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Spontaneous cell proliferation is associated with poor sensitivity to glucocorticoids in patients infected with HTLV

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Abstract. *Background*: Human T-cell lymphotropic viruses (HTLV)-I/II have a special tropism for infecting T cells and inducing spontaneous lymphocyte proliferation. Leukaemia and neurological manifestations are associated with HTLV-I/II infections, and treatment is usually based on anti-inflammatory drugs including glucocorticoids. Although steroid resistance has been reported, it is unknown whether this condition is related to the infection itself or to the treatment. *Objective*: We investigated whether spontaneous cell proliferation is associated with T-cell sensitivity to glucocorticoids. *Materials and Methods*: Twenty-eight HTLV-I/II patients and 11 healthy age-matched controls took part in this study. Lymphocytes were isolated and cultured *in vitro* to measure spontaneous and mitogen-induced proliferation as well as cellular sensitivity to dexamethasone. *Results*: Patients with HTLV-I/II infection showed similar stimulated and unstimulated T-cell proliferation as well as comparable sensitivity to dexamethasone *in vitro*. There were no group differences in the frequency of glucocorticoid responders versus non-responders. However, T cells of patients with spontaneous proliferation were unresponsive to mitogenic stimulation and were remarkably more resistant to dexamethasone than cells of patients with normal proliferation. *Conclusion*: These data suggest that the poor clinical response to steroids may be associated with spontaneous cell proliferation during HTLV infection.

INTRODUCTION

Human T-cell lymphotropic virus, type I (HTLV-I) and type II (HTLV-II), are retroviruses with a special tropism to infect T cells, inducing spontaneous cell proliferation (Itoyama *et al*. 1988; Prince *et al*. 1990; Wiktor *et al*. 1991; Prince & Swanson 1993; Mann *et al*. 1994; Prince *et al*. 1994). First isolated in 1980 (Poiesz *et al*. 1980), HTLV-I is the most prevalent type worldwide and is related to several pathological states, characterized by local or systemic chronic inflammation. Within its related diseases, HTLV-I is known to induce adult T-cell leukaemia/lymphoma (ATL/L) (Uchiyama *et al*. 1977; Blattner *et al*. 1983; Uchiyama 1988) and HTLV-I–associated

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myelopathy (HAM), also known as 'tropical spastic paraparesis' (TSP) (Gessain *et al*. 1985; Osame *et al*. 1987). ATL/L is a pathogenic process caused by T-cell proliferation and has a neoplastic outcome, regardless of treatment, that often leads to death within a few months (Uchiyama *et al*. 1977; Franchini 1995). HAM/TSP is a chronic myelopathy that presents as an inflammatory and demyelinating process, mainly located in the thoracic spinal cord (Iwasaki 1990; Bhigjee *et al*. 1991; Gessain & Gout 1992; Cartier *et al*. 1997; Umehara *et al*. 2000), where a high concentration of T cells and monocytes are typically found (Murphy & Blattner 1988; Piccardo *et al*. 1988; Ijichi *et al*. 1989). This process leads to spasticity of the lower body, bladder disorders and distinct sensory disturbances (Gessain *et al*. 1985; Osame *et al*. 1986).

HTLV-II is epidemic among intravenous drug users (IDUs) in the United States (Khabbaz *et al*. 1991), Brazil (Alcantara *et al*. 2003) and western Europe (Zanetti & Galli 1992) and is also endemic among some native populations from the Americas (Heneine *et al*. 1991; Maloney *et al*. 1992; Hjelle *et al*. 1993) and sub-Saharan Africa (Goubau *et al*. 1993). Some case reports have described HTLV-II–associated neurological manifestations (Menna-Barreto 2003; Orland *et al*. 2003).

