UNIVERSIDADE FEDERAL DA BAHIA

INSTITUTO DE CIÊNCIAS DA SAÚDE

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MODULAÇÃO DA RESPOSTA IMUNE EM INDIVÍDUOS INFECTADOS PELO HTLV-I E EXPRESSÃO DE DOENÇA

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"A coisa mais bela que o homem pode

experimentar é o mistério. É essa emoção

fundamental que está na raiz de toda ciência e arte".

(Albert Einstein)

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meus filhos André Luis e Luis Felipe!

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(Cora Coralina)

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B7.1 e B7.2	Moléculas co-estimulatórias
CD	"Cluster of differentiation" – marcador de membrana de células
CMSP	Células mononucleares do sangue periférico
EAE	Encefalomielite experimental auto-imune
G e GM-CSF	Fator estimulador de colônias de granulócitos e macrófagos
HLA	Antígeno leucocitário humano
ICAM – 1	Molécula de adesão intracelular -1
IFN-γ	Interferon - γ
IL	Interleucina
IL-R	Receptor de Interleucina
LFA-3	Antígeno 3 associado à função de leucócitos
LTC	Linfócito T citotóxico
LTRs	Longas terminações repetidas
MIP-1α e 1β	Proteína inflamatória de macrófagos 1α e 1β
MMP-2 e 9	Metaloproteinases 2 e 9
mRNA	Ácido ribonucleíco mensageiro
NK	Células "Natural Killer"
NOD	"Non obese diabetic" - camundongos diabéticos não obesos
PPD	"Purified protein derivative"
T CD4+	Linfócitos T auxiliares CD 4 positivo
T CD8+	Linfócitos T citotóxicos CD 8 positivo
TCR/MHC	Receptor da célula T / Complexo maior de Histocompatibilidade
TFG-β	Fator de crescimento e transformação β
Th1	Linfócitos T auxiliar tipo 1
Th2	Linfócitos T auxiliar tipo 2
TNFα	Fator de necrose tumoral - α
SNC	Sistema nervoso central
SWAP	Antígeno bruto do verme adulto do *Schistosoma sp*.
VCAM -1	Molécula de adesão celular vascular – 1

SUMÁRIO

RESUMO

O vírus linfotrópico de células T humanas tipo 1 (HTLV-I) é um retrovírus que infecta preferencialmente células T, causando grandes alterações na resposta imune. A maioria dos indivíduos infectados é assintomática (portadores do HTLV-I) e somente uma minoria desenvolve doenças como a leucemia/linfoma de células T do adulto (LLcTA) ou a mielopatia associada ao HTLV-I / paraparesia espástica tropical (HAM/TSP). A resposta imune nos indivíduos infectados pelo HTLV-I é caracterizada por uma ativação intensa e persistente das células T com proliferação e produção espontânea de citocinas pro-inflamatórias como IFN-γ e TNF-α. Este estudo avalia a resposta imune de indivíduos infectados pelo vírus e a capacidade de citocinas, antagonistas de citocinas e infecções causadas por helmintos de modular esta resposta. A resposta imune foi determinada pelo perfil de citocinas presentes no sobrenadante de cultura de células mononucleares não estimuladas, descrição das populações de células envolvidas na resposta imune e na avaliação da freqüência e tipo de células que apresentam marcadores de ativação celular e secretam citocinas. Pacientes com HAM/TSP apresentaram uma ativação celular intensa caracterizada por uma maior proliferação e produção espontânea de IFN-γ e TNF-α, maior freqüencia de linfócitos T CD4+ e CD8+ produzindo IFN-γ e TNF-α e um aumento de células T CD8+/CD28- quando comparados com indivíduos assintomáticos. Observou-se, entre os portadores assintomáticos, uma grande variabilidade na produção de IFN-γ, com alguns indivíduos apresentando concentrações similares às observadas nos pacientes com HAM/TSP. A adição de citocinas supressoras da resposta imune (IL-10 e TGF-β) e antagonistas de citocinas (anti-IL-2, anti-IL-12 e anti-IL-15) às culturas de células dos indivíduos infectados pelo HTLV-I mostrou que a IL-10 e a adição simultânea de anti-IL-2 e anti-IL-15 reduzem significantemente a síntese espontânea de IFN-γ em indivíduos assintomáticos quando comparados com HAM/TSP. Adicionalmente,

foi identificado um sub-grupo de indivíduos assintomáticos, freqüentemente alto produtores de IFN-γ, que não apresentam atividade imunoregulatória após adição de citocinas e antagonistas de citocinas, comportando-se imunologicamente como os pacientes com HAM/TSP. A co-infecção de indivíduos assintomáticos infectados pelo HTLV-I com helmintos (*Strongyloides stercoralis e Schistosoma mansoni)* resultou na diminuição da síntese de IFN-γ, menor freqüência de células T CD4+ e CD8+ produzindo IFN-γ e uma maior freqüência de células produzindo IL-5 e IL-10. Quando comparados com portadores assintomáticos, sem co-infecção por helmintos, indivíduos co-infectados apresentaram uma carga proviral menor. Adicionalmente, observou-se uma maior prevalência de infecções por helmintos entre os portadores assintomáticos do que nos pacientes com HAM/TSP. Estes resultados sugerem que a co-infecção por helmintos reduz a ativação das células tipo 1 e influencia a expressão clínica da infecção pelo HTLV-I.

INTRODUÇÃO

O vírus linfotrópico de células T humanas tipo I (HTLV-I) é um vírus oncogênico pertencente à família *Retroviridae*. O HTLV-I foi inicialmente isolado de um paciente com linfoma cutâneo de células T e posteriormente descrito como o primeiro retrovírus humano associado com quadros de leucemia/linfoma de células T do adulto, LLcTA (ATLL, a*dult T cell leukemia/lymphoma*) (Poieszi *et al.*, 1980). O envolvimento do HTLV-I com doenças neurológicas humanas foi relatado primeiro na Martinica, em um grupo de pacientes com paraparesia espástica tropical (Gessain *et al.*, 1985), e depois no Japão, em pacientes com mielopatia (Osame *et al.*, 1986). Estabeleceu-se finalmente que se tratava de uma mesma doença, que tinha como agente etiológico o HTLV-I, passando então a ser denominada de paraparesia espástica tropical/mielopatia associada ao HTLV-I (HAM/TSP). A maioria dos indivíduos infectados pelo HTLV-I é assintomática e somente uma pequena proporção desenvolve ATLL ou HAM/TSP (Hollsberg & Hafler,1993). Em menor freqüência são descritas outras doenças inflamatórias associadas ao HTLV-I como artrite (Nishioka et *al.*,1989), polimiosite (Morgan *et al.*,1989), uveíte (Mochizuki *et al.*,1992), alveolite (Sugimoto *et al.*,1987), dermatite infectiva em crianças (Lagrenade *et al.*, 1990) e Sindrome de Sjögren (Terada *et al.*, 1994).

Aproximadamente 20 milhões de pessoas em todo o mundo estão infectadas pelo HTLV-I (Edlich *et al.*, 2000). O Sul do Japão, as ilhas do Caribe (Jamaica e Trinidad-Tobago), a América do Sul e várias regiões do continente africano são reconhecidamente áreas de grande endemicidade da infecção. Na Europa e na América do Norte a infecção pelo HTLV-I é descrita somente entre imigrantes de áreas endêmicas e usuários de drogas injetáveis. No Brasil, soroprevalências muito variadas são detectadas nas várias regiões do país. Recentemente, na Bahia, um estudo de base populacional revelou uma prevalência de

1,76% na população geral (Dourado *et al.,* 2003). Entre doadores de sangue, a cidade de Salvador na Bahia, registra uma das mais altas soroprevalências da infecção pelo HTLV-I (1,35%) (Galvao-Castro *et al.*, 1997), fazendo desta infecção um problema relevante de saúde pública.

O HTLV-I é transmitido de forma mais eficiente pela transferência de linfócitos infectados. A principal rota de transmissão da infecção é da mãe para o filho no período pós-natal, pelo aleitamento materno, enquanto que a transmissão pré-natal (transplacentária) não é muito comum. Segue-se então, a transmissão pelo contato sexual e a transmissão por meio de sangue contaminado, em transfusões sanguíneas ou objetos contaminados e compartilhados entre usuários de drogas.

O HTLV-I tem a capacidade de infectar *in vitro* grande quantidade de células incluindo linfócitos B, macrófagos, células NK, células da glia, células endoteliais e fibroblastos (Ho *et al.*, 1984; Yoshikura *et al*., 1984; De Revel *et al*., 1993). I*n vivo*, este vírus apresenta um tropismo especial por células T CD4+ (Richardson *et al.*, 1990) e células T CD8+ (Nagai *et al.*, 2001a). A capacidade de infectar vários tipos celulares (Trejo & Ratner, 2000) sugere que o HTLV-I possua um receptor específico cuja natureza (Nath *et al.*, 2003; Manel *et al*., 2003) e a forma de como permite a entrada do vírus na célula somente agora começam a ser esclarecidos (Igakura *et al.*, 2003).

INTERAÇÃO DO HTLV-I COM O SISTEMA IMUNE

O HTLV-I, como outras retroviroses, é um vírus envelopado constituído de duas fitas de RNA que utiliza a transcriptase reversa para transcrever o RNA viral em DNA proviral e integrar-se no genoma da célula hospedeira. O HTLV-I possui 9.032 pares de base e apresenta no genoma, regiões comuns a outras retroviroses, além de uma região que codifica produtos gênicos únicos do vírus. Nas extremidades do genoma do HTLV-I observam-se as longas terminações repetidas (LTR 5`e 3`) e no centro, regiões que codificam um grupo de genes estruturais (*gag*, *pro/pol* e *env* que codificam respectivamente proteínas da matriz viral,

capsídeo e nucleocapsídeo, enzimas como transcriptase reversa, integrases, proteases e as proteínas do envelope viral) e a região *pX*, que codifica produtos gênicos únicos do HTLV-I (*tax e rex*). O principal deles é o gene tax que ativa a transcrição das LTRs, aumentando a replicação viral e a expressão de vários genes celulares. A proteina viral, tax, utiliza vários mecanismos para amplificar o genoma viral (Yoshida, 2001). O aumento da expressão de importantes fatores de transcrição celular como, por exemplo, o NFkB, transativa genes que codificam a expressão de citocinas e receptores de citocinas envolvidos no crescimento e proliferação de células T (Buckle *et al.*, 1996). A ativação do NFkB pela proteína transativadora tax, induz a transcrição da IL-2 e IL-2R (Ballard *et al.*, 1988), IL-6 (Muraoka *et al.*, 1993), IL-15 (Azimi *et al.*, 1998), IL-15R (Mariner *et al.*, 2001), G-CSF e GM-CSF (Himes *et al.*, 1993) criando um ambiente que favorece uma intensa proliferação das células T e a produção espontânea de citocinas. O tax também induz, via NFkB, a expressão de um inibidor da apoptose (Bcl-X_L) tornando as células infectadas resistentes ao processo de morte celular programada (Tsukahara *et al.*, 1999). Desta forma, além da proteina tax aumentar a expressão de genes relacionados ao crescimento celular, ela age também reprimindo a expressão de genes envolvidos no reparo do DNA e apoptose (*apud* Grant *et al.*, 2002). Adicionalmente, tax inibe proteinas supressoras de tumores (Yoshida, 2001) e bloqueia a interação de proteinas envolvidas na sinalização celular, inativando a função do TGF-β (Mori *et al.*, 2001; Lee *et al.*, 2002). Todos estes mecanismos utilizados por tax, para induzir proliferação e transformação das células infectadas e impedir a apoptose, são estratégias utilizadas pelo vírus para amplificar seu genoma proviral.

Este estado de ativação permanente observado nas células infectadas pelo HTLV-I escapa dos mecanismos normais de controle da ativação celular. Normalmente, a ativação das células T necessita de dois sinais. O primeiro é antígeno específico e dependente da interação do receptor da célula T com as moléculas do complexo principal de histocompatibilidade (TCR/MHC). O segundo sinal co-estimulatório não é antígeno específico e depende de

moléculas expressas nas células T, como o CD28, e de seus ligantes (B7.1 e B7.2) expressos nas células apresentadoras de antígeno. Na infecção pelo HTLV-I, a presença de sinais co-estimulatórios envolvendo o CD28 e B7.1/B7.2 e a restrição ao MHC não são necessários para que as células infectadas proliferem ou secretem citocinas (Popovic *et al.*,1984; Scholz *et al.,* 1996), caracterizando uma proliferação desregulada. Adicionalmente, o HTLV-I utiliza vias de ativação que são insensíveis à ação supressora do TGF-β (Hollsberg *et al.*, 1994).

A ativação das células T pelo HTLV-I ocorre por diferentes mecanismos (Hollsberg, 1999). O mais estudado é o mecanismo que envolve a ativação da própria célula infectada pelo vírus. A ativação de fatores de transcrição celular, pela proteína viral tax, aumenta a expressão constitutiva do IL-2R e a produção de citocinas como IL-2 e IL-15, envolvidas na manutenção e proliferação celular. Outra via alternativa de ativação celular é representada pelo CD2 e seu ligante CD58 (LFA-3), capazes de aumentar a propagação do vírus pelo contato das células T infectadas com células T não infectadas (Kimata *et al.*, 1993; Wucherpfenning *et al.,* 1992; Guyot *et al.*, 1997). Após este contato célula-célula, ocorre também indução de uma produção intensa de citocinas que faz com que o sistema imune fique sempre ativado e propagando continuamente o vírus. Como conseqüência desta ativação persistente, os linfócitos ficam hiperativados e proliferam intensamente. Esta linfoproliferação espontânea, na ausência de estímulos exógenos, caracteriza a infecção pelo HTLV-I e é demonstrada, *in vitro*, por uma maior incorporação de timidina no DNA das células (Jacobson *et al.*, 1988; Itoyama *et al*., 1988). A alta expressão de marcadores de ativação celular (MHC classe II e IL-2Rα) são alterações secundárias ao processo de replicação ativa do HTLV-I e também fornecem evidências diretas desta ativação persistente (Hollsberg & Hafler, 1993; Macchi *et al.,* 1988).

Outra alteração importante observada após interação das células T com HTLV-I é o aumento da expressão de moléculas de adesão sobre as células infectadas. ICAM-1, VCAM-1, LFA-3 e L-selectina (CD62L) são algumas das moléculas induzidas após ativação

transcricional de seus genes pela proteína viral (Valentin *et al.*, 1997; Al-Fahim *et al.*, 1999).

RESPOSTA IMUNE NA INFECÇÃO PELO HTLV-I

A resposta imune ao HTLV-I difere da resposta observada para outras infecções crônicas causadas por vírus. Os mecanismos naturais de controle baseados na produção de Interferons (IFN) do tipo 1 (α e β) e na lise mediada pelo complemento não são suficientes para controlar a infecção pelo HTLV-I.

Com relação à resposta imune humoral tem sido bem documentado que apesar do estabelecimento de uma intensa resposta humoral, o vírus normalmente persiste. Anticorpos contra glicoproteínas do envelope viral, principalmente gp46, são detectados em mais de 95% dos indivíduos infectados (*apud* Lal, 1996), entretanto, a maioria destes anticorpos não são capazes de neutralizar a atividade viral (*apud* Carrington *et al*., 1996). É provável que por estar integrado no DNA das células e raramente ser encontrado no meio extracelular, o HTLV-I não seja destruído nem neutralizado pelos anticorpos.

A resposta imune celular é dependente da geração de células T auxiliares CD4+ e também de linfócitos T citotóxicos CD8+ com a finalidade de lisar células infectadas. A ativação dos linfócitos T citotóxicos (LTC) pelos antígenos do HTLV-I, principalmente tax, associado com a secreção de citocinas antivirais como IFN-γ e TNF-α são etapas importantes para diminuição da carga proviral do HTLV-I.

A resposta imune no curso da infecção pelo HTLV-I apresenta-se de forma diferente nos pacientes que desenvolvem doenças associadas a este vírus. Quando comparado com indivíduos assintomáticos, pacientes com HAM/TSP apresentam uma resposta imune humoral e celular muito mais intensa. A HAM/TSP é uma doença desmielinizante crônica e progressiva cujos sintomas iniciais incluem fraqueza muscular, espasticidade de membros inferiores, hiperreflexia do tendão patelar, sinal de Babinsky e distúrbios sensoriais. Disfunção autonômica caracterizada por distúrbios urinários (aumento da freqüência urinária,

urgência, incontinência, disúria e sensação de esvaziamento incompleto da bexiga), distúrbios intestinais e disfunção erétil são também descritas (Araujo *et al.*, 1998).

Indivíduos com HAM/TSP apresentam no soro e fluido cérebro espinhal, títulos elevados de anticorpos anti-HTLV-I (Osame *et al.*, 1986). Quando comparados com indivíduos assintomáticos, pacientes com HAM/TSP apresentam uma resposta mais intensa das células T, evidenciada por uma maior linfoproliferação (Itoyama *et al.*, 1988; Sakai *et al.*, 2001) e produção espontânea de citocinas pró-inflamatórias como IFN-γ, TNF-α e IL-2 (*apud* Jacobson, 1996).

Por induzir a proliferação clonal de células infectadas pelo HTLV-I, a tax tem sido considerada o principal alvo da resposta imune mediada por células T citotóxicas (Jacobson *et al.*,1990; Bieganowska *et al.,* 1999). Grande quantidade de linfócitos T citotóxicos CD8+ específicos para tax tem sido demonstrada no sangue periférico, líquor e nas lesões inflamatórias de pacientes com HAM/TSP quando comparada com indivíduos assintomáticos (Umehara *et al*., 1994; Kubota *et al.,* 1998; Sakai *et al.,* 2001; Nagai *et al*., 2001b). Esta alta proporção de células T CD8+, continuamente ativadas pelos antígenos do HTLV-I, é mantida pela IL-15, uma citocina importante na manutenção das células T CD8+ de memória (Zhang *et al.,* 1998) e na redução da apoptose (Bulfone-Paus *et al.,* 1997). A expressão de mRNA para IL-15 está aumentada nas células mononucleares de pacientes com HAM/TSP (Azimi *et al.*, 1999) e esta citocina tem um papel importante na persistência de células T CD8+ específicas para tax (Azimi *et al.*, 2001).

Um dos aspectos importantes na infecção pelo HTLV-I é o papel dos LTC na resposta imune contra os antígenos virais. Apesar desta resposta ser de um lado benéfica, ela pode também ser deletéria para o indivíduo infectado. Os LTCs utilizam tanto o mecanismo de lise dependente de perfurina, quanto sua capacidade de produzir grande quantidade de metaloproteinases (MMP-2 e MMP-9), quimiocinas (MIP-1α e MIP-1β) e citocinas pro-inflamatórias (Bissiaon *et al.,* 1997; Kubota *et al.,* 1998) para eliminarem diretamente células

alvos expressando antígenos do HTLV-I, independentes destas serem linfócitos T CD4+, CD8+ ou células residentes do SNC. Entretanto, apesar desta intensa resposta citotóxica inicial, estes LTC CD8+ não são capazes de eliminar totalmente o vírus e a permanência destes no líquor, aumenta a quantidade de citocinas pro-inflamatórias com capacidade de lesar diretamente o sistema nervoso central caracterizando uma resposta deletéria (Nagai & Osame, 2003).

Embora não totalmente elucidada, a patogênese da HAM/TSP parece estar associada a esta intensa resposta imune do hospedeiro (Bangham, 2000; Jacobson, 2002). Acredita-se que a carga proviral influencie o desenvolvimento e progressão para HAM/TSP uma vez que esta se apresenta mais alta em pacientes com mielopatia do que em indivíduos assintomáticos (Nagai *et al.,* 1998; Yamano *et al.,* 2002). Fatores relacionados ao vírus e/ou fatores próprios do hospedeiro também participam deste processo (Nakane *et al.*, 2000). Tem sido descrita uma possível susceptibilidade genética para o desenvolvimento da HAM/TSP (Sonoda *et al.*,1996). Em portadores assintomáticos a presença do haplótipo HLA-A*02 ou HLA-Cw*08 foi associado com uma redução significativa tanto na carga proviral quanto no risco para desenvolver HAM/TSP (Odds ratio [OD]=0.42, enquanto a expressão de HLA-B*5401 foi associada com alta carga proviral e maior susceptibilidade para desenvolver HAM/TSP (OD=0.6), (Jeffery *et al.*, 1999; Jeffery *et al.,* 2000; Vine *et al.*, 2002).

Embora a maioria dos estudos evidenciem o papel dos LTC CD8+ na patogênese da mielopatia pelo HTLV-I (Nagai & Jacobson, 2001d; Jacobson, 2002) outros trabalhos sugerem que as células T CD4+ também apresentam um papel importante, principalmente na fase inicial do processo patogênico (Goon *et al.*, 2003). A população de células T CD4+ além de ser a mais infectada pelo vírus é também a que mais secreta espontaneamente citocinas pró-inflamatórias (Richardson *et al.*,1990; Hanon *et al.,* 2000). Adicionalmente tem sido descrita uma maior quantidade de células T CD4+ específicas para HTLV-I produzindo IL-2,

IFN-γ e TNF-α em pacientes com HAM/TSP do que em indivíduos com infecção assintomática (Goon *et al.*, 2003; Goon *et al.*, 2002).

