

Cutaneous Biology

Acute immobilization stress induces clinical and neuroimmunological alterations in experimental murine cutaneous leishmaniasis

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Summary

Background The skin is an important component of the neuroendocrine-immune axis. Several studies have shown that stress exacerbates skin disorders, affecting the function of sebaceous glands, keratinocytes, epidermal Langerhans cells and other cells, having an impact on the pathogenesis of many immunologically associated skin diseases. In American cutaneous leishmaniasis, we have shown the importance of the epidermis as a regulatory site, with the key participation of Langerhans cells.

Objectives To analyse the effect of acute immobilization stress on Langerhans cells, substance P (SP), calcitonin gene-related peptide (CGRP) and the natural course of infection in a murine model of cutaneous leishmaniasis.

Methods BALB/c mice, susceptible to *Leishmania* infection, were placed under acute stress by immobilization (confinement) for 2 or 8 h before inoculation with *L. mexicana* (MHOM/BZ/82/BEL21). An avidin–biotin immunoperoxidase technique was used for cell and neuropeptide identification.

Results The stressed animals became more susceptible to the parasite infection, which was manifested by acceleration and exacerbation of the lesions. In addition, the stressed animals showed morphological alterations (spherical bodies and shortened dendrites) and decreased numbers of epidermal Langerhans cells, when compared with control *L. mexicana*-infected mice. Mice stressed for 8 h showed greater and antidromic immunoreactivity to CGRP and SP at the time of infection. Moreover, the single inoculation of parasites caused a decrease of CGRP innervation.

Conclusions Acute immobilization stress induces an immunosuppressive state that further favours *Leishmania* invasion in susceptible animals.

Key words: immunosuppression, Langerhans cells, leishmaniasis, neuropeptides, stress

The skin is an important component of the neuroendocrine-immune axis that is characterized by a common communication language governed by bioactive polypeptides termed neurotransmitters, hormones or cytokines. Corticotrophin-releasing hormone and locus ceruleus–noradrenaline (LC-NA) autonomic (sympathetic) neurones regulate the peripheral activities of the

hypothalamic–pituitary–adrenal (HPA) axis and the systemic/adrenomedullary sympathetic nervous system, respectively. Activation of the HPA axis and LC-NA/autonomic system results in systemic elevation of glucocorticoids and catecholamines, respectively, which act in concert to maintain homeostasis.¹ Thus, stress is mediated by the HPA axis through the release of adrenocortical hormones regulated by the adrenocorticotrophic hormone secreted from the pituitary gland under the control of the hypothalamus. Several studies have shown that stress exacerbates skin disorders,

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affecting the function of sebaceous glands, keratinocytes, Langerhans cells and other cell compartments.² Langerhans cells are mobile antigen-presenting cells that take up and process antigen in the skin, and migrate to peripheral lymph organs where they stimulate naïve T cells, thus initiating primary T-cell responses.³ The exogenous administration of glucocorticoids affects the function of Langerhans cells, explaining part of the alterations caused by stress on these cells.⁴ Stress induced by confinement or immobilization influences epidermal Langerhans cells and contact hypersensitivity responses.^{2,5} Also, the incubation of Langerhans cells with sera from stressed mice down-regulates the expression of major histocompatibility complex (MHC) class II molecules.⁵

The principal mediators of neurogenic inflammation, a term first used to describe the contribution of sensory peptides to local cutaneous inflammatory processes, are calcitonin gene-related peptide (CGRP) and substance P (SP).⁵ These neuropeptides can be released in response to proinflammatory cytokines produced by inflammatory cells at local sites.⁶ CGRP, which is usually present in close association with Langerhans cells, can inhibit their antigen-presenting capacity.⁷

We and others have shown the importance of Langerhans cells in the pathogenesis of cutaneous leishmaniasis.^{4,8–12} Also, we have shown the importance of epidermal cell signalling in determining the type of cytokine-related immune response to be generated against *Leishmania* parasites.^{10–12} In the present study, we examine the effect of immobilization stress on epidermal Langerhans cells, SP, CGRP and the natural course of infection in a murine model of cutaneous leishmaniasis.