Because of its property of inappropriately activating T cells and inducing diseases characterized by a chronic inflammatory state (Franchini 1995; Hollsberg 1997), treatment of HTLV infections is usually based on anti-inflammatory drugs such as synthetic glucocorticoids (GCs). These steroids exert their actions through specific binding to two distinct intracellular receptor subtypes: the mineralocorticoid and GC receptors. After being bound, the receptor-ligand complex translocates to the nucleus, where it either binds to GC response elements on DNA or interacts with other transcription factors and regulates (positively or negatively) the genes to which they are linked (Juruena *et al*. 2003). Although the management of HTLV-I/II–associated diseases often include steroidal drugs, clinical responses to GCs have been reported to be varied, with some patients responding poorly to them (Araujo *et al*. 1993; Nakagawa *et al*. 1996; Matsushita *et al*. 2002). However, it is largely unknown to what extent poor clinical response correlates to spontaneous proliferation and peripheral T-cell sensitivity to GCs. The understanding of patients' T-cell sensitivity to GCs prior to treatment would be of valuable clinical significance as it would enable physicians to discriminate steroid responders from non-responders. The objectives of this study are (1) to determine patients' peripheral T-cell sensitivity to GCs (2) to discriminate steroid responders from non-responders *in vitro* and (3) to evaluate whether spontaneous cell proliferation is associated with T-cell sensitivity to GCs (dexamethasone, DEX) among HTLV-I/II–infected drug-free patients. We hypothesized that HTLV patients would be more resistant to both mitogenic and steroid signalling *in vitro.*

MATERIALS AND METHODS

Subjects

Twenty-eight, untreated HTLV-I– and HTLV-II–infected subjects were recruited for this study from the HTLV Unit (Department of Neurology, Hospital São Lucas, Porto Alegre, Brazil). Eighteen HTLV-I infected patients (14 women), ages 15 to 62 years (mean ± SD, 44.89 ± 12.9 years) and 10 HTLV-II–infected patients (5 women), ages 30 to 75 years (49.40 ± 13.94 years) took part in this study. The diagnosis of HTLV infection was confirmed by Western blot analysis. To discriminate steroid responders from non-responders, 11 healthy subjects (7 women), ages 21 to 73 years (39.81 ± 18.17 years) were also recruited as a control group. Exclusion criteria included presence of infection, acute or chronic inflammatory conditions, heart disease, under-nourishment,

anaemia, leucopaenia, neoplasia and drug use (including GCs). There were no differences in gender distribution ($\chi^2 = 2.30$, d.f. = 2, $P = 0.32$) or age ($\chi^2 = 1.11$, d.f. = 2, $P = 0.34$) between patients and controls. The study protocol was approved by both scientific and ethics committees (Pontifical Catholic University of Rio Grande do Sul, PUCRS, Porto Alegre, Brazil) and written informed consent was obtained from all subjects.

Collection of peripheral blood and isolation of mononuclear cells

Ten millilitres of peripheral blood was collected by venepuncture in the morning (between 9:00 h and 10:00 h) and samples were stored in lithium-heparin tubes prior to analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque (Sigma, St. Louis, MO, USA) gradient (900 ***g***, 30 min). After washing, the cells were counted then viewed microscopically (100×) and viability always was found to exceed 95%, as judged from the cells' ability to exclude trypan blue (Sigma). PBMCs were resuspended in complete culture medium (RPMI-1640, supplemented with gentamicin 0.5%, glutamine 1%, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid 1%, fungizone 0.1% and foetal calf serum 10% all from Sigma) and concentration in fluid was adjusted to 3×10^6 cells/ml.

Lymphocyte proliferation/viability assays

PBMCs were cultured in flat-bottomed 96-well microplates at a final concentration of 1.5×10^5 cells/well in complete culture medium for 96 h at 37 °C in an atmosphere with 5% CO_2. Stimulation by the selective T-cell mitogen phytohaemagglutinin (PHA; from Gibco, Grand Island, NY, USA) was performed in triplicates (100 ml/well) to yield an optimal concentration (1%). In non-stimulated cultures (PHA 0), mitogen was substituted by complete culture medium. To assess *in vitro* sensitivity to GCs, 10^{-9}–10^{-4} M of DEX (a synthetic GC receptor agonist) was added in duplicates (50 µl/well; water soluble, Sigma) to mitogen-stimulated (PHA 1%) cultures. GC concentrations were used in a range so that free endogenous GCs during resting state would reach (10^{-9} M), stress (10^{-6} M) and under pharmacological treatment (10^{-5} M) *in vivo*.