A ausência de modulação da resposta imune faz com que a produção exagerada de citocinas pró-inflamatórias (IFN-γ e TNF-α) pelas células T CD4+ e CD8+ infectadas pelo HTLV-I sejam fatores contribuintes para a patogênese da doença (Nishiura *et al.*, 1996; Hanon *al.*, 2001). Estas citocinas são encontradas em concentrações mais altas no sangue, líquor e lesões da medula espinhal de pacientes com HAM/TSP quando comparados com indivíduos assintomáticos (Nakamura *et al.*, 1993; Umehara *et al.*,1994; Nagai *et al.*, 2001d). Embora essas alterações imunológicas sejam descritas principalmente em pacientes com HAM/TSP são também observadas em uma parcela dos portadores assintomáticos, tornando este sub-grupo de indivíduos infectados clinicamente importante para o estudo da evolução da infecção pelo HTLV-I (Daisley *et al.*,1993; Kramer *et al.*,1989; Daenke *et al.*,1996; Nagai *et al.*,1998). O perfil de citocinas produzidas em resposta ao HTLV-I evidencia um padrão predominantemente Th1, com aumento da produção de IFN-γ e TNF-α. Entretanto, células de portadores assintomáticos infectados pelo HTLV-I mostram que tanto citocinas do tipo 1 quanto citocinas do tipo 2 encontram-se elevadas quando comparadas com controles negativos para HTLV-I. A despeito da produção de IFN-γ ser mais elevada nos indivíduos assintomáticos quando comparadas com outras citocinas, existe uma grande variabilidade na produção desta citocina, fazendo com que estes indivíduos sejam divididos em alto e baixo produtores de IFN-γ (Carvalho *et al.*, 2001). Como os indivíduos infectados pelo HTLV-I, mesmo ainda assintomáticos, apresentam alterações significativas da resposta imune**,** *a primeira hipótese deste estudo é que apesar dos pacientes com HAM/TSP apresentarem mais proliferação celular e produção de citocinas que os indivíduos assintomáticos, existe uma sub-população de indivíduos com infecção assintomática que apresentam uma resposta imune semelhante aos pacientes com HAM/TSP.*

A proteina viral tax induz uma variedade de genes (Buckle *et al.,* 1996). Entre eles, genes que vão ativar a produção de IL-2 e a expressão do IL-2Rα e conduzir à ativação policlonal das células T infectadas. Este processo autócrino desregulado inicia um mecanismo de ativação e proliferação persistente da célula T, que contribui para as lesões inflamatórias observadas no SNC de pacientes que desenvolvem HAM/TSP. Apesar da descrição inicial do envolvimento da alça autócrina da IL-2/IL-2R neste processo, foi também demonstrado que a proliferação espontânea das CMSP de pacientes com HAM/TSP não são totalmente inibidas pela ação bloqueadora de drogas imunossupressoras ou anticorpos anti-IL-2 e anti-IL-2Rα (Tendler *et al.*, 1990). Estes dados levantaram a possibilidade de existir outras citocinas capazes de manter a proliferação constante das células nos indivíduos infectados pelo HTLV-I. Por compartilhar funções biológicas com a IL-2, a IL-15 que é capaz de se ligar às cadeias β e γ do IL-2R, foi sugerida como uma citocina participante deste processo (Azimi *et al.*, 1999). A IL-15 é uma citocina inflamatória cujo mRNA encontra-se elevado nas CMSP de pacientes com HAM/TSP (Azimi *et al.*, 1998). O bloqueio da atividade da IL-15, utilizando anticorpos neutralizantes, inibe parcialmente a proliferação espontânea das CMSP de pacientes com HAM/TSP enquanto que inibição significativa só é obtida com a combinação de anticorpos anti-IL-2 e anti-IL-15 ou de seus respectivos receptores. Estes dados confirmam a participação de alças autócrinas e parácrinas envolvendo tanto a IL-2 quanto a IL-15 no processo de ativação celular pelo HTLV-I e seus prováveis papéis na patogênese da HAM/TSP (Tendler *et al.*,1990; Azime *et al.*, 1999; *apud* Mariner *et al.*, 2001).

A infecção pelo HTLV-I ativa o sistema imune levando a uma resposta persistente e exacerbada das células T, onde a produção espontânea de grandes quantidades de citocinas pró-inflamatórias (IFN-γ e TNF-α) é observada. Durante a resposta imune, vários mecanismos imunoregulatórios são montados pelo organismo com o objetivo de modular a resposta imunológica e impedir a replicação viral. Citocinas regulatórias da resposta imune

como a IL-10 e o TGF-β são sintetizadas para controlar o processo de diferenciação e proliferação de células T ativadas. Estas citocinas são produzidas principalmente por células T do tipo 2 e apresentam funções importantes na modulação de várias vias de ativação celular. A IL-10 reduz a proliferação de células T por meio da diminuição da expressão MHC e da inibição de eventos metabólicos necessários à ativação celular, que resultam na diminuição da produção de IFN-γ (Moore *et al.*, 1993; Fiorentino *et al.*,1989), representando assim uma boa alternativa para reduzir a produção espontânea de IFN-γ na infecção pelo HTLV-I. Outra alternativa é a utilização do TGF-β. O TGF-β possui uma variedade de efeitos imunológicos incluindo ações bloqueadoras da produção de IFN-γ (Letterio & Roberts,1998). Considerando que a IL-12 induz a produção de IFN-γ e promove a diferenciação das células T tipo 1 (German *et al.*, 1993), a neutralização desta citocina, em adição ao bloqueio da IL-2 e da IL-15 constitui-se também em uma alternativa. Como o dano tecidual na infecção pelo HTLV-I está relacionado com um aumento espontâneo da síntese de citocinas pró-inflamatórias, principalmente IFN-γ, é de grande relevância o entendimento da regulação da resposta imune nesta infecção e de como esta resposta imunológica exagerada e não controlada pode ser modulada. *A segunda hipótese deste estudo é que citocinas como IL-10 e TGF-β e antagonistas de citocinas (anti-IL-2, anti-IL-12 e anti-IL-15) têm capacidade de inibir a intensa produção espontânea de IFN-γ nas culturas de células dos indivíduos infectados pelo HTLV-I.*

ASSOCIAÇÃO ENTRE INFECÇÃO PELO HTLV-I E DOENÇAS CAUSADAS POR HELMINTOS

Diferente da infecção pelo HTLV-I que é caracterizada por uma alta produção de citocinas pró-inflamatórias como IFN-γ e TNF-α (Biddison *et al.*, 1997; Carvalho *et al.*, 2001), as infecções por helmintos são fortes indutores de uma resposta imune do tipo 2, caracterizada por produção elevada de IL-4, IL-5, IL-10 e IL-13 (Pearce *et al.*, 1991;

Finkelman *et al.*, 1997), diminuição na produção de IFN-γ (Araujo *et al.*, 1996) e altas concentrações de IgE (Dunne *et al.*, 1992). É também reconhecido que na regulação da resposta imune, os efeitos das citocinas produzidas pelas células Th1 e Th2 se opõem (Fiorentino *et al.*, 1989). Desta forma, é possível que a forte resposta Th1 observada em pacientes com HTLV-I modifique a resposta imune na infecção por helmintos e influencie na apresentação clínica da doença. Esta hipótese tem sido comprovada pela documentação de uma maior prevalência de *S. stercoralis* e *S. mansoni* em pacientes infectados pelo HTLV-I e pelos relatos de casos de formas graves de estrongiloidíase em indivíduos infectados pelo HTLV-I.

O *S. stercoralis* é um nematódeo intestinal que produz uma infecção gastrointestinal crônica, porém assintomática na maioria dos indivíduos. Os mecanismos de defesa contra o *S. stercoralis* envolvem a expulsão das larvas junto com as fezes e a destruição das larvas que penetram na corrente sanguínea. A presença de infiltração eosinófilica em torno das larvas desintegradas do *S. stercoralis* evidencia a importância do mecanismo de citotoxicidade celular dependente de anticorpos no controle da infecção. A ligação da imunoglobulina E (IgE), que está presa aos mastócitos, com antígenos de superfície do parasito e conseqüente degranulação destas células é reconhecidamente uma forma de destruição de helmintos. Após estímulo com antígenos do parasito, células mononucleares de pacientes infectados com *S. stercoralis* produzem altas concentrações de IL-4, IL-5 e uma quantidade reduzida de IFN-γ. Este padrão de resposta imune observado em pacientes com estrongiloidíase é completamente modificado em pacientes co-infectados pelo HTLV-I. Concentrações menores de IL-4, IL-5, IgE total e IgE específica contra antígeno de *S. stercoralis* são encontradas em pacientes co-infectados (Hayashi *et al.*, 1997; Porto *et al.*, 2001a; Porto *et al.*, 2001b; Neva *et al.*, 1998). Por outro lado, estes pacientes produzem mais IFN-γ do que os pacientes somente com *S. stercoralis* e o aumento desta citocina é inversamente relacionado com as concentrações de IgE total, IgE específica e IL-5 (Porto *et al.* 2001a; Porto *et al.*, 2001b).

Como os mecanismos de defesa contra helmintos são dependentes da síntese de IL-4, IL-5, IL-13 e consequente produção de IgE, eosinofilia e mastocitose, a diminuição dessas moléculas, que também alteram as funções destas células, pode se constituir na base para a ocorrência de maior prevalência de estrongiloidíase em indivíduos com HTLV-I (Nakada *et al.*, 1984; Sato *et al*., 1989), na falha da resposta terapêutica à drogas anti-parasitárias em indivíduos co-infectados (Sato *et al.*, 1994) e na maior maior gravidade da infecção nos pacientes infectados pelo HTLV-I (Gotuzzo *et al.*, 1999; Patey *et al*., 1992).

A infecção causada pelo *S. mansoni* tem um quadro clínico muito variado, apresentando desde casos pouco sintomáticos até quadros graves, como observado na forma aguda e na forma crônica hepatoesplênica da doença. A esquistossomose hepatoesplênica é a manifestação clínica mais importante da infecção pelo *S. mansoni* cujo mecanismo patogênico está relacionado à formação de granuloma ao redor dos ovos do parasito presos no sistema porta. O desenvolvimento de intensa fibrose periportal resulta em hipertensão portal, esplenomegalia, formação de circulação colateral e sangramentos gastrointestinais que elevam intensamente a morbidade e a mortalidade dos indivíduos que desenvolvem este quadro.

Tanto resistência à infecção pelo *S. mansoni*, como o desenvolvimento de fibrose estão relacionados predominantemente com uma resposta tipo 2. Resistência à infecção pelo *S. mansoni* é correlacionada com concentrações elevadas de IgE total, IgE específica contra antígenos parasitários e principalmente com o aumento da relação IgE:IgG4 (Dunne *et al.*, 1992; Caldas *et al.*, 2000; Rihet *et al.*, 1991). Com relação à formação de granulomas e o desenvolvimento de fibrose hepática, evidências experimentais têm sido acumuladas sobre o papel da IL-4 e principalmente da IL-13 nestes fenômenos (Cheever *et al.*, 1994; Chiaramonte *et al.*, 1999).

No homem, os aspectos patogênicos da fibrose causada pelo S. *mansoni* ainda não estão totalmente elucidados. Em pacientes com infecção crônica pelo *S. mansoni*, um estudo recente, avaliando o papel de citocinas e sua relação com o grau de fibrose hepática,

encontrou concentrações mais altas de IL-5, IL-10 e IL-13 em pacientes hepatoesplênicos com grau mais elevado de fibrose (grau III) quando comparado com os pacientes de grau I e II, fortalecendo o papel das citocinas tipo 2 no desenvolvimento da fibrose hepática (De Jesus *et al.*, 2004).

Diferente da forte associação descrita entre o HTLV-I e o *S. stercoralis*, até recentemente não havia informações sobre a resposta imunológica e os aspectos clínicos da co-infecção HTLV-I e *S. mansoni*. O encontro de uma freqüência maior de infecção pelo *S. mansoni* entre indivíduos infectados pelo HTLV-I quando comparados com indivíduos com sorologia negativa para HTLV-I, direcionaram estudos clínicos e imunológicos para avaliar esta co-infecção (Porto *et al.*, 2004; Santos *et al.*, 2004). Nestes estudos, a determinação do perfil de citocinas no sobrenadante de culturas de células estimuladas com SWAP mostrou um aumento na concentração de IFN-γ e uma diminuição de IL-5 e IL-10 nos pacientes com esquistossomose co-infectados pelo HTLV-I quando comparado com pacientes somente com infecção pelo *S. mansoni*. A redução na concentração de IgE específica para SWAP no grupo de pacientes co-infectados confirmou a diminuição da resposta tipo 2 nos indivíduos com esquistossomose após infecção pelo HTLV-I. Adicionalmente foi observado uma menor eficácia do praziquantel nos pacientes com esquistossomose co-infectados pelo HTLV-I. Ainda nestes estudos, a maioria dos pacientes co-infectados pelo *S. mansoni* e HTLV-I apresentaram grau leve de fibrose hepática (grau I). Como pacientes co-infectados com *S. mansoni* e HTLV-I produzem menos citocinas do tipo 2, a descrição de uma menor intensidade de fibrose nestes pacientes dão suporte aos estudos de que a resposta Th2 é importante no desenvolvimento das lesões hepáticas na esquistossomose (De Jesus *et al.*, 2004). Desta forma, a despeito da infecção pelo HTLV-I aumentar a prevalência de infecções pelo *S. mansoni*, pacientes co-infectados com HTLV-I não desenvolvem formas graves da esquistossomose.

MODULAÇÃO DA RESPOSTA IMUNE PELO *Schistosoma mansoni*

Enquanto na esquistossomose aguda existe uma forte resposta inflamatória com uma predominante resposta Th1 (Pearce *et al.,* 1991; De Jesus *et al.,* 2002), após ocorrência da ovoposição existe um desvio da resposta imune com diminuição ou ausência de produção de IFN-γ (De Jesus *et al.*, 1993; Araújo *et al.*, 1994) e grande produção de IL-4, IL-5, IL-10 e IL-13 (Araujo *et al.*, 1996; Pearce *et al.*, 2004). Esta resposta Th2 não só controla a reação inflamatória observada na fase aguda como também exerce efeito regulatório sobre outros antígenos. Esta modulação da resposta tipo 1 é uma característica da fase crônica da esquistossomose e é dependente de IL-10, desde que a neutralização desta citocina com anticorpo monoclonal anti-IL-10 aumenta a produção de IFN-γ *in vitro* em culturas estimuladas com antígenos do *S. mansoni* (Araujo *et al.,* 1996). A capacidade da infecção pelo *S. mansoni* de suprimir a produção de IFN-γ foi também observada em indivíduos imunizados com toxóide tetânico. Enquanto indivíduos sadios, sem esquistossomose e imunizados pelo toxóide tetânico, produziram tanto IFN-γ como IL-4, pacientes com esquistossomose após imunização com este antígeno sintetizavam predominantemente IL-4 (Sabin *et al.*, 1996).

Tem sido bem documentado, em modelos experimentais, o papel do *S. mansoni* em modular uma resposta Th1 e modificar o curso de doenças inflamatórias e auto-imunes. Na encefalomielite experimental auto-imune (EAE), a lesão tecidual é dependente de citocinas do tipo 1. A infecção pelo *S. mansoni* retarda o início da doença e reduz a gravidade da lesão neurológica (La Flamme *et al.*, 2003). Em camundongos infectados com *Toxoplasma gondii*, o aumento da produção de TNF-α causa efeitos que comprometem a função dos hepátócitos enquanto a infecção destes animais com *S. mansoni* atenua a lesão hepática (Marshall *et al.*, 1999). Um potente efeito modulatório provocado pelo *S. mansoni* tem sido também observado em camundongos NOD (n*on obese diabetic*), que desenvolvem diabetes do tipo 1 a partir da 5ª semana de vida. Nestes animais, a infecção com *S. mansoni* logo após o nascimento ou a

injeção com ovos do parasito impede o desenvolvimento espontâneo do quadro de diabetes melitus dependente de insulina (Cooke *et al.*, 1999).

Também em infecções virais tem sido descrita a capacidade da infecção pelo *S. mansoni* de influenciar a resposta imune contra estes patógenos. Camundongos infectados pelo *S. mansoni* e co-infectados com o vírus da vaccínia, quando desafiados com proteínas virais recombinantes, não são capazes de induzir uma resposta característica tipo 1 com produção de IFN-γ e IL-2. Adicionalmente observa-se também neste modelo, uma diminuição da atividade citotóxica dos linfócitos T CD8+ e um conseqüente retardo na remoção das partículas virais (Actor *et al.*, 1993).

Infecções causadas por vírus têm na lise efetuada pelos linfócitos T citotóxicos CD8+, um dos principais mecanismos de controle da replicação viral. Na infecção pelo HTLV-I, caracterizada por uma intensa resposta tipo 1 com produção exagerada de IFN-γ, embora existam evidências da participação destes linfócitos na lise das células T CD4+ infectadas (Hanon *et al.*, 2000), estas células estão também envolvidas na lesão tecidual, sendo, portanto a modulação desta resposta um objetivo importante a ser alcançado. Considerando as informações sobre o papel da infecção pelo *S. mansoni* na redução da resposta do tipo 1, *a terceira hipótese deste estudo é que co-infecção por helmintos (S. stercoralis e S. mansoni) pode interferir na resposta imune de indivíduos infectados pelo HTLV-I.*

Geral

Avaliar a resposta imune em indivíduos assintomáticos infectados pelo HTLV-I e em pacientes com HAM/TSP e a capacidade de citocinas, antagonistas de citocinas e infecção por helmintos de modular a resposta imune na infecção pelo HTLV-I.

Específicos

1 – Comparar a resposta imune de pacientes com mielopatia pelo HTLV-I com a resposta de indivíduos assintomáticos infectados pelo HTLV-I.

2 – Descrever as populações de células envolvidas na resposta imune dos indivíduos infectados pelo HTLV-I, a freqüência e o tipo de células que apresentam marcadores de ativação celular e secretam citocinas.

3 - Avaliar a capacidade de citocinas e antagonistas de citocinas de modular a resposta imune dos indivíduos assintomáticos infectados pelo HTLV-I e dos pacientes com HAM/TSP.

4 – Determinar a influência de infecções por helmintos (*S. mansoni* e *S. stercoralis)* sobre a resposta imune e carga proviral dos indivíduos infectados pelo HTLV-I.

5 - Comparar a prevalência de infecções por helmintos em indivíduos assintomáticos infectados pelo HTLV-I com a observada em pacientes com HAM/TSP.

ARTIGO 1

Exacerbated inflammatory cellular immune response characteristic of HAM/TSP is observed in a large proportion of HTLV-I asymptomatic carriers

Silvane Braga Santos, Aurélia Fonseca Porto, André Luis Muniz, Amélia Ribeiro de Jesus, Elza Magalhães, Ailton Melo, Walderez Dutra, Keneth Gollob & Edgar M. Carvalho.

Este estudo teve como objetivo comparar a resposta imune de pacientes com HAM/TSP e indivíduos assintomáticos infectados pelo HTLV-I. A determinação do perfil de citocinas produzido por células mononucleares do sangue periférico de indivíduos infectados pelo HTLV-I mostrou que apesar da intensa variabilidade, a produção espontânea de IFN-γ foi significativamente maior nos pacientes com mielopatia do que nos indivíduos assintomáticos. As concentrações de IL-5 e IL-10 não diferiram entre os grupos, e somente uma tendência de maior produção de TNF-α foi observado nos pacientes com mielopatia. A descrição das populações de células envolvidas na resposta imune dos indivíduos infectados pelo HTLV-I mostrou uma maior freqüência de células T CD4+ e CD8+ expresando IFN-γ e TNF-α nos pacientes com HAM/TSP, quando comparado com indivíduos assintomáticos. Nos pacientes com mielopatia, o TNF-α foi sintetizado tanto por células T CD4+ quanto por células CD8+, enquanto a produção de IFN-γ foi observada principalmente pelas células T CD8+. Não houve diferença na expressão de CD69 e CD62L entre os grupos, enquanto nos pacientes com mielopatia observou-se uma maior freqüência de linfócitos T com fenótipo CD8+/ CD28-. Adicionalmente, estes resultados mostraram que aproximadamente 40% dos indivíduos assintomáticos, apresentaram linfoproliferação e produção espontânea de IFN-γ similares aos observados nos pacientes com HAM/TSP.

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Exacerbated inflammatory cellular immune response characteristics of HAM/TSP is observed in a large proportion of HTLV-I asymptomatic carriers

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Abstract

Background: A small fraction of Human T cell Leukemia Virus type-I (HTLV-I) infected subjects develop a severe form of myelopathy. It has been established that patients with HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) show an exaggerated immune response when compared with the immunological response observed in HTLV-I asymptomatic carriers. In this study the immunological responses in HAM/TSP patients and in HTLV-I asymptomatic carriers were compared using several immunological assays to identify immunological markers associated with progression from infection to disease.

Methods: Immunoproliferation assays, cytokine levels of unstimulated cultures, and flow cytometry analysis were used to evaluate the studied groups. Nonparametric tests (Mann-Whitney U test and Wilcoxon matched-pairs signed ranks) were used to compare the difference between the groups.

Results: Although both groups showed great variability, HAM/TSP patients had higher spontaneous lymphoproliferation as well as higher IFN-γ levels in unstimulated supernatants when compared with asymptomatic carriers. Flow cytometry studies demonstrated a high frequency of inflammatory cytokine (IFN-γ and TNF-α) producing lymphocytes in HAM/TSP as compared to the asymptomatic group. This difference was accounted for mainly by an increase in CD8 cell production of these cytokines. Moreover, the HAM/TSP patients also expressed an increased frequency of CD28-/CD8+ T cells. Since forty percent of the asymptomatic carriers had spontaneous lymphoproliferation and IFN-γ production similar to HAM/TSP patients, IFN-γ levels were measured eight months after the first evaluation in some of these patients to observe if this was a transient or a persistent situation. No significant difference was observed between the means of IFN-γ levels in the first and second evaluation.

Conclusions: The finding that a large proportion of HTLV-I carriers present similar immunological responses to those observed in HAM/TSP, strongly argues for further studies to evaluate these parameters as markers of HAM/TSP progression.

