day during the first 15 days. Blood samples were collected from patients with LL after confirmation of diagnosis, as well as before onset of treatment. All five patients with DL underwent multiple previous treatments every 4 months since DL diagnosis had been established; two of five patients with DL had no active lesions when blood samples were taken.

Controls. Twenty healthy individuals, living in the same neighborhoods as the patients, were invited to participate as controls. After obtaining informed approval, 20 mL of venous blood was drawn on the same day and time as patients, and samples were processed in parallel.

DHEA and cortisol determinations. DHEA was measured in serum by using a solid-phase 125I radioimmunoassay (Diagnostic Products, Los Angeles, CA). Cortisol was also measured in serum by using a competitive chemoluminescent immunoassay kit (Diagnostic Products). Both determinations of DHEA and cortisol were blinded with respect to their source (patient or control) and tested in triplicate in two separate assays, performed on different days. To avoid influence of sex as a confounding variable, statistical analysis was performed separately. The average of individual determinations was used for statistical analysis.

Analysis of cytokines produced by T cells. White blood cells (WBCs) from patients or controls were incubated over 4 hours at 37°C with or without phorbol myristate acetate (PMA) at 25 ng/mL, final concentration; and Ionomycin (Ion) at 1 μg/mL, final concentration. In addition, Brefeldin A (Sigma, St. Louis, MO) was added at 10 μg/mL, final concentration. All samples were incubated for 15 minutes at room temperature with the following antibodies: anti-CD4-FITC or anti-CD8-FITC and anti-CD3-PC5 (Immunotech Research, Lakewood, NJ). Subsequently, 250 μL of diluted fluorescence-activated cell sorter (FACS) lysis solution (Becton-Dickinson, Franklin Lakes, NJ) was added, and cells were incubated for 10 minutes at room temperature. Cell suspension was washed by centrifugation at 900 g for 5 minutes at room temperature, and the pellet was resuspended in 500 μL of 1:10 diluted FACS permeabilizing solution (Becton-Dickinson). All samples were incubated for 10 minutes at room temperature, washed, and resuspended for staining with the following antibodies: anti-hemocyanin IgG2-FITC/anti-hemocyanin IgG1-PE (Pharmergen, San Diego, CA), anti-IFN-γ-PE, or anti-IL-4-PE (Pharmergen). The cell suspension was incubated 30 minutes at room temperature in the dark, washed, and analyzed in an EPICS-ALTRA flow cytometer (Beckman-Coulter, Fullerton, CA). Data were analyzed using the EXPO software (Beckman-Coulter). PMT-5 (PE-CY5) versus PMT-1 (granularity) was used to select the population of CD3-positive cells, and 10,000 events were acquired. The difference between stimulated and unstimulated values was used for statistical analysis.

Serum IL-6. A capture ELISA (Immunotech, Marseille, France) was used to measure IL-6 concentration in serum samples from patients and controls; samples were tested in triplicate, using the means for statistical analysis. Positive and negative controls were assayed at the same time.

Specific anti-Leishmania serum antibodies. Leishmania mexicana mexicana M379 reference strain parasites were used to determine specific anti-Leishmania serum antibodies titers by dot blot. Parasites growing in Schneider insect medium were lysed by five freeze-thawing cycles in phosphate-buffered saline (PBS; pH 7.2) plus a protease inhibitor cocktail for eukaryotic cells (Sigma); debris was eliminated, and protein concentration was adjusted to 1 mg/mL. Two microfilters of these preparations was placed on nitrocellulose (NC) membranes, blocked with 2% skim milk, reacted with 1:1,000, 1:2,000, 1:5,000, and 1:10,000 dilutions of serum from patients or controls, incubated overnight at 4°C, reacted with anti-human alkaline phosphatase label secondary antibody (Bio-Rad Laboratories, Hercules, CA), and developed with 5-Bromo-4chloro-3-indolyl phosphate/NitroBlue Tetrazolium (BCIP/NBT) substrate (Sigma).

Leishmania isolates. Parasites were isolated in Seneckjie media by tissue culture from nodules or ulcers of patients with LL or DL; samples were obtained by needle biopsies, taken before the onset of treatment. When parasites reached 10⁶/mL in culture, samples were prepared on glass slides for indirect immunofluorescent staining (IFF) and incubated with RPMI medium supplemented with 10% fetal calf serum (FCS) and 1:100 penicillin-streptomycin solution (Sigma) for axenic culture.