Proliferative responses were estimated using a modified colourimetric assay that correlates with the number of viable cells (Mosmann 1983; Collaziol *et al*. 2002). In the last 4 h of culture, 100 µl of supernatant was gently discarded and 40 µl of freshly prepared MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) (Sigma) solution (5 mg/ml in sterile PBS) was added to each well. Cell cultures were incubated for 4 h at 37 °C in 5% CO_2 atmosphere. After complete removal of the supernatant, 120 µl of dimethyl sulfoxide (Sigma) was added to each well. Optical density (OD) was determined using a Biorad enzyme-linked immunosorbent assay plate reader at wavelengths of 492 and 630 nm. Spontaneous cell proliferation was determined by visual identification of several cellular clusters (light microscopy, 40×) in unstimulated cells following 96 h of culture. Proliferation data are presented as OD. Difference between the OD of stimulated and non-stimulated cultures indicates the non-specific T-lymphocyte proliferation induced by PHA. Results of T-lymphocyte sensitivity to GCs are presented as percentage proliferation, where 100% (basal) represents maximum proliferation, obtained by OD means from cell cultures of PHA 1% without steroids.

Steroid responsiveness

Glucocorticoid responders and non-responders were identified through analysis of dose–response curves of control subjects. PBMCs of healthy control donors were cultured with PHA and DEX, as described in the previous section. The area under the curve (AUC) for each control subject was then calculated by the trapezoidal rule (PRISM 4.0, GraphPad Software), and the group median of the sample was determined (366.6 M). The same AUC determination was

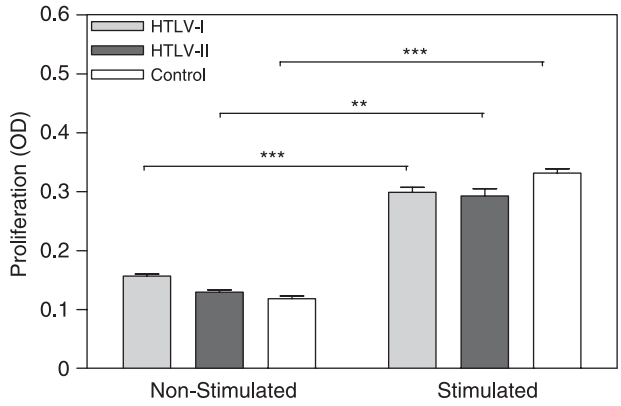

Figure 1. Evaluation of non-stimulated and mitogen-stimulated T-cell proliferation (HTLV-I: $n = 18$; HTLV-II: $n = 10$; control: $n = 11$). PBMCs were cultured with and without 1% PHA for 96 h and cell proliferation/viability was estimated by MTT assay. OD was determined at wavelengths of 492 and 630 nm. Statistical significance differences are indicated: $^{**}P < 0.01$; $^{***}P < 0.001$.

performed for each HTLV-I/II patient individually. Patients with AUC higher than the median AUC from the control group (366.6 M) were classified as GC non-responders, indicating that their dose–response curve to varied DEX concentrations maintained itself closer to basal proliferation (100%). Patients with an AUC lower than this value were considered to be sensitive to DEX *in vitro*, as their dose–response curve indicated lower proliferation percentages, and were thus classified as responders.

Statistical analysis

All variables were tested for homogeneity of variances and normality of distribution by means of the Levene and Kolmogorov-Smirnov tests, respectively. Proliferation data were analysed by repeated measures ANOVA that included one between-subjects variables (groups) and one within-subjects variables (mitogen or GC levels). One way ANOVA was performed to analyse cell proliferation (non-stimulated versus stimulated) data. Multiple comparisons among levels (mitogen or GC) were checked with Tukey's *post hoc* test. Differences between variables were assessed by Student's *t*-test. Statistical interactions between group distributions were compared by means of chi-squared (χ^2) test. Data are expressed as mean ± SE in all figures. A statistical software package (SPSS 11.5, SPSS, Chicago, IL, USA) was used to perform the analyses. The significance level was set at $\alpha = 0.05$ (two-tailed).