Materials and methods

Animals and experimental design

Female BALB/c mice were obtained from Taconic (Germantown, NY, U.S.A.). The experimental animals ($n = 100$) aged 4–6 weeks were divided into the following groups: (i) mice ($n = 20$) placed under immobilization stress for 2 h before infection with *L. mexicana*; (ii) mice ($n = 20$) placed under immobilization stress for 8 h before infection with *L. mexicana*; (iii) mice ($n = 20$) only infected with *L. mexicana* (infection control); (iv) mice ($n = 20$) placed under immobilization stress for 2 h (stress control); (v) healthy mice ($n = 20$).

Stress procedure

Mice were exposed to a single homotypic stressor just before the inoculation of *Leishmania* parasites. The stressor consisted of immobilization by placement, for 2 or 8 h, in restraining cages ($11 \times 2.5 \times 2.5$ cm) made from 50 mL polypropylene centrifuge tubes. Control animals were maintained in standard cages. During the stress regimen animals had no access to food and water.

Leishmania infection

Mice were inoculated subcutaneously in the left footpad with 10^3 amastigotes of *L. mexicana* (MHOM/BZ/82/BEL21). Briefly, amastigotes were extracted from footpad nodules of hamsters infected a month earlier with 10^6 amastigotes. The nodules were aseptically dissected out and washed in phosphate-buffered saline (PBS, pH 7.4) with antibiotics, and finely cut and ground in a Petri dish containing cold PBS. The suspension was filtered through a sterile sieve to remove large debris; the parasites were counted in a haemocytometer and adjusted to 4×10^4 mL⁻¹.

On the day of infection and every 4 weeks until the twelfth week, groups of four mice were killed by cervical dislocation and the experimental footpad removed. Two animals were used for the identification of Langerhans cells on epidermal sheets, and two for neuropeptide characterization on cryopreserved tissue.

Analysis of cutaneous lesions

The cutaneous lesion was evaluated by measuring the footpad thickness with a dial gauge caliper every week for 12 weeks. The presence of parasites was confirmed by Giemsa staining of smears from longitudinal sections of infected footpad tissues.

The percentage increase of lesions in the experimental groups was calculated by subtracting the starting footpad thickness of healthy control mice (1.74 mm) from each experimental footpad measurement.

Epidermis separation

Footpad skin was taken and cut into 1 mm² pieces; about four pieces were obtained from each footpad. The skin pieces were immersed in PBS containing 20 mmol L⁻¹ tetrasodium ethylenediamine tetraacetic acid at pH 7.4 for 150 min at 37 °C. After washing in PBS, the epidermis was separated from the dermis with

the aid of wooden toothpicks under a dissecting microscope. Epidermal sheets were placed for 5 min in PBS at room temperature until immunoperoxidase staining.

Monoclonal antibodies

A rat monoclonal antibody used to identify epidermal Langerhans cells (AMS-32.1, Ia^d, MHC class II at 1 : 300) was purchased from Pharmingen (San Diego, CA, U.S.A.); murine monoclonal antibodies to recognize CGRP (PEPA27 at 1 : 1000) and SP (PEPA40 at 1 : 1000) were purchased from Serotec Inc. (Oxford, U.K.).

Immunohistology

Characterization of Langerhans cells and neuropeptides was performed at weeks 0 (before *Leishmania* infection), 4 and 8 after infection. Immunoperoxidase staining was carried out as previously described,⁹ with modifications. Briefly, after fixation in fresh acetone for 5 min, the epidermal pieces were transferred to round-bottomed microplates. Both cryostat sections for the detection of neuropeptides and the epidermal sheets for identification of Langerhans cells were hydrated in PBS and sequentially incubated for 90 min with primary rat or murine monoclonal antibodies, biotinylated goat antirat IgG or horse antimouse IgG (50 µg mL⁻¹) (Vector Laboratories, Burlingame, CA, U.S.A.) for 45 min, and Vectastain[®] Elite ABC kit (Vector Laboratories) at 1 : 100 for 30 min. Five-minute washes with PBS were done between incubations. The reactions were developed for 10 min with 90 µmol L⁻¹ H₂O₂ and 3-amino-9-ethylcarbazole (final concentration 0.88 mmol L⁻¹), which was dissolved in 50 mmol L⁻¹ N,N-dimethylformamide in 0.1 mol L⁻¹ acetate buffer, pH 5.2. The epidermal sheets were then washed and mounted on glass slides with glycerin-gelatin. Controls consisted of omission of the primary antibody or the use of an antibody of irrelevant specificity at the same protein concentration.

