Pontifícia Universidade Católica do Rio Grande do Sul
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ASSOCIAÇÃO DE RESISTÊNCIA A GLICOCORTICÓIDES E PROLIFERAÇÃO ESPONTÂNEA EM LINFÓCITOS DE PACIENTES INFECTADOS COM HTLV-I/II

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RESUMO

Introdução e Objetivos: As infecções por HTLV-I/II se caracterizam pelo tropismo viral por infectar células T humanas e promover um estado de proliferação espontânea deste tipo celular. Leucemia e doenças inflamatórias graves, com destaque para as doenças neurológicas, estão fortemente associadas às infecções por HTLV-I/II e o tratamento usual destas patologias envolve a administração de antiinflamatórios da classe dos glicocorticóides. Embora haja relatos de resistência farmacológica à terapia com glicocorticóides, não se sabe ao certo se essa condição se deve à infecção viral ou ao hospedeiro. Neste estudo, avaliou-se a relação entre proliferação celular espontânea e sensibilidade de linfócitos T aos efeitos dos glicocorticóides (dexametasona - DEX). *Materiais e Métodos:* Células mononucleares de sangue periférico foram isoladas de pacientes assintomáticos e livres de tratamento, com infecção por HTLV-I (n=18) e HTLV-II (n=10), e foram cultivadas na presença e na ausência do mitógeno fitohemaglutinina (PHA 1%). Células foram também cultivadas com PHA 1% e concentrações variadas de DEX (10^{-9} a 10^{-4} M). Para fins comparativos, as mesmas avaliações foram realizadas em sujeitos saudáveis (Controle, n=11). Resultados e Discussão: Os pacientes com HTLV-I/II apresentaram taxas similares de proliferação estimulada (PHA 1%) e não-estimulada (PHA 0%), bem como sensibilidade comparável à DEX. Não houve diferença na freqüência de indivíduos resistentes versus sensíveis à DEX entre os grupos HTLV-I e HTLV-II. Entretanto, linfócitos T de pacientes com proliferação espontânea não responderam ao estímulo mitogênico por PHA e foram mais resistentes à modulação por DEX do que as células de pacientes com proliferação normal. Os resultados aqui apresentados sugerem que a baixa resposta clínica à terapia com glicocorticóides pode estar associada a um estado de proliferação celular espontânea decorrente da infecção por HTLV.

ABSTRACT

Introduction and Objectives: HTLV-I/II viruses have a special tropism for infecting T cells and inducing spontaneous lymphocyte proliferation. Leukemia and inflammatory states such as neurological manifestations are associated with HTLV-I/II infections and treatment is usually based on antiinflammatory drugs including glucocorticoids. Although steroid resistance has been reported, it is unknown whether this condition is related to the viral infection itself or treatment. Here, we investigated whether spontaneous cell proliferation is associated to T-cell sensitivity to glucocorticoids (dexamethasone – DEX). *Materials ad Methods:* Human peripheral blood mononuclear cells (PBMCs) were isolated from assinthomatic drug-free HTLV-I (n=18) and HTLV-II (n=10) infected patients, as well as from healthy subjects (Control, n=11) and cultivated with and without phytohemmaglutinin (PHA 1%). Cells were also cultivated with PHA 1% and several concentrations of DEX (10^{-9} a 10^{-4} M). *Results and Discussion:* Patients with HTLV-I/II showed similar unstimulated and stimulated 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 remarkably more resistant to dexamethasone than cells of patients with normal proliferation. These data suggest that the poor clinical response to steroids may be associated to spontaneous cell proliferation during HTLV infection.

1. APRESENTAÇÃO DO TEMA

1.1. Introdução

1.1.1. HTLV-I e HTLV-II

A infecção pelos vírus linfotrópicos de células T humanas dos tipos I e II (HTLV-I/II) tem chamado a atenção e despertado o interesse dos meios clínicos e científicos. São retrovírus humanos geneticamente relacionados, pertencentes à subfamília *Oncovirinae* e que possuem um tropismo especial por infectar células T e induzir proliferação espontânea deste tipo celular (1-6).

A infecção por HTLV-I/II caracteriza-se por: agrupamento da infecção em áreas geográficas definidas; variação espacial das taxas de soroprevalência, dentro de áreas de prevalência reconhecidamente elevadas; aumento da soroprevalência com a idade e soroprevalência mais elevada em mulheres, mais acentuada após os 40 anos (7).

O vírus HTLV-I tem distribuição geográfica esparsa, com soroprevalências mais elevadas nas ilhas do sul do Japão, na região sudeste dos EUA, nas ilhas do Caribe, nas Américas Central e do Sul, em regiões da África e na Melanésia (8-15).

O HTLV-II tem sido encontrado entre usuários de drogas injetáveis nos EUA, Europa e Brasil (16-18). Altas prevalências de anticorpos anti-HTLV-II têm sido também encontradas em populações nativas das Américas e da África (19-26).

Embora a maior parte dos indivíduos infectados por HTLV-I ou HTLV-II permaneça assintomática por toda a vida, reconhece-se o papel etiológico destes vírus em algumas doenças. Isolado em 1980 (27), o HTLV-I é o mais prevalente dos dois

tipos virais em todo o mundo e está associado a um maior número de patologias, tais como: a leucemia/linfoma de células T do adulto (ATL/L) (28-30) e a paraparesia espástica tropical ou mielopatia associada ao HTLV-I (HAM/TSP) (31, 32). A ATL/L é um processo patogênico causado pela ativação e proliferação descontrolada de linfócitos T, com conseqüências neoplásicas e sem tratamento, resultando (normalmente) na morte do paciente em alguns meses (30, 33). A HAM/TSP é uma patologia neurológica crônica resultante de um processo inflamatório desmielinizante localizado, principalmente, na medula espinhal, onde grandes quantidades de linfócitos T e monócitos são comumente encontrados (34-36). Este processo leva à fraqueza e espasticidade dos membros inferiores, além de distúrbios sensoriais e esfincterianos (31, 32).

Embora as patologias associadas ao HTLV-I mais estudadas sejam as neurológicas e hematológicas, devido à gravidade de seus sintomas e à severidade de suas conseqüências, a infecção por HTLV-I está associada também a estados inflamatórios importantes tais como: uveítes; polimiosites; artropatias; síndrome de Sjögren e dermopatias, entre outras (7, 37, 38). À medida que os estudos se desenvolvem, mais se evidencia que os efeitos da infecção podem ser sistêmicos, evoluindo provavelmente para o conceito de síndrome, abrangendo diferentes campos da medicina como neurologia, hematologia, dermatologia, oftalmologia e imunologia (7).

Descoberto em 1982 (39), o HTLV-II, apesar de ter grande homologia com o HTLV-I, não está consistentemente associado a nenhuma patologia em especial, ao contrário do que acontece com o outro tipo viral. Contudo, alguns estudos clínicos verificaram a participação do HTLV-II em casos de mielopatias crônicas (40-42), semelhantes à HAM/TSP, demonstrando seu potencial neuropatogênico.

1.1.2. Glicocorticóides

Tendo em vista o fato de que as patologias ocasionadas por infecções pelos vírus HTLV-I/II provém da propriedade destes vírus em induzir ativação e proliferação exacerbada de células T, caracterizando um estado inflamatório crônico, as terapias farmacológicas utilizadas usualmente baseiam-se no uso de antiinflamatórios e imunossupressores. Uma classe de fármacos antiinflamatórios amplamente utilizado pela clínica médica no tratamento de doenças inflamatórias, inclusive nas associadas às infecções por HTLV, é a classe dos antiinflamatórios glicocorticóides (GCs).

Os GCs são potentes drogas imunossupressoras e antiinflamatórias e representam alguns dos medicamentos mais importantes utilizados na terapêutica nos últimos anos (43, 44). Entretanto, sabe-se que, mesmo em indivíduos saudáveis, existe variação na sensibilidade linfocitária aos efeitos dos GCs (45) e que, portanto, esta droga não representa sempre uma solução satisfatória no tratamento de enfermidades inflamatórias (46).

No organismo, os GCs são reguladores essenciais do desenvolvimento, do metabolismo, da homeostasia e de funções efetoras do sistema imune inato e adaptativo (47-50). Eles alteram a ativação, diferenciação e maturação de muitos tipos de células imunes, bem como exercem múltiplos papéis na regulação da sensibilidade imune celular à apoptose (51, 52). Os GCs são hormônios naturais antiinflamatórios produzidos pelas glândulas adrenais, segundo o controle do hipotálamo, que podem reduzir efetivamente parâmetros de inflamação como a velocidade de sedimentação globular (VSG) e a proteína C reativa (CRP), induzindo a remissão da doença (53). Na espécie humana, o principal GC secretado pelas glândulas adrenais é o cortisol.

Os mecanismos de ação dos GCs são baseados na ligação desta molécula a receptores para glicocorticóides (GCR). A magnitude dos efeitos biológicos é determinada, entre outros fatores, pela quantidade de receptores das células-alvo e pela afinidade dos receptores aos GCs (54). Embora os mecanismos de ação dos GCs possam ser subdivididos em efeitos genômicos e não-genômicos (55), a maioria dos efeitos antiinflamatórios e imunomodulatórios dos GCs é mediada predominantemente por mecanismos genômicos (56). Isso acontece após a ligação do hormônio com seu respectivo receptor na membrana celular (mGCR) ou no citoplasma (cGCRα), onde há um maior número de receptores. O complexo hormônio-receptor geralmente induz transativação ou inibe a síntese de proteínas regulatórias (57). Os GCRs constituem um complexo multiprotéico consistindo de várias proteínas de choque térmico (HSPs), incluindo: HSP90, HSP70 e HSP56 (58). Após a ligação dos GCs aos GCRs, ocorre uma mudança conformacional na molécula do receptor, resultando na dissociação das HSPs. Ocorre então a translocação do complexo GC-GCR para o núcleo celular onde aquele se ligará a sítios de DNA específicos: os elementos responsivos aos GCs (GREs) (57). Dependendo do gene alvo, a transcrição é então ativada (transativação via GRE positivo) ou inibida (GRE negativo) (56).