RESULTS

Lymphocyte proliferation

Mitogen-induced T-cell proliferation was evaluated as an index of cell-mediated immunity. Non-stimulated cell proliferation was found to be marginally increased in HTLV-I patients compared to HTLV-II–infected individuals ($t = 1.43$, d.f. = 25.98, $P = 0.17$) and healthy control subjects ($t = 1.79$, d.f. = 25.42, $P = 0.09$), although it only approached statistical significance (Fig. 1). Stimulation with PHA yielded significant T-cell proliferation in all groups. However, mitogen-induced proliferative responses were found similar in both HTLV groups.

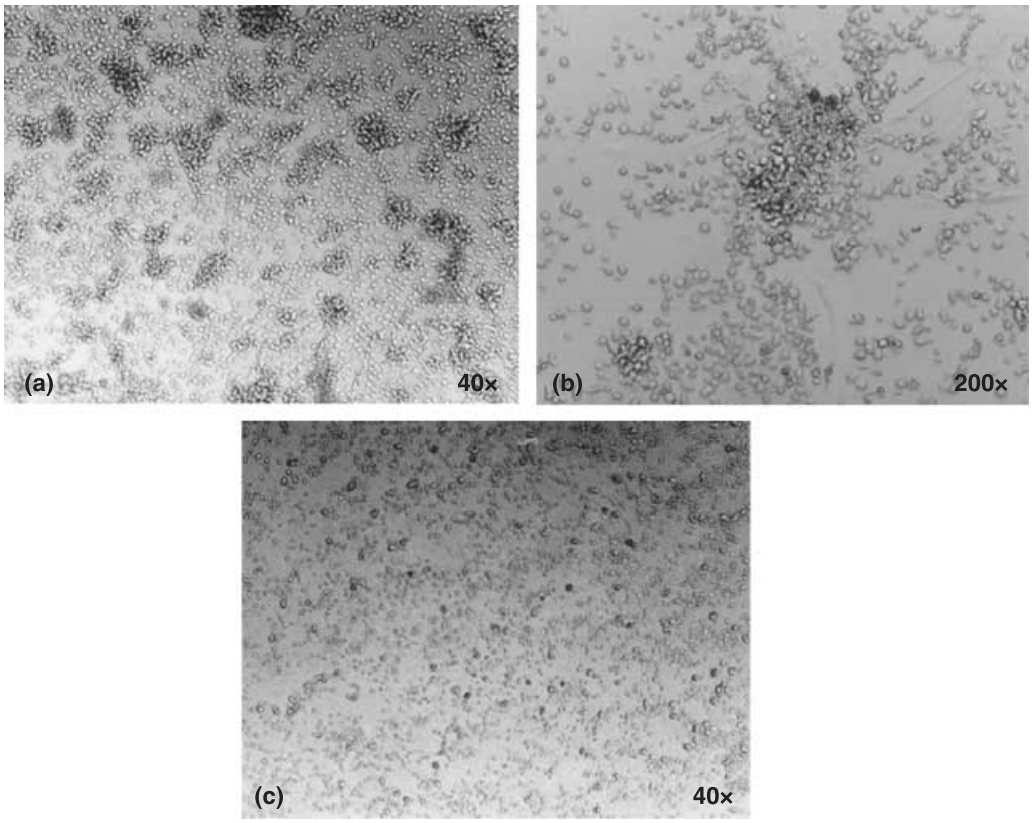

Figure 2. Spontaneous cell proliferation in HTLV-I infection. Representative photographs of unstimulated cultures of two HTLV-I patients. (a) Spontaneous cell proliferation as demonstrated by cellular clusters (40×) that can be seen magnified in (b) (200×). (c) Cells with a normal proliferation level.