Cell quantification

Cells were counted using a light microscope (Leica, Wetzlar, Germany) connected to a colour video monitor. Only cells with a visible nucleus and showing red immunostaining were counted as positive. All fields were counted in each epidermal sheet at a magnification of ×400. For neuropeptide detection on frozen

tissue, four nonserial alternate sections were immunostained for each marker. All the fields of interest were counted in each section at a magnification of ×1000, giving 2–4 × 10⁴ cells per section.

Statistical analysis

Langerhans cell density results are presented as mean ± SEM. Comparisons between groups were made with the nonparametric Mann–Whitney test. *P* = 0.05 was considered significant. The Kruskal–Wallis nonparametric test was used for the analysis of variance (ANOVA) to compare variability within groups. A repeated measures ANOVA and a Pearson correlation test were used for the percentage increase in size of the lesions. All tests were performed using GraphPad InStat 3.02 (GraphPad Software, San Diego, CA, U.S.A.; <http://www.graphpad.com>).

Results

Cutaneous lesions in the experimental groups

Both *L. mexicana*-infected BALB/c mice (infection control) and mice stressed for 2 h and infected showed a progressive and statistically significant increase in footpad thickness from the third week after inoculation to the twelfth week (*P* ≤ 0.05). In contrast, mice stressed for 8 h and infected showed a significant increase from the very first week (*P* ≤ 0.05) to the twelfth week (*P* ≤ 0.05) as compared with stressed and noninfected mice. From the fifth week onwards, although not statistically significant, the differences in footpad thickness between infected mice and those stressed for 2 or 8 h and infected were greater than those between the stress control and healthy control groups until the end of the study (12 weeks) (Fig. 1a, Table 1).

Most mice stressed for 8 h and infected presented a premature ulcer as early as the eighth week of infection, while the rest of the groups manifested it during the tenth week.

The effect of the stress on lesion size was clearly appreciated when comparing the percentage increase from the starting size during the 12 weeks of evaluation (Fig. 1b). The statistical analysis using ANOVA of these percentage increase values showed significant differences for mice stressed for 2 h (*P* ≤ 0.05) or 8 h (*P* ≤ 0.001) and infected as compared with the infection control group. Moreover, very significant Pearson correlations were observed between the infection