Background

Human T cell leukemia virus-type 1 (HTLV-I) infects an estimated 10 to 20 million people worldwide, making it a serious public healthy problem. The HTLV-1 infection has a high prevalence in Brazil, and Salvador, the capital of the state of Bahia, has the highest prevalence of HTLV-1 in the country in blood donors (1,35%) [1,2]. It is estimated that 95% of HTLV-I infected individuals are asymptomatic carriers. A small percentage of infected individuals (2 to 5%) develop adult T cell leukemia/lymphoma (ATL) [3,4] or a chronic inflammatory disease, involving the central nervous system, termed HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5,6]. One of the most important immunological observations of HTLV-I infection is the demonstration that lymphocytes spontaneously proliferate *in vitro* in the absence of stimulus [7]. It has been shown that both infected CD4+ and CD8+ lymphocytes infiltrate spinal cord and peripheral blood and produce cytokines such IFN-γ, TNF-α and IL-6, which are considered important inflammatory mediators of the tissue damage in HAM/TSP [8-10]. Extensive previous studies have compared the immunological response of asymptomatic HTLV-I carriers to that of HAM/TSP patients [11,12]. Additionally, a recent study [13] has emphasized that the percentage of HTLV-I carriers that develop other immunological abnormalities, including HAM/TSP, is much higher than that previously cited in the literature. The aim of the present study is to compare in HTLV-I asymptomatic carriers, and in HAM/TSP patients, the spontaneous lymphoproliferative response and cytokine production, the overall *ex vivo* T cell activation states, and the production of immunoregulatory cytokines by CD4+ and CD8+ T cells. The documentation that some HTLV-I carriers have immunological alteration similar to that observed in HAM/TSP suggests that potential markers of disease progression may be determined in HTLV-I infection.

Methods

Patients selection and neurological exam

Patients were selected from the HTLV-I clinic of the Hospital Universitário Professor Edgard Santos, Federal University of Bahia, Brazil. The diagnosis was confirmed by Western blot (HTLV blot 2.4, Genelabs, Singapore). Seventeen patients with HAM/TSP were selected based on WHO criteria and thirty-six HTLV-I asymptomatic carriers were referred from two blood banks. Exclusion criteria included the use of antiviral drugs or immunomodulators in the previous 90 days, helminth infection, co-infection with HIV, HCV or hepatitis B and presence of other neurologic diseases. Motor dysfunction was determined by Osame's Motor Disability Score (OMDS) [14] and Expanded Disability Status Scale (EDSS) [15]. Patients with HAM/TSP had a marked neurological impairment with EDSS ≥ 3 and OMDS ≥ 1 and all asymptomatic subjects had OMDS and EDSS of zero. Seronegative normal donors were also referred from the same blood banks and used as negative controls. The Ethical Committee of the Hospital Universitário Professor Edgard Santos approved this study and informed consent was obtained from all prospectively enrolled patients.

Cell preparation and proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated and cultivated in RPMI 1640 (Gibco, Grand Island, NY, USA) plus 10 % heat inactivated human AB Rh+ serum (Sigma Chemical Co., St. Louis, MO), antibiotics and glutamine. A total of 2×10^5 cells/mL were incubated at 37°C in 5 % CO_2 atmosphere in a 96 well flat-bottom microtiter plates. The cells were kept unstimulated (media alone) and after 5 days, the cultures were pulsed with ^{3}H-Thymidine (1 μCi/well) for a final 6–16 hours period, and then harvested. The ^{3}H-Thymidine uptake was measured using a LKB beta scintillation counter. The average of counts per minute (cpm) was plotted and PHA (1.0 μg/mL, Sigma) was used as a positive control in the proliferation assay.

Cytokine determination

PBMC were adjusted to 3×10^6 cells/mL in RPMI 1640 plus 10 % serum AB Rh+. The cells were cultured unstimulated or stimulated with PHA (5 μg/mL) and all cultures were incubated at 37°C in 5 % CO_2 atmosphere for 72 hours until supernatants were collected. IFN-γ, TNF-α, IL-5 and IL-10 levels were measured by sandwich ELISA technique (R & D system, Minneapolis, MN).

Ex vivo staining of lymphocyte profiles

2×10^5 PBMC from HTLV-I patients were incubated with FITC, PE, or Cychrome-labeled antibody solutions for 20 minutes at 4°C. After staining, preparations were washed with 0.1% sodium-azide PBS, fixed with 2% formaldehyde in PBS and kept at 4°C until data acquisition using a FACScalibor (Becton Dickinson, San Jose, CA). The antibodies used were all directly conjugated either for FITC, PE, or Cychrome and consisted of: Ig controls, anti-CD4, CD8, CD28, CD69 (Pharmingen, San Diego, CA) and anti-CD62L (Caltag, Burlingame, CA).

Single cell cytoplasmic cytokine staining

Briefly, 2×10^5 PBMC were cultured in RPMI 1640 plus 5% AB Rh+ serum in 96 well plates. Based on preliminary results all the cytokine staining was performed after 20 hours of incubation. During the last 4 hours of culture, Brefeldin-A (1 μg/mL) was added to the culture. The cells were then washed and centrifuged using ice-cold PBS plus sodium-azide, stained for surface markers and fixed using 2% formaldehyde. The fixed cells were then permeablized with a solution of Saponin and stained, for 30 minutes at 4°C, using anti-cytokine mAbs directly conjugated with

PE (IFN-γ, TNF-α, IL-4 and IL-10) (Pharmingen). Preparations were then washed, fixed and analyzed using a FACScalibor. In all cases the cells were double stained for cytokine and for cell surface markers. In all cases, 30,000 gated events were acquired for later analysis due to the low frequency of positive events being analyzed.

Statistical analysis

A nonparametric Mann-Whitney U test and Wilcoxon matched-pairs signed rank tests were used to evaluate differences between the groups. An alpha (α) of 5% ($p < 0.05$) was considered for statistical significance. Lymphocytes were analyzed for their intracellular cytokine expression patterns and frequencies and for surface markers using the program Cell Quest. Statistical analysis was performed using the ANOVA "comparison of all pairs" contained in the statistical program from SAS, JMP.

Results

The mean age of the seventeen myelopathy patients was 53 ± 16 (mean ± SD) years and of the thirty-six HTLV-I healthy carriers was 39 ± 11 years. To determine if HAM/TSP patients and asymptomatic subjects produce different levels of secreted cytokines, IFN-γ, TNF-α, IL-10 and IL-5 were measured in supernatants of unstimulated cultures of HTLV-I infected groups and compared with negative controls. There was a high variability in IFN-γ levels in asymptomatic carriers (Figure 1A). The mean and SD of IFN-γ levels in myelopathy patients (4,246 ± 2,924 pg/mL, range: 375 to 10,750), was higher than that observed in asymptomatic carriers (1,362 ± 1,408 pg/mL range: 15 to 6,995) or in negative controls (1 ± 4 pg/mL), $p = 0.0001$, Mann-Whitney U test. There was also a tendency for higher TNF-α levels in HAM/TSP patients (378 ± 316 pg/mL) when compared with levels observed in asymptomatic carriers (259 ± 366 pg/mL) or in negative controls (60 ± 63 pg/mL), $p = 0.06$. No differences between IL-5 levels (151 ± 141 versus 166 ± 231 pg/mL), $p = 0.17$ and IL-10 levels (70 ± 66 versus 94 ± 110 pg/mL), $p = 0.9$, were observed between HTLV-I infected groups or negative controls (2 ± 2 versus 2.6 ± 10), Figure 1B.

The lymphoproliferative assays performed using PBMC from ten HAM/TSP patients and eleven asymptomatic individuals showed that lymphoproliferation was higher in HAM/TSP than HTLV-I asymptomatic carriers. The five day spontaneous proliferation of the HAM/TSP group gave a mean and SD of 21,404 ± 30,859 cpm (range: 919 to 102,242), while the asymptomatic HTLV-I group had a mean and SD of 3,365 ± 5,188 cpm (range: 139 to 18,169). The magnitude of the responses was higher in HAM/TSP than in HTLV-I carriers ($p = 0.006$) although a great variability of the spontaneous lymphoproliferation had been observed in both groups (date not shown).

Based on IFN-γ production, the immunological responses in 40% of the HTLV-I carriers were similar to that observed in patients with HAM/TSP ($p > 0.05$). These individuals had IFN-γ levels higher than 1,322 pg/mL, representing the mean minus one standard deviation of the IFN-γ levels obtained in HAM/TSP patients. To determine if the cytokine levels in HTLV-I carriers was reflecting a transient or persistent situation, IFN-γ levels were measured between 6 months to one year (with a mean of eight months) after the first evaluation. No significant differences were observed between the means of IFN-γ levels from fourteen asymptomatic carriers in the first and second evaluation (1,723 ± 450 and 1,670 ± 1,396, respectively), $p = 0.67$. HAM/TSP patients (n = 11) also had similar levels in the first and second evaluation (5,771 ± 3,365 versus 4,718 ± 2,427, respectively), $p = 0.17$, Wilcoxon matched-pairs signed ranks test (Figure 2).

To determine the activation states and relative lymphocyte proportions, as well as the cellular sources of immunoregulatory cytokines, four HAM/TSP patients and eight asymptomatic carriers were randomly selected and analyzed using flow cytometry. To measure lymphocyte activation and regulation, the markers CD69 and CD28 respectively, where used in conjunction with CD4 and CD8 in separate staining protocols. The relative proportions of CD4 and CD8 cells expressing the adhesion molecule, CD62L was also determined for both groups. Figure 3A demonstrates that the HAM/TSP group expressed a significantly higher frequency of CD8+ T cells (21.4 ± 3.3) than the asymptomatic group (9.8 ± 1.7). A higher frequency (51.9 ± 3.5) of the hyper-activated, CD28-/CD8+ T cells within the CD8+ T cell population in HAM/TSP than in asymptomatic group (35.2 ± 7.4) was also observed. There was no difference in CD69 and CD62L lymphocyte populations between both HTLV-I infected groups (data not shown).

To further investigate the differences in cytokine profile between the HAM/TSP and asymptomatic groups, flow cytometry was performed to determine the frequency of T cells producing IFN-γ, TNF-α, IL-4 and IL-10. A significant increase in the frequency of lymphocytes expressing TNF-α and IFN-γ was seen in the HAM/TSP group (12 ± 6 and 5 ± 0.3, respectively) as compared to the asymptomatic group (4 ± 2 and 1 ± 0.5, respectively) (Figure 3B). Moreover, CD8+ T cells where the major cellular source responsible for the difference in IFN-γ producing cells between the two groups. The frequency of CD8+ T cells producing TNF-α was higher in HAM/TSP as compared to asymptomatic carriers, with both CD4+ and CD8+ T cells contributing equally to the difference seen in TNF-α production (Figure 3C). In contrast, no difference was seen for the frequency of cells producing IL-4 or IL-10 (data not shown). While there was no difference regarding the frequency of

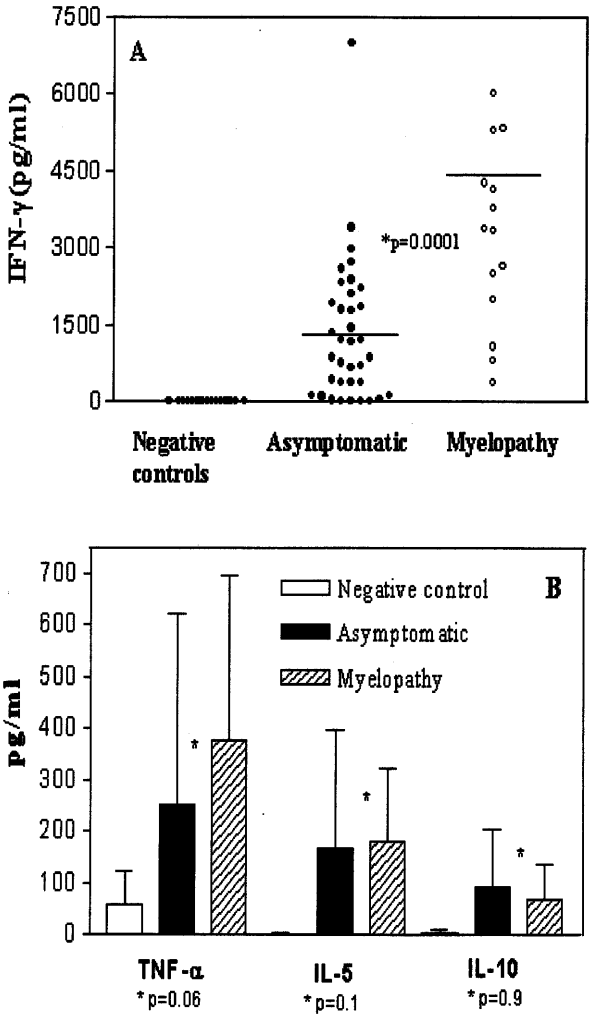

Figure 1

HAM/TSP patients display higher production of IFN-γ as compared with others cytokines synthesis. Unstimulated cultures supernatants of PBMC from 17 HAM/TSP patients and 36 asymptomatic carriers were compared with negative controls (n = 15) and assayed by ELISA to observe IFN-γ, TNF-α, IL-5 and IL-10 synthesis. Figure 1A shows IFN-γ levels (pg/ml) in HAM/TSP patients as compared with asymptomatic carries or negative controls. Figure 1B shows TNF-α, IL-5 and IL-10 levels in both groups. The bars represent the median of IFN-γ concentrations and the difference were considered significant when $p < 0.05$ (Mann-Whitney U Test).

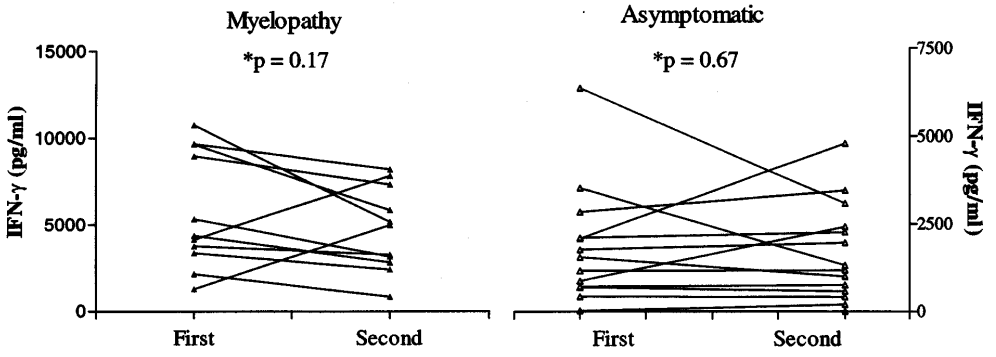

Figure 2
IFN-γ levels are relatively constant when evaluated in two different periods. Unstimulated cultures supernatants of PBMC from 11 HAM/TSP patients and 14 asymptomatic carriers were assayed by ELISA and analyzed at the initial evaluation and eight months following the first evaluation. The data represent IFN-γ levels (pg/ml) of each patient in first and second evaluation. The differences were not statistically significant ($p > 0.05$, Wilcoxon matched-pairs signed ranks test).

CD4+ and CD8+ T cells producing IFN-γ within asymptomatic carriers (0.5 ± 0.4 vs. 0.5 ± 0.3), there was a significant difference ($p < 0.05$) in the frequency of CD4 cells producing IFN-γ in HAM/TSP (1.4 ± 0.5) compared to the CD8+ T cells (3.6 ± 0.5).

Discussion

The present study shows that lymphocytes from patients with HAM/TSP displayed higher spontaneous proliferation and IFN-γ synthesis, a higher frequency of TNF-α and IFN-γ producing lymphocytes and a significant increase in the deregulated T cell population, CD28-/CD8+, as compared to those from HTLV-I asymptomatic carriers.

The pathogenesis of HAM/TSP is not completely understood. Although initially considered a rare and late complication of infection, the disease has been identified in children, and in some cases, a rapidly progressive disease has been observed [16]. Increased proviral load, proinflammatory cytokines and the expansion of HTLV-I tax-specific CD8+ cytotoxic T lymphocytes, both in cerebrospinal fluid and in peripheral blood, have been associated with the central nervous system involvement in patients with HAM/TSP [17-21]. A recent report [13] established a cohort of HTLV-I asymptomatic carries to study clinical events and documented an increased frequency of abnormalities, including a case of HAM/TSP.

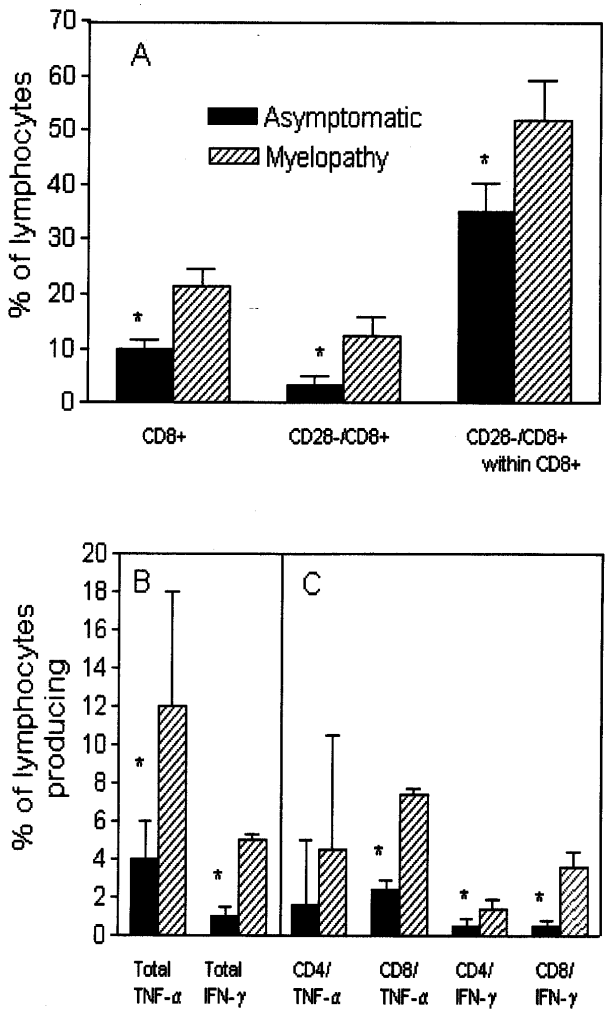

Figure 3

HAM/TSP patients display a higher frequency of CD28-/CD8+ T cells and inflammatory cytokine producing T cells than do asymptomatic carriers. PBMC from 4 HAM/TSP and 8 asymptomatic carriers were analyzed ex vivo or following a 20 hour media alone culture for the expression of CD4+ and CD8+ T cell subpopulations using flow cytometry. Figure 3A shows the frequency of CD8 cells and the subpopulations defined by CD28 expression. Figure 3B shows the frequency of total lymphocytes producing TNF-α and IFN-γ. Figure 3C shows the relative contribution of CD4+ or CD8+ T cells to the overall cytokine producing population shown in Figure 3B. The data represent the mean ± S.D and the asterisk represents differences with a $p < 0.05$ (Mann-Whitney U Test).

Evaluation of immunological responses in patients with HAM/TSP and asymptomatic carries is important for the understanding of the pathogenesis of the HAM/TSP, and to identify early immunological markers associated with progression from infection to disease. This study indicates a higher and significant spontaneous lymphoproliferation and IFN-γ production in HAM/TSP patients as compared to HTLV-I asymptomatic carriers. Additionally, lymphocyte responses were quite variable, and in some asymptomatic carriers the lymphocyte proliferation and IFN-γ production were similar to those found in patients with myelopathy. These data induce us to observe if the parameters could be considered good markers of disease progression. This is in agreement with our previous observations that asymptomatic carriers can be divided in high and low producers according to IFN-γ levels [22]. A great variability of immune response observed in about 40% of HTLV-I infected subjects was not reflecting a temporary situation, since the IFN-γ levels were relatively constant within an individual over time (eight months). During this period of time no neurological manifestation was documented and no other disease that could alter the immune response was observed.

Many studies have demonstrated that IFN-γ and TNF-α produced in large quantities contribute to tissue damage of the central nervous system [23,24], and are likely involved in the pathogenesis of several infections and non-infections disease. The flow cytometric determination of cytokine producing lymphocytes extend the observations made in supernatants of lymphocytes cultures showing a significant increase in the frequency of IFN-γ and TNF-α producing cells in patients with HAM/TSP. This difference was accounted for both CD4+ and CD8+ T cells for the TNF-α producing cells, and mainly by CD8+ T cells for IFN-γ producing lymphocytes. Since CD4+ T cells are the main source of IFN-γ in HTLV-I carriers [22,25,26] this data may indicate that during the evolution from asymptomatic to myelopathy there is a switch from CD4+ to CD8+ in relation to the main cell producing IFN-γ. These findings support studies suggesting that CD8+ T cells may play an important role in the pathogenesis of HAM/TSP [27].

Previous studies have shown that CD28-/CD8+ cells display high cytotoxic activity, playing an important role in the pathology associated with viral diseases [28,29]. Additionally, recent studies have shown that HIV-1 can incorporate CD28 and the acquisition of this specific host surface glycoprotein modulates the virus life cycle [30]. Moreover, the role of CD28-/CD8+ T cells in HAM/TSP needs to be further investigated since these cells may induce cell damage and / or death in infections disease [31].

In conclusion, these results show an exacerbated type 1 immune response in HAM/TSP patients, characterized by elevated IFN-γ production, an increased frequency of TNF-α and IFN-γ producing lymphocytes, and by an increase in the frequency of CD28-/CD8+ T cells. Given that cellular activation and pro-inflammatory cytokine production are likely directly involved in the pathogenesis of HAM/TSP disease, longitudinal studies of HTLV-I infected asymptomatic carriers who present with high lymphoproliferative response, high production of IFN-γ and TNF-α and high expression of CD28-/CD8+ T cells should be conducted to determine the frequency of disease progression in this group. The identification of markers of HAM/TSP progression will allow for earlier initiation of current therapeutic interventions, and hopefully delay the fast and progressive development of the motor disability observed in HTLV-I infected individuals.

Competing interests

None declared.

Authors' contributions

SBS carried out the immunological studies, performed statistical analysis and drafted the manuscript. ALM, EM and AM participated in the coordination of neurological evaluations. AFP was involved in clinical evaluation of the patients. ARJ participated in the design and statistical analysis. WOD and KJG carried out FACS analysis and EMC conceived the study and participated in its design and coordination.

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ARTIGO 2

Modulation of T cell response in HTLV-I asymptomatic carriers and in patients with myelopathy associated to HTLV-I

Silvane Braga Santos, Aurélia Fonseca Porto, André Luis Muniz, Jamary Oliveira-Filho, Tania Luna, Jaqueline B. Guerreiro & Edgar M. Carvalho.