Spontaneous cell proliferation

We investigated the frequency of patients with spontaneous T-lymphocyte proliferation. HTLV-I/II patients presented similar proportions of subjects with spontaneous proliferation, 33.3% (six patients) with HTLV-I and 10% (one patient) with HTLV-II, respectively ($\chi^2 = 1.87$, d.f. = 1, $P = 0.17$). Spontaneous cell proliferation was confirmed by the presence of several cellular clusters in unstimulated cultures of HTLV-I subjects (Fig. 2). We then assessed the extent cells to which patients who developed spontaneous T-lymphocyte proliferation responded to mitogenic stimulation. Interestingly, it was observed that T cells with spontaneous proliferation were unresponsive to PHA stimulation (Fig. 3). This was similarly described for patients with HTLV-I and -II infections. However, no statistical analysis could be performed within HTLV-II subjects as only one patient presented spontaneous proliferation in that group (Fig. 2b).

Lymphocyte sensitivity to GCs

In view of evidence that some patients with HTLV-I/II infections respond poorly to GC treatment (Araujo *et al*. 1993; Nakagawa *et al*. 1996), we examined the peripheral T-cell sensitivity to DEX, *in vitro* prior to treatment. DEX produced dose-dependent suppression of T-cell

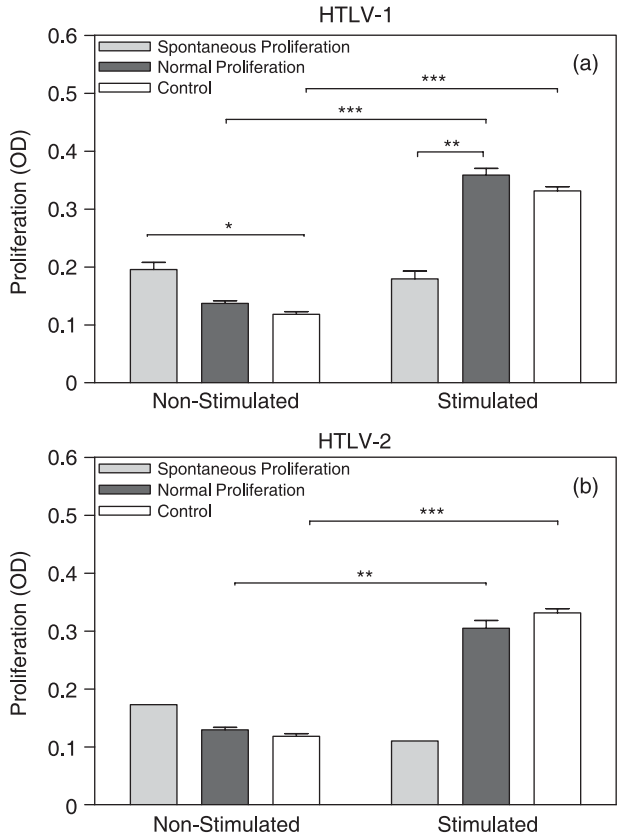

Figure 3. Evaluation of non-stimulated and mitogen-stimulated T-cell proliferation in HTLV-infected patients with normal and spontaneous proliferation levels. PBMCs were cultured with and without 1% PHA for 96 h and proliferation/viability was estimated by MTT assay. OD was determined at wavelengths of 492 and 630 nm (a) HTLV-I–infected subjects (normal: $n = 12$; spontaneous: $n = 6$; control: $n = 11$); (b) HTLV-II–infected subjects (normal: $n = 9$; spontaneous: $n = 1$; control: $n = 11$). Statistical significance differences are indicated: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

proliferation, ($F_{5,130} = 38.24$, $P < 0.001$) (Fig. 4). However, T-cell sensitivity to DEX did not differ between HTLV groups ($F_{1,26} = 0.60$, $P = 0.44$).