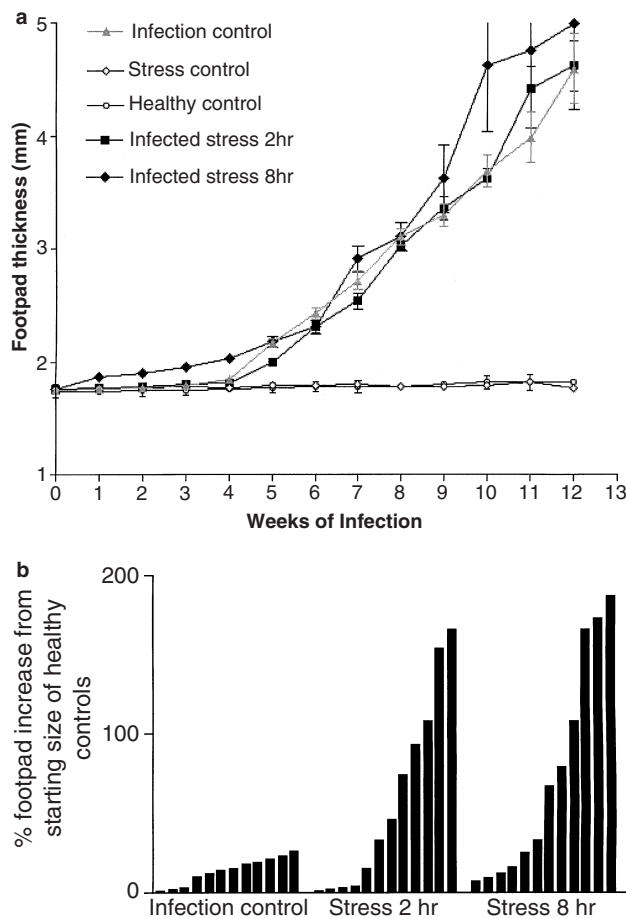


Figure 1. (a) Progression of *Leishmania mexicana* infection in experimental groups of BALB/c mice. Results are presented as mean \pm SEM. (b) Percentage increase from the starting footpad thickness during the 12 weeks of evaluation in mice from the infection control group and mice stressed for 2 h and 8 h prior to infection.

control group and mice stressed for 2 h and infected ($r = 0.906$, $P = 0.001$) and mice stressed for 8 h and infected ($r = 0.899$, $P = 0.001$).

Density of Ia^d+ Langerhans cells

Immediately after stress and inoculation of parasites, mice stressed for 2 or 8 h and infected with *L. mexicana* showed decreased numbers of Langerhans cells (1456 ± 30 and 1062 ± 49 cells mm^{-2} , respectively), as compared with nonstressed and infected mice (1575 ± 23 cells mm^{-2}) and healthy control mice (1501 ± 22 cells mm^{-2}). This decrease was greater in the animals stressed for 8 h than in those stressed for 2 h ($P \leq 0.05$) (Figs 2 and 3). Healthy control animals showed significant differences from mice stressed for 8 h but not from mice stressed for 2 h prior to infection. The group of mice stressed for 2 h and noninfected also showed a lower density of Langerhans cells (1000 ± 44 cells mm^{-2}) than nonstressed and infected mice and healthy control mice. At week 4 of infection, the density of Langerhans cells in mice stressed for 2 or 8 h and infected was lower (1018 ± 44 and 819 ± 58 cells mm^{-2} , respectively) than nonstressed and infected mice (1975 ± 37 cells mm^{-2}) and healthy control mice (1217 ± 23 cells mm^{-2}). Nonstressed and infected mice presented an increase in the numbers of Langerhans cells as compared with stressed and noninfected mice (1750 ± 50 cells mm^{-2}) (Fig. 2).

At week 8 of infection, mice stressed for 2 or 8 h and infected maintained a significantly diminished population of Langerhans cells (981 ± 82 and 1237 ± 45 cells mm^{-2} , respectively) as compared with nonstressed and infected mice (1806 ± 49 cells mm^{-2})

Table 1. Footpad thickness during the 12 weeks of evaluation for the different experimental groups