Os efeitos dos GCs se devem principalmente à inibição da liberação de citocinas por células imunes. Os GCs, após sua ligação com os receptores, induzem a transcrição do inibidor de proteínas IκB (IκBα) que mantém o fator nuclear-κB (NFκB) no citoplasma em sua forma inativa, impedindo o NFκB de migrar para o núcleo, onde se ligaria ao elemento de resposta apropriado no DNA e ativaria a produção/secreção de citocinas e contribuiria, dessa forma, para a imunossupressão (59, 60). Eles também suprimem a adesão celular, a marginação e migração, ativação dos macrófagos, apresentação de antígenos, expressão de receptores de células T,

ativação dos linfócitos T, proliferação, diferenciação e função das células maduras, incluindo citotoxicidade e função das células B como a produção de anticorpos (48). Os GCs também têm a propriedade de induzir a apoptose de linfócitos e timócitos, mas estes efeitos podem ser secundários, pela inibição da produção de citocinas e fatores de proliferação (48) (**Figura 1**).

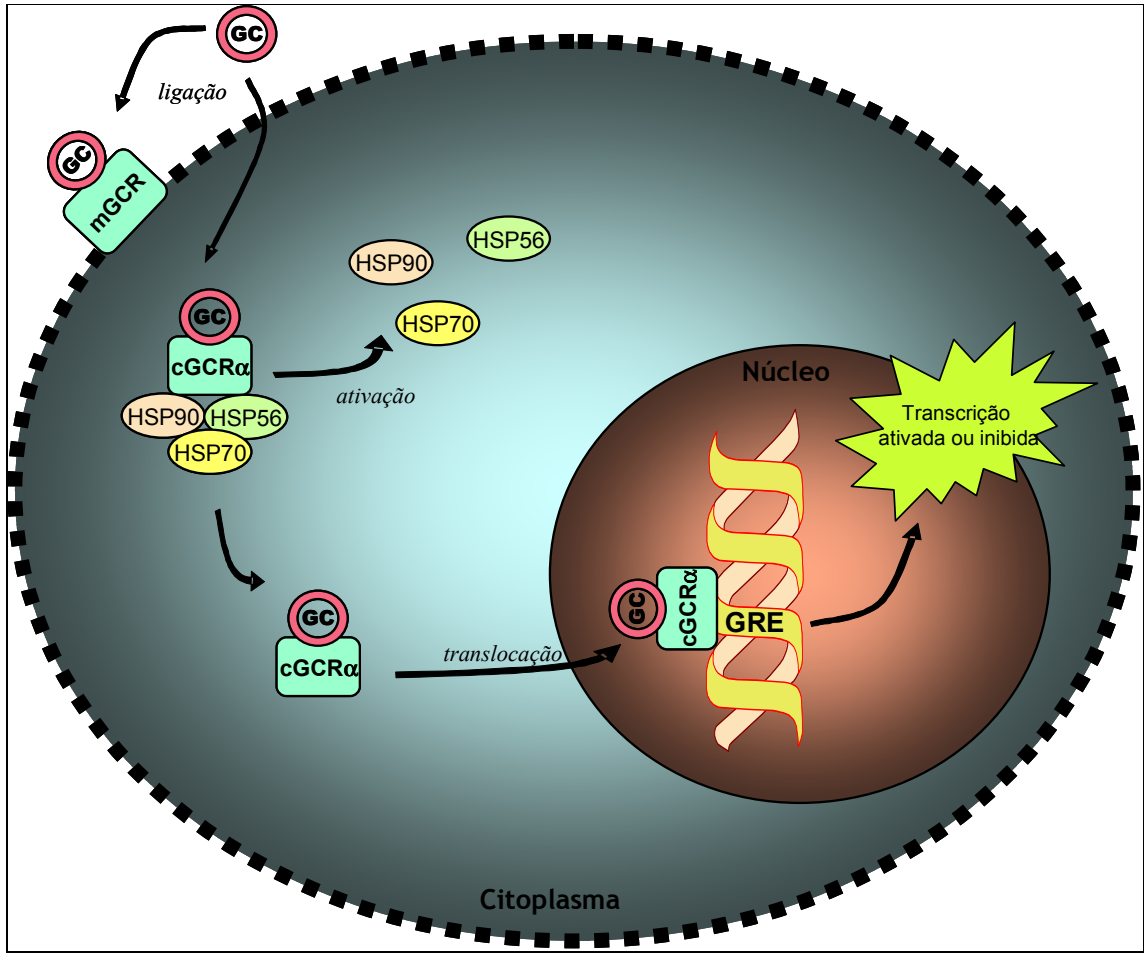

Figura 1: Ilustração demonstrando o mecanismo de ação dos glicocorticóides.

1.1.3. Sensibilidade e Resistência aos Glicocorticóides

A variabilidade de respostas ao uso dos GCs no tratamento de diversas doenças inflamatórias é um efeito conhecido no meio clínico. Embora muitos pacientes apresentem respostas satisfatórias à terapia com GCs, uma pequena subpopulação

de indivíduos fracassa em responder aos efeitos terapêuticos desta classe de medicamentos. Com base nisso, os pacientes podem ser classificados como resistentes aos corticosteróides (CR) ou sensíveis (CS) (61, 62). A resistência ao tratamento farmacológico à base de corticosteróides pode ser notada em muitos pacientes que requerem grandes quantidades e/ou períodos de administração prolongados de GCs (46) para apresentar melhoras significativas em seus respectivos quadros clínicos. Acredita-se que a propensão ao desenvolvimento de resistência aos GCs pode ser uma propriedade intrínseca de cada indivíduo (61), provavelmente com bases genéticas (63).

A sensibilidade periférica aos GCs é regulada por diversos mecanismos envolvendo células e tecidos. Por exemplo, alterações na produção/secreção de citocinas e hormônios (64). A disponibilidade de GC no meio extracelular pode ser determinada por aspectos como: alterações na expressão tecido-dependente de 11β-hydroxysteroid dehydrogenases (11β-HSD), catalisador da conversão de glicocorticóides ativos (cortisol) para suas formas inativas (cortisona) e vice-versa (65); e por alterações nos níveis plasmáticos da globulina ligante de corticosteróides (CBG), molécula carreadora de glicocorticóides biologicamente ativos e responsável pela distribuição do hormônio para tecidos periféricos. A sensibilidade intracelular aos GCs pode ser modulada por diversos mecanismos envolvendo anormalidades nas vias de sinalização, defeitos no complexo proteína/receptor dos GCs e alterações na função do cGCRα e na expressão celular de cGCRβ (64). Estudos relatam, por exemplo: diferenças na quantidade de receptores funcionais para glicocorticóides de membrana (mGCR) e citoplasmáticos (cGCRα) e mudanças na afinidade dos receptores (66); expressão alterada de HSPs, responsáveis pela estabilização da molécula de cGCRα (67); expressão alterada de cGCRβ, antagonista do cGCRα (68); pro-

blemas na translocação do complexo GCR-GC para o núcleo (69); expressão alterada de citocinas (70, 71); além da expressão alterada de fatores de transcrição AP-1 e NF-κB (72). Ainda, vale citar a existência de mecanismos adaptativos de resistência envolvidos com resistência a múltiplas drogas (MDR), como os mediados pela glicoproteína-P (P-gp), molécula de membrana responsável pela expulsão de substâncias nocivas à célula do meio intracelular (73-76), dentre as quais glicocorticóides sintéticos como a dexametasona (**Figura 2**).