We also investigated the frequency of GC responders within HTLV groups. Eight HTLV-I (44.4%, 42.38 ± 16.10 years, 5 women) and four HTLV-II (40%, 43.25 ± 11.23 years, 2 women) patients were classified as GC non-responders. There were no group differences in the frequency of GC responders/non-responders ($\chi^2 = 0.05$, d.f. = 1, $P = 0.82$). However, GC non-responders (in both HTLV groups) were similarly more resistant to DEX *in vitro* than cells of GC responders (Fig. 5).

Finally, we assessed whether spontaneous cell proliferation is associated with T-cell sensitivity to GCs. Interestingly, it was observed that T cells of HTLV-I patients with spontaneous cell proliferation were significantly more resistant to DEX than cells of patients with normal proliferation levels (Fig. 6a) ($F_{1,16} = 6.4$, $P < 0.05$). No statistical analysis could be performed within HTLV-II subjects because only one patient presented spontaneous proliferation in that group (Fig. 6b).

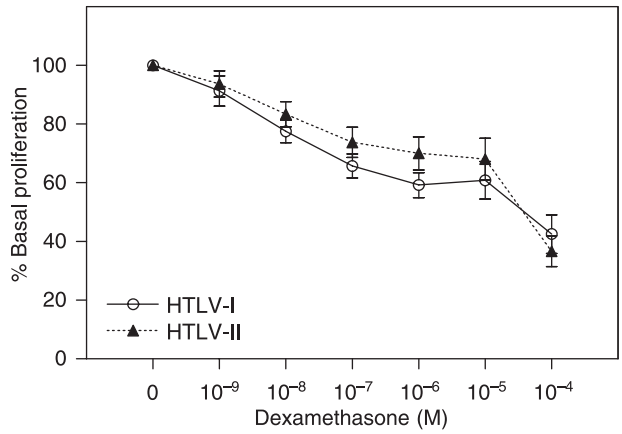

Figure 4. Peripheral T-cell sensitivity to dexamethasone (HTLV-I: $n = 18$; HTLV-II: $n = 10$). Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX for 96 h. Cell proliferation was estimated by MTT assay. OD was determined at wavelengths of 492 and 630 nm. Data are shown as percentage of base line cell proliferation (100% = PHA 1% without steroids).

DISCUSSION

Human T-cell lymphotropic virus infections are known to induce the appearance of inflammatory diseases by activating T lymphocytes and inducing spontaneous cell proliferation (Itoyama *et al*. 1988; Prince *et al*. 1990; Wiktor *et al*. 1991; Prince & Swanson 1993; Mann *et al*. 1994; Prince *et al*. 1994). HTLV-I has more disease associations then HTLV-II and is known to cause ATL/L (Uchiyama *et al*. 1977; Blattner *et al*. 1983; Uchiyama 1988) and HAM/TSP (Gessain *et al*. 1985; Osame *et al*. 1987). However, HTLV-II infections may also lead to neuropathological states (Hjelle *et al*. 1992; Menna-Barreto 2003; Orland *et al*. 2003).

Because of its ability to mediate the appearance of diseases with severe prognosis, such as ATL/L and HAM/TSP, we initially anticipated that HTLV-I–infected lymphocytes would proliferate more intensively than HTLV-II–infected cells. However, T-cell proliferation was found to be similar in both groups of infected patients (Fig. 1). These results suggest that the capacity of HTLV-I virus to induce a greater number of inflammatory diseases than HTLV-II may not necessarily be associated with a greater peripheral T-cell response.

As previously reported (Itoyama *et al*. 1988; Prince *et al*. 1990; Wiktor *et al*. 1991; Prince & Swanson 1993; Mann *et al*. 1994; Prince *et al*. 1994), we also have observed a significant proportion of subjects with spontaneous T-cell proliferation within both HTLV-I– (33.3%, $n = 6$) and HTLV-II–infected (10%, $n = 1$) patients. Here we have investigated to what extent the cells of patients with spontaneous T-cell proliferation would respond to mitogenic stimulation. It was observed for the first time that T cells of patients with spontaneous proliferation were completely unresponsive to PHA stimulation. These results suggest that HTLV-infected T lymphocytes that had become activated and proliferate as a result of the viral infection do not respond to unspecific activation. Indeed, it has been shown previously that spontaneous cell proliferation is associated with increased proviral load (Prince & Swanson 1993); this clinical parameter was not assessed here. Further studies should investigate whether mitogen unresponsiveness would be related to proviral load. It is reasonable to speculate that these patients would be more