Este estudo teve como objetivo avaliar a capacidade de citocinas (IL-10 e TGF-β) e antagonistas de citocinas (anti-IL-2, anti-IL-12 e anti-IL-15) de suprimir a produção espontânea de IFN-γ nas culturas de indivíduos assintomáticos infectados pelo HTLV-I e de pacientes com HAM/TSP. Os resultados mostraram que a adição de IL-10 reduziu a produção espontânea de IFN-γ nas culturas dos indivíduos assintomáticos quando comparada com pacientes com mielopatia. Em ambos os grupos, a síntese de IFN-γ não foi suprimida pela adição de TGF-β ou IL-12. A média de supressão da produção de IFN-γ após adição de anti-IL-2, anti-IL-15 e a adição simultânea de anti-IL-2 e anti-IL-15, foi maior nos indivíduos assintomáticos do que nos pacientes com HAM/TSP, sugerindo que as culturas de indivíduos assintomáticos são mais facilmente moduladas por citocinas e antagonistas de citocinas do que as culturas de pacientes com HAM/TSP. Adicionalmente, foi registrado um sub-grupo de indivíduos assintomáticos, alto produtores de IFN-γ, que apresentavam parâmetros imunológicos similares aos dos pacientes com HAM/TSP. Estes resultados sugerem que este sub-grupo de indivíduos, cuja síntese espontânea de IFN-γ não foi suprimida pela adição de citocinas e anti-citocinas, devem ser acompanhados para detectar precocemente alterações clínicas que sejam características de pacientes que desenvolvem HAM/TSP.

(Manuscrito em preparação)

(Modulation of HTLV-I immune response)

Modulation of T Cell Response in HTLV-I Asymptomatic Carriers and in Patients with Myelopathy Associated to HTLV-I

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ABSTRACT

Human T lymphotropic virus-type I (HTLV-I) infection activates the immune system leading to a persistent and exacerbated T cell response with high production of IFN-γ and TNF-α. Overproduction of pro-inflammatory cytokines is correlated with development of HAM/TSP although some HTLV-I asymptomatic carriers also register high levels of these pro-inflammatory cytokines. In this study the ability of regulatory cytokines (IL-10 and TGF-β) and cytokine antagonists (anti-IL-2, anti-IL-12 and anti-IL-15) in inhibiting spontaneous IFN-γ production was investigated. Addition of IL-10 significantly reduced spontaneous IFN-γ synthesis in cell cultures from asymptomatic carriers ($p=0.03$) while no differences were observed in HAM/TSP patients ($p=0.1$). TGF-β and anti-IL-12 failed to decrease spontaneous IFN-γ levels in both groups**.** Neither anti-IL-2 nor anti-IL-15 alone suppressed IFN-γ synthesis in asymptomatic carriers or HAM/TSP patients. Simultaneous addition of anti-IL-2 and anti-IL-15 decreased IFN-γ synthesis in 58% of asymptomatic carriers whereas only by 29% in patients with HAM/TSP ($p=0.002$). Moreover, in about 15-20% of the asymptomatic carriers, IL-10, anti-IL-2 and the combination of anti-IL-2 plus anti-IL-15 did not inhibit IFN-γ production. Together, these data suggest that IL-10 and cytokine antagonists combined can modulate IFN-γ production in asymptomatic carriers. However they are not effective in decreasing the synthesis of these cytokine in patients with HAM/TSP. Furthermore, there were a small percentage of HTLV-I carriers in whom no modulatory action with these immunomodulators was observed, making the immunological response of this sub-group similar to patients with HAM/TSP.

INTRODUCTION

Human T-cell lymphotropic virus-type I (HTLV-I) infection modifies the cellular and humoral immune response. Activated T cells incorporate the virus in their genome, where regulatory proteins (Tax) alter activation and cell death pathways leading to a persistent activation and an exacerbated T cell response. In a small percentage of infected individuals, HTLV-I causes adult T-cell leukemia-lymphoma (ATL) or a chronic inflammatory disease of the central nervous system (HTLV-I-associated myelopathy/tropical spastic paraparesis, HAM/TSP). Uveitis, polyarthritis and infective dermatitis in children have also been associated with HTLV-I [1]. HTLV-I infection induces *in vitro* spontaneous proliferation of lymphocytes [2], a persistent and high titer of anti-HTLV-I antibodies [3], high HTLV-I proviral load with an increased number of Tax specific CD8+ T lymphocytes [4,5], and also an increased expression of pro-inflammatory cytokines and chemokines in peripheral blood and in the cerebral spinal fluid [6-8]. These immunological abnormalities are more pronounced in HAM/TSP patients but evidence of enhanced T cell activation is also detected in HTLV-I asymptomatic carriers [8,9]. HTLV-I Tax activates Interleukin-2 (IL-2) and Interleulin-15 (IL-15) genes, the products of which participate of the spontaneous lymphoproliferation observed in HTLV-I infected patients [10,11]. Moreover, overproduction of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-15 contribute to and are responsible for the persistent inflammatory reaction observed in HAM/TSP patients [12,13]. Therefore, the control of the exacerbated T cell response in HTLV-I infection is highly desirable in such patients. Interleukin-10 (IL-10) and Transforming growth factor β (TGF-β) have been recognized as important cytokines that down regulate the type 1 immune response. In this study, the ability of regulatory cytokines (IL-10 and TGF-β) and antagonists of cytokines (anti-IL-2, anti-IL-12 and anti-IL-15) to down regulate the high spontaneous IFN-γ production observed in unstimulated cell cultures of HTLV-I infected patients were analyzed.

Additionally, a comparative analysis of the role of these modulators that down regulates the immune response in HTLV-I asymptomatic carriers and in HAM/TSP patients was performed. The overall data showed that IL-10 in high concentrations and anti-IL-2 plus anti-IL-15 reduce significantly IFN-γ production in asymptomatic carriers but is not able to down regulate cells from HAM/TSP. Moreover, a small sub-group of HTLV-I carriers have immunological abnormalities similar to HAM/TSP without evidence of neurological disease.

MATERIAL AND METHODS

Study subjects

The study population consisted of twenty (20) patients with HAM/TSP and twenty (20) HTLV-I asymptomatic carriers that were randomly selected from the HTLV-I clinic of the Hospital Universitário Professor Edgard Santos, Federal University of Bahia, Brazil. The diagnosis of HTLV-I infection was detected by enzyme-linked immunosorbent assay (Murex HTLV-I + II, England) and confirmed by Western blot analysis (Genelabs HTLV 2.3 - 2.4, Singapore). The diagnosis of HAM/TSP was made according to the World Health Organization guidelines. All of the HAM/TSP patients were independently observed by two neurologists and had HTLV-I antibodies in their cerebral spinal fluid (CSF). Neurological and motor dysfunctions were measured by two scales: Expanded Disability Status Scale (EDSS) [14] and Osame's Motor Disability Score (OMDS) [15]. All HAM/TSP patients had OMDS ≥1 and EDSS ≥ 3. Individuals who did not fulfill the criteria for HAM/TSP were classified as HTLV-I carriers. All subjects were also screened for HIV-1 and 2 and hepatitis virus type B and C. Samples were taken under informed consent, and the study was conducted with approval of the Ethical Committee of the Hospital Universitário Professor Edgard Santos.

Cell cultures

Peripheral blood mononuclear cells (PBMCs) from 20 HAM/TSP patients and 20 asymptomatic carriers were isolated from heparinized blood by density gradient centrifugation with Ficoll-Hypaque. The cells were cultured in RPMI 1640 (Life Technologies Gibco BRL, Grand Island, N.Y), 10% human AB serum (Sigma, St. Louis, MO), glutamine, HEPES and antibiotics. A total of 3×10^{6} cells/mL were plated in 24 well flat-bottom microtiter plates (Falcon, Becton Dickinson, Lincoln Park, N.J). The cell cultures were kept unstimulated (media alone) or stimulated with IL-10 100 ng/mL (DNAX Institute, Palo Alto, CA) and rTGF-β1 50 ng/mL (R&D System, Minneapolis, MN). A total of 20

μg/mL of anti-IL-2, anti-IL-12, anti-IL-15 (R&D System, Minneapolis, MN) or a combination of both anti-IL-2 and anti-IL-15 were also used. Cell cultures were incubated at 37ºC with 5% CO_2 and 95% air for 72 hours. Supernatant fluid of cell cultures were collected and stored at -20º C until use. IFN-γ level was assayed and the percentage of IFN-γ synthesis reduction in the presence and absence of each neutralizing antibody or IL-10 and TGF-β were calculated (percentage of suppression). The activity of the cytokines and cytokines antagonists was demonstrated by the suppression of IFN-γ production in seronegative HTLV-I cultures stimulated with purified protein derivative of tuberculin (PPD) (2 μg/mL). Before all of the experiments, a curve dose response, with concentrations ranging from 0,5 to 20,0 μg/mL, was performed to determinate the best concentrations of monoclonal antibody to be used.

IFN-γ ELISA

Enzyme-linked immunosorbent assay (ELISA) sandwich techniques following instructions described by the manufacturers (PhaMingen, San Diego, CA) were used to measure IFN-γ levels. Briefly, microtiter plates were coated with purified anti-human IFN-γ at 3 μg/mL. After the blockage, 100μL of culture supernatant or standard of recombinant IFN-γ were added and incubated. After incubation, plates were incubated again with biotin mouse anti-human IFN-γ at 1.0 μg/mL. Finally, streptavidin conjugated to horseradish-peroxidade (Sigma Chemical Co. St. Louis, MO) at a 1/4000 dilution was used for developed with tetramethylbenzidine (Calbiochen, La Jolla, CA). Absorbance was measured at 450 nm using Labsystem Multiskan ELISA Reader**.** Supernatants of cell cultures from HTLV-I asymptomatic carriers were measured without dilution while samples from HAM/TSP patients were diluted 5X before IFN-γ assay. The results were expressed as picograms per milliliter based on a standard curve generated using recombinant human IFN-γ.

Statistical analysis

Mann-Whitney U test, Student t test and Wilcoxon signed rank test were used to compare data between patients with HAM/TSP and asymptomatic carriers. GraphPad InStat program (San Diego, CA) carried out the statistical evaluation and p values < 0.05 were considered to indicate a significant difference.

RESULTS

Demographic data and IFN-γ production

Twenty HAM/TSP patients and 20 HTLV-I asymptomatic subjects participated of the study. The age of patients with myelopathy ranged from 39 to 72 years with the mean and Standard Deviation (SD) of 51 ± 9 years; 12 were male and 8 females. One HAM/TSP patient was co-infected with HCV. The Osame's score ranged from 3 to 11 and the EDSS was higher than 3 in all cases. The age of asymptomatic carriers ranged from 22 to 66 years (mean ± SD = 45 ± 12 years); 11 were male and 9 females. In the asymptomatic group, two subjects had positive serological test for viral hepatitis B and C and one was co-infected with HTLV-2. The mean and SD of spontaneous IFN-γ levels in myelopathy patients was (2,629 ± 2,245 pg/mL ranging from 364 to 8,215; median 2,108). It was higher than that observed in asymptomatic carriers (819 ± 950 pg/mL ranging from 62 to 3,515, median 391).

Ability of IL-10 to inhibit IFN-γ production in PBMCs cultures of asymptomatic carriers and HAM/TSP patients

IL-10 and TGF-β are the best-studied down-regulatory cytokines. To evaluate the ability of these modulatory cytokines to decrease the high spontaneous IFN-γ production observed in HTLV-I infected patients, PBMCs of HAM/TSP patients and asymptomatic HTLV-I carriers were cultured in the presence of these cytokines (Figure 1). IFN-γ levels in unstimulated supernatants of HTLV-I asymptomatic carriers and HAM/TSP patients were 819

± 950 pg/mL and 2,629 ± 2,245 pg/mL, respectively, p= 0.0005, Mann-Whitney U test. While the addition of 100 ng/mL of IL-10 decreased IFN-γ production to 453 ± 682 pg/mL in cell cultures from asymptomatic carriers (p=0.03), there was no difference in IFN-γ production after addition of IL-10 in HAM/TSP patients (1,581 ± 1,555 pg/mL, p=0.1). TGF-β (50 ng/mL) failed to significantly down regulate IFN-γ production in cell cultures from both asymptomatic carriers (519 ± 654 pg/mL, p=0.1) and in HAM/TSP patients (2,170 ± 1,864 pg/mL, p=0.4). While IL-10 decreased spontaneous IFN-γ synthesis in asymptomatic carriers cell cultures by 50±26%, in HAM/TSP patients the reduction in IFN-γ production was only of 33±30% (p=0.1). Addition of TGF-β decreased spontaneous IFN-γ production by 37±30% in asymptomatic HTLV-I cell cultures and by 22±17% in myelopathy patients. Both IL-10 and TGF-β were able to down regulate IFN-γ synthesis of PBMC from HTLV-I negative healthy individuals stimulated with PPD (2μg/mL). Interleukin-10 (20 ng/mL) and TGF-β (20 ng/mL) suppressed IFN-γ synthesis by 97% and 47%, respectively, in PPD stimulated cultures from HTLV-I negative controls (data not shown).

Ability of antagonists of cytokines (anti-IL-2, anti-IL-12 and anti-IL-15) to down regulate IFN-γ production in PBMC of HTLV-I asymptomatic carriers and HAM/TSP patients

IL-2 and IL-15 are cytokines participants of T cell activation and proliferation and both are involved in the spontaneous lymphoproliferation observed in HTLV-I infected patients. A dose response curve of the suitable concentration of neutralizing antibodies to down regulate IFN-γ synthesis was assayed. After this evaluation, the effects of neutralizing antibodies against IL-2, IL-12 and IL-15 (20μg/mL) were assayed in PBMCs cultures of HTLV-I asymptomatic subjects and myelopathy patients as described in Material and Methods. Figure 2 shows that anti-IL-2 neutralizing antibody decreased IFN-γ production by 30±26% in HAM/TSP cultures and 41±26% in asymptomatic carriers (p=0.2). After anti-IL-2

addition the IFN-γ levels in unstimulated cell cultures decreased from 819 ± 950 pg/mL to 565 ± 739 pg/mL in asymptomatic carriers (p=0.1) and from 2,629 ± 2,245 pg/mL to 1,845 ± 1,813 pg/mL in HAM/TSP patients (p=0.2). Anti-IL-15 addition reduced IFN-γ production by 38±33% (819 ± 950 pg/mL to 611 ± 886 pg/mL, p=0.1) in HTLV-I asymptomatic cultures. In HAM/TSP patients a 19±24% of reduction (2,629 ± 2,245 pg/mL to 1,896 ± 1,648 pg/mL, p=0.3) was observed. To evaluate the role of anti-IL-2 plus anti-IL-15 on IFN-γ production of HTLV-I asymptomatic carriers and HAM/TSP, both anti-cytokine antibodies were added to PBMCs cultures. No significant decrease in IFN-γ levels was observed in HAM/TSP cell cultures (2,629 ± 2,245 pg/mL to 1,767 ± 1,765 pg/mL, p=0.2). However, these two anti-cytokines combined significantly reduced IFN-γ levels in asymptomatic HTLV-I carriers from 819 ± 950 pg/mL to 389 ± 545 (p=0.02). Addition of antibodies against IL-2 plus IL-15 inhibited the IFN-γ synthesis in asymptomatic carriers by approximately 58±26% while IFN-γ production was reduced only 29±26% in HAM/TSP cell cultures (p=0.002). The addition of anti-IL-12 was not able to decrease spontaneous IFN-γ production. Both HTLV-I asymptomatic carriers (819 ± 950 pg/mL to 684 ± 822 pg/mL, p=0.5) as well HAM/TSP patients (2,629 ± 2,245 pg/mL to 2,667 ± 1,766 pg/mL, p=0.7) were not modulated by anti-IL-12.

Comparison of IFN-γ levels before and after addition of immunomodulators

In both groups, an expected individual variation was observed (Figure 3). Making a paired analysis of IFN-γ production without and with addition of regulating molecules it was found that IL-10 in high concentrations (100 ng/mL) significantly decreased spontaneous IFN-γ levels both in HTLV-I asymptomatic carriers (p=0.0001) and HAM/TSP patients (p=0.001, Wilcoxon signed rank test). Only two individuals in the asymptomatic group did not change IFN-γ levels after IL-10 addition (Figure 3A). After anti-IL-2 addition, IFN-γ

levels significantly decreased in 16 asymptomatic carriers and remained at similar levels in 4 of the 20 subjects evaluated (p=0.0001). In HAM/TSP patients, the addition of anti-IL-2 did not change IFN-γ levels in 8 of the 20, 40% of the patients (p=0.01), Figure 3B. The levels of IFN-γ were significantly decreased after the addition of anti-IL-2 plus anti-IL-15 in PBMC cultures from 17 HTLV-I asymptomatic carriers (p=0.0001), however 3 individuals remained at similar IFN-γ levels to the initial culture. The addition of anti-IL-2 plus anti-IL-15 in PBMC from HAM/TSP did not decrease IFN-γ in 25% (5 of the 20) of the patients, p=0.004 (Figure 3C). The levels of IFN-γ were significantly decreased after anti-IL-15 addition in PBMC from asymptomatic carriers (p=0.0007) and HAM/TSP (p=0.06) (data not shown).

Different response to the addition of IL-10 and antagonists of cytokines in PBMC cultures from HTLV-I asymptomatic carriers: high and low IFN-γ producers

As observed the IL-10 and cytokine antagonists do not homogeneously modulate PBMC cultures from HTLV-I asymptomatic carriers. We have previously shown that HTLV-I asymptomatic carriers show a great variability in the spontaneous IFN-γ levels and these individuals can be divided in high and low IFN-γ producers [8]. In this study when the asymptomatic carriers were divided in high (>400 pg/mL) and low IFN-γ producers (<400 pg/mL) these sub-groups showed a significant difference of IFN-γ suppression after addition of anti-IL-2 and anti-IL-2 plus anti-IL-15 (Table 1). HTLV-I asymptomatic carriers who produce low spontaneous IFN-γ levels (n=14) decreased significantly IFN-γ production after anti-IL-2 and anti-IL-2 plus anti-IL-15 addition (49±25% and 64±23%, respectively, p<0.04, Student t test). The means of individual suppression of asymptomatic carriers high producer (n=06) were 23±21% to anti-IL-2 and 42±18% to anti-IL-2 plus anti-IL-15. However, IL-10 added to PBMCs cultures equally suppressed HTLV-I asymptomatic carriers who were high and low IFN-γ producers (p>0.05, Student t test). No difference was observed in the means of

individual IFN-γ suppression after addition of IL-10 and anti-IL-2 between asymptomatic high producer and HAM/TSP were compared ($p>0.05$, data not shown).

DISCUSSION

The present study evaluated the ability of immunomodulatory cytokines and cytokine antagonists to inhibit spontaneous IFN-γ production in PBMCs cultures of HTLV-I infected subjects. Addition of IL-10, anti-IL-2 and simultaneous addition of anti-IL-2 and anti-IL-15 significantly down regulated spontaneous IFN-γ synthesis in asymptomatic carriers compared with HAM/TSP. Moreover, we observed a small sub-group of HTLV-I asymptomatic carriers that behaved similar to HAM/TSP patients in which no modulatory actions with these immunoregulatory cytokines were observed.

Activation of the host T cells by Tax protein coded by HTLV-I may induce a number of cytokines [16], especially pro-inflammatory cytokines as IFN-γ and TNF-α [6,17]. Although IFN-γ and TNF-α synthesis are important controls to virus replication, they are also involved in HAM/TSP pathogenesis [18-20]. Moreover, T cells from HAM/TSP patients produce higher IFN-γ levels when compared with asymptomatic carriers [21,22]. In HTLV-I infection, regulatory mechanisms based in inhibition of pro-inflammatory cytokines can be an important key to modulate the exaggerated immune response and to prevent development of inflammatory mediated disease.

An overview of our results showed that IL-10, anti-IL-2 and simultaneous addition of anti-IL-2 and anti-IL-15 down modulated IFN-γ production in asymptomatic HTLV-I carriers but failed to decrease the levels of this cytokine in HAM/TSP patients. These finding indicate that HAM/TSP cultures are more difficult to be modulated in vitro than cells from HTLV-I asymptomatic carriers. Interleukin-10 has been shown to be an important immunoregulatory cytokine and a potential clinical tool to reduce exacerbated inflammatory response in various

diseases [23]. The data that this regulatory cytokine decrease spontaneous IFN-γ production in PBMC cultures from asymptomatic carriers are in agreement with previous observations [8], although the inhibitory effect of IL-10 were only observed when this cytokine was added in high concentrations to the cultures. The TGF-β has potent immunoregulatory properties on human lymphocyte functions [23]. The failure of TGF-β in down-regulate IFN-γ synthesis in both HTLV-I infected groups is consistent with the data demonstrating that the T cell activation pathway by HTLV-I is insensitive to TGF-β action [25]. Recently it was described that Tax inhibits the signal of TGF-β binding to nuclear regulatory proteins and makes HTLV-I infected cells able to escape TGF-β-mediated growth inhibition [26,27].

In addition to IL-10 and TGF-β, other molecules such as IL-2, IL-12 and IL-15 are important in the differentiation and multiplication of T cells. Up-regulation of these cytokines, especially IL-15, are associated with ATL and HAM/TSP [28]. Because IL-12 induces IFN-γ production and promotes differentiation of type 1 T cells [29], we also tried to block T cell activation using a neutralizing anti-IL-12. Our results showed that the blockage of IL-12 failed to decrease IFN-γ production in both HTLV-I studied groups, suggesting that this cytokine does not participate in the spontaneous T cell activation observed after HTLV-I infection. In HTLV-I infection the persistent T cell activation is due to an existence of two autocrine loops including IL-2, IL-15 and their respective receptors (IL-2Rα and IL-15Rα), which are transcriptionally regulated by Tax [11,13]. Previous studies have shown that neutralization of IL-2 and IL-15 reduces spontaneous lymphocyte proliferation in HAM/TSP patients, although a complete inhibition was observed only when monoclonal antibodies against both cytokines and their receptors were added simultaneously [10,13]. This study demonstrates that anti-IL-2 plus IL-15 significantly decreased IFN-γ production in asymptomatic carriers but were not able to inhibit IFN-γ synthesis in most patients with myelopathy.