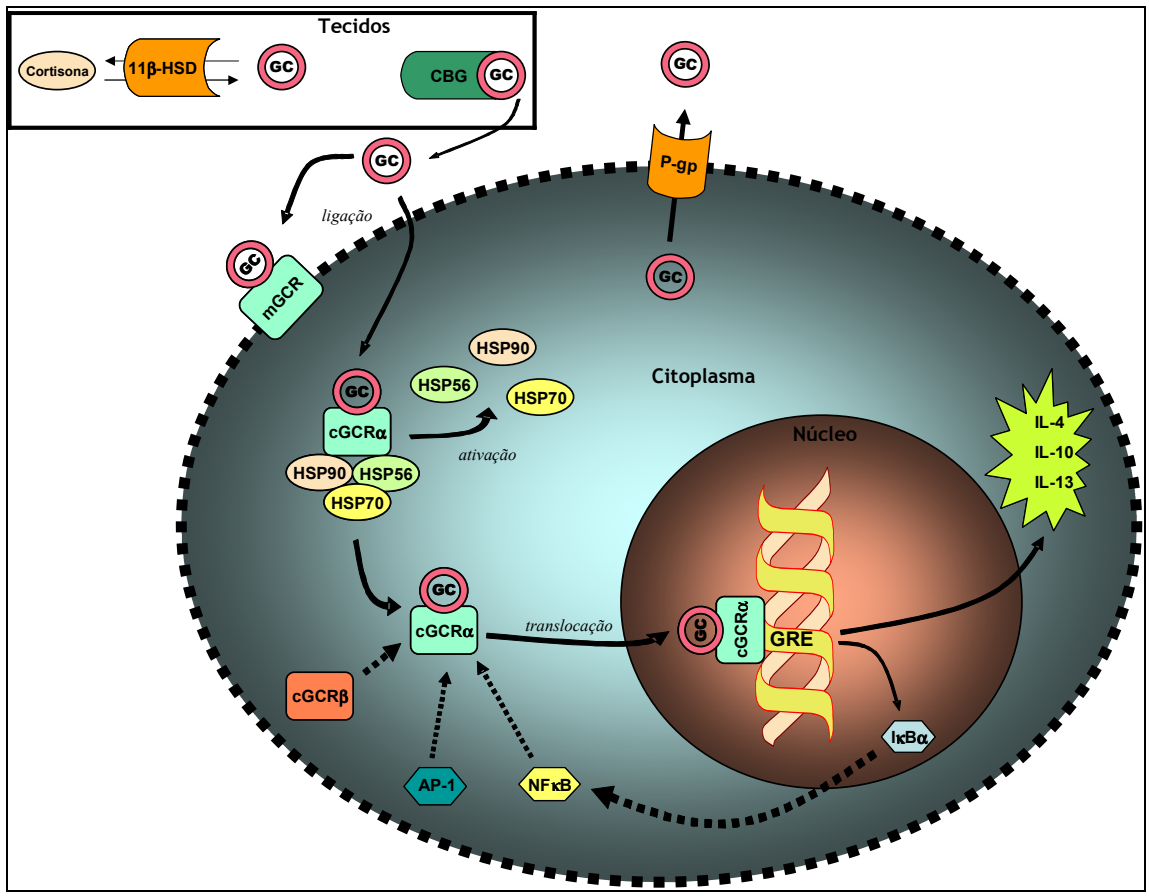

Figura 2: Mecanismos de resistência celular aos GCs. As setas pontilhadas indicam vias inibitórias do cGCRα.

Existem dados conflitantes em relação às respostas clínicas ao uso de corticóides no tratamento farmacológico de estados inflamatórias relacionadas à infecção

por HTLV, especialmente no tratamento de HAM/TSP (77, 78), onde apenas alguns pacientes respondem satisfatoriamente ao tratamento com GCs. Em doenças inflamatórias menos severas relacionadas ao HTLV, a terapia com GCs é utilizada com mais sucesso (79, 80), mas também há casos de pacientes que não respondem ao tratamento (81, 82). Entretanto, a literatura carece de trabalhos avaliando os efeitos de sensibilidade e resistência de tipos celulares de pacientes com infecção por HTLV à modulação por GCs. Estudos como este, voltados aos aspectos celulares, são importantes para auxiliar na busca por terapias cada vez mais eficazes para o tratamento de patologias associadas às infecções por HTLV.

1.2. Objetivos

1.2.1. Hipótese

Acredita-se que os estados patológicos associados às infecções virais por HTLV estejam relacionados à proliferação celular descontrolada promovido pelos vírus. Portanto, por estar mais consistentemente associado ao desenvolvimento de doenças graves do que o HTLV-II, espera-se que as células de pacientes com infecção por HTLV-I apresentem maior capacidade de proliferação do que as células de pacientes com infecção por HTLV-II. Além disso, espera-se que aquelas sejam menos susceptíveis aos efeitos imunomoduladores dos GCs do que estas.

1.2.2. Objetivos Gerais

Avaliar os fenômenos de proliferação celular espontânea e resistência linfocitária a GCs em pacientes infectados com HTLV-I ou HTLV-II.

1.2.3. Objetivos Específicos

- Avaliar a proliferação celular (espontânea e induzida por mitógeno) em pacientes assintomáticos, com diagnóstico positivo para HTLV-I ou HTLV-II, e em sujeitos saudáveis;
- Avaliar a sensibilidade de linfócitos T periféricos de pacientes HTLV-I/II aos efeitos *in vitro* da dexametasona (DEX);
- Classificar os pacientes com HTLV-I/II em sensíveis ou resistentes ao tratamento *in vitro* com DEX;
- Avaliar os resultados de proliferação celular e de sensibilidade à DEX em pacientes HTLV-I/II.

2. ARTIGO CIENTÍFICO – Conforme submissão para CELL PROLIFERATION

Spontaneous cell proliferation is associated to poor sensitivity to glucocorticoids in patients infected with human T-cell lymphotropic virus (HTLV)

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Running title: Spontaneous proliferation and HTLV infection

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Abstract. HTLV-I/II viruses have a special tropism for infecting T cells and inducing spontaneous lymphocyte proliferation. Leukemia and neurological manifestations are associated with HTLV-I/II infections and treatment is usually based on antiinflammatory drugs including glucocorticoids. Although steroid resistance has been reported, it is unknown whether this condition is related to the infection itself or treatment. Here, we investigated whether spontaneous cell proliferation is associated to T-cell sensitivity to glucocorticoids. Patients with HTLV-I/II showed similar unstimulated and stimulated 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 remarkably more resistant to dexamethasone than cells of patients with normal proliferation. These data suggest that the poor clinical response to steroids may be associated to spontaneous cell proliferation during HTLV infection.

2.1. Introduction

Human T-cell lymphotropic virus type I (HTLV-I) and type II (HTLV-II) are retroviruses with a special tropism for infecting 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 it's related diseases, HTLV-I is known to induce adult T-cell leukemia/lymphoma (ATL/L) (Uchiyama *et al.* 1977; Blattner *et al.* 1983; Uchiyama 1988) and HTLV-I–associated 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 a T-cell proliferation with a neoplasic 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 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 found (Murphy & Blattner 1988; Piccardo *et al.* 1988; Ijichi *et al.* 1989). This process leads to spasticity of the lower members, 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 endemic among some native populations from America (Heneine *et al.* 1991; Maloney *et al.* 1992; Hjelle *et al.* 1993; Levine *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 to inappropriately activate T cells and induce diseases characterized by a chronic inflammatory state (Franchini 1995; Hollsberg 1997), treatment of HTLV infections is usually based on antiinflammatory drugs such as synthetic glucocorticoids (GCs). These steroids exert their actions through specific binding to two distinct intracellular receptor subtypes: the mineralocorticoid (MR) and glucocorticoid receptors (GR). After being bound, the receptor-ligand complex translocates to the nucleus, where it either binds to glucocorticoid response elements (GREs) on DNA or interacts with other transcription factors and regulate positive 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 correlate to spontaneous proliferation and peripheral T-cell sensitivity to GCs. The understanding of patient's T-cell sensitivity to GCs prior treatment would be of valuable clinical significance since it will enable physicians to discriminate steroid responders from non-responders. The objectives of this study are (a) to determine patient's peripheral T-cell sensitivity to GCs, (b) to discriminate *in vitro* steroid responders from non-responders and (c) to evaluate whether spontaneous cell proliferation is associated to 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.*

2.2. Materials and Methods

2.2.1. Subjects

Twenty eight, non-medicated 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 females), aged from 15 to 62 years (mean ± SD, 44.89 ± 12.90 yrs) and 10 HTLV-II infected patients (5 females), aged from 30 to 75 years (49.40 ± 13.94 yrs) took part in this study. The diagnosis of HTLV infections was confirmed by Western blots. To discriminate steroid responders from non-responders, 11 healthy subjects (7 females), aged from 21 to 73 years (39.81 ± 18.17 yrs) were also recruited as a control group. Exclusion criteria included infections, acute or chronic inflammatory diseases, heart disease, under nourishment, anaemia, leucopoenia, neoplasia and drug use (including GCs). There were no differences in gender distribution ($\chi^2 = 2.30$, df = 2, $p = 0.32$) or age ($\chi^2 = 1.11$, df = 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.

2.2.2. Collection of peripheral blood and isolation of mononuclear cells

Ten millilitres of peripheral blood was collected by venepuncture in the morning (between 9-10am) and samples stored into lithium-heparin tubes prior to analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over a Ficoll-Hypaque (Sigma) gradient (900 x G, 30 min). After washing, cells were counted by means of microscopy (100x) and viability always exceeded 95%, as judged from their ability to exclude Trypan Blue (Sigma). PBMCs were resuspended in complete

culture medium (RPMI-1640, supplemented with gentamicine 0.5%, glutamine 1%, hepes 1%, fungizone 0.1%, and fetal calf serum 10%; all from Sigma) and adjusted to $3x10^6$ cells/mL.

2.2.3. Lymphocyte proliferation/viability assays

PBMCs were cultured in flat bottomed 96-well microplates in a final concentration of $1.5x10^5$ cells/well in complete culture medium for 96 h at 37ºC in 5% CO_2 atmosphere. Stimulation by the selective T-cell mitogen phytohemagglutinin (PHA; from Gibco) was performed in triplicates (100 μL/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} to 10^{-4} M of DEX (a synthetic GC receptor agonist) was added in duplicates (50μL/well; water-soluble, Sigma) to mitogen-stimulated (PHA 1%) cultures. Glucocorticoid concentrations were used in a range that free endogenous GCs would reach during resting state (10^{-9} M), stress (10^{-6} M) and under pharmacological treatment (10^{-5} M) *in vivo*.