Identification of Leishmania species. Strains isolated from patients were typed by IIF, using the monoclonal antibodies (mAb) M2, M7, M9, M11, B2, B4, B5, B16, B19, T1, and T3, which were produced and kindly provided by Diane McMahon-Pratt,22–25 M7 reacts with L. mexicana strains, M2 identifies L. mexicana amazonensis, B4 reacts with L. braziliensis braziliensis, B19 reacts with L. braziliensis guyanensis; and T1 and T3 react with L. major and L. tropica, and weakly with members of the L. mexicana complex (D. McMahon-Pratt, personal communication, 2003). M9 and M11 react with L. mexicana complex, B2 reacts with the L. braziliensis complex, and B5 reacts with L. braziliensis panamensis and L. braziliensis braziliensis. The reference strains used were MNYC/BZ/62/M379 for L. mexicana, MHOM/VE/74/PMH3 for L. mexicana venezuelensis, MHOM/BR/75/M2903 for L. braziliensis, MHOM/PA/71/LS94 for L. panamensis, MHOM/BZ/82/ BEL21 for L. mexicana, IFLA/BR/67/P8 for L. amazonensis, and HOM/BR/74/PP75 for L. donovani chagasi. Briefly, 10 μL of 10⁶ parasites/mL were added per well and air-dried. Slides were fixed with −20°C methanol for 15 minutes and stored at −70°C until tested. Slides were rehydrated in PBS (pH 7.2) and reacted with the WHO anti-Leishmania reference mAb diluted 1:1,000 for 1 hour at 37°C in a humid atmosphere. Slides were washed three times and reacted with a second anti-human FITC-labeled antibody (Sigma) for 1
hour. Results were assessed by observed on an epifluorescence microscope.

**Statistical analysis.** DHEA and cortisol data were analyzed by one-way ANOVA, Tukey multiple comparison test. Pearson $r$ was applied for DHEA, cortisol, IFN-$\gamma$ or IL-6 correlations. Cox and Steward trend tests were used to compare DHEA values per age ranges. Comparisons between two groups were assessed using Student $t$ test.

**RESULTS**

**Patients.** The main characteristics of the patients studied are shown in Table 1. Most patients with LL were young men with an age range of 20—50 years old; only three were younger than 20 years of age and two were older than 50 years of age. In the DL group, one patient younger than 20 years of age (19-year-old woman), exhibiting persistent lesions since the age of 6, was unresponsive to multiple glucantime treatment. One man, 39 years old, having inactive DL lesions for the previous 3 years, was diagnosed when he was 27 years old. The other three DL cases were men older than 50 years of age, with active lesions for 4, 12, and 15 years, respectively. While presenting active DL, the five patients received cyclic glucantime treatment. The clinical evolution for patients with DL was chronic (4–15 years; mean, 11 ± 4.18 years), whereas LL was shorter (1–24 months; mean, 7 ± 5.8 months). The patients with LL that were younger than 20 or older than 50 years of age had lesions with longer evolution (12 ± 7.35 months), whereas patients between 20 and 50 years of age had lesions with shorter progression (4.25 ± 2.25 months). Controls were 12–78 years old (mean, 35.82 ± 17.23 years).

**DHEA.** Serum concentrations of DHEA were lower in patients with DL (1.68 ± 0.45 ng/mL; range, 0.7–2.66 ng/mL) compared with patients with LL (3.32 ± 0.58 ng/mL; range, 0.31–6.01 ng/mL) or controls (5.28 ± 0.79 ng/mL; range, 1.13–12.96 ng/mL; Figure 1A; ANOVA, $P = 0.038$). Because DHEA reaches peak values in adults and declines as age advances, we considered it necessary to ascertain if DHEA was differentially distributed according to age in the studied patients. In fact, DHEA was lower in patients with LL compared with age-matched controls ($P = 0.031$, Cox and Steward trend test). DL cases were not included in this analysis because there were no cases for each age interval (Figure 1B). Two patients with LL older than 50 years of age had lesions lasting at least 10 and 24 months, respectively, when they were studied. The youngest patient with LL, an 11-year-old boy, had serum DHEA $< 1$ ng/mL, with 12-month lesions and no spontaneous resolution. A female patient with DL younger than 20 years old showed low DHEA (2.71 ng/mL) compared with her matched control (4.21 ng/mL). Two other women, 34 and 39 years old, from the LL group also had DHEA values below their matched controls.

**Cortisol.** Similar to DHEA, serum cortisol levels were lower in patients with DL compared with controls: 76.0 ± 24.7 versus 140.9 ± 10.65 ng/mL (ANOVA, $P = 0.013$), whereas patients with LL had intermediate values (101.5 ± 11.82 ng/mL; Figure 2A). There was a positive correlation between DHEA and cortisol serum concentrations (Figure 2B; Pearson $r = 0.441$; $P = 0.013$).