One important question is why IL-10 and cytokine antagonists, who normally have immunoregulatory actions, were unable to control T cell response of HAM/TSP patients. In a patient with HAM/TSP, in contrast to asymptomatic HTLV-I carriers, the high production of pro-inflammatory cytokines induced by abundant Tax protein could diminish the actions of these immunomodulatory cytokines. In addition, proviral load, genetic polymorphisms or the action of an unknown lymphoproliferative cytokine are possibilities to investigate [9,21].

Although IL-10 and anti-IL-2 plus anti-IL-15 significantly decreased IFN-γ production in PBMCs from asymptomatic HTLV-I subjects, the modulation was not homogeneously observed in all of patients. While in a majority of asymptomatic carriers these molecules suppressed IFN-γ production, in a small group of asymptomatic carriers (15-20%), who produce high levels of spontaneous IFN-γ (>400pg/mL), the inability to down modulate IFN-γ production was similar to that observed with HAM/TSP patients**.** Previously we showed that about 40% of the HTLV-I asymptomatic carriers have a lymphoproliferative response and high production of IFN-γ and TNF-α [22]. These findings, that immunoregulatory molecules do not significantly decrease IFN-γ production in all asymptomatic carriers, supports and extends our observation that asymptomatic carriers who present similar immunological abnormalities as the observed in patients with HAM/TSP could, in the future, develop neurological alterations similar to ones observed in patients with myelopathy.

Various research groups have tried therapeutic approaches based in the use of immunomodulators to control diseases associated to HTLV-I infection [30,31]. The association of these immunomodulators with news drugs should be an alternative. We believe that HTLV-I asymptomatic carriers who produce high levels of spontaneous IFN-γ and show *in vitro* no modulatory action with immunoregulatory cytokines or cytokine antagonists should receive special attention. In summary, we demonstrated that PBMCs from HTLV-I asymptomatic carriers are more easily modulated by immunoregulatory cytokines and

cytokine antagonists than PBMC from HAM/TSP patients. Moreover, we identified a sub-group of asymptomatic HTLV-I carriers who have a pattern of cytokine and lymphoproliferative response similar to HAM/TSP patients. This sub-group of asymptomatic carriers who are high IFN-γ producers with poor modulation for IFN-γ by cytokines and cytokine antagonists should be followed closely for evidence of early manifestations of myelopathy such as neurological changes, urinary disturbances or sexual dysfunction.

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FIGURES LEGEND

Figure 1

Immunoregulatory effects of IL-10 and TGF-β on the spontaneous IFN-γ production in HTLV-I asymptomatic carriers (n=20) and HAM/TSP patients (n=20). PBMCs were cultured in presence or absence of IL-10 (100ng/mL) and TGF-β (50ng/mL) for 72 h. IFN-γ levels were determined by ELISA and each bar represents the mean and SD from each group.

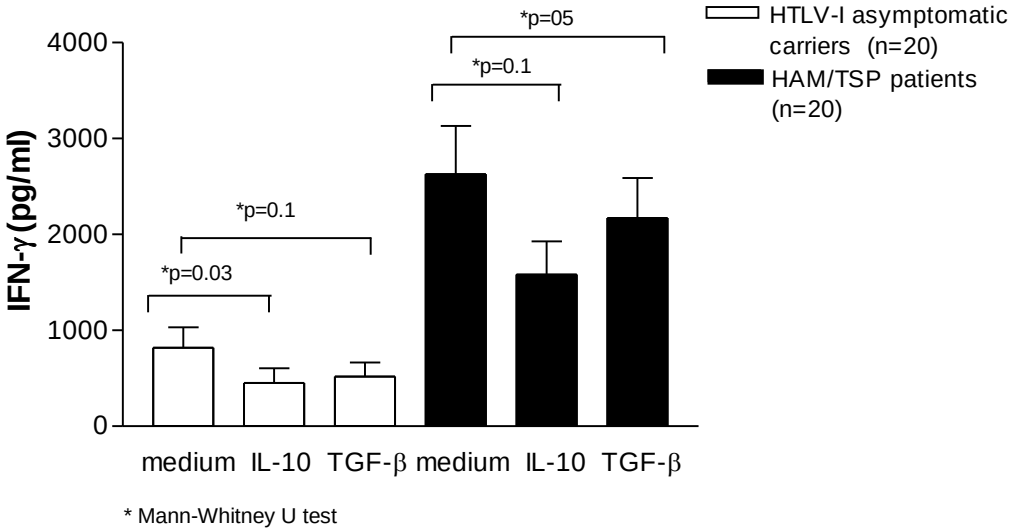
Figure 2

Inhibition of spontaneous IFN-γ production after addition of neutralizing cytokines in PBMCs cultures of HTLV-I asymptomatic carriers (n=20) and HAM/TSP patients (n=20). The percentage of suppression was calculated: [(spontaneous IFN-γ - treated IFN-γ / spontaneous IFN-γ) x 100] and the results expressed as mean and SD of % suppression from each group.

Figure 3

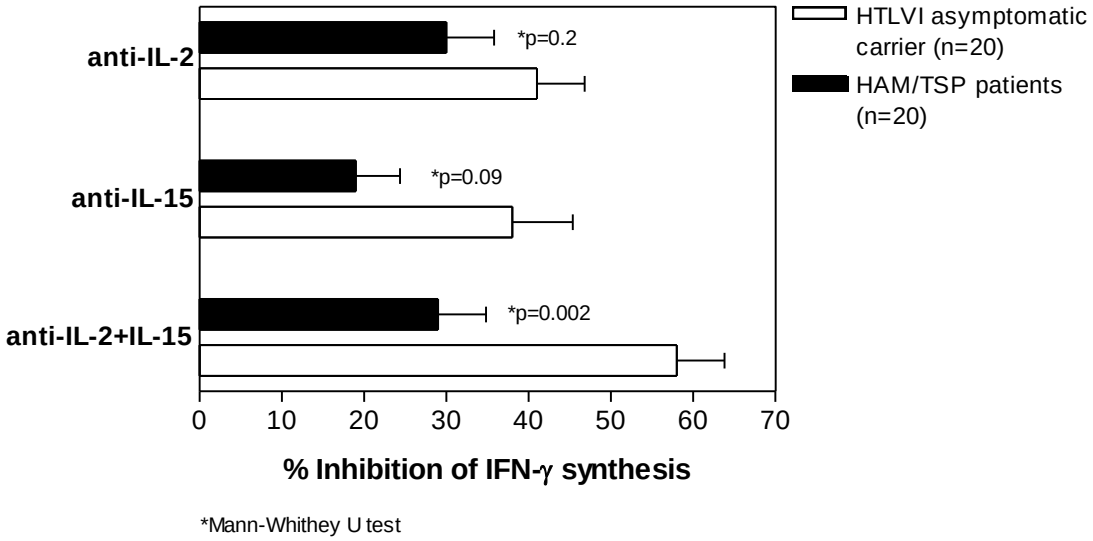
Effects of the addition of IL-10 (A), anti-IL-2 (B) and anti-IL-2 plus anti-IL-15 (C) on the spontaneous IFN-γ synthesis in PBMCs from HTLV-I asymptomatic carriers (n=20) and HAM/TSP patients (n=20).

Figure 1



* Mann-Whitney U test

Figure 2



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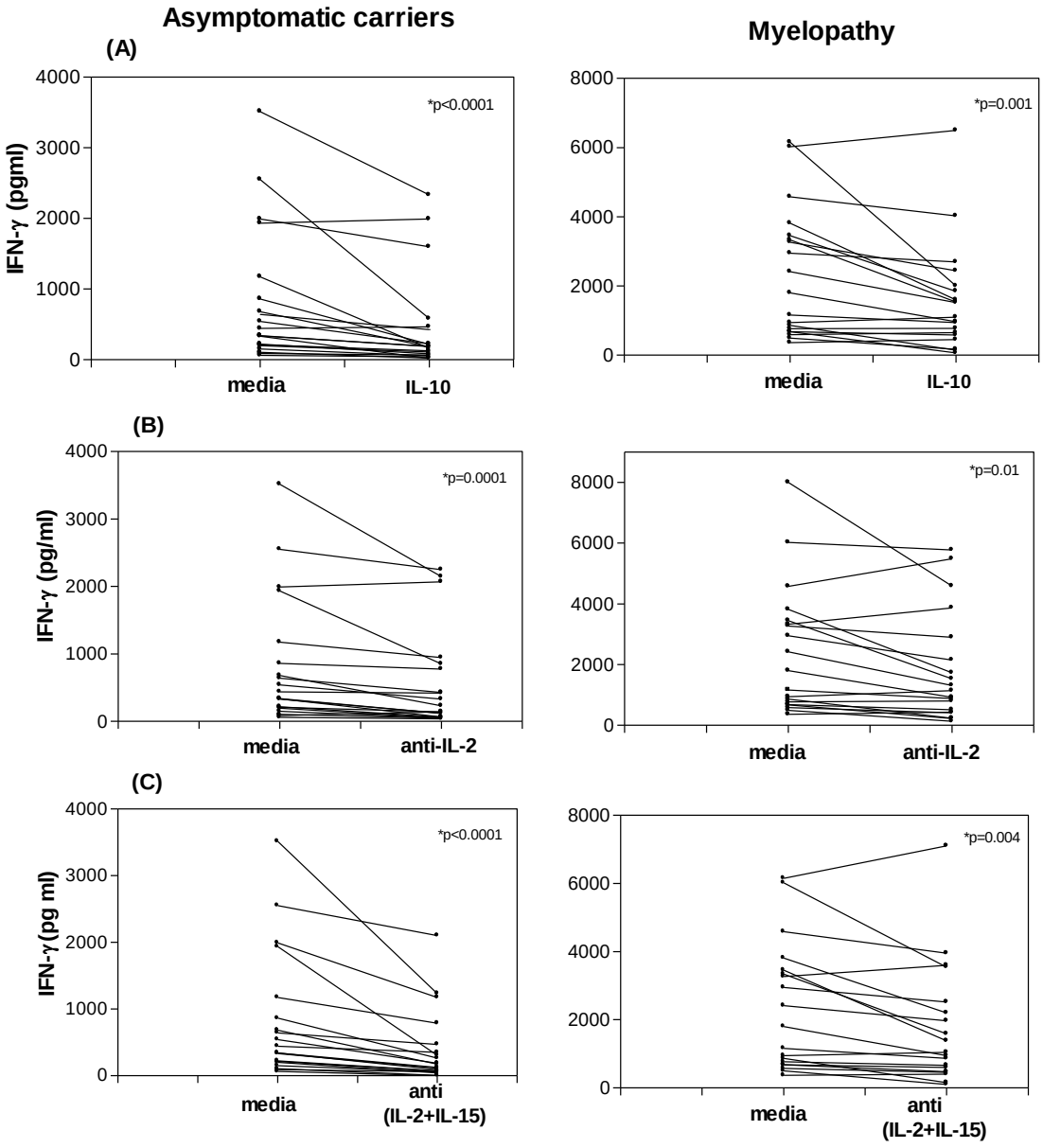

*Wilcoxon signed rank test

Table1. Mean of Inhibition of IFN-γ Production by Cytokines and Antagonists of Cytokines between HTLV-I Asymptomatic Carriers High and Low IFN-γ producers.

	HTLV-I carrier Low Producer (n =14)	HTLV-I carrier High Producer (n = 06)	p value
Spontaneous IFN-γ (pg/mL)	311 ± 201	2,005 ± 957	0.0001[1]
% Inhibition of IFN-γ production by exogenous addition of			
IL-10	50 ± 23	49 ± 35	0.91 [2]
anti-IL-2	49 ± 25	23 ± 21	0.04 [2]
anti-IL-2 + anti-IL-15	64 ± 23	42 ± 18	0.04 [2]

Data represent the Mean ± Standard Deviation of individual percentage of IFN-γ suppression. [1] Mann-Whitney U test; [2] Student t test.

ARTIGO 3

Helminthic infections down modulate type 1 immune response in HTLV-I patients and are more prevalent among HTLV-I carriers than patients with HTLV-I associated myelopathy/tropical spastic paraparesis

Aurélia Fonseca Porto, Silvane Braga Santos, André Luis Muniz, Vanessa Basílio, Waldyr Rodrigues Jr., Franklin Neva, Walderez Dutra, Kenneth Gollob, Steven Jacobson & Edgar Carvalho.

Este estudo teve como objetivo principal avaliar a influência das infecções por helmintos (*S. stercoralis e S. mansoni)* sobre a resposta imune e carga proviral de indivíduos infectados pelo HTLV-I. A resposta imune nas infecções por helmintos é caracterizada pela produção de citocinas tipo 2 enquanto indivíduos infectados pelo HTLV-I apresentam uma intensa resposta tipo 1, com produção aumentada de IFN-γ. Culturas de células de indivíduos assintomáticos HTLV-I positivo co-infectados por helmintos apresentam uma diminuição da síntese espontânea de IFN-γ quando comparados com indivíduos HTLV-I positivo sem co-infecção. Os resultados da citometria de fluxo mostraram uma menor frequência de células T CD4+ e CD8+ produzindo IFN-γ e uma maior freqüência de células T produzindo IL-5 e IL-10 nos indivíduos co-infetados por helmintos, quando comparado com os indivíduos sem co-infecção. Apesar da grande variabilidade, a carga proviral do HTLV-I foi menor nos indivíduos co-infectados com helmintos do que nos indivíduos somente com infecção pelo HTLV-I. Adicionalmente, observou-se que a prevalência de infecções por helmintos é maior entre os portadores assintomáticos do vírus do que nos pacientes com HAM/TSP, sugerindo que helmintos podem proteger indivíduos infectados pelo HTLV-I de desenvolverem mielopatia.

MAJOR ARTICLE

Helminthic Infection Down-Regulates Type 1 Immune Responses in Human T Cell Lymphotropic Virus Type 1 (HTLV-1) Carriers and Is More Prevalent in HTLV-1 Carriers than in Patients with HTLV-1–Associated Myelopathy/Tropical Spastic Paraparesis

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Human T cell lymphotropic virus type 1 (HTLV-1) infection is associated with an exacerbated type 1 immune response and secretion of high levels of proinflammatory cytokines. In contrast, helminthic infection induces a type 2 immune response. In the present study, the cytokine profile in HTLV-1 carriers coinfected with helminths (*Strongyloides stercoralis* and/or *Schistosoma mansoni*) was compared with that in HTLV-1 carriers not coinfected with helminths. Levels of interferon (IFN)–γ were higher in HTLV-1 carriers not coinfected with helminths than in HTLV-1 carriers coinfected with helminths ($P < .05$). The overall frequency of IFN-γ–expressing $CD8^+$ and $CD4^+$ cells was decreased in HTLV-1 carriers coinfected with helminths ($P < .05$). The percentage of interleukin (IL)–5– and IL-10–expressing T cells in HTLV-1 carriers coinfected with helminths was higher than that in HTLV-1 carriers not coinfected with helminths ($P < .05$). Moreover, we found that the prevalence of helminthic infection was 7-fold higher in HTLV-1 carriers than in patients with HTLV-1–associated myelopathy/tropical spastic paraparesis ($P < .05$). These data show that helminthic infection decreases activation of type 1 cells, which may influence the clinical outcome of HTLV-1 infection.

Human T cell lymphotropic virus type 1 (HTLV-1) infection is associated with spontaneous activation of T cells, uncontrolled proliferation of lymphocytes, and an exacerbated type 1 immune response including secretion of high levels of proinflammatory cytokines [1–3]. The great majority of individuals infected with HTLV-1 display an asymptomatic form of the infection and are referred to as "HTLV-1 carriers." HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T cell leukemia/lymphoma (ATLL) are the main clinical manifestations associated with HTLV-1 infection. HAM/TSP is characterized by hyperreflexia, muscle weakness, and spasticity in the lower extremities. Evidence that the immunological response participates in the pathogenesis of HAM/TSP includes the following: (1) cytotoxic activity against viral Tax protein is present in patients with HAM/TSP [4, 5]; (2) an increase in proinflammatory cytokines—such as tumor necrosis factor (TNF)–α, interleukin (IL)–1, and IL-6—has been observed in the cerebral spinal fluid (CSF)

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of patients with HAM/TSP [6–8]; and (3) spinal cord lesions are associated with infiltration of $CD4^+$ and $CD8^+$ T cells, presence of macrophages, proliferation of astrocytes, and fibrillary gliosis [9]. Although proinflammatory cytokines are more prominent in patients with HAM/TSP, HTLV-1 carriers also have high production of proinflammatory cytokines such as interferon (IFN)–γ and TNF-α [2].

In contrast to HTLV-1 infection, helminthic infection is associated with a type 2 immune response and with high levels of IL-4, IL-5, and IL-10 and low levels of IFN-γ [10, 11]. It has been shown that, as a regulatory mechanism of the immune response, cytokines secreted by type 2 cells may down-regulate type 1 immune responses, and vice versa. For instance, IL-4 and IL-10 may down-regulate the IFN-γ response [12], and IFN-γ decreases the secretion of type 2 cytokines [13]. We and others have previously shown a high frequency of strongyloidiasis [14–17] and an increased susceptibility to develop disseminated *Strongyloides stercoralis* infection in HTLV-1 carriers [18–20]. It is also well known that helminthic infection—in particular, schistosomiasis—down-regulates type 1 immune responses and decreases the severity of autoimmune disease in experimental animals [21, 22]. To evaluate whether helminthic infection influences the immunological response in patients infected with HTLV-1, the cytokine profile and HTLV-1 proviral DNA load were determined in both HTLV-1 carriers coinfected with helminths (*S. stercoralis* and/or *Schistosoma mansoni)* and HTLV-1 carriers not coinfected with helminths. Additionally, the prevalence of helminthic infection in patients with HAM/TSP and that in HTLV-1 carriers were compared.

PATIENTS, MATERIALS, AND METHODS

Patients. The present study included 310 HTLV-1 carriers and 32 patients with HAM/TSP from the HTLV-1 multidisciplinary clinic located at Hospital Universitário Prof. Edgard Santos in Salvador, Bahia, Brazil. A clinical history was obtained from and a physical examination was performed on all patients. For all patients, HTLV-1 infection was confirmed by Western-blot analysis, and 3 examinations of stool specimens were performed (Hoffman and Baermann techniques). Twenty-five percent of the patients infected with *S. stercoralis* had complained of diarrhea. Patients with schistosomiasis were asymptomatic and had <25 eggs/g of stool. Immunological evaluation was performed for 35 HTLV-1 carriers coinfected with helminths (*S. stercoralis* and/or *S. mansoni*) and for a control group of 35 HTLV-1 carriers matched by age and sex but without evidence of helminthic infection. Immunological studies were also performed for 18 patients with HAM/TSP, including the 1 with HAM/TSP and helminthic infection. Immunological evaluation consisted of measurement of cytokines (IFN-γ and IL-5) in supernatants of unstimulated peripheral blood mononuclear cell (PBMC) cultures by ELISA and measurement of intracellular cytokines (IFN-γ, IL-10, and IL-5) and phenotypic immunological markers by flow-cytometric analysis. Moreover, HTLV-1 proviral DNA load was determined. The mean ± SD ages of HTLV-1 carriers coinfected with helminths and of HTLV-1 carriers not coinfected with helminths were 45 ± 17 and 46 ± 12 years, respectively, and the male:female ratios were 6:1 and 5:1, respectively. This was the naturally occurring bias found in the sample population. The criterion for a diagnosis of strongyloidiasis or schistosomiasis was a positive identification of either *S. stercoralis* larvae (Baermann technique) or *S. mansoni* eggs (Hoffman technique) in a stool specimen. By the examination of stool specimens, 13 patients were found to have *S. stercoralis* infection alone, 15 patients were found to have *S. mansoni* infection alone, and 7 patients were found to have both *S. stercoralis* and *S. mansoni* infection. After collection of blood, all patients infected with *S. stercoralis* were treated with cambendazol (5 mg/kg of weight), and those infected with *S. mansoni* were treated with praziquantel (50 mg/kg of weight, divided into 2 doses) or oxaminiquine (20 mg/kg of weight, in a single dose).

For evaluation of the prevalence of helminthic infection, 342 patients attending the HTLV-1 clinic were included in the present study. These patients had been evaluated by 2 neurologists and were divided into 2 groups according to the Osames' Motor Disability Score [23] and the Expanded Disability Status Scale [24]: (1) patients with HAM/TSP and (2) HTLV-1 carriers who did not fulfill the World Health Organization criteria for HAM/TSP. Informed consent was obtained from all participants, and the human-experimentation guidelines of the Hospital Universitário Prof. Edgard Santos were followed in the conduct of this clinical research.

Immunological Studies

Determination of levels of cytokines. Levels of cytokines (IFN-γ and IL-5) in supernatants of unstimulated PBMCs were measured by ELISA. Briefly, PBMCs were obtained by density-gradient centrifugation by use of lymphocyte separation media (Organon Teknika). After being washed in saline, the cells were adjusted to 3×10^6 cells/mL in RPMI 1640 medium (Gibco) supplemented with 10% AB^+ serum containing 100 U of penicillin/g and 10 μg/mL streptomycin. All cultures were incubated without stimulus, for 72 h at 37°C in 5% CO_2. Supernatants were collected and stored at −20°C. Levels of IFN-γ (Genzyme) and IL-5 (PharMingen) were measured by the ELISA sandwich technique, and the results were expressed in picograms per milliliter on the basis of a standard curve generated by use of recombinant cytokines.