The proliferative responses were estimated by a modified colorimetric 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 the 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. The cell cultures were incubated for 4 h at 37ºC in 5% CO_2 atmosphere. After completely removal of the supernatant, 120 μL of dimethyl sulfoxide (Sigma) was added to each well. The optical density (OD) was determined using Biorad ELISA plate reader at a wavelength of 492 and 630 nm. Spontaneous proliferation was determined by visual identification of several cellular clusters (light microscopy, 40x) in unstimulated cells

following 96h of culture. Proliferation data are presented as OD. The difference between the OD of stimulated and non-stimulated cultures indicates the non-specific T lymphocyte proliferation induced by PHA. Results regarding T-lymphocyte sensitivity to GCs are presented as proliferation percentage, where 100% (basal) represents maximum proliferation, obtained by OD means from cell cultures of PHA 1% without steroids.

2.2.4. Steroid responsiveness

Glucocorticoid responders and non-responders were identified through analysis of dose-response curves of control subjects. PBMCs of healthy controls were cultured with PHA and DEX, as described in the previous section. The area under the curve (AUC) for each control subject was than 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 performed for each HTLV-I/II patient individually. Patients with AUC higher than the median AUC from 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 sensitive to DEX *in vitro*, since their dose-response curve indicate lower proliferation percentages, and were thus classified as responders.

2.2.5. 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 was analyzed by repeated measures ANOVA that included one between-subjects variables (groups) and one within-subjects variables (mitogen or GC levels). Oneway

ANOVA was performed to analyze proliferation (non-stimulated vs. 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-square (χ^2) test. Data are expressed as mean $\pm$ SE in all figures. A statistical software (SPSS 11.5, USA) was used for the analyses. The significance level was set at $\alpha = 0.05$ (two-tailed).

2.3. RESULTS

2.3.1. Lymphocyte proliferation

Mitogen-induced T-cell proliferation was evaluated as an index of cell-mediated immunity. Non-stimulated proliferation was found marginally increased in HTLV-I patients compared to HTLV-II infected individuals (t = 1.43, df = 25.98, *p* = 0.17) and healthy control subjects (t = 1.79, df = 25.42, *p* = 0.09), although it only approached statistically 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.

INSERT FIGURE 1 HERE

2.3.2. 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% (6 patients) of HTLV-I and 10% (1 patient) of HTLV-II respectively (χ^2 = 1.87, df = 1, p = 0.17). Spontaneous proliferation was confirmed by the

presence of several cellular clusters in unstimulated cultures of HTLV-I subjects (Fig. 2). We then assessed to what extent cells of patients who developed spontaneous T lymphocyte proliferation responded to mitogenic stimulation. Interestingly, it was observed that T cells of patients 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 since only one patient presented spontaneous proliferation in that group (Fig. 2B).

INSERT FIGURES 2 and 3 HERE

2.3.3. Lymphocyte sensitivity to glucocorticoids

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 a dose-dependent suppression of T-cell proliferation, $F(5,130) = 38.24$, $p < 0.001$ (Fig. 4). However, T-cell sensitivity to DEX did not differ between HTLV groups, $F(1,26) = 0.60$, $p = 0.44$.

INSERT FIGURE 4 HERE

We also investigated the frequency of GC responders within HTLV groups. Eight HTLV-I (44.4%, 42.38 ± 16.10 years, 5 females) and 4 HTLV-II (40%, 43.25 ± 11.23 years, 2 females) patients were classified as GC non-responders. There were no group differences in the frequency of GC responders/non-responders ($\chi^2 = 0.05$, df = 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).

INSERT FIGURE 5 HERE

We finally assessed whether spontaneous cell proliferation is associated to T-cell sensitivity to GCs. Interestingly, it was observed that T cells of HTLV-I patients with spontaneous proliferation were significantly more resistant to DEX than cells of patients with normal proliferation (Fig. 6A), $F(1,16) = 6.4$, $p < 0.05$. No statistical analysis could be performed within HTLV-II subjects since only one patient presented spontaneous proliferation in that group (Fig. 6B).

INSERT FIGURE 6 HERE

2.4. 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 property to mediate the appearance of diseases with severe prognosis such as ATL/L and HAM/TSP, we initially expected that HTLV-I infected lymphocytes would proliferate more intensively than HTLV-II infected cells. However, T cell proliferation was found similar in both groups of infected patients (Fig 1). These results suggest that HTLV-I virus' capacity of inducing more inflammatory diseases

than HTLV-II may not necessarily be associated to 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 observed a significant proportion of subjects with spontaneous T-cell proliferation within both HTLV-I (33.3%, n=6) and HTLV-II (10%, n=1) infected patients. Here, we investigated to what extent cells of patients with spontaneous T-cell proliferation responded 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 due to the viral infection, do not respond to unspecific activation. Indeed, it has previously been shown that spontaneous proliferation is associated with increased proviral load (Prince & Swanson 1993). This clinical parameter was not assessed here. Further studies should investigate if mitogen unresponsiveness is related to proviral load. It is reasonable to speculate that these patients would be more 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 antiinflammatory drugs such as synthetic GCs. However, some HTLV patients respond poorly to this treatment (Araujo *et al.* 1993) and the 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 the treatment with these steroids may be limited to the peripheral in-

flamed tissues. Interestingly, we observed for the first time that T lymphocytes from HTLV-I patients with spontaneous proliferation were significantly more resistant to DEX than cells of 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 which may justify this discrepancy. For instance, we evaluated the ability of DEX to suppress T-cell proliferation to assess steroid sensitivity of activated lymphocytes whereas Yamano and col. analysed the steroid sensitivity of non-stimulated PBMCs. Therefore, it remains difficult to precise the cellular targets responding 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 since only one subject from the evaluated group of HTLV-II infected patients presented *in vitro* spontaneous proliferation.

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

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2.6. LEGENDS AND FIGURES

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%-phytohemagglutinin (PHA) for 96h and proliferation/viability estimated by MTT assay. Optical density (OD) was determined at a wavelength of 492 and 630 nm. Statistical significance differences are indicated: ** $p < 0.01$; *** $p < 0.001$.

Figure 2. Spontaneous cell proliferation in HTLV-I. Representative photographs of unstimulated cultures of two HTLV-I patients. Figure 2A shows spontaneous proliferation as demonstrated by cellular clusters (40X) that can be seen magnified in 2B (200X). Figure 2C shows normal proliferation.

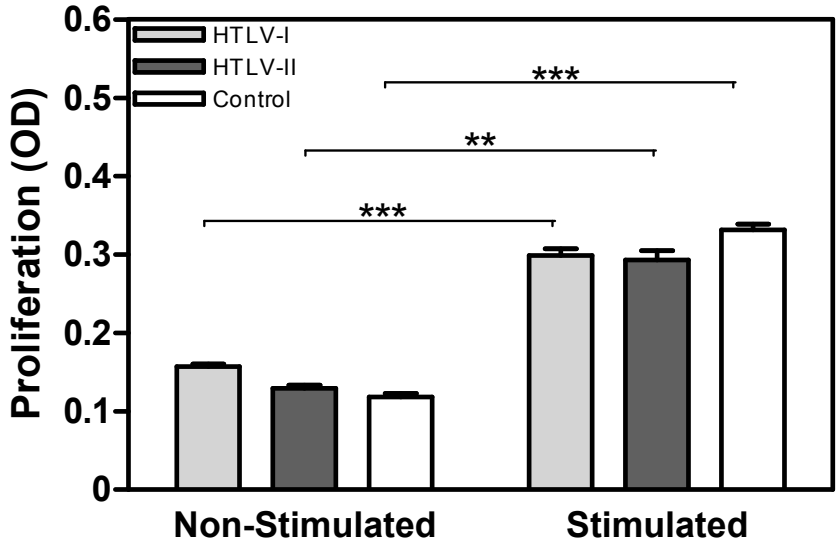
Figure 3. Evaluation of non-stimulated and mitogen-stimulated T-cell proliferation in HTLV infected patients with normal and spontaneous proliferation. PBMCs were cultured with and without 1%-phytohemagglutinin (PHA) for 96h and proliferation/viability estimated by MTT assay. Optical density (OD) was determined at a wavelength 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: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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 during 96h. Proliferation was estimated by MTT assay. Optical density (OD) was determined at a wavelength of 492 and 630 nm. Data are shown as percentage of basal proliferation (100% = PHA 1% without steroids).

Figure 5. Peripheral T-cell sensitivity to dexamethasone (DEX) in responders/non-responders HTLV infected patients. Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX during 96h. Proliferation was estimated by MTT assay. Optical density (OD) was determined at a wavelength of 492 and 630 nm. Data are shown as percentage of basal 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: * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$. Statistically interaction of T-cell sensitivity to the variation of DEX concentrations between groups are indicated: ## $p < 0.01$; ### $p < 0.001$.

Figure 6. Peripheral T-cell sensitivity to dexamethasone (DEX) in HTLV patients with spontaneous/normal proliferation. Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX during 96h. Proliferation was estimated by MTT assay. Optical density (OD) was determined at a wavelength of 492 and 630 nm. Data are shown as percentage of basal 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: * $p < 0.05$; ** $p = 0.01$. Statistically interaction of T-cell sensitivity to the variation of DEX concentrations between groups are indicated: # $p < 0.05$.

Figure 1.