Weeks	A	B	C	D	E	$P \leq 0.05$
	Infection control	Stress control	Healthy control	Infected, stressed 2 h	Infected, stressed 8 h	
0	1.75 \pm 0.005	1.76 \pm 0.005	1.74 \pm 0.064	1.76 \pm 0.006	1.76 \pm 0.005	NS
1	1.76 \pm 0.004	1.77 \pm 0.004	1.74 \pm 0.008	1.77 \pm 0.005	1.87 \pm 0.100	A-E, B-E, D-E, C-E
2	1.77 \pm 0.004	1.77 \pm 0.160	1.75 \pm 0.060	1.78 \pm 0.003	1.90 \pm 0.016	A-E, B-E, D-E, C-E
3	1.79 \pm 0.003	1.78 \pm 0.009	1.75 \pm 0.050	1.80 \pm 0.004	1.95 \pm 0.021	A-E, A-B, B-D, C-D, D-E
4	1.85 \pm 0.007	1.77 \pm 0.007	1.76 \pm 0.004	1.81 \pm 0.003	2.03 \pm 0.007	A-E, A-B, D-E, B-D, B-E, C-D, C-E
5	2.17 \pm 0.040	1.79 \pm 0.010	1.77 \pm 0.040	2.00 \pm 0.280	2.18 \pm 0.047	A-B, A-D, B-D, B-E, C-D, C-E, D-E
6	2.43 \pm 0.048	1.79 \pm 0.010	1.78 \pm 0.040	2.31 \pm 0.045	2.31 \pm 0.059	A-B, B-D, B-E, C-E
7	2.72 \pm 0.082	1.80 \pm 0.014	1.78 \pm 0.050	2.54 \pm 0.069	2.91 \pm 0.111	A-B, B-D, B-E, C-E, D-E
8	3.11 \pm 0.070	1.78 \pm 0.013	1.78 \pm 0.010	3.03 \pm 0.043	3.11 \pm 0.126	A-B, B-D, B-E, C-E
9	3.30 \pm 0.096	1.78 \pm 0.014	1.80 \pm 0.010	3.36 \pm 0.106	3.62 \pm 0.298	A-B, B-D, B-E, C-E
10	3.69 \pm 0.144	1.79 \pm 0.019	1.82 \pm 0.060	3.63 \pm 0.083	4.63 \pm 0.591	A-B, B-D, B-E, C-E
11	3.99 \pm 0.228	1.82 \pm 0.011	1.82 \pm 0.070	4.42 \pm 0.200	4.76 \pm 0.688	A-B, B-D, B-E, C-E
12	4.60 \pm 0.312	1.77 \pm 0.006	1.82 \pm 0.010	4.63 \pm 0.224	5.00 \pm 0.767	A-B, B-D, B-E, C-E

Measurements in mm are expressed as mean \pm SEM. NS, not statistically different.

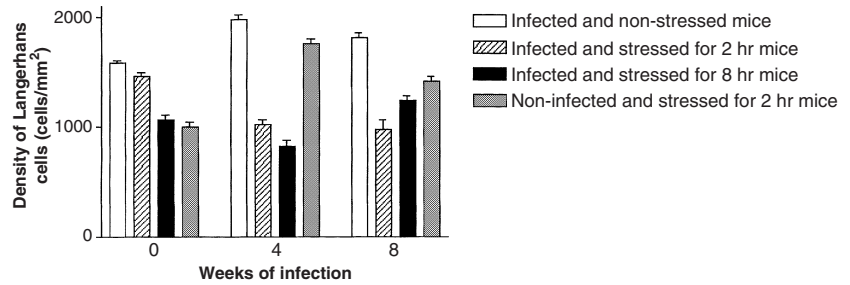


Figure 2. Langerhans cell density in experimental groups of BALB/c at weeks 0, 4 and 8. Results are presented as mean \pm SEM.