Single-cell cytoplasmic cytokine staining. Briefly, 2×10^5 PBMCs were cultured in RPMI 1640 medium supplemented with 5% AB Rh^+ serum, in 96-well plates. On the basis of preliminary results, all the cytokine staining was performed

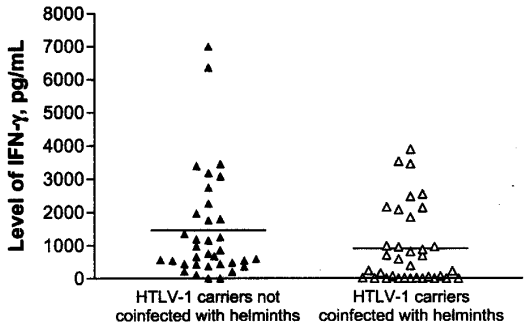

Figure 1. Levels of interferon (IFN)–γ in human T cell lymphotropic virus type 1 (HTLV-1) carriers coinfected or not coinfected with helminths *(Strongyloides stercoralis* and/or *Schistosoma mansoni).* Levels of IFN-γ were determined in unstimulated 72-h culture supernatants of peripheral blood mononuclear cells. The horizontal lines represent the means of the populations.

after 20 h of incubation with or without αCD3/CD28 stimulus. During the last 4 h of incubation, brefeldin A (1 μg/mL) was added to the culture. The cells were then washed and centrifuged by use of ice-cold PBS plus sodium-azide, were stained for surface markers, and were fixed by use of 2% formaldehyde. The fixed cells were then permeabilized with a solution of saponin and stained for 30 min at 4°C by use of anticytokine monoclonal antibodies directly conjugated with phosphatidylethanolamine (IFN-γ, IL-5, and IL-10) (Pharmingen). Preparations were then washed, fixed, and analyzed by use of a FACSCalibur flow cytometer (Becton Dickinson). In all cases, the cells were double-stained for cytokines and for cell-surface markers. In all cases, because of the low frequency of positive events being analyzed, 30,000 gated events were acquired for later analysis.

Proviral Load

Patients and cells. The sample population consisted of 12 HTLV-1 carriers not coinfected with helminths and 17 HTLV-1 carriers coinfected with helminths from whom frozen PBMCs were available; determination of HTLV-1 proviral DNA load was performed only in a subgroup of patients, because of the limited number of available samples.

Real-time polymerase chain reaction analysis of DNA. The HTLV-1 proviral DNA load in PBMCs was measured by use of an ABI PRISM 7700 Sequence Detector (Applied Biosystems), as described elsewhere [25]. DNA was extracted from 10^6 cells by use of a Puregene DNA Isolation Kit (Gentra), in accordance with the manufacturer's instructions, and 100 ng of sample DNA solution/well was analyzed. The HTLV-1 proviral DNA load was calculated by use of the following formula: copy number of HTLV-I (pX) per 100 cells = (copy number of pX)/(copy number of β-actin/2) × 100.

Serological Testing for HTLV-1

All serum samples were screened for HTLV-1 and HTLV-2 antibodies by ELISA (Cambridge Biotech). Repeatedly reactive samples were subjected to Western-blot analysis, to distinguish between HTLV-1 and HTLV-2, by use of HTLV blot 2.4 (Genelabs), in accordance with the manufacturer's instructions.

Statistical Analysis

The Wilcoxon rank sum test was used to compare means. Fisher's exact test was used to compare proportions. The χ^2 test was used to compare the prevalence of helminthic infection.

RESULTS

The levels of IFN-γ in supernatants of lymphocyte cultures from HTLV-1 carriers coinfected with helminths and those not coinfected with helminths are shown in figure 1. The levels (mean ± SD) of IFN-γ were higher in 35 HTLV-1 carriers not coinfected with helminths (1465 ± 1648 pg/mL) than in 35 HTLV-1 carriers coinfected with helminths (913 ± 1163 pg/mL) ($P < .05$). Both *S. mansoni* and *S. stercoralis* infections contribute to the decreasing levels of IFN-γ, but the down-regulation of IFN-γ was mainly observed in HTLV-1 carriers coinfected with *S. mansoni* (474 ± 838 pg/mL). Although the levels (mean ± SD) of IL-5 did not differ between the 2 groups, there was a tendency for higher levels of IL-5 in HTLV-1 carriers coinfected with helminths (199 ± 476 pg/mL), compared with those in HTLV-1 carriers not coinfected with helminths (132 ± 258 pg/mL) ($P > .05$) (data not shown). As we have reported elsewhere [2], levels of IFN-γ in HTLV-1 carriers were quite variable, and patients could be divided into low-level (<400 pg/mL; range, 0–370 pg/mL) and high-level (>400 pg/mL; range, 430–6995 pg/mL) producers of IFN-γ. The frequencies of low-level producers of IFN-γ were 52% in the HTLV-1 carriers not coinfected with helminths and 80% in the HTLV-1 carriers coinfected with helminths ($P < .05$) (table 1). The mean production of IFN-γ in patients with HAM/TSP was 4246 ± 2924 pg/mL. Because only 1 patient with HAM/TSP was in-

Table 1. Frequency of high-level and low-level producers of interferon-γ among human T cell lymphotropic virus type 1 (HTLV-1) carriers coinfected or not coinfected with helminths *(Strongyloides stercoralis* and/or *Schistosoma mansoni).*

	Infection, no. (%) of patients	
Level	HTLV-1 alone (n = 35)	HTLV-1 plus helminths (n = 35)
High (>400 pg/mL)	17 (48)	7 (20)
Low (<400 pg/mL)	18 (52)	28 (80)[a]

[a] $P < .05$, Fisher's exact test.

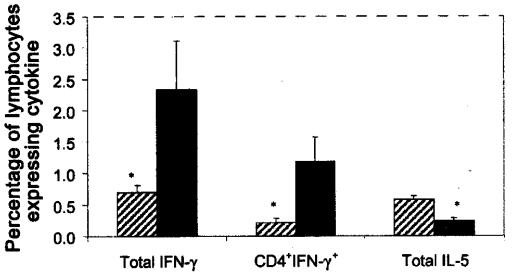

Figure 2. Lower frequency of $CD4^+$ interferon (IFN)–γ^+ cells and higher frequency of cells secreting interleukin (IL)–5 in human T cell lymphotropic virus type 1 (HTLV-1) carriers coinfected with helminths *(striped bars)* than in HTLV-1 carriers not coinfected with helminths *(black bars)*. The columns and error bars represent the mean ± SD, respectively. $n = 3$ patients/group. $^*P < .05$

fected with helminths, no comparison could be performed between patients with HAM/TSP infected with helminths and patients with HAM/TSP not infected with helminths.

The frequencies of cytokine-producing cells in HTLV-1 carriers not coinfected with helminths and HTLV-1 carriers coinfected with helminths, after stimulation with αCD3/CD28, were determined by fluorescence-activated cell sorter analysis. Figure 2 shows that, although the frequency of total cells secreting IFN-γ was 2.33% in 3 HTLV-1 carriers not coinfected with helminths, in 3 HTLV-1 carriers coinfected with helminths, only 0.70% of cells secreted IFN-γ ($P < .05$). In contrast, the frequency of cells secreting IL-5 was 2-fold higher in HTLV-1 carriers coinfected with helminths (0.58%) than in HTLV-1 carriers not coinfected with helminths (0.24%) ($P < .05$). Most of the IFN-γ was secreted by $CD4^+$ T cells, and the frequency of IFN-γ–expressing $CD4^+$ T cells (1.18%) was higher in HTLV-1 carriers not coinfected with helminths than in HTLV-1 carriers coinfected with helminths (0.22%) ($P < .05$) (figure 2).

We have previously shown that most of the IFN-γ–producing cells in HTLV-1 carriers were $CD4^+$ T cells, although both $CD4^+$ and $CD8^+$ T cells are responsible for the high levels of IFN-γ observed in HTLV-1 carriers [26]. Figure 3 shows the frequency of $CD8^+$ T cells secreting IFN-γ or IL-10 and the frequency of total cells secreting IL-10 in unstimulated cultures. Coinfection with HTLV-1 and helminths significantly decreases the frequency of $CD8^+$ T cells secreting IFN-γ ($P < .05$). In contrast, the frequency of total cells secreting IL-10 and the frequency of $CD8^+$ T cells secreting IL-10 were higher in 4 HTLV-1 carriers coinfected with helminths (0.58%) than in 7 HTLV-1 carriers not coinfected with helminths (0.21%) ($P < .05$). Moreover, the frequency of total $CD8^+$ T cells was higher in HTLV-1 carriers coinfected with helminths (data not shown).

Figure 4 shows the HTLV-1 proviral DNA load in a subset of patients from the 2 groups of patients from whom frozen PBMCs were available. Although the number of copies were quite variable in both groups, the HTLV-1 proviral DNA load was significantly lower in the 17 HTLV-1 carriers coinfected with helminths (2.2 ± 1.5 copies/100 cells) than in the 12 HTLV-1 carriers not coinfected with helminths (3.7 ± 1.2 copies/100 cells) ($P < .05$).

The frequency of infection with the intestinal helminths *S. stercoralis* and *S. mansoni* is higher in patients infected with HTLV-1 than in seronegative individuals [27]. By comparing the prevalence of these helminths in HTLV-1 carriers with that in patients with HAM/TSP, we found that HTLV-1 carriers had a 7-fold higher prevalence of infection with intestinal helminths than did patients with HAM/TSP (table 2).

DISCUSSION

The present study has shown that helminthic infection decreases both production of IFN-γ and the overall frequency of IFN-γ–expressing $CD8^+$ and $CD4^+$ cells in HTLV-1 carriers. In contrast, the percentage of IL-10–expressing cells in HTLV-1 carriers coinfected with helminths was higher than that in HTLV-1 carriers not coinfected with helminths. Moreover, the prevalence of helminthic infection was significantly lower in patients with HAM/TSP than in HTLV-1 carriers.

Coinfection with HTLV-1 and helminths has clinical and immunological implications. It is known that the prevalence of strongyloidiasis and schistosomiasis is higher in HTLV-1 carriers than in seronegative control subjects [14–17, 27] and that coinfection with HTLV-1 and *S. stercoralis* is associated with dissemination of parasites and development of severe forms of strongyloidiasis [18–20]. We have previously shown that HTLV-1 infection decreases the type 2 immune response in patients with strongyloidiasis and schistosomiasis [27–29].

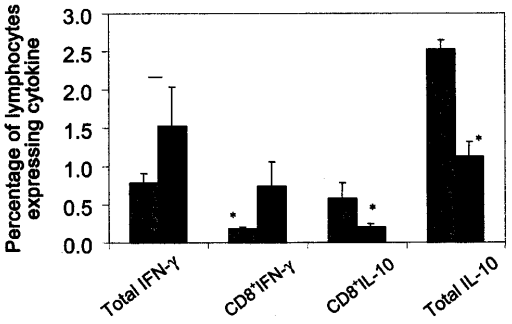

Figure 3. Lower frequency of $CD8^+$ T cells secreting interferon (IFN)–γ and higher frequency of cells secreting interleukin (IL)–10 in human T cell lymphotropic virus type 1 (HTLV-1) carriers coinfected with helminths ($n = 4$) *(gray bars)* than in HTLV-1 carriers not coinfected with helminths ($n = 7$) *(black bars)*. The columns and error bars represent the mean ± SD, respectively. $^*P < .05$.

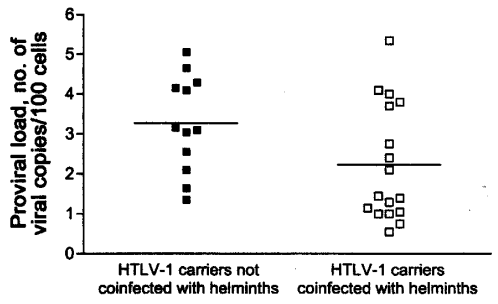

Figure 4. Proviral load in human T cell lymphotropic virus type 1 (HTLV-1) carriers coinfected or not coinfected with helminths (*Strongyloides stercoralis* and/or *Schistosoma mansoni*). The horizontal lines represent the means of the populations.

Here, we have shown that helminthic infection can down-regulate the exaggerated inflammatory response observed in HTLV-1 carriers. Additionally, coinfection with HTLV-1 and helminths was associated with a decreased HTLV-1 proviral DNA load and a decreased frequency of myelopathy.

HTLV-1 infects predominantly T cells, leading to spontaneous proliferation of lymphocytes and increased secretion of cytokines. Although levels of both type 1 and type 2 cytokines are increased in unstimulated lymphocyte cultures from HTLV-1 carriers, compared with those from control subjects, the most striking finding in this regard is the high levels of IFN-γ secreted by both CD4 and $CD8^+$ T cells [26]. Because helminthic infection is associated with increasing levels of IL-4, IL-5, and IL-10 [10–11], the immunological consequences of the association between HTLV-1 infection and helminthic infection was evaluated. That levels of IFN-γ and numbers of $CD4^+$ and $CD8^+$ T cells were decreased in HTLV-1 carriers coinfected with helminths indicates that helminthic infection may down-regulate production of IFN-γ in HTLV-1 carriers.

We have previously shown that exogenous IL-10 can decrease production of IFN-γ in lymphocyte cultures from HTLV-1 carriers [26]. That HTLV-1 carriers coinfected with helminths have an increased frequency of cells secreting IL-10, compared with HTLV-1 carriers not coinfected with helminths, indicates that, in HTLV-1 infection, helminths may down-regulate production of IFN-γ through the induction of IL-10.

Although little is known about defense mechanisms against HTLV-1, killing of infected T cells by $CD8^+$ T cells participates in this phenomenon [5]. Given that helminthic infection down-regulates the type 1 immune response, it is plausible that helminthic infection increases the HTLV-1 proviral DNA load. In fact, a previous study [30] showed that coinfection with *S. stercoralis* increases the HTLV-1 proviral DNA load. Here, we have shown that the proviral load in HTLV-1 carriers coinfected with helminths is lower than that in HTLV-1 carriers not coinfected with helminths, suggesting that helminths may inhibit HTLV-1 transcription. Since the spread of the virus is accelerated by activation of T cells [31], it is possible that the low proviral load in HTLV-1 carriers coinfected with helminths may be due to down-regulation of the immune system. Interestingly, a study of patients with T cell non-Hodgkin lymphoma and patients with ATLL showed that there was a better response to treatment and longer survival in HTLV-1 carriers coinfected with *S. stercoralis* than in HTLV-1 carriers not coinfected with *S. stercoralis* [32].

HAM/TSP is one of the most important consequences of HTLV-1 infection and is characterized by weakness, hyperreflexia, urinary manifestations, and spastic paraparesis. Several studies have emphasized the role of the immune response in the pathogenesis of HAM/TSP, with the following observations: (1) infiltration of the spinal cord by T cells with an increasing number of $CD8^+$ T cells expressing *tax* [9]; (2) increasing levels of proinflammatory cytokines in lymphocyte cultures and CSF [6–8]; and (3) occurrence of fibrosis of the neurological tissue associated with inflammation [9]. We have previously shown that the frequencies of *S. stercoralis* and *S. mansoni* infections were higher in HTLV-1 carriers than in HTLV-1–seronegative blood donors [17, 27]. In the present study, we found that the frequencies of *S. mansoni* and *S. stercoralis* infections were much lower in patients with HAM/TSP than in HTLV-1 carriers. Although it can be argued that patients with HAM/TSP are potentially less frequently exposed to *S. stercoralis* and *S. mansoni*, because of their physical limitations, the group of HTLV-1 carriers coinfected with helminths reported here had no recent exposure to these helminths. In fact, all of the HTLV-1–infected patients in the present study now live in urban areas, where *S. mansoni* transmission is not documented and contamination of the adult population with *S. stercoralis* is less likely. These observations, together with the data suggesting that HTLV-1 infection increases the failure rate of antihelminthic drugs [33, 34], suggest that most of the HTLV-1 carriers coinfected with *S. stercoralis* and/or *S. mansoni* acquired the helminthic infection during childhood. In such cases, the low frequency of helminthic infection in patients with HAM/TSP may suggest that helminths,

Table 2. Frequency of helminths (*Strongyloides stercoralis* and/or *Schistosoma mansoni*) in human T cell lymphotropic virus type 1 (HTLV-1) carriers and patients with HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP).

Clinical form of HTLV-1 infection	No. of patients with helminths/ no. of patients tested (%)
HTLV-1	71/310 (23)[a]
HAM/TSP	1/32 (3)

[a] $P < .05$, χ^2 test.

by decreasing the production of IFN-γ and the HTLV-1 proviral DNA load, protect HTLV-1 carriers from development of myelopathy. Interestingly, the majority of the studies of coinfection with HTLV-1 and *S. stercoralis* in Japan have been performed in Okinawa, and there are no data in the literature on the prevalence of HAM/TSP in this area of Japan [34].

The present study has clearly demonstrated that HTLV-1 carriers coinfected with helminths display an immune phenotype consistent with suppression of the type 1 response, resulting in decreased proviral load. These findings, together with the finding of a lower prevalence of helminthic infection in the patients with more-severe cases of HAM/TSP, aid in understanding the events that lead to the development of this more severe clinical outcome of HTLV-1 infection. Lastly, they highlight an important interaction, between viral and parasitic pathogens, within the infected host.

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DISCUSSÃO

O presente estudo teve como objetivo principal caracterizar a resposta imune dos indivíduos infectados pelo HTLV-I e avaliar a capacidade de citocinas, antagonistas de citocinas e infecções por helmintos (*S. stercoralis e S. mansoni*) de modular esta resposta. O estudo comparativo da resposta imune mostrou que pacientes com mielopatia apresentam uma resposta celular mais intensa, caracterizada por uma maior linfoproliferação e produção espontânea de IFN-γ e TNF-α do que os indivíduos assintomáticos. Foi também observado que IL-10 e anticorpos monoclonais anti-IL-2 e anti-IL-15 possuem a capacidade de suprimir a produção de IFN-γ, mas esta supressão foi maior nas culturas de células dos indivíduos assintomáticos do que nos pacientes com mielopatia. Finalmente foi documentado que co-infecção com helmintos diminue a produção de IFN-γ, aumenta a frequência de células T produzindo IL-10 e IL-5 e reduz a carga proviral nos indivíduos assintomáticos infectados pelo HTLV-I. Adicionalmente, registrou-se uma prevalência sete vezes maior de infecções por helmintos entre portadores assintomáticos do vírus do que nos pacientes com HAM/TSP.

Indivíduos infectados pelo HTLV-I apresentam uma ativação persistente e contínua das células T que resulta em uma resposta imune celular exacerbada. Esta resposta é caracterizada pela produção de altos títulos de anticorpos anti-HTLV-I e uma frequência elevada de LTC CD8+ específicos para tax, apesar da persitência de uma alta carga proviral. Nestes pacientes, os linfócitos ativados têm função comprometida (Popovic *et al.*, 1984) e sintetizam grandes quantidades de citocinas pró-inflamatórias como TNF-α e o IFN-γ (Nishiura *et al.*, 1996; Carvalho *et al.*, 2001).

Embora a maioria dos indivíduos infectados pelo HTLV-I seja assintomática, ainda se desconhecem as razões de uma pequena parcela destes indivíduos evoluírem para as diferentes doenças associadas ao HTLV-I como HAM/TSP, ATLL, uveítes, dermatite

infectiva, etc. Vários estudos têm buscado identificar marcadores prognósticos evolutivos que possam estar associados ao risco de desenvolver estas doenças, principalmente a HAM/TSP. Tem sido mostrado que o risco de uma pessoa infectada pelo HTLV-I desenvolver HAM/TSP, esta relacionado ao polimorfismo gênico que influencia a eficiência da resposta dos LTC específicos contra antígenos do HTLV-I (Jeffery *et al.*, 1999; Vine *et al.*, 2002), a elevação da carga proviral (Nagai *et al.*, 1998; Matsuzaki *et al.*, 2001) e a produção de grandes quantidades de citocinas pro-inflamatórias e neurotóxicas como IFN-γ e TNF-α (Nishiura *et al.*, 1996; Carvalho *et al.*, 2001; Umehara *et al.*, 1994; Kubota *et al.*, 1998*)*. Adicionalmente, tem sido sugerido que a rota inicial da infecção viral (sangue periférico ou mucosas) direciona o curso das doenças associadas ao HTLV-I (*apud* Barmak *et al.*, 2003).

O efeito transativador de tax, principal proteína viral, causa expressão desregulada de vários genes relacionados ao crescimento e regulação do ciclo celular como, por exemplo, os genes que codificam as citocinas e seus receptores. O provírus do HTLV-I infecta, i*n vitro*, linfócitos T CD4+ e CD8+. A presença destes linfócitos infectados, continuamente ativados e produzindo grande quantidade de citocinas inflamatórias, tanto no sangue periférico quanto nas lesões do SNC de pacientes com HAM/TSP, reforçam a hipótese de que a exacerbação da resposta imune celular é um dos principais fatores responsáveis pelo desenvolvimento das lesões neurológicas descritas na mielopatia.

Apesar da descrição de Levin et al. (Levin *et al.*, 1998; Levin *et al.*, 2002), da existência de um mimetismo molecular entre proteínas do HTLV-I e autoantígenos presentes no SNC de indivíduos infectados e o possível envolvimento de auto-anticorpos na patogênese da HAM/TSP (hipótese auto-imune), a hipótese citotóxica, com a presença de linfócitos produtores de citocinas pro-inflamatórias, sugere explicações que refletem melhor os danos neurológicos observados na mielopatia: linfócitos T ativados específicos ou não para o HTLV-I, migram do sangue periférico para o SNC, quebram a integridade da barreira hemato-encefálica, acumulam-se nas lesões e liberam grandes quantidades de citocinas pro-

inflamatórias. Estas citocinas fazem com que os linfócitos T infectados se expandam e produzam quantidades cada vez maiores de citocinas lesivas para as células residentes no SNC como neurônios e células da glia (Nagai & Osame, 2003). Adicionalmente, a descrição de uma maior produção de citocinas pro-inflamatórias em pacientes com HAM/TSP apresentando carga proviral semelhante e expressando a mesma quantidade de tax que os indivíduos assintomáticos (Furukawa *et al.*, 2003) reforçam o papel das citocinas inflamatórias no processo patogênico da HAM/TSP.