HTLV-I
HTLV-II
Control
Proliferation (OD)
0.6
0.5
0.4
0.3
0.2
0.1
0
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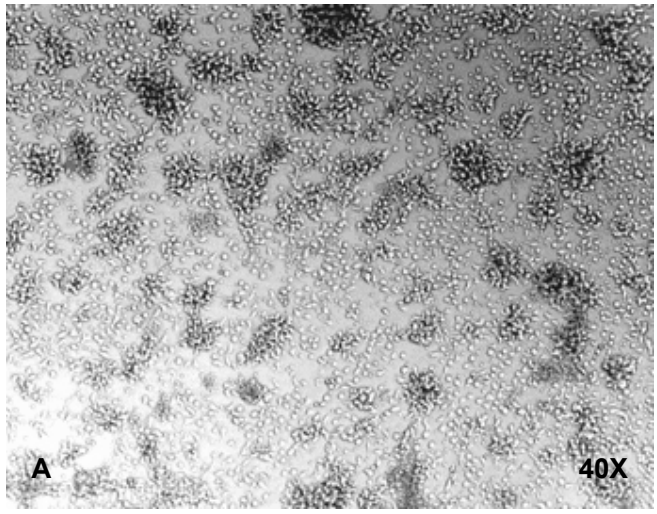
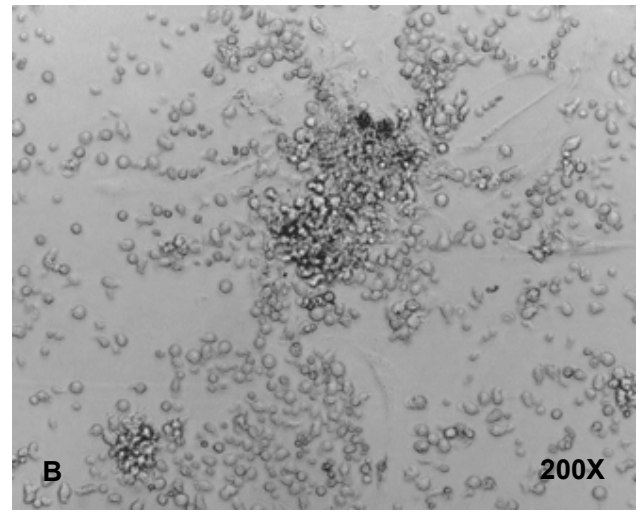
**
***
Non-Stimulated
Stimulated

Figure 2.





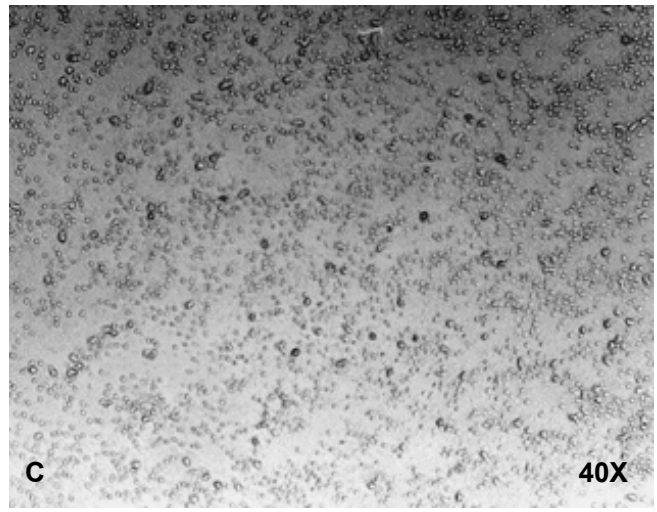
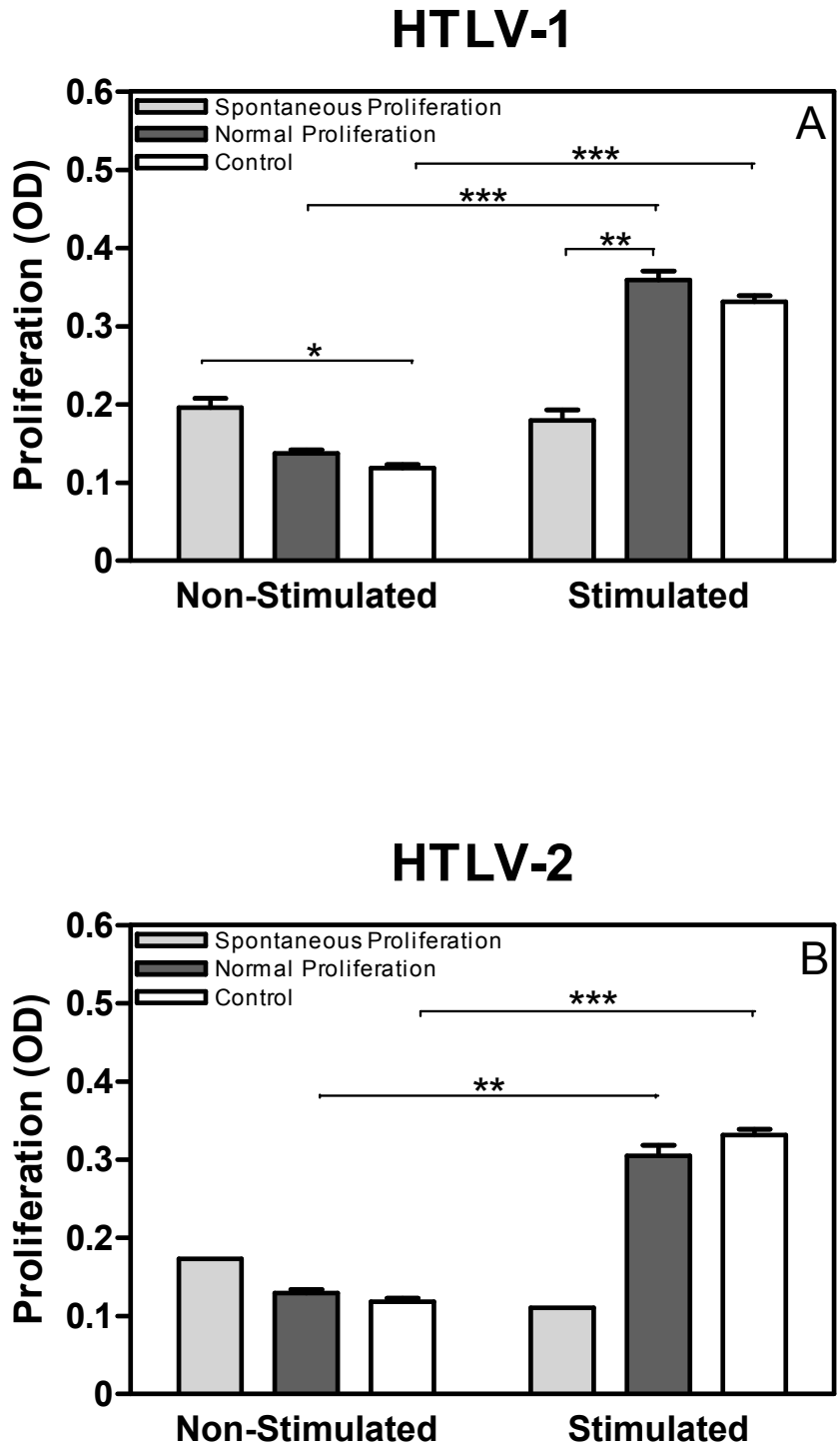


Figure 3.


HTLV-1
Spontaneous Proliferation
Normal Proliferation
Control
A
***
***
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*
Proliferation (OD)
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0.3
0.2
0.1
0
Non-Stimulated
Stimulated
HTLV-2
Spontaneous Proliferation
Normal Proliferation
Control
B
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Proliferation (OD)
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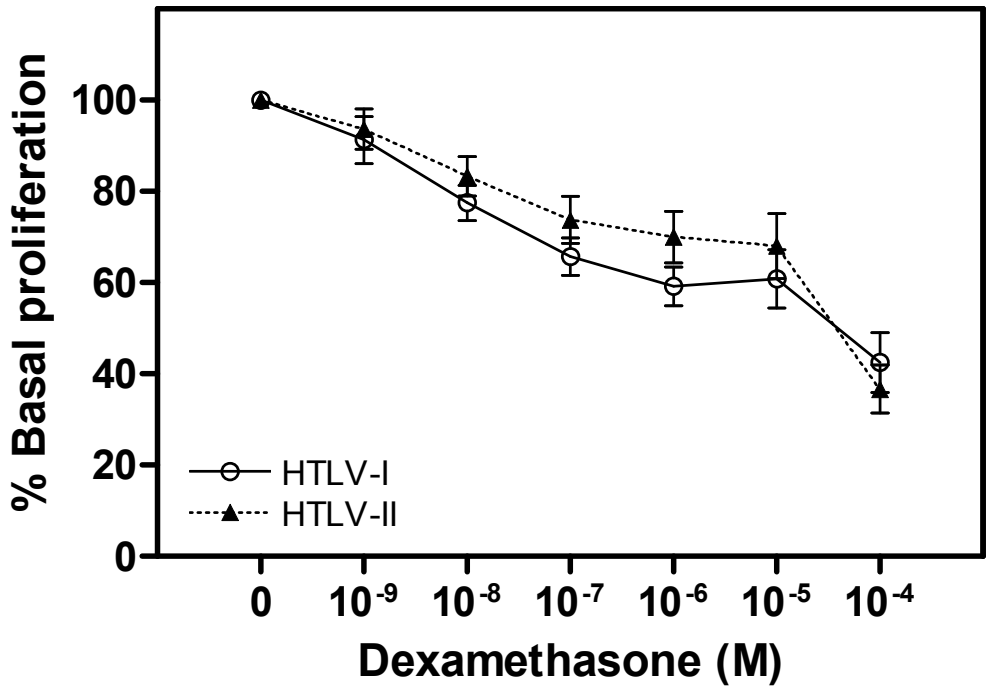
0
Non-Stimulated
Stimulated

Figure 4.