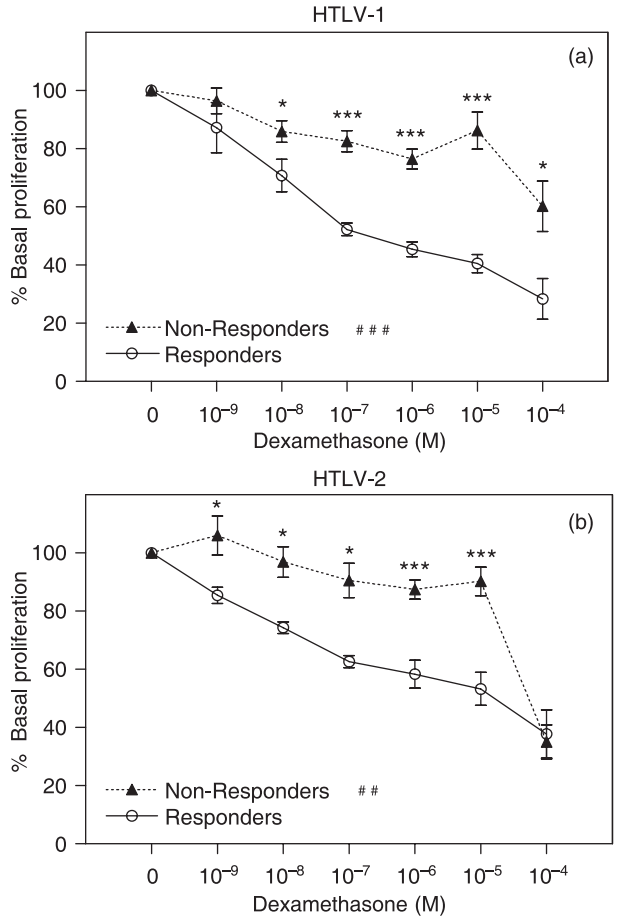

Figure 5. Peripheral T-cell sensitivity to DEX in responders/non-responders, HTLV-infected patients. Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX for 96 h. Cell proliferation was estimated by MTT assay. OD was determined at wavelengths of 492 and 630 nm. Data are shown as percentage of base line cell proliferation (100% = PHA 1% without steroids). (a) HTLV-I–infected subjects (responders: $n = 10$; non-responders: $n = 8$); (b) HTLV-II–infected subjects (responders: $n = 6$; non-responders: $n = 4$). Statistical significance differences in T-cell sensitivity to isolated DEX concentrations are indicated: $*P < 0.05$, $**P < 0.01$; $***P < 0.001$. Statistical interaction of T-cell sensitivity to variation of DEX concentrations between groups indicated: $\#\#P < 0.01$; $\#\#\#P < 0.001$.

susceptible to other infectious diseases, which are under control of effective T-cell responses. The underlying mechanisms of this mitogenic unresponsiveness are still completely obscure.

Treatment of HTLV infections usually involves the administration of anti-inflammatory drugs such as synthetic GCs. However, some HTLV patients respond poorly to this treatment (Araujo *et al*. 1993) and concomitant therapy with other immunosuppressive drugs is often required (Nakagawa *et al*. 1996). In this study, patients with HTLV-I/II showed comparable T-cell sensitivity to DEX *in vitro* and similar frequency of GC responders versus non-responders. We speculate that clinical resistance to treatment with these steroids may be limited to the peripheral inflamed tissues. Interestingly, we observed for the first time that T lymphocytes from