Avaliar comparativamente a resposta imune de pacientes com mielopatia e a resposta imune de indivíduos infectados pelo HTLV-I, mas que não desenvolvem doença (portadores assintomáticos), é uma das formas de compreender o processo patogênico da doença. Os resultados que compõem a Publicação 1 mostram que, quando comparados com controles normais, os linfócitos dos indivíduos infectados pelo HTLV-I apresentam um aumento significativo na produção de todas as citocinas. Dentre as citocinas sintetizadas após ativação viral, o IFN-γ foi produzido espontaneamente em maior quantidade. Não houve diferenças nas concentrações de TNF-α, IL-5 e IL-10 entre os indivíduos infectados, apesar da tendência de uma síntese mais elevada de TNF-α entre os pacientes com mielopatia. Nestes pacientes, observou-se uma frequência maior de linfócitos T CD4+ e CD8+ secretando IFN-γ e TNF-α, quando comparado com indivíduos assintomáticos, além de um aumento significante da população de linfócitos T CD8+/CD28-. Adicionalmente, foi documentado que, em aproximadamente 40% dos indivíduos assintomáticos avaliados, a proliferação linfocitária e a produção espontânea de IFN-γ apresenta-se de forma similar à descrita nos pacientes com mielopatia. A grande variabilidade na produção de IFN-γ entre os assintomáticos, já tinha sido anteriormente observada. De acordo com a capacidade de produzirem espontânemente IFN-γ, os portadores assintomáticos da infecção pelo HTLV-I foram classificados em altos e baixos produtores de IFN-γ (Carvalho *et al*., 2001). O presente trabalho realizado com um número

maior de indivíduos assintomáticos confirma estes achados e extende o conhecimento sobre o perfil de citocinas sintetizado pelas células dos indivíduos infectados pelo HTLV-I.

A célula T CD4+ foi inicialmente descrita como a principal fonte de citocinas pro-inflamatórias envolvidas na patogênese da HAM/TSP (Biddison *et al.*, 1997). Atualmente, é reconhecido que tanto as células T CD4+ quanto às células T CD8+ produzem e contribuem de forma similar para a produção das citocinas inflamatórias envolvidas no processo lesivo do SNC de pacientes que desenvolvem mielopatia pelo HTLV-I (Bangham, 2000; Jacobson, 2002). Em estudo anterior, com base na depleção de células com anticorpo monoclonal, Carvalho *et al.* descreveram que a fonte principal de produção de IFN-γ na infecção pelo HTLV-I eram as células T CD4+ (Carvalho *et al.*, 2001). Os resultados descritos na Publicação 1, utilizando citometria de fluxo, mostram que ambas as células (CD4+ e CD8+) produzem IFN-γ. Estes achados sugerem que durante a evolução de portador assintomático da infecção para a instalação da HAM/TSP, ocorre uma mudança quanto à principal fonte de célula produtora de IFN-γ e que as células T CD4+, consideradas inicialmente a principal fonte de célula secretora de IFN-γ, sejam gradativamente substituidas pelas células T CD8+. Estes dados estão de acordo com vários trabalhos que descrevem a importância das células T CD8+, principalmente as células específicas para antígenos do HTLV-I na patogênese da HAM/TSP (Kubota *et al.*, 2000; Jacobson *et al.*, 2002).

Apesar de nossos resultados descreverem uma maior frequência de células T CD8+ IFN-γ+ nos pacientes com HAM/TSP, ainda existe um grande questionamento quanto aos danos que estas células podem causar e sua participação efetiva neste processo patogênico. Na maioria das infecções virais, as células T CD8+ têm um papel importante na redução da replicação viral, ou lisando as células infectadas ou produzindo IFN-γ. Deve-se, portanto, considerar o papel benéfico destas células na obtenção do equilibrio entre a carga viral e a resposta imune do hospedeiro. Na infecção pelo HTLV-I estas duas hipóteses não são

exclusivas. Provavelmente o que existe é uma ausência de controle entre os efeitos benéficos e os efeitos deletérios das células T CD8+ (Bangham, 2003).

Os efeitos das células T CD4+ devem ser também considerados. As tentativas de avaliar a resposta das células T CD4+ contra o HTLV-I têm sido difíceis por causa do fenômeno da proliferação espontânea e da produção também espontânea de citocinas nas culturas de células de pacientes, após alguns dias de incubação. Ensaios de curta duração, utilizando peptídios do HTLV-I, para estimular diretamente as células dos pacientes, contornaram este problema e Goon *et al.* demonstraram que as células T CD4+ tipo 1 (produtoras de IFN-γ) predominavam entre as células T específicas para o HTLV-I, tanto em pacientes com HAM/TSP, quanto em portadores assintomáticos da infecção (Goon *et al.*, 2002) e que quando ativadas causam um processo inflamatório poderia resultar nas lesões teciduais descritas nos pacientes com HAM/TSP (Goon *et al.*, 2003).

A análise do estado de ativação celular não mostrou diferenças significativas na expressão de CD69, um antígeno de ativação aguda, ou na expressão de uma molécula de adesão (CD62L), entre os indivíduos infectados pelo HTLV-I. Entretanto, foi observada uma freqüência maior de células T CD8+/CD28- em pacientes com mielopatia quando comparados com os indivíduos assintomáticos. A expansão desta população de células de fenótipo atípico tem sido descrita em estágios avançados de maturação celular, sugerindo que elas estejam envolvidas no processo de senescência imune (Nociari *et al.*, 1999). Esta sub-população de células hiper-ativada apresenta uma atividade citotóxica intensa e produzem grandes quantidades de IFN-γ (Azuma *et al.*, 1993; Weekes *et al.*, 1999). Adicionalmente, estas células (CD8+/CD28-) também têm sido relacionadas com a função supressora (Lum *et al.*, 1982) e com a capacidade de tornarem-se anérgicas e mais propensas a sofrer apoptose (Lewis *et al.*, 1994). Embora as células T CD8+/CD28- não tenham ainda um papel definido na progressão da HAM/TSP (*apud* Matsui *et al.*, 1995), a presença desta população tem sido

associada com numerosas doenças, onde observa-se o envolvimento do sistema imune, como por exemplo, lupus eritematoso sistêmico, artrite reumatóide, doença de Chagas, transplantes e infecção pelo HIV (Dutra *et al., 1*996). A expressão de CD28 constitui-se numa etapa importante para o processo de ligação do vírus com a célula hospedeira. Adicionalmente, tem sido descrito que a incorporação deste antígeno pelo vírus do HIV diminui a replicação viral sugerindo que a aquisição do CD28 pelo HIV modula o ciclo viral e sua consequente infectividade (Giguere *et al.*, 2002). Observamos neste estudo uma maior freqüência de células CD8+ que não expressam o CD28 entre os pacientes com mielopatia. Estudos posteriores são necessários para determinar se estas células representam uma população com grau reduzido de responsividade ao vírus (anérgicas), contribuindo assim para o aumento do número de células infectadas ou se representam uma população com intensa capacidade citotóxica e provável envolvimento na patogenia da HAM/TSP.

A exacerbação da resposta imune celular é considerada o principal fator desencadeador do processo patogênico que se instala nos pacientes com HAM/TSP (Nagai & Osame, 2003). A utilização de mecanismos regulatórios baseados na inibição de citocinas inflamatórias envolvidas no processo de ativação celular, constitui-se numa alternativa para modular esta resposta imune anormal e prevenir o desenvolvimento da doença. A avaliação da capacidade de citocinas imunomodulatórias como IL-10 e TGF-β e antagonistas de citocinas (anti-IL-2, anti-IL-12 e anti-IL-15) de inibir a produção espontânea de IFN-γ nas culturas de células de indivíduos infectados pelo HTLV-I (portador assintomático e HAM/TSP), mostraram que dentre as moléculas estudadas, somente IL-10 e anti-IL-2 combinado com anti-IL-15 foram capazes de reduzir significativamente a síntese espontânea de IFN-γ. Após adição destas moléculas às culturas, esta supressão foi observada principalmente nos indivíduos assintomáticos do que nos pacientes com HAM/TSP (Publicação 2).

A Interleucina-10 é uma citocina com funções imunorregulatórias importantes (Del Prete *et al.*, 1993). A capacidade desta citocina de suprimir a expressão de mediadores inflamatórios produzidas por células imune ativadas e células da glia (Moore *et al.*, 1993) e o aumento na expressão de mRNA para IL-10 na EAE e na esclerose múltipla (Issazadeh *et al.*, 1996), mostram a importância desta citocina no controle de uma resposta imune exacerbada. Apesar alguns trabalhos experimentais apresentarem resultados controversos com relação à ação imunorregulatória da IL-10, (Cannella *et al.*, 1996) a maioria dos dados reforçam o papel da IL-10 no controle dessas doenças. A adição de IL-10, nas culturas de células de indivíduos assintomáticos infectados pelo HTLV-I, reduz de forma significativa a produção espontânea de IFN-γ, sugerindo e confirmando dados anteriores de que esta citocina representa uma ferramenta útil no controle da resposta imune exagerada, observada nos indivíduos infectados pelo HTLV-I (Carvalho *et al.*, 2001). Apesar da IL-10 suprimir a produção espontânea de IFN-γ nas culturas de indivíduos assintomáticos quando comparada com pacientes com mielopatia, essa redução não foi tão significantiva quanto à supressão observada após adição de IL-10 nas células de controles estimulados com PPD. Somente em altas concentrações (100 ng/ml) a IL-10 é capaz de suprimir a síntese espontânea de IFN-γ, mostrando a dificuldade desta citocina para controlar a intensa ativação celular, característica da infecção pelo HTLV-I (Publicação 2).

O TGF-β, reconhecido por sua ação modulatória sobre o sistema imune quando utilizado para suprimir a síntese espontânea de IFN-γ nas culturas de células de indivíduos infectados pelo HTLV-I, mostra-se totalmente ineficaz. A falta de controle do TGF-β sobre a ativação das células infectadas pelo HTLV-I foi anteriormente descrita (Hollsberg *et al.*, 1994) e provavelmente está relacionada à capacidade da proteina viral tax que, após interação com proteinas nucleares que regulam a ativação celular, inibe as vias de sinalização do TGF-β (Mori *et al.*, 2001; Lee *et al.*, 2002).

O sistema envolvendo a IL-2 e seu receptor (IL-2R) é um dos principais mecanismos promotores do crescimento celular das células T maduras presentes no sangue periférico. A IL-2 liga-se às sub-unidades de seus receptores (cadeias α,β e γ), polimeriza estas cadeias e ativa fatores de transcrição que induzem as células a sairem do seu estado de repouso. Na infecção pelo HTLV-I, a indução destes fatores de transcrição, pela ação transativadora de tax, aumenta a expressão de inúmeros genes celulares, entre eles os genes que codificam a IL-2 e seu receptor, iniciando uma ativação e proliferação persistente das células infectadas. Este sistema (IL-2/IL-2R) continuamente ativado contribui para a evolução das lesões inflamatórias do SNC de pacientes com HAM/TSP (Tendler *et al.*, 1990). Tendler et al. também demonstraram que, apesar da adição de anticorpos anti-IL-2 e anti-IL-2R inibirem a proliferação espontânea das células mononucleares do sangue periférico de pacientes com HAM/TSP, esta inibição não era completa. Como a ação neutralizadora da anti-IL-2 e anti-IL-2R era parcial, especulou-se que outra citocina poderia estar contribuindo para a proliferação espontânea das células dos pacientes com HAM/TSP. A Interleucina 15 é, além da IL-2, outra citocina promotora de crescimento celular (Bamford *et al.*, 1994). A descrição de que esta citocina utiliza a cadeia β do IL-2R; e que anticorpos anti-IL-2Rβ bloqueiam a interação da IL-15 com a cadeia β do receptor compartilhado pela IL-2 e IL-15 (Giri *et al.*, 1995), direcionaram vários estudos com o objetivo de avaliar também a participação da IL-15 na linfoproliferação espontânea de pacientes com HAM/TSP (Azimi *et al.*, 1999).

Por serem reconhecidamente importantes na diferenciação e multiplicação das células envolvidas na resposta imune, neste estudo (Publicação 2), as ações da IL-2, IL-12 e da IL-15 foram neutralizadas com o objetivo de bloquear a intensa produção espontânea de IFN-γ observada nas culturas dos indivíduos infectados pelo HTLV-I. O bloqueio da IL-12 não interferiu na síntese espontânea de IFN-γ, sugerindo que esta citocina não participa deste processo de ativação viral. Adicionalmente, nossos dados mostraram a incapacidade da anti-

IL-2 e da anti-IL-15 de suprimir a produção de IFN-γ nas culturas de pacientes com HAM/TSP. Nos indivíduos assintomáticos, observamos somente uma supressão significante após adição de anti-IL-2 mais anti-IL-15. Estes dados confirmam o envolvimento de alças autócrinas envolvendo os sistemas IL-2/IL-2R e IL-15/IL-15R na ativação da resposta imune dos indivíduos com mielopatia pelo HTLV-I (*apud* Mariner *et al.*, 2001; Azimi *et al.*, 1999). Devemos ressaltar que a utilização de anticorpos monoclonais humanizados como anti-IL-2 e anti-IL-2R, têm sido utilizados com relativo sucesso, em associação com esquemas padrões de immunossupressão, para controle de rejeição de enxertos após transplantes renais, sendo sugerida como uma alternativa promissora na terapia de desordens auto-imunes mediada pela célula T, como observado na HAM/TSP (Waldmann & O'shea, 1998; Waldman, 2000). Nossos resultados não mostrarem uma inibição significativa da produção de IFN-γ após adição destes anticorpos em pacientes com HAM/TSP embora esta ação modulatória tenha sido documentada nos indivíduos assintomáticos HTLV-I positivo.

Partindo da observação de que no grupo de indivíduos infectados pelo HTLV-I e assintomáticos existem altos e baixos produtores de IFN-γ (Carvalho *et al.*, 2001), na Publicação 2 foi descrito que entre os portadores assintomáticos existia uma diferença na susceptibilidade das células T de serem moduladas por IL-10, anti-IL-2 e anti-IL-15. Foi observado que células dos portadores do HTLV-I alto produtores de IFN-γ são predominantemente as que não conseguem ser modulada, *in vitro*, pela adição de citocinas e anti-citocinas, evidenciando um comportamento imunológico similar ao descrito para pacientes com mielopatia.

Se esta ausência de modulação pode ser considerada um evento inicial no processo patogênico da mielopatia, ainda não podemos afirmar. Somente o acompanhamento clínico, associado a avaliações neurológicas e imunológicas periódicas, poderá confirmar se estes indivíduos, alto produtores de IFN-γ, cujas células são incapazes de serem moduladas *in vitro*

pela adição de citocinas ou anti-citocinas, serão os que desenvolverão manifestações clínicas de HAM/TSP.

A Publicação 3 descreve a influência das infecções por helmintos (*S. stercoralis* e/ou *S. mansoni*) sobre a resposta imune de indivíduos infectados pelo HTLV-I. De modo geral, os resultados mostram uma redução na síntese de IFN-γ nos indivíduos co-infectados por helmintos, quando comparados com indivíduos sem co-infecção. Estes resultados foram acompanhados de uma diminuição na frequência de células T CD4+ e CD8+ sintetizando IFN-γ e de um aumento na frequência de células produzindo IL-5 e IL-10. Foi também descrita uma diminuição da carga proviral nos indivíduos infectados pelo HTLV-I e co-infectados por helmintos. Finalmente, a prevalência de infecções por helmintos entre os indivíduos infectados pelo HTLV-I mostrou-se significantemente menor nos pacientes com HAM/TSP do que nos portadores assintomáticos do vírus.

Vários trabalhos descrevem a existência de associação entre HTLV-I e infecção pelo *S. stercoralis.* O desenvolvimento de formas graves da doença e o comprometimento da resposta imune dos indivíduos co-infectados são algumas das alterações que indicam que o HTLV-I modifica a resposta imune de pacientes com estrongiloidíase (Porto *et al.*, 2002). A descrição recente dos aspectos clínicos e imunológicos da co-infecção HTLV-I e *S. mansoni* sugere que o HTLV-I também interfere na resposta imune dos pacientes com esquistossomose (Porto *et al.*, 2004). Considerando que uma resposta imune é regulada pela produção de citocinas cujas ações se opõem, a resposta tipo 2, característica das infecções por helmintos, poderia inibir a intensa resposta imune tipo 1 descrita na infecção pelo HTLV-I.

Alguns trabalhos sugerem que infecções por helmintos podem agir como potentes modificadores do padrão de resposta imune. A produção intensa de citocina tipo 2, descrita na infecção por *S. mansoni*, reduz, em indivíduos imunizados com toxóide tetânico (TT), a produção de IFN-γ específica para este antígeno que é um forte indutor de resposta Th1

(Sabin *et al.,* 1996). Adicionalmente, estudos experimentais utilizando modelos murinos de diabetes e encefalomielite demonstram que a infecção pelo *S. mansoni,* reduz a severidade e a intensidada da resposta imune tipo 1 estimulada por estas doenças, dando suporte a esta observação (La Flamme *et al.,* 2003; Cooke *et al.,* 1999). Os resultados da Publicação 3 mostram que portadores assintomáticos da infecção pelo HTLV-I quando co-infectados por helmintos apresentam uma diminuição da produção de IFN-γ e um aumento na freqüência de células produzindo IL-5 e IL-10 quando comparados com portadores assintomáticos sem co-infecção por helmintos. Embora tanto *S. stercoralis* quanto *S. mansoni* tenham contribuido para a diminuição da síntese de IFN-γ nos indivíduos co-infectados, a redução da concentração desta citocina foi principalmente decorrente da ação modulatória do *S. mansoni*. A fase crônica da esquistossomose é caracterizada por uma resposta tipo 2 com diminuição da produção de IFN-γ e concentrações elevadas de IL-4, IL-5 e IL-10 (De Jesus *et al.*, 2004; Joseph *et al.,* 2004). A Interleucina-10 é uma citocina com intensas propriedades imunoregulatórias e capacidade de inibir a proliferação celular e a síntese de citocinas produzidas tanto pelas células Th1 quanto Th2 (Del Prete *et al.*, 1993; Araujo *et al.,* 2004). Devido às propriedades imunorregulatórias da IL-10 e a indução de sua síntese por helmintos, principalmente *S. mansoni,* vários estudos têm avaliado a influência deste helminto sobre a resposta imune e o desenvolvimento de várias doenças (Curry *et al.*, 1995; Correa-Oliveira *et al.*, 2002; Cooke *et al.,* 1999; La Flamme *et al.,* 2003). O aumento da frequência de células T produzindo IL-10, nas culturas de portadores assintomáticos co-infectados por helmintos, sugere que esta citocina seja um dos agentes reguladores da grande produção de IFN-γ, e que o *S. mansoni* é capaz de modular a forte resposta Th1 característica da infecção pelo HTLV-I.

Apesar da importância da população de células Th2, produzindo IL-10 e regulando a resposta imune dos indivíduos infectados pelo *S. mansoni* (Araujo *et al.,* 1996), é provável que outros mecanismos também contribuam para esta regulação. A descrição recente das funções imunossupressivas das células T regulatórias (CD4+CD25+) e sua intensa atividade

supressiva *in vitro* e em modelos de doenças auto-imune, sugere que estas células atuem também no controle da resposta imune das doenças mediadas por células (Zaccone *et al.*, 2003). Embora existam muitos questionamentos sobre as células T regulatórias, já está bem estabelecido que estas células suprimem a resposta imune via interação célula-célula e/ou produção de IL-10 e TGF-β (Roncarolo *et al.*, 2003). Desta forma, podemos especular que estas células possam adicionalmente participar dos mecanismos que controlam a resposta imune exacerbada na infecção pelo HTLV-I.

O aumento da carga proviral do HTLV-I reflete a atividade da infecção e também o comprometimento da resposta imune do hospedeiro infectado (Yamano *et al.*, 2002). A presença de infecções helmínticas crônicas bem estabelecidas pode interferir na progressão e na resposta imune contra o HTLV-I. Gabet *et al.* descreveram anteriormente uma carga proviral maior entre indivíduos HTLV-I positivo co-infectados por *S. stercoralis* do que em indivíduos somente com infecção pelo *S. stercoralis* (Gabet *et al.*, 2000). Os dados da Publicação 3 mostraram que, apesar de apresentarem grande variabilidade, a carga proviral dos portadores assintomáticos co-infectados com helmintos foi menor do que a carga proviral dos portadores assintomáticos sem co-infecção, sugerindo que infecções por helmintos podem diminuir a transcrição e a propagação do HTLV-I.

A propagação do HTLV-I ocorre pelo contato célula T infectada com célula T não infectada. Embora o mecanismo molecular envolvido neste contato célula-célula ainda não tenha sido identificado, ele é necessário para que as células e os mecanismos de defesa contra o vírus sejam ativados e este se propague pelo organismo (Hollsberg, 1999; Igakura *et al.*, 2003). Indivíduos infectados pelo HTLV-I e co-infectados com helmintos desregulam a resposta imune, fato este evidenciado pela redução na síntese de IFN-γ e aumento da produção de IL-10. É possível que estas alterações reduzam a carga proviral como resultado de uma menor intensidade de resposta das células T, diminuindo o contato célula-célula e conseqüente redução da propagação do vírus.

As principais complicações da infecção pelo HTLV-I são a ATLL e HAM/TSP. Alguns estudos sugerem que a infecção pelo *S. stercoralis* induz o desenvolvimento de doenças como ATLL (O'doherty *et al.*, 1984; Plumelle *et al.*, 1996). Outros descrevem uma melhor resposta terapêutica e maior sobrevida nos pacientes ATLL co-infectados com *S. stercoralis,* quando comparado com pacientes não infectados (Agape *et al.*, 1999), podendo ser um indicativo de que a co-infecção por helmintos altera de forma positiva a resposta imune dos indivíduos infectados pelo HTLV-I. Estudos posteriores devem ser conduzidos para esclarescer o real papel das infecções por helmintos sobre a carga proviral e prognóstico das doenças associadas ao HTLV-I.