% Basal proliferation
100
80
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40
20
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HTLV-I
HTLV-II
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10-9
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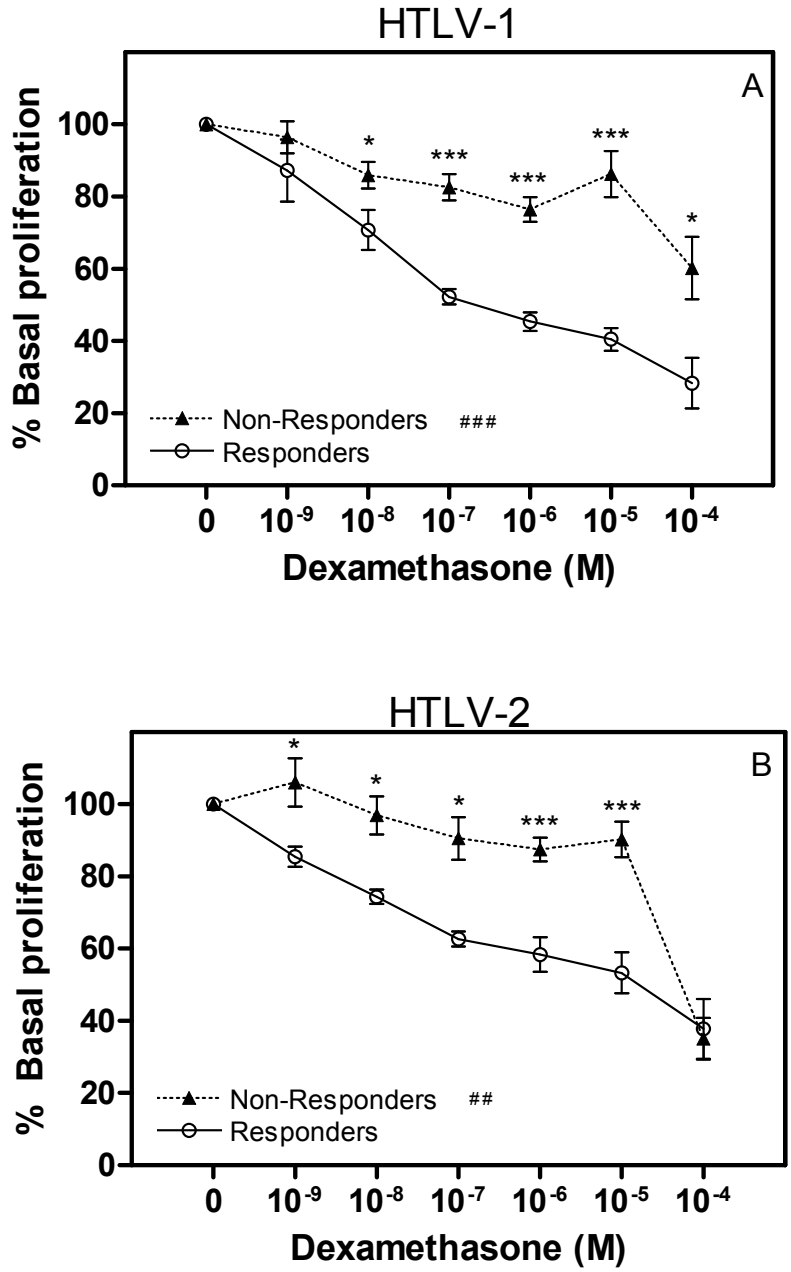
10-5
10-4
Dexamethasone (M)

Figure 5.


HTLV-1
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% Basal proliferation
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*
Non-Responders ###
Responders
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10-8
10-7
10-6
10-5
10-4
Dexamethasone (M)
HTLV-2
B
% Basal proliferation
100
80
60
40
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Non-Responders ##
Responders
0
10-9
10-8
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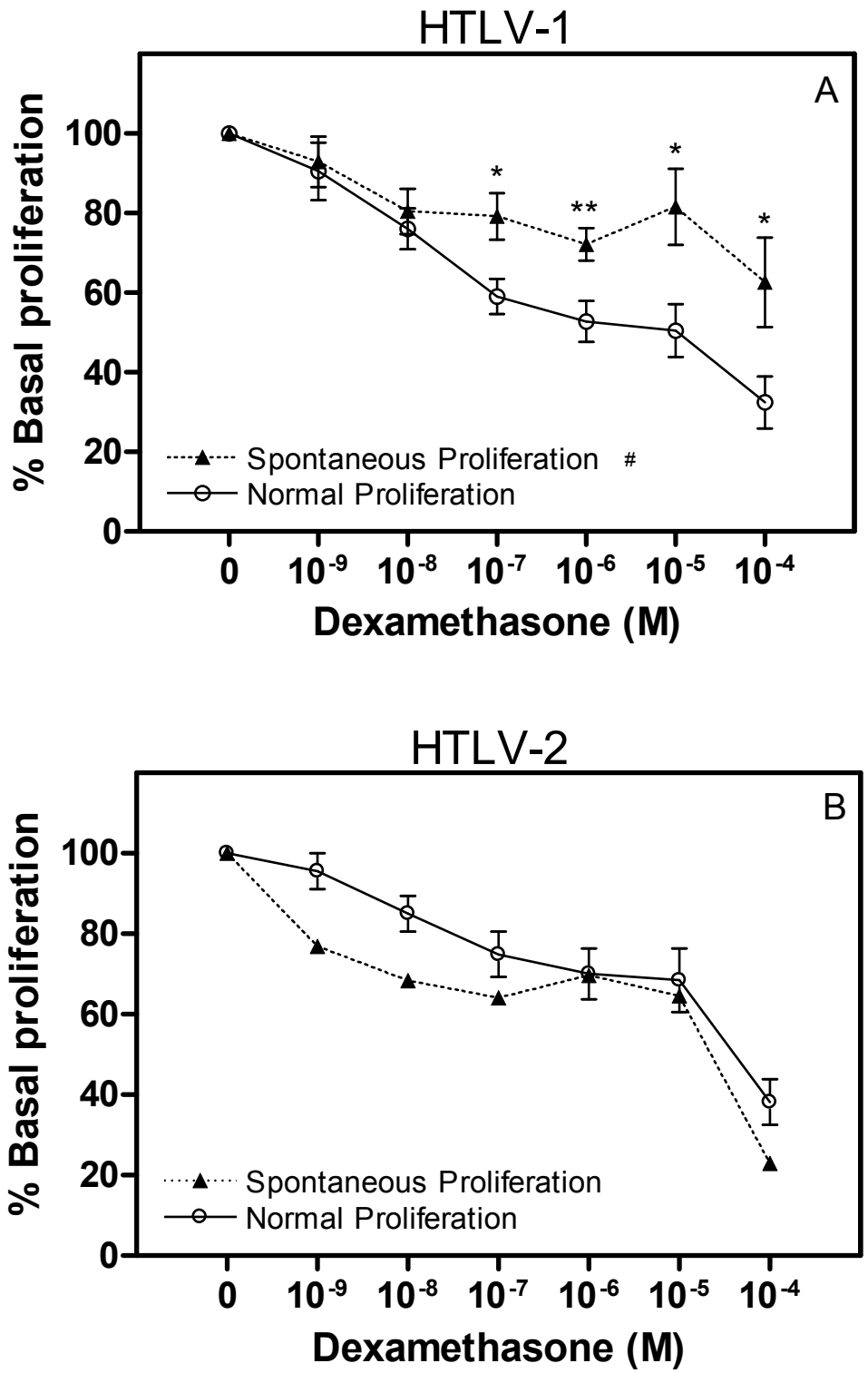
10-4
Dexamethasone (M)

Figure 6.


HTLV-1
A
% Basal proliferation
100
80
60
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*
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Spontaneous Proliferation #
Normal Proliferation
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10-9
10-8
10-7
10-6
10-5
10-4
Dexamethasone (M)
HTLV-2
B
% Basal proliferation
100
80
60
40
20
0
Spontaneous Proliferation
Normal Proliferation
0
10-9
10-8
10-7
10-6
10-5
10-4
Dexamethasone (M)

3. CONSIDERAÇÕES FINAIS

Estados inflamatórios relacionadas a infecção pelos vírus HTLV-I/II são decorrentes do tropismo viral por infectar linfócitos T, promovendo sua ativação e, subseqüente, proliferação descontrolada (1-4, 6, 83). O HTLV-I possui maior distribuição geográfica e está mais associado a doenças do que o HTLV-II, sendo apontado como o agente etiológico de duas doenças graves: a leucemia/linfoma de células T do adulto (ATL/L) (28-30) e a parapareseia espástica tropical ou mielopatia associada ao HTLV-I (HAM/TSP) (31, 32). Embora não haja registros relacionando fortemente o HTLV-II à alguma doença específica, sabe-se que a infecção por este tipo viral pode levar ao desenvolvimento de estados neuropatológicos importantes, com conseqüências clínicas semelhantes às da HAM/TSP (40-42).

Devido à sua propriedade de promover o surgimento de doenças inflamatórias com prognóstico tão grave quanto a ATL/L e a HAM/TSP, era esperado que a proliferação de linfócitos de pacientes com diagnóstico confirmatório de infecção pelo vírus HTLV-I fosse maior que a de células de pacientes com diagnóstico positivo para HTLV-II. Contudo, os resultados obtidos neste estudo demonstraram que tanto nas culturas celulares estimuladas com o mitógeno fitohemaglutinina (proliferação estimulada) quanto nas culturas de células sem mitógeno (proliferação não-estimulada), não houve diferença entre os tipos virais. Ainda, não foi observada diferença entre os resultados de proliferação celular dos pacientes HTLV-I quando comparados a pacientes HTLV-II e a sujeitos saudáveis (controles). Estes resultados sugerem que a maior predisposição de pacientes infectados pelo vírus HTLV-I em desenvolver estados inflamatórios importantes, em relação ao vírus HTLV-II, não se deva necessariamente a uma maior capacidade deste tipo viral em induzir a proliferação de linfócitos T periféricos.