**Cytokine-producing cells.** Low levels of IFN-$\gamma$ have been found consistently when DHEA levels are low. IFN-$\gamma$ was evaluated as the difference between the percentage of CD4$^+$ or CD8$^+$ IFN-$\gamma$-positive cells under stimulation and unstimulated cells. The percentage of CD4$^+$ IFN-$\gamma^-$ cells tended to be lower in patient with DL compared with controls or patients with LL (Table 2; ANOVA, $P = 0.42$). Although differences were not statistically significant, a positive correlation was

<table>
<thead>
<tr>
<th>Table 1</th>
<th>General characteristics of the studied patients with leishmaniasis</th>
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<tr>
<td>Clinical form</td>
<td>LL</td>
</tr>
<tr>
<td>Age (years)</td>
<td>33.87 (11–79) *</td>
</tr>
<tr>
<td>Sex</td>
<td>13 Male, 2 Female</td>
</tr>
<tr>
<td>Duration of disease</td>
<td>7 months (1–24)</td>
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</table>

* Mean (range).
† Student $t$-test.
‡ Fisher exact $x^2$ test.

Figure 1. Serum DHEA in patients with leishmaniasis. Samples were tested in triplicate by EIA using a standard curve and positive and negative internal controls (A). Histograms show the mean ± SE from 17 controls, 11 LL, and 4 DL. Analysis was performed by ANOVA Tukey test. *DHEA was significantly lower in DL compared with controls, $P = 0.038$. Even if values were age distributed (B), DHEA remained lower in LL and DL groups compared with controls.
found between serum DHEA and IFN-γ–positive cells in patients with DL and LL (Figure 3A; Pearson $r = 0.687; P = 0.007$). Interestingly, these differences were not observed in controls. There were no differences in the percentage of CD8$^+$ IFN-γ$^+$ cells between groups. Differences between stimulated and unstimulated IL-4–positive cells were close to zero, both in patients and controls. There were no differences in the CD4/CD8 ratio between groups (data not shown).

**IL-6.** Serum IL-6 tended to be higher in patients with DL compared with controls or patients with LL (Table 2; ANOVA, $P = 0.12$). Statistical analysis between the three groups, performed by ANOVA, was not significant, but if only patients with DL were compared with controls by $t$ test with $\alpha = 0.05$, significant differences were found ($P = 0.019$).

IL-6 correlated inversely to DHEA in both patients with LL and DL (Figure 3B; Pearson $r = -0.561; P = 0.1481$). No correlation was seen in the control group (data not shown).

**Anti-Leishmania antibodies.** Specific anti-Leishmania antibodies could not be found in young patients with LL, even when tested against the Leishmania strain isolated from the same patient. However, high serum titers of specific anti-Leishmania antibodies (up to 1:10,000) were found in all patients with DL when assayed by dot blot. Only one elderly patient with LL, 78 years old, showed a 1:5,000 antibody titer.

**Isotyping of isolates.** The Leishmania strains that were isolated from patients with DL or LL were identified as *L. mexicana* using the reference mAb M7. All the strains were also reactive to the T3 mAb by IIF but not to the T1 mAb. Low reactivity to mAb B5 was observed for some strains, mostly when incubated overnight at 4°C. Serum from patients with DL did not react to *L. braziliensis* reference strains tested, as they did with *L. mexicana* reference strain M379 or Bel21.

**TABLE 2**

<table>
<thead>
<tr>
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<th>Controls</th>
<th>LL</th>
<th>DL</th>
<th>ANOVA $P$</th>
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<tr>
<td>IFN-γ (%CD4$^+$ cells)</td>
<td>18.67 ± 2.7*</td>
<td>18.52 ± 2.85</td>
<td>12.7 ± 3.5</td>
<td>0.42</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>90 ± 57</td>
<td>129 ± 145</td>
<td>259 ± 155†</td>
<td>0.12</td>
</tr>
</tbody>
</table>

$^*$Mean ± SEM.

† $P = 0.019$ for DL against controls (Student $t$ test).

**DISCUSSION**

DL is a rare disease, with only 300–500 cases reported by the WHO throughout the world. DL is regarded as an extreme of natural evolution of cutaneous leishmaniasis, which grants us an opportunity to study the state of variables in such an advanced state of the disease, where it is extremely difficult to eliminate the parasite. A description of low levels DHEA and cortisol, as presented in this paper, may contribute to our understanding, modify the outcome of the disease, and perhaps, offer new possibilities for novel therapies for its management.