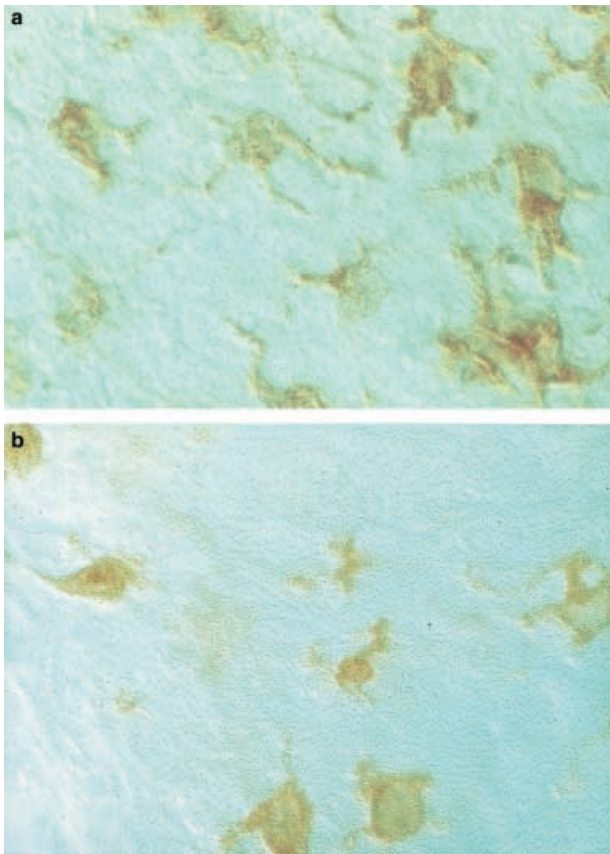


Figure 3. Ia^d+ Langerhans cells in epidermal sheets of *Leishmania mexicana*-infected BALB/c mice. (a) Nonstressed mice; (b) mice stressed for 2 h. Avidin–biotin immunoperoxidase procedure.

(Fig. 2). At week 12, the quantification of Langerhans cells in the infected groups was impossible to carry out due to the abundant presence of polymorphonuclear cells in the epidermis.

Morphological changes of Ia^d+ epidermal Langerhans cells in stressed mice

In the epidermis of mice stressed for 2 or 8 h and infected, Langerhans cells were smaller and more

spherical with shortened and fewer dendrites than the cells from nonstressed and infected mice (Fig. 3). The density and morphology of these Langerhans cells returned to the control value after 4–8 weeks of infection in all stressed animals.

Neuropeptide expression (calcitonin gene-related peptide and substance P) in cutaneous sensory nerves of stressed mice

Acute immobilization generated an increase in the innervations by CGRP and SP. In addition, the single infection by *L. mexicana* produced a decrease in expression of CGRP, while SP remained unaltered.

Immediately after stress and in the first week of infection, immunoreactivity to CGRP was observed in the epidermis, papillary dermis, sebaceous glands and nerves of mice stressed for 8 h and infected (Fig. 4), whereas animals stressed for 2 h and infected and those nonstressed and infected showed few CGRP+ nerve fibres in the papillary dermis. CGRP expression was not observed during weeks 4 and 8 of evaluation.

SP immunoreactivity was more prominent during the first week after infection in the group of mice stressed for 8 h and infected, with preferential localization in the papillary dermis and around sweat glands and vascular endothelia. At week 4 of infection, SP+ neurofibrillia were observed in the periphery of the granuloma and in the vascular endothelia (Fig. 5).

Discussion

In this study, we analysed the effect of acute stress by immobilization on epidermal Langerhans cells, sensorial innervation and the clinical course in the susceptible BALB/c model of cutaneous leishmaniasis. Our results showed that immobilization stress affected the course of *L. mexicana* infection and epidermal Langerhans cells in the susceptible BALB/c mouse model. In BALB/c mice stressed for 8 h and infected, the lesion