Da mesma forma que em outros trabalhos (1-4, 6, 83), observou-se neste estudo uma significativa proporção de indivíduos com proliferação espontânea entre os pacientes com infecção pelos vírus HTLV-I (33.3%, n=6) e HTLV-II (10%, n=1). Pela primeira vez, foi investigado em pacientes com proliferação celular espontânea, o potencial de proliferação de linfócitos T estimulados pelo mitógeno fitohemaglutinina (PHA). Foi verificado que as células T de pacientes com proliferação espontânea não respondem aos estímulos mitogênicos por PHA. Os dados aqui apresentados sugerem que linfócitos T de pacientes infectados por HTLV, em estado de proliferação espontânea, sejam incapazes de proliferar em resposta a um estímulo inespecífico. Com isso, pode-se especular que pacientes em tais situações tenham respostas imunológicas comprometidas, tornando-se mais suscetíveis a outras infecções cujos mecanismos de defesa sejam dependentes de células T efetoras (ativação e expansão clonal). Estudos realizados com células mononucleares isoladas de sangue periférico de pacientes com HTLV-I com HAM/TSP (84) e de pacientes com HTLV-II (4) mostram correlação entre proliferação espontânea e carga viral. Seria conveniente, no entanto, realizar avaliação semelhante com o objetivo de relacionar carga viral e resposta celular ao estímulo mitogênico.

Conforme citado anteriormente, o tratamento de estados inflamatórios decorrentes de infecções por HTLV envolvem, normalmente, a administração de fármacos da classe dos GCs. Contudo, existem relatos de alguns pacientes com baixas respostas ao tratamento com GCs (81), tornando necessário o emprego de outras drogas imunossupressoras concomitantemente (77). Neste estudo, foi verificado o grau de sensibilidade de linfócitos T periféricos de pacientes HTLV-I/II aos efeitos imunomodulatórios *in vitro* de doses variadas de dexametasona (DEX), um potente GC sintético. Dos indivíduos avaliados neste estudo, tanto os pacientes HTLV-I, quanto

os pacientes HTLV-II apresentaram níveis de resposta similares aos efeitos da DEX. Estes resultados sugerem que a resistência farmacológica de pacientes com estados inflamatórios decorrentes de infecção por HTLV-I/II, freqüentemente observada na clínica, pode estar limitada ao foco inflamatório e não representar um efeito sistêmico, com conseqüências se estendendo às células periféricas.

Ainda, com o auxílio da realização de análises específicas da mesma avaliação de resposta celular à DEX em indivíduos saudáveis (grupo controle), foi possível classificar os pacientes HTLV-I/II como: sensíveis a DEX, com um padrão normal de resposta *in vitro* à variação de doses empregadas; e resistentes à DEX, com células refratárias ao GC, apresentando um padrão de resposta atípico. Mais uma vez, não houve diferença entre a freqüência de sujeitos sensíveis e resistentes à DEX encontrada na avaliação dos pacientes HTLV-I e HTLV-II. Com a combinação destas informações, foi possível correlacionar resistência/sensibilidade de linfócitos T periféricos à dexametasona, com padrão de proliferação celular (espontânea/normal). Pela primeira vez, foi verificado que linfócitos T de pacientes HTLV-I com proliferação espontânea apresentaram maior resistência à imunomodulação por DEX quando comparadas às células de pacientes HTLV-I com proliferação normal. Estes resultados diferem de um estudo similar publicado em 1997 (85), onde células mononucleares de sangue periférico de pacientes com HAM/TSP e com proliferação espontânea *in vitro*, eram sensíveis aos efeitos da prednisolona (redução da proliferação celular e alteração na produção de citocinas). Contudo, diferenças no delineamento dos estudos são capazes de justificar as discrepâncias entre os resultados aqui apresentados e os do referido estudo. Por exemplo, enquanto Yamano e colaboradores avaliaram os efeitos da prednisolona na proliferação de células mononucleares de sangue periférico, este trabalho avaliou os efeitos da dexametasona na proliferação de um

tipo celular específico (linfócitos T), por meio da utilização de um mitógeno seletivo (fitohemaglutinina). Ainda, existe uma diferença crucial entre as populações avaliadas em ambos os estudos. Yamano e colaboradores avaliaram pacientes HTLV-I com diagnóstico de uma severa patologia relacionada à infecção (HAM/TSP). O estudo aqui apresentado avaliou pacientes com diagnóstico confirmatório recente de infecção por HTLV, mas com ausência de sintomas clínicos, sem doença inflamatória relacionada ao vírus e livres de tratamento farmacológico.

Não foi possível observar interação entre os padrões de proliferação celular (espontânea/normal) e sensibilidade *in vitro* à DEX em pacientes HTLV-II. Contudo, a análise foi comprometida pelo baixo número amostral, já que somente um indivíduo do grupo de pacientes avaliados com diagnóstico de infecção por HTLV-II apresentou proliferação celular espontânea. Estudos complementares que se beneficiem de um maior número de pacientes deveriam realizar tal avaliação.

4. CONCLUSÕES

Os dados aqui apresentados apontam para a possibilidade de que a baixa resposta clínica à terapia farmacológica com GCs em pacientes com doenças inflamatórias decorrentes de infecção por HTLV (especialmente HTLV-I) pode estar relacionada a um estado de proliferação espontânea nestes indivíduos. A proliferação linfocitária espontânea por infecção pelos vírus HTLV parece comprometer tanto os mecanismos de ativação de células T frente a um estímulo inespecífico quanto os mecanismos de regulação por GCs. Estes resultados levam a crer que as infecções por HTLV podem levar a um estado de exaustão clonal que leve ao comprometimento de respostas imunológicas frente a outros agentes patogênicos, tornando o paciente mais suscetível a infecções secundárias e oportunistas. De fato, estudos anteriores citam a ocorrência de doenças secundárias e infecções oportunistas em pacientes com infecção por HTLV-I que desenvolveram estados de imunodeficiência: gammopatias monoclonais; falência renal crônica; hiperinfecção por *Strongyloides stercoralis* além de infecções oportunistas pulmonares por *Mycobacterium tuberculosis*, *Pneumocystis carinii*, cytomegalovirus, *Aspergillus fumigatus* e *Cryptococcus neoformans* (37, 38, 86-89).

Pacientes com infecção por HTLV e quadro de proliferação espontânea são, possivelmente: mais susceptíveis ao desenvolvimento de doenças mais severas relacionadas à infecção viral, podem ser menos responsivos às terapias farmacológicas com GCs e podem vir a apresentar quadros de infecções secundárias e oportunistas. Portanto, identificar dentre os pacientes infectados pelos vírus HTLV, aqueles que apresentem padrão de proliferação celular espontânea, pode vir a ter um grande papel no monitoramento clínico desses indivíduos.

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6. ANEXO 1: Cópia do artigo científico publicado

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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

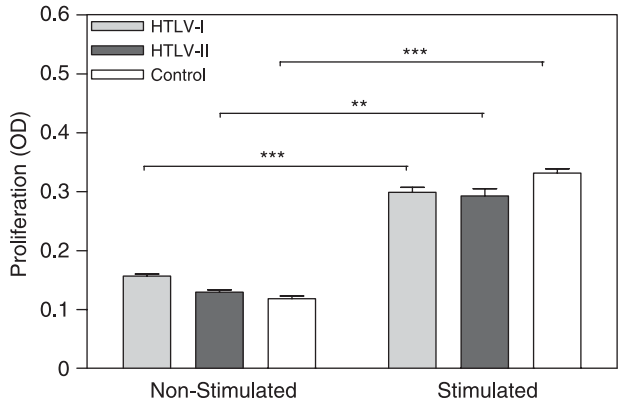

Figure 1. Evaluation of non-stimulated and mitogen-stimulated T-cell proliferation (HTLV-I: $n = 18$; HTLV-II: $n = 10$; control: $n = 11$). PBMCs were cultured with and without 1% PHA for 96 h and cell proliferation/viability was estimated by MTT assay. OD was determined at wavelengths of 492 and 630 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.