In this study, both DHEA and cortisol were low in patients with DL, who are considered to have chronic inflammation. To understand the results, we may first hypothesize that a common stimulator of these two hormones is altered, as proopiomelanocortin (POMC) or one of its products, such as the adrenocorticotrophic hormone. However, there is no explanation for why the number of patients with DL is higher for men than women. A second explanation is given, if independent mechanisms for DHEA or cortisol diminution are considered. For instance, modifications of DHEA could be explained by hormonal changes developed by an organism during normal growth, for example, the young and elderly, considering that DHEA is naturally low at both extremes of life. Furthermore, low DHEA serum levels have been associated with immunosenescence, meaning that normal decrease of DHEA in elderly people seems to increase the risk of severity of many diseases and susceptibility to infections.

A decrease in serum levels of dehydroepiandrosterone sulfate (DHEAS) and DHEA is typical for noninfectious chronic inflammation where TNF and IL-6 have a predictive role for such changes as in autoimmune disorders, including inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, and pemphigus. These low levels are also seen in infectious diseases with chronic inflammation, such as tuberculosis, where DHEA prevents experimental infection in mice when given within the first few days or before *M. tuberculosis* inoculum.

We reported here that patients with DL were young or elderly, which was also observed in other studies, al-
though age groups have not yet been analyzed. Furthermore, LL lesions remained active longer in both young and old patients compared with patients between 20 and 50 years old.

An increase in host resistance to Plasmodium falciparum has been described at the beginning of puberty, a stage that is characterized by increased levels of DHEAS. However, because of the fact that populations in endemic areas are highly exposed to this parasite, the immunologic memory may account for the increased host resistance.

DHEA may exert direct and indirect effects over the immune system. The effect may be direct if DHEA is able to regulate the gene expression of several mediators of the immune system, for instance, inhibiting IL-6 and TNF production by monocytes.

DHEA is also able to modulate other cellular types, such as rat liver, where it diminishes the expression of transporter associated with antigen processing (TAP)-1 and β2-microglobulin and enhances gene expression of TAP-2. It can affect endothelial cells as well, which respond by increasing their in vitro proliferation and genomic and non-genomic regulation of NO synthesis, independently of androgen or estrogen receptors. The recent description of a receptor for DHEA signaling thought a G protein lends support to understanding direct effects of DHEA.

On the other hand, DHEA may also have indirect actions during its conversion to sexual hormones, as is the case of 17β-estradiol, which inhibits TNF and IL-6 production. Even if this indirect action could explain greater male susceptibility to Leishmania, additional effects on diverse cells participating in the immune response, as mentioned before, should not be ignored, considering the effect exerted by DHEA over a microorganism. For instance, DHEA seems to extend its actions directly on the protozoa, because a negative effect over the growth of Plasmodium have been recently reported.

An independent decrease of cortisol levels could be explained if we consider that free cortisol has been reported to be low in critically ill patients with severe infections, such as septic shock, trauma, burns, or surgery. Low cortisol in our patients may be caused by the chronic inflammation state associated with DL.

Cytokine production during infection by Leishmania has been extensively studied in murine animal models but is poorly studied in human patients. Despite the few papers on cytokine production made in humans with DL, they have confirmed low IFN-γ and high IL-4 production in DL in contrast to usually normal production of these cytokines in LL patients or during in vitro stimulation of mononuclear cells in Leishmania-naïve humans. Low IFN-γ production in patients with DL has been determined by ELISA on supernatants of stimulated cells by mRNA expression, and by percentage of IFN-γ-producing CD4+ cells. Our data show that high levels of IL-6 are associated with DL, which is consistent with previous findings on elderly people and confirms a direct correlation of low DHEA with low numbers of IFN-γ-producing T cells. Elderly or very young patients with naturally low levels of DHEA who become infected with Leishmania could be at major risk of developing DL. This potential risk may be particularly important in endemic areas.

Murine models have shown important differences in IFN-γ responses depending on the species used to infect the animals. However, the contribution of different Leishmania species does not seem to be a major determinant for the development of either LL or DL, because, in this study, only L. mexicana mexicana was found in patients with DL and LL. According to these data, the development of DL could be a consequence of a high initial parasite inoculum on susceptible hosts, as well as the amount and types of cytokines induced in response to infection.

In conclusion, DHEA and cortisol were found to be low in patients with DL, correlating with low IFN-γ and high IL-6 levels. Supplementation with DHEA and cortisol, in addition to specific treatment, could provide additional benefits to patients, biasing the immune response toward a Th1 response, and perhaps, completely eliminating the parasites.

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DHEA IN HUMAN DIFFUSE CUTANEOUS LEISHMANIASIS

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