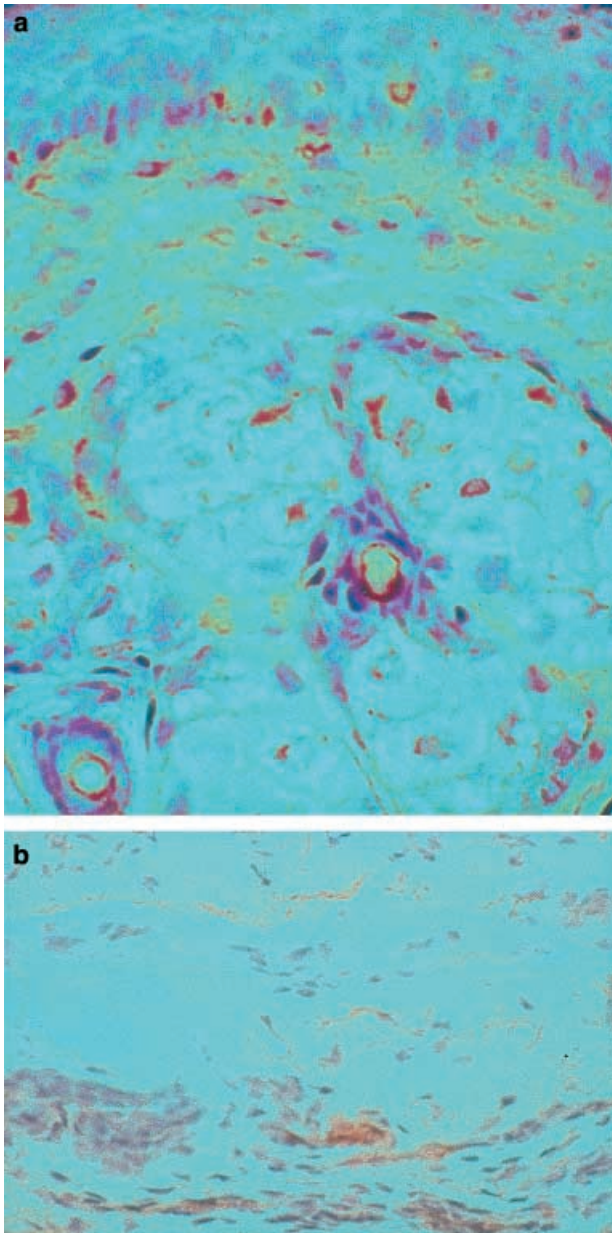


Figure 4. Calcitonin gene-related peptide (CGRP) immunoreactivity in cutaneous lesions of *Leishmania mexicana*-infected BALB/c mice stressed for 8 h. (a) Immunostaining in epithelium and sebaceous glands; (b) CGRP+ neurofibrillia in a nerve ending. Avidin–biotin immunoperoxidase procedure.

appeared earlier than in the groups of animals stressed for 2 h and infected or nonstressed and infected animals.

Previous studies have shown a decrease in density of epidermal Langerhans cells by the topical application of steroids, thus altering the course of infection by *Leishmania* sp. in susceptible BALB/c mice, and