A prevalência de infecções por *S. stercoralis* e *S. mansoni* é mais alta nos indivíduos infectados pelo HTLV-I do que controles com sorologia negativa para HTLV-I (Porto *et al.*, 2002; Porto *et al.,* 2004). Os dados da Publicação 3 mostram que os portadores assintomáticos do HTLV-I apresentam uma freqüência mais alta de infecções por helmintos do que os pacientes com mielopatia. Entre os portadores assintomáticos a freqüência de infecções por helmintos foi maior nos indivíduos que produzem espontaneamente quantidade menores de IFN-γ (baixo produtores) do que nos alto produtores. Evidentemente, as limitações físicas dos pacientes com mielopatia devem ser consideradas, por causa da menor exposição destes pacientes aos agentes causais das helmintíases. Entretanto, é importante salientar que os indivíduos infectados pelo HTLV-I negavam exposições recentes com estes agentes, todos eram adultos e viviam há muito tempo em áreas urbanas, sugerindo que a aquisição das infecções por helmintos tenha ocorrido durante a infância. Apesar destes dados serem representativos apenas de um pequeno número de pacientes, é interessante registrar que a maioria dos estudos de co-infecção HTLV-I e *S. stercoralis,* foram feitos em locais onde se registravam baixa prevalência da HAM/TSP.

A HAM/TSP é a conseqüência mais importante da infecção pelo HTLV-I. Embora o mecanismo patogênico não esteja totalmente esclarecido, a exacerbação da resposta imune

tem sido considerada como um fator desencadeador deste processo (Nagai & Osame, 2003). Este estudo mostra que indivíduos infectados pelo HTLV-I, principalmente os pacientes com HAM/TSP, apresentam uma intensa resposta imune celular caracterizada pela produção espontânea de citocinas pró-inflamatórias (Publicação 1) e uma maior dificuldade de serem modulados por citocinas imunoregulatórias ou antagonistas de citocinas (Publicação 2). A descrição de uma menor freqüência de infecções por helmintos em pacientes com HAM/TSP sugere que helmintos, principalmente o *S. manson*i, diminui a produção de IFN-γ e a carga proviral do HTLV-I, protegendo os portadores assintomáticos da infecção pelo HTLV-I de desenvolverem mielopatia (Publicação 3). Estudos futuros devem ser conduzidos no sentido de determinar a prevalência de infecções por helmintos em um maior número de indivíduos infectados e avaliar a influência dos helmintos sobre o desenvolvimento de mielopatia visando uma melhor compreensão da interação HTLV-I e helmintos.

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RESUMO DOS RESULTADOS

1 – Indivíduos infectados pelo HTLV-I (portadores assintomáticos e pacientes com mielopatia) sintetizam espontâneamente uma quantidade maior de citocinas como IFN-γ, TNF-α, IL-5 e IL-10 do que controles com sorologia negativa para HTLV-I.

2 – Quando comparados com portadores assintomáticos, pacientes com HAM/TSP apresentam uma resposta imune mais intensa, caracterizada por linfoproliferação espontânea, produção elevada de IFN-γ, alta frequência de linfócitos T CD4+ e CD8+ produtores de IFN-γ e TNF-α e aumento de células T CD8+/CD28-.

3 – Indivíduos assintomáticos apresentam uma intensa variabilidade na produção espontânea de IFN-γ, sendo que, aproximadamente 40% destes indivívudos apresentam concentrações de IFN-γ similares aos descritos para pacientes com HAM/TSP.

4 - Citocinas imunorregulatórias (IL-10) e antagonistas de citocinas (anti-IL-2 e anti-IL-15) modulam mais facilmente as culturas de células de indivíduos assintomáticos infectados pelo HTLV-I do que as culturas de pacientes com mielopatia.

5 – Portadores assintomáticos da infecção pelo HTLV-I podem ser classificados em alto e baixo produtores de IFN-γ.

6 – Células de indivíduos assintomáticos, alto produtores de IFN-γ, não conseguem ser moduladas pela adição de citocinas ou anti-citocinas, mostrando características imunológicas semelhantes às apresentadas pelas células dos pacientes com HAM/TSP.

7 – Portadores assintomáticos do HTLV-I, co-infectados com *S. stercoralis* e *S. mansoni*, apresentam uma redução da produção de IFN-γ, uma menor frequência de células T CD4+ e CD8+ produzindo IFN-γ e um aumento na frequência de células sintetizando IL-5 e IL-10, quando comparado com portadores assintomáticos não infectados com helmintos.

8 – Indivíduos co-infectados com HTLV-I e helmintos apresentam uma carga proviral menor do que os não co-infectados com helmintos, apesar de apresentarem uma diminuição da produção de IFN-γ e maior produção de IL-10.

9 – O aumento de sete vezes na frequência de infecções por helmintos entre portadores assintomáticos e a frequência reduzida de co-infecção entre os pacientes com HAM/TSP, sugere que helmintos protegem indivíduos infectados pelo HTLV-I de desenvolverem HAM/TSP.

CONSIDERAÇÕES ÉTICAS

Ainda como projeto, este estudo foi submetido ao Comitê de Ética em Pesquisa do Hospital Universitário Professor Edgard Santos e aprovado pelos seus representantes legais, conforme anexo I.

Antes de participarem do estudo, todos os pacientes foram informados sobre a naturuza da pesquisa e a voluntariedade fez-se mediante a assinatura do termo de consentimento, seguindo as normas preconizadas pelo Ministério da Saúde. Foram respeitados aqueles pacientes que não quiseram participar ou que não consentiram na realização dos exames necessários ao estudo. Os pacientes que não participaram receberam tratamento e atenção médica similar aos pacientes que foram favoráveis a sua inclusão. Os métodos utilizados não trouxeram prejuízos aos pacientes, além daqueles inerentes aos procedimentos usuais para seu diagnóstico clínico. Além de não oferecer riscos, o estudo proporcionou aos participantes atendidos, um acompanhamento clínico completo. Este é feito por meio de avaliações nas diversas especialidades médicas (Clínica Médica, Neurologia, Reumatologia, Urologia e Psicologia) que compõem o Ambulatório Multidisciplinar de HTLV-I (Ambulatório Magalhães Neto – HUPES – UFBA). A inclusão no estudo fez-se mediante a realização de três parasitológicos de fezes, colhidos em dias alternados. Os pacientes que apresentavam parasitológico positivo eram tratados gratuitamente. Na presença de queixas clínicas os pacientes eram orientados a utilizar drogas cuja prescrição era adequada à disponibilidade comercial do medicamento. As coletas de sangue e o manuseio de material biológico foram realizados no Serviço de Imunologia, utilizando medidas de segurança já padronizadas e em prática nos laboratórios do Serviço.

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HTLV-1 modifies the clinical and immunological response to schistosomiasis

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SUMMARY

The immunological response in HTLV-1 infected individuals is characterized by a prominent Type-1 cytokine response with high production of IFN-γ and TNF-α. In contrast, helminthic infections and in particular chronic schistosomiasis are associated with a predominant production of IL-4, IL-5, IL-10 and IL-13. Liver fibrosis is the main pathological finding in schistosomiasis that occurs after many years of infection. This pathology is T cell dependent but the immune response mechanisms are not completely understood. The North-east region of Brazil is endemic for both HTLV-1 and schistosomiasis. In the present study the immune response, clinical severity, and therapeutic response to praziquantel of patients with schistosomiasis coinfected with HTLV-1 were compared with patients infected only with *S. mansoni*. Patients with HTLV-1 and *S. mansoni* had lower levels of IL-5 ($P < 0{\cdot}05$) and higher levels of IFN-γ ($P < 0{\cdot}05$) in cultures stimulated with *S. mansoni* antigen and decreased *S. mansoni* antigen specific IgE levels when compared with patients with schistosomiasis without HTLV-1 coinfection. Liver fibrosis was mild in all HTLV-1 coinfected patients and efficacy of praziquantel was lower in patients dually infected than in patients infected only with *S. mansoni*.

Keywords HTLV-1 schistosomiasis liver fibrosis

INTRODUCTION

Human T cell leukaemia virus type-1 (HTLV-1) infects an estimated 20 million people worldwide [1]. Adult T cell leukaemia and lymphoma (ATLL) and HTLV-1 associated myelopathy (HAM/TSP) are the main diseases caused by HTLV-1, but other diseases such us Sjögren syndrome, polyarthritis, uveitis, infective dermatitis and lymphocytic alveolitis are documented clinical manifestation of HTLV-1 infection. The immunologic response in HTLV-1 infection is characterized by an exaggerated T cell response with high production of IFN-γ and TNF-α [2,3]. The predominant IFN-γ production and lymphocyte proliferation occur even in unstimulated cultures [2,4]. In contrast, helminthic infections and in particular chronic schistosomiasis are associated with a predominant type-2 immune response [5,6]. The main pathological finding in schistosomiasis is liver fibrosis, and late severe fibrosis occurs in about 6% of chronically *S. mansoni* infected patients [7]. The hepatic pathology of schistosomiasis is T cell dependent but the immunplogic mechanisms mediating liver damage are not completely understood.

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The impact of HTLV-1 on helminthic infection has been reported in patients coinfected with *Strongyloides stercoralis* [8–10]. In such cases, coinfection with HTLV-1 decreases the predominant type-2 immune response observed in strongyloidiasis [11] as well as *S. stercoralis* specific and total IgE antibodies [12,13]. In addition, disseminated and recurrent strongyloidiasis are associated with HTLV-1 coinfection [14–16].

Salvador, the capital of the state of Bahia, located in the North-east of Brazil has the highest (1·35%) prevalence of HTLV-1 infection as reported in blood donors throughout Brazil [17]. The North-east region of Brazil is also endemic for schistosomiasis making it possible to evaluate the association of these two diseases. In the present study the immune response in patients with schistosomiasis coinfected with HTLV-1 was compared with that patients only infected with *S. mansoni*. Additionally, clinical and ultrassonography evaluation of liver fibrosis and therapeutic response to praziquantel were determined.

MATERIALS AND METHODS

Patients

Patients infected with HTLV-1 were recruited in the HTLV-1 multidisciplinary clinic, located in Hospital Universitário Prof Edgard Santos (HUPES) in Salvador, Bahia, Brazil. The clinic started in

Clinical and Immunological Consequences of Human T Cell Leukemia Virus Type-I and *Schistosoma mansoni* Co-infection

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Human T cell leukemia virus type-I (HTLV-I) infection is associated with spontaneous T cell activation and uncontrolled lymphocyte proliferation. An exacerbated type-1 immune response with production of pro-inflammatory cytokines (interferon-γ and tumor necrosis factor-α) is significantly higher in patients with myelopathy associated to HTLV-I than in HTLV-I asymptomatic carriers. In contrast with HTLV-I, a chronic Schistosoma mansoni *infection is associated with a type-2 immune response with high levels of interleukin (IL-4, IL-5, and IL-10) and low levels of IFN-γ. In this study, clinical and immunological consequences of the HTLV-I and* S. mansoni *infection were evaluated. The immune response in patients with schistosomiasis co-infected with HTLV-I showed low levels of IL-5 ($p < 0.05$) in peripheral blood mononuclear cells cultures stimulated with* S. mansoni *antigen (SWAP) and decreased SWAP-specific IgE levels when compared with patients with only schistosomiasis ($p < 0.05$). Liver fibrosis was mild in all HTLV-I co-infected patients. Immunological response was also compared in individuals who had only HTLV-I infection with those who were co-infected with HTLV-I and helminths* (S. mansoni *and* Strongyloides stercoralis). *In patients HTLV-I positive co-infected with helminths the IFN-γ levels were lower than in individuals who had only HTLV-I. Moreover, there were fewer cells expressing IFN-γ and more cells expressing IL-10 in individuals co-infected with HTLV-I and helminths. These dates indicate that HTLV-I infection decrease type 2-response and IgE synthesis and are inversely associated with the development of liver fibrosis. Moreover, helminths may protect HTLV-I infected patients to produce large quantities of pro-inflammatory cytokines such as IFN-γ.*

Key words: human T cell leukemia virus type-1 - *Schistoma mansoni* - co-infection

The human T cell leukemia virus type-I (HTLV-I) is an oncogenic exogenous retrovirus that infects between 10 and 20 million people worldwide (Edlich et al. 2000). HTLV-I is the recognized cause of adult T-cell leukemia (ATL) as well as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Osame et al. 1986, Uchiyama 1997), but other disorders have been associated with HTLV-I infection. The immunological response in HTLV-I infection is characterized by a spontaneous lymphoproliferation and an exaggerated T cell response with high production of important inflammatory mediators of tissue damage as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin (IL-6) (Nishimoto et al. 1990, Kubota et al. 1998, Carvalho et al. 2001). Although the pathogenesis of neurological disease associated to HTLV-I is not completely understood, there are various evidences that immunological response participate and is responsible by inducing tissue damage (Hanon et al. 2000, Nagai & Jacobson 2001, Osame 2002). By the other hand, helminthes infections such as strongyloidiasis and in particular a chronic disease caused by infection with *Schistosoma mansoni* are associated with a predominant anti-inflammatory type-2 immune response with increased levels of IL-4, IL-5 and IL-10 and low levels of IFN-γ (Araujo et al. 1996, Finkelman et al. 1997). The high degree of infection and the host's immune reaction to parasite eggs contribute to granuloma formation. Liver fibrosis is the most important pathological finding in schistosomiasis, being registered in about 5% of chronically *S. mansoni* infected patients (Bina & Prata 2003). Although initial experimental studies suggested that type-1 cytokines were associated with granulomatous reaction to *S. mansoni* infection (Leptak & McKerrow 1997, Rezende et al. 1997), its clear from current data that type-2 cytokines play a primary role in inducing fibrosis, whereas the IFN-γ (type-1 cytokine) acts as an endogenous down regulator of the response (Wynn et al. 1994, Chiaramonte et al. 1999a, Jankovic et al. 1999). Simultaneous infection between HTLV-I and *Strongyloides stercoralis* decreases the predominant type-2 immune response in patients with strongyloidiasis (Neva et al. 1998, Porto et al. 2001a) as well as *S. stercoralis*-specific and total IgE antibodies (Neva et al. 1998, Porto et al. 2001b). Moreover, co-infection with HTLV-I is also associated with disseminated and recurrent strongyloidiais (Phelps et al. 1991, Newton et al. 1992). It is known that the prevalence of strongyloidiasis is higher in HTLV-I infected patients than in seronegative controls (Robinson et al. 1994, Hayashi et al. 1997). Based on these observations one of the aims of this study was to determine if HTLV-I infection decrease the type-2 im-

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Juvenile HAM/TSP of Subacute Evolution: Case Report and Literature Review

HAM/TSP Juvenil de Evolução Subaguda: Relato de Caso e Revisão de Literatura

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Abstract

Human T cell lymphotropic virus type I (HTLV-I) is an exogenous retrovirus that has shown to be the etiological agent in adult T cell leukemia (ATL) and a progressive neurological disease called HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The physiopathogenesis of this disease is not completely understood, although it has been suggested that virus-host interactions play a role in the pathogenesis of the disorder. The prevalence of this retrovirus is 1.35% among blood donors in Salvador, Bahia, Brazil. HAM/TSP may present a rapid evolution, with acute or subacute onset, even in young patients with a history of vertical transmission. The aim of the current study is to present a report of a probable juvenile HAM/TSP case and its clinical, epidemiological, and immunological features. A 15-year-old black male was admitted to the HTLV-I outpatient clinic because of large joint pain, paraparesis with signs of pyramidal liberation in the lower limbs, and a HTLV-I positive blood test. His mother, a 54-year-old woman, is also a HTLV-I carrier. An immunological analysis of the boy was performed attempting to evaluate type 1 (IFN-γ and TNF-α) and type 2 (IL-5 and IL-10) responses in supernatants of unstimulated peripheral blood mononuclear cells. In this report, we extend previous observations of high IFN-γ production in unstimulated cultures of HTLV-I-infected patients, showing that other cytokines such as TNF-α, IL-5, and IL-10 are also increased in cell supernatants of these patients.

Key words: human T-lymphotropic virus 1; paraparesis, tropical spastic; cytokines.

Resumo

O vírus linfotrópico humano de células T tipo I (HTLV-I) é um vírus exógeno que já foi demonstrado ser o agente etiológico na leucemia de células T do adulto (ATL) e em uma doença neurológica chamada de mielopatia associada ao HTLV-I/paraparesia espástica tropical (HAM/TSP). A fisiopatogenia desta doença não está completamente entendida, embora interações vírus-hospedeiro possam desempenhar um papel na patogênese da doença. A prevalência deste retrovírus é de 1,35% dentre os doadores de sangue em Salvador, Bahia, Brasil. HAM/TSP pode apresentar uma

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Cytokine Profile and Immunomodulation in Asymptomatic Human T-Lymphotropic Virus Type 1–Infected Blood Donors

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Summary: The modulation of the immune response has been used as therapy for clinical disorders associated with human T-lymphotropic virus type 1 (HTLV-1) infection. In this study, the cytokine profile was evaluated in 26 asymptomatic HTLV-1 blood donors. Additionally, both the cell responsible for producing interferon-γ (IFN-γ) and the role of exogenous interleukin (IL)-10 in downregulating IFN-γ production were studied. Cytokine levels were determined in supernatants of unstimulated lymphocyte cultures by enzyme-linked immunosorbent assay. The levels of IFN-γ, tumor necrosis factor-α, IL-5, and IL-10 were higher in supernatants of the lymphocyte cultures taken from HTLV-1–infected donors than in those taken from healthy subjects. Although depletion of $CD8^+$ T cells and natural killer cells did not affect IFN-γ production, depletion of $CD4^+$ T cells significantly decreased IFN-γ production. Furthermore, at a concentration of 2 ng/ml, IL-10 had only a minimum effect on IFN-γ production, although at high concentrations (100 ng/ml), IL-10 decreased IFN-γ production by 50% in HTLV-1–infected individuals. These data indicate that both T helper 1 and T helper 2 cytokines are elevated in HTLV-1 infection and that IL-10 in high concentrations modulates IFN-γ production in these patients. **Key Words:** Cellular immunity in HTLV-1—Cytokines in HTLV-1—HTLV-1—Immune response in HTLV-1—Immunomodulation in HTLV-1.

The immunologic response in human T-lymphotropic virus type 1 (HTLV-1) infection is characterized by spontaneous T-cell proliferation with increasing secretion of interleukin (IL)-2 and expression of the IL-2 receptor (1–3). Abnormalities in the response have been shown in patients with HTLV-1–associated myelopathy (tropical spastic paraparesis) compared with asymptomatic HTLV-1–positive individuals, including elevated levels of cytokines such as tumor necrosis factor-α (TNF-α), IL-6, and IL-2 in sera and cerebrospinal fluid (4,5). Both $CD4^+$ and $CD8^+$ T cells are infected by HTLV-1 (6,7). Although only a small percentage of infected individuals develop clinical manifestations associated with HTLV-1, the prevalence of this retrovirus is high in endemic areas such as Salvador, Bahia, Brazil, where 1.35% of blood donors are infected with HTLV-1 (8). Although high levels of IL-2 and interferon-γ (IFN-γ) have been documented in supernatants of lymphocyte cultures from asymptomatic HTLV-1 carriers (3), little is known about the secretion of T helper (Th) 2 cytokines or the ability of cytokines and cytokine antagonists to modulate the lymphocyte function in the course of this viral infection. The major aim of the current study was to evaluate the cytokine profile in asymptomatic subjects infected with HTLV-1, to determine which cells are secreting IFN-γ in such patients, and to evaluate the ability of IL-10 to downregulate IFN-γ production in patients infected with HTLV-1.

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ABSTRACT

Human T-cell lymphotropic virus type 1 (HTLV-1) is a retrovirus that predominantly infects T cells leading to alterations in the immune response. The majority of infected individual are asymptomatic (HTLV-I carriers) and only a minority develop diseases such as adult-T cell leukemia/lymphoma (ATLL) or a progressive neurological disease called HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). The immune response in HTLV-I infected patients is characterized by intense and persistent T cell activation with proliferation and spontaneous production of pro-inflammatory cytokines such as IFN-γ and TNF-α. This study evaluates the immune response in HTLV-I infected individual and the ability of cytokines, cytokine antagonists and helminth infection to modulate this exacerbated immune response. The HTLV-I immune response was evaluated by the profile of cytokines in the supernatant of peripheral blood mononuclear cell cultures, description of cell populations present in the immune response and the frequency and type of cells that show markers of cellular activation and produce cytokines. HAM/TSP patients showed a more intense cellular activation characterized by higher spontaneous lymphoproliferation as well as higher spontaneous IFN-γ and TNF-α production than HTLV-I asymptomatic carriers. It was also observed that IFN-γ production showed great variability between HTLV-I asymptomatic carriers with some individuals showing similar IFN-γ concentrations to those observed in HAM/TSP patients. The addition of immunossupressive cytokines (IL-10 and TGF-β) and cytokine antagonists (anti-IL-2, anti-IL-12 and anti-IL-15) to peripheral blood mononuclear cells from HTLV-1 infected individuals showed that IL-10 and the addition of combined IL-2 plus IL-15 significantly reduced spontaneous IFN-γ synthesis in HTLV-I asymptomatic carriers as compared with HAM/TSP patients. Additionally, a sub-group of HTLV-1 asymptomatic carriers who have a profile of cytokines and a lymphoproliferative response

similar to HAM/TSP patients was identified. This sub-group of asymptomatic carriers is high IFN-γ producer with poor modulation of IFN-γ by cytokines and cytokine antagonists. Additionally, HTLV-1 carriers co-infected with helminths (*Strongyloides stercoralis and Schistosoma mansoni)* showed a decrease in IFN-γ synthesis and a low frequency of CD4+ and CD8+ T cell expressing IFN-γ and a high frequency of cell expressing IL-5 and IL-10. When compared with asymptomatic carriers without helminthic infection, HTLV-I infected subjects co-infected with helminthes showed a low proviral load. Moreover, a higher prevalence of helminth infections was observed in asymptomatic carriers than HAM/TSP patients. These results suggests that co-infection with helminths reduces type 1 T cell activation and influences the clinical expression of HTLV-I infection.

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ANEXO

Anexo 1