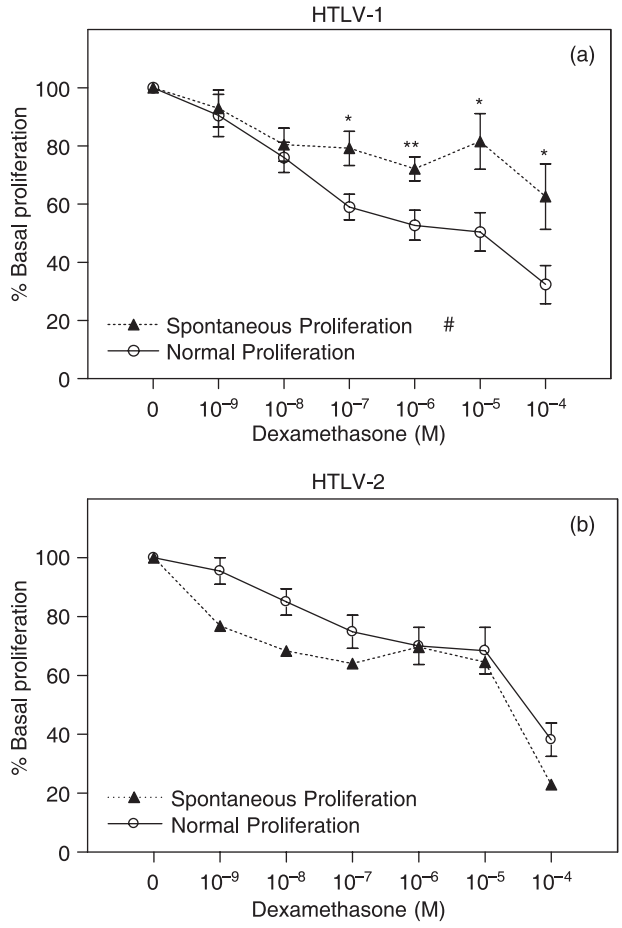

Figure 6. Peripheral T-cell sensitivity to DEX in HTLV patients with spontaneous/normal cell proliferation. Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX for 96 h. Cell proliferation was estimated by MTT assay. OD was determined at wavelengths of 492 and 630 nm. Data are shown as percentage of base line cell proliferation (100% = PHA 1% without steroids). (a) HTLV-I–infected subjects (normal: $n = 12$; spontaneous: $n = 6$); (b) HTLV-II–infected subjects (normal: $n = 9$; spontaneous: $n = 1$). Statistical significance differences in T-cell sensitivity to isolated DEX concentrations are indicated: $*P < 0.05$; $**P = 0.01$. Statistical inter-action of T-cell sensitivity to variation of DEX concentrations between groups indicated: $\#P < 0.05$.

HTLV-I patients showing spontaneous cell proliferation were significantly more resistant to DEX than cells from patients with normal proliferation. These results differ from a previous study (Yamano *et al*. 1997) in which PBMCs from HAM/TSP patients with spontaneous proliferation were highly sensitive to prednisolone's modulatory effects (reduced proliferation and altered cytokine production). However, there are methodological differences between our and Yamano's work that may justify this discrepancy. For example, we evaluated the ability of DEX to suppress T-cell proliferation to assess steroid sensitivity of activated lymphocytes, whereas Yamano and colleagues analysed the steroid sensitivity of non-stimulated PBMCs. Therefore, it remains difficult to discern precisely the cellular targets that respond to steroids in the former study. The cellular activation state is of paramount importance to steroid sensitivity.

No interaction between cellular spontaneous/normal proliferation and GC sensitivity was observed within HTLV-II. However, this evaluation was compromised because only one subject from the evaluated group of HTLV-II–infected patients presented spontaneous proliferation *in vitro*.

Taken together, these data indicate that poor clinical response to steroid treatment may be related to spontaneous cell proliferation during HTLV infection, especially HTLV-I. We confirm our main hypothesis and speculate that spontaneous cell proliferation would render lymphocytes resistant to both mitogenic and steroid signalling, as a result of repeated polyclonal T-cell infections. These chronic infections may lead to clonal exhaustion and further disease vulnerability in HTLV-infected people. Thus, the identification of HTLV-infected patients with spontaneous cell proliferation will be of clinical value.

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