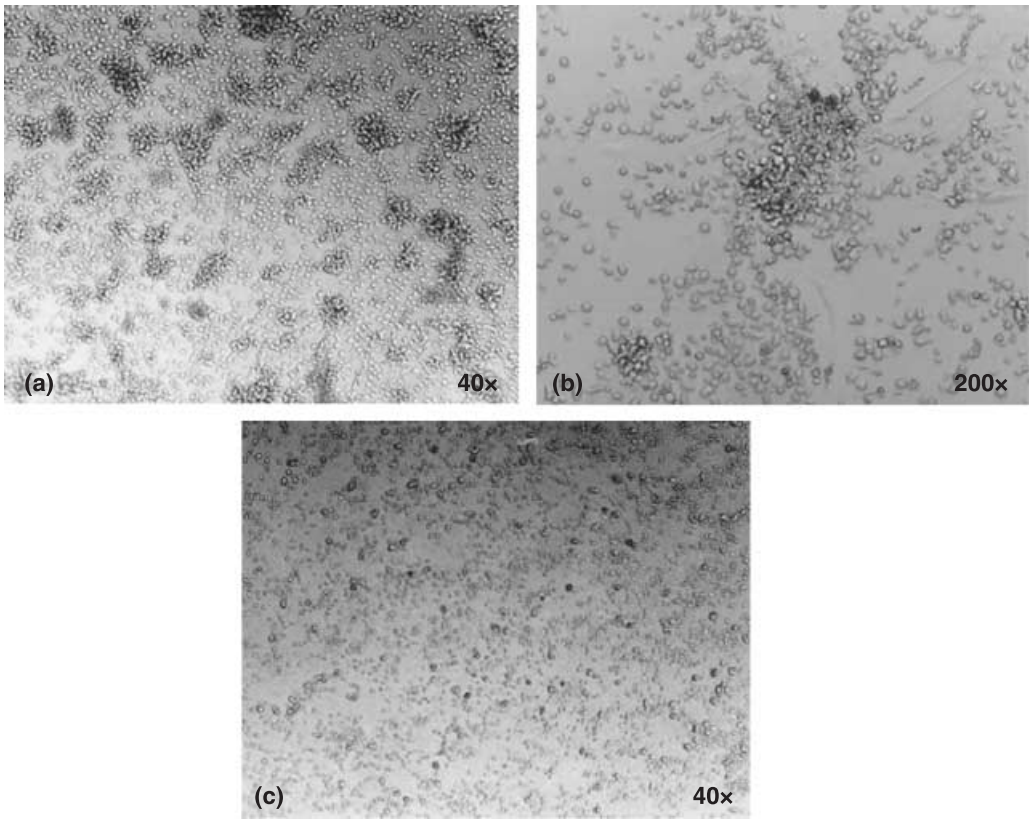

Figure 2. Spontaneous cell proliferation in HTLV-I infection. Representative photographs of unstimulated cultures of two HTLV-I patients. (a) Spontaneous cell proliferation as demonstrated by cellular clusters (40×) that can be seen magnified in (b) (200×). (c) Cells with a normal proliferation level.

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

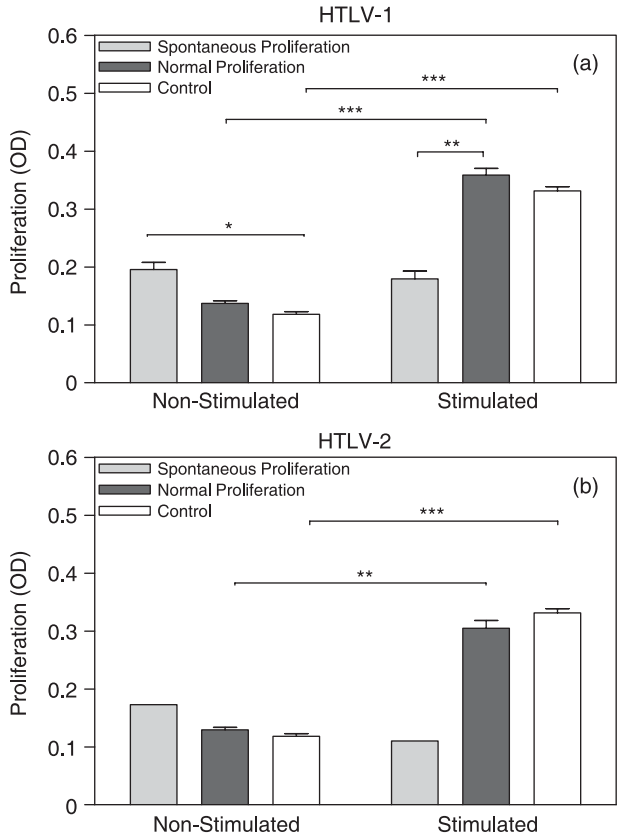

Figure 3. Evaluation of non-stimulated and mitogen-stimulated T-cell proliferation in HTLV-infected patients with normal and spontaneous proliferation levels. PBMCs were cultured with and without 1% PHA for 96 h and proliferation/viability was estimated by MTT assay. OD was determined at wavelengths 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).

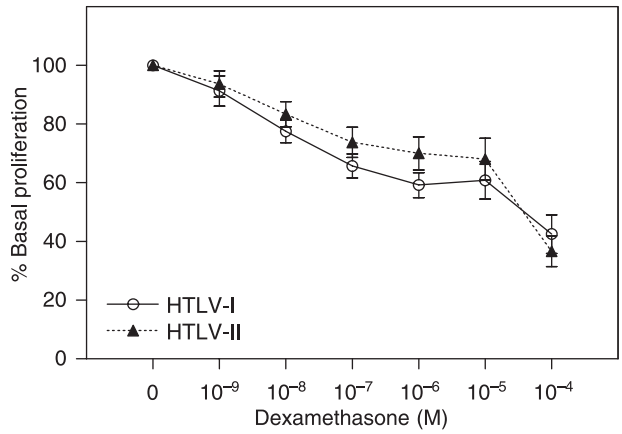

Figure 4. Peripheral T-cell sensitivity to dexamethasone (HTLV-I: $n = 18$; HTLV-II: $n = 10$). Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX for 96 h. Cell proliferation was estimated by MTT assay. OD was determined at wavelengths of 492 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

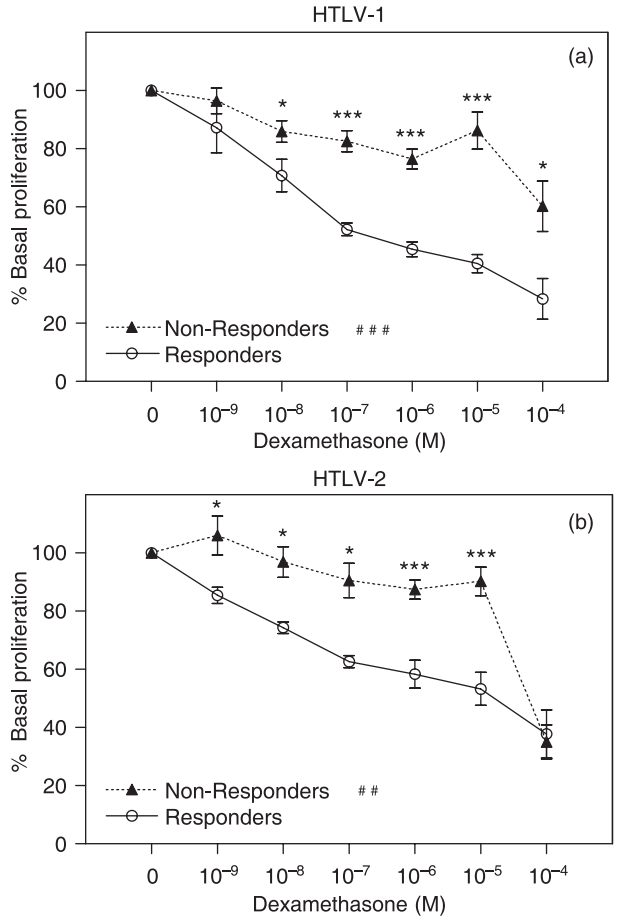

Figure 5. Peripheral T-cell sensitivity to DEX in responders/non-responders, HTLV-infected patients. Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX for 96 h. Cell proliferation was estimated by MTT assay. OD was determined at wavelengths of 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

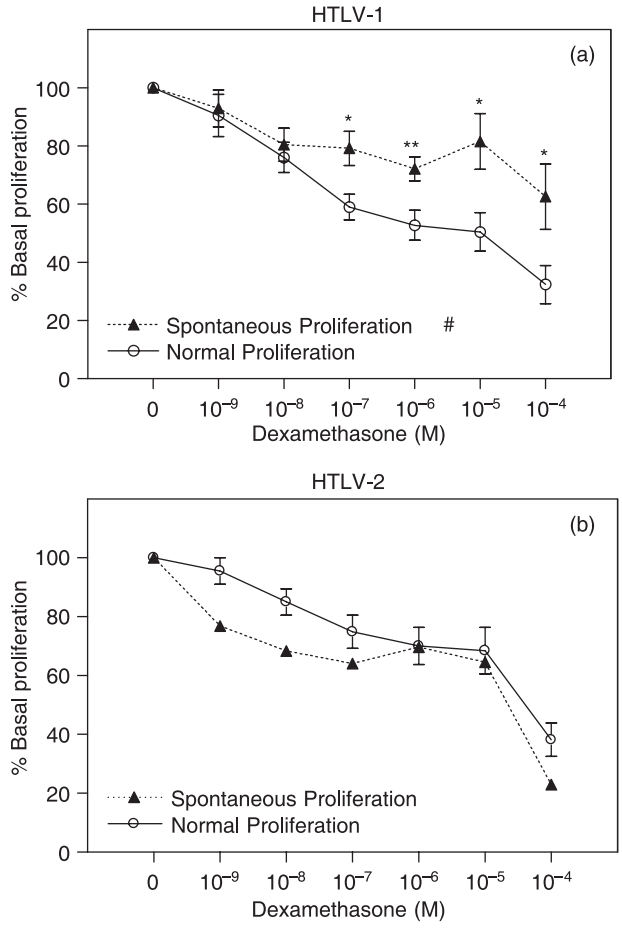

Figure 6. Peripheral T-cell sensitivity to DEX in HTLV patients with spontaneous/normal cell proliferation. Glucocorticoid sensitivity was assessed by incubating PBMCs with PHA 1% and increasing concentrations of DEX for 96 h. Cell proliferation was estimated by MTT assay. OD was determined at wavelengths 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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