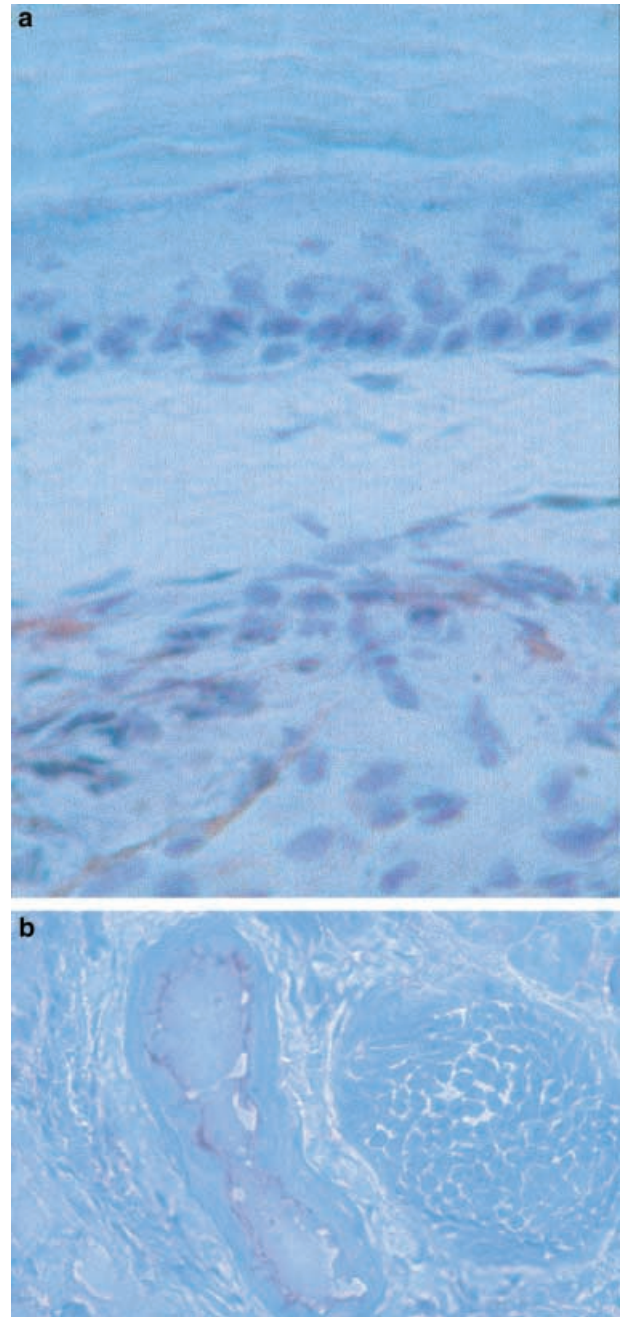


Figure 5. Substance P (SP) innervation in cutaneous lesions of stressed and *Leishmania mexicana*-infected BALB/c mice. (a) SP+ neurofibrillia in the periphery of inflammatory infiltrate, week 4, 2 h stress; (b) SP in vascular endothelium, week 4, 8 h stress. Avidin–biotin immunoperoxidase procedure.

accelerating the clinical manifestation of the illness.⁴ Here, Langerhans cells were affected by immobilization stress, as a decrease in density and morphological changes with fewer and shortened dendrites were

observed, coinciding with results described by other investigators.^{2,5} These alterations in Langerhans cells may compromise their function as antigen-presenting cells or parasite targets, thus affecting the formation of a T-helper 1 immune response.^{4,9} In addition, these changes in Langerhans cells may be related to the liberation of glucocorticoids by the adrenal glands in response to acute immobilization stress. This statement is supported by a recent study showing that adrenalectomized C57BL/6 mice exposed to the same stressor failed to show alterations in Langerhans cells.⁵ At weeks 4 and 8 after infection Langerhans cells recovered their normal morphology, but a smaller number remained in the infected and stressed groups as compared with infected and nonstressed mice, although the clinical course was very similar among the groups. These results suggest that mice partially recover from the effect of acute immobilization stress. However, the sustained decrease in Langerhans cell density may be due to alterations in the proliferative and migratory capacities of cell precursors travelling from the bone marrow to the epidermis.

Previous studies have demonstrated that stress increases the liberation of neuropeptides including CGRP, which can inhibit the antigen-presenting function of Langerhans cells.⁷ In the present study, acute immobilization stress caused an increase in CGRP innervation but we also observed that the single inoculation of *L. mexicana* induced a decrease of this peptide. SP remained unaltered after infection. The expression of CGRP was more evident in the first week after inoculation in mice stressed for 8 h and infected than in those stressed for 2 h and infected and nonstressed and infected animals, coinciding with the observations of Kawaguchi *et al.*,² who used a murine model of delayed-type hypersensitivity. CGRP was not observed in *L. mexicana*-infected mice at weeks 4 and 8 after infection. Ahmed *et al.*¹³ observed a significant decrease of CGRP in susceptible BALB/c mice as compared with resistant C57BL/6 mice, from the very first week of infection with *L. major*. This difference in CGRP concentration among mouse strains may contribute to the susceptibility or resistance to *Leishmania* infection, and explain the nociceptive alterations observed in this illness.¹³ CGRP can stimulate the migration of macrophages induced by *L. major*,¹⁴ and paradoxically, may inhibit some immunostimulatory functions of Langerhans cells.¹⁵ Overall, a stressor such as immobilization aggravates the susceptibility of BALB/c mice to infection by *Leishmania* spp. In this

microenvironment, CGRP may favour the migration of macrophages, which by the action of steroids have a diminished phagocytic capacity, triggering a rapid but not very effective inflammatory response against the parasite. SP was observed in the periphery of the granuloma and in the vascular endothelia of *L. mexicana*-infected mice, indicating its possible participation in the inflammatory process.

The clinical and neuroimmunological differences observed among the groups of *L. mexicana*-infected and restraint-stressed mice clearly demonstrate that the effect of the stressor depends on its duration and intensity.

Further understanding of the relationship between Langerhans cells and sensory neuropeptides will clarify the biological basis of many cutaneous diseases. The present study shows the impact of stress on the skin immune system, triggering or exacerbating illnesses. Future investigations may contribute to the development of therapeutic schemes targeting Langerhans cells and neuropeptides.

Acknowledgments

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