BRIEF REPORT

Majority of Interferon-γ–Producing CD4+ Cells in Patients Infected with Human T Cell Lymphotrophic Virus Do Not Express Tax Protein

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The present study was conducted to determine whether interferon (IFN)–γ production by CD4+ cells in patients infected with human T cell lymphotropic virus (HTLV) is associated with expression of Tax, an HTLV type 1 (HTLV-1) transactivator. The frequency of IFN-γ production from CD4+ cells was greater in HTLV-1–infected patients ($n = 21$) than in uninfected ($n = 3$) and *Strongyloides stercoralis*–infected patients ($n = 4$), and greater in patients with HTLV-1 with detectable Tax than in patients with HTLV-1 with undetectable Tax. In the patients with HTLV-1 with detectable Tax, the majority of CD4+ cells making IFN-γ did not express Tax.

Human T cell lymphotropic virus type 1 (HTLV-1) infection predisposes patients to severe and refractory *Strongyloides stercoralis* (Ss) infections [1, 2]. This appears to be the result of the down-regulation of the usual Th2 response to *Strongyloides* by interferon (IFN)–γ produced in the setting of HTLV-1 infection and is reflected by increased levels of IFN-γ and decreased levels of interleukin (IL)–4, IL-5, and antigen-specific IgE in individuals coinfected with HTLV-1 and Ss [3, 4].

Although it is known that most of the IFN-γ produced in HTLV-1–infected patients comes from CD4+ T cells [5, 6], it is not known whether this IFN-γ production is induced directly by HTLV-1 or whether it occurs as part of the human immune response to HTLV-1 infection. To gain insight into this question, we sought to determine whether IFN-γ production by CD4+ T cells in HTLV-1–infected patients is associated with expression of Tax, an HTLV-1 gene product present in most HTLV-1–infected cells [7], which has the ability to induce IFN-γ transcription [8].

Patients and methods. Participants were recruited from 3 sources: HTLV-1–infected seroreactors from blood donors in Salvador, Bahia, Brazil; residents from a rural endemic area near Salvador with positive fecal examination for Ss infection; and uninfected control subjects from donors at the Department of Transfusion Medicine (National Institutes of Health, Bethesda, MD). HTLV-1 serologic analysis was performed by ELISA (Cambridge Biotech) and confirmed by Western blot test (HTLV Blot 2.4, Genelabs). The criterion for a diagnosis of strongyloidiasis was a positive fecal examination for larvae by the Baermann concentration technique. Twenty-eight patients were included in the study: 20 with HTLV-1 infection (4 of whom were symptomatic with HTLV-1–associated myelopathy [HAM] and 16 of whom were asymptomatic carriers), 4 with Ss infection, 1 with both HTLV-1 and Ss infection, and 3 uninfected control subjects.

Heparinized venous blood samples were collected, and peripheral blood mononuclear cells (PBMCs) were separated with a Ficoll diatrizoate gradient (LSM; ICN Biomedicals). Cells were washed and then cultured at 2×10^6/mL in RPMI 1640 medium (BioWhittaker) supplemented with 20 mmol L-glutamine (BioWhittaker), 10% heat-inactivated fetal calf serum (Harlan Bioproducts for Science), 0.01 mol HEPES (BioWhittaker), and 50 μg/mL gentamicin (Mediatech). Culture supernatants were harvested after 72 h for IFN-γ production, as detected by use of a human IFN-γ ELISA kit (Biosource).

PBMCs were split into 2 wells and cultured in either medium alone or with phorbol myristate acetate (Sigma Chemical) at 50 μg/mL plus ionomcyin (CalBiochem) at 1 μg/mL. After 2 h, 2 mmol monensin (Sigma) was added. After 24 h, cells were washed, fixed with 4% paraformaldehyde, and cryopreserved, as described elsewhere [9].

Fixed cells were thawed, washed with PBS/0.1% bovine serum albumin (BSA), permeabilized, and blocked for 1 h by incubating in PBS containing 0.1% saponin (CalBiochem)/1% BSA at 4°C. Cells then were stained with anti–HTLV-1 Tax monoclonal antibodies (168A51-42, obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Insitute of Allergy and Infectious Diseases, and provided by Dr. Steven Jacobson, National Institute of Neurological Disorders and Stroke, National Institutes of Health). After washing, cells were stained with phycoerythrin-conjugated mouse anti–human IFN-γ mouse IgG1 (BD PharMingen), allo-

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phycocyanine-conjugated anti–human CD4 mouse IgG1 (Immunotech), and fluorescein isothiocyanate–conjugated goat F(ab′)2 anti–mouse IgG2a (Southern Biotechnology Associates). Unlabeled mouse IgG2a (Sigma) was used as the isotype control for anti–HTLV-1 Tax staining, and phycoerythrin-conjugated mouse IgG1 (BD PharMingen) was used as the isotype control for IFN-γ staining. Cells were again washed twice, resuspended in PBS, and analyzed with a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest software. Comparisons were performed with the nonparametric Mann-Whitney rank test, and correlations were analyzed by Spearman's rank correlation test.

Results. PBMCs from 20 patients with HTLV-1, 4 with Ss, 1 with both infections, and 3 with neither were cultured in medium alone for 24 h and then were evaluated by intracellular flow cytometry for expression of Tax protein in $CD4^+$ cells. A frequency >0.15% was defined as the threshold for detectable Tax, because this frequency was the maximum background measured by isotype staining after titration of the anti–Tax antibody. Tax expression was evaluated after 6, 24, and 48 h of in vitro culture in 6 patients with detectable Tax. Tax expression was found to be highest at the 24-h time point in 4 of these 6 patients, and this time point was used for all subsequent analyses.

As expected, none of the PBMCs from the uninfected control subjects or Ss-only–infected patients had detectable Tax expression. However, 10 of 21 patients infected with HTLV-1 did have detectable Tax expression by their $CD4^+$ cells (figure 1*A*). The Tax^+ $CD4^+$ cells comprised the bulk of all the Tax^+ PBMCs in these patients (range, 53%–90%; geometric mean [GM], 73%; data not shown), and the percentage of $CD4^+Tax^+$ cells ranged from 0.18% to 1.95%. Three of the 4 patients with HAM had detectable Tax expression by their $CD4^+$ cells, whereas only 6 of the 16 asymptomatic carriers had detectable Tax. The 1 patient coinfected with HTLV-1 and Ss had Tax expression in 0.56% of his $CD4^+$ cells.

The percentage of $CD4^+$ cells spontaneously producing IFN-γ was significantly greater in HTLV-1–infected patients than in patients with Ss infection (GM, 0.15% vs. 0.01%; $P = .01$). It is of interest that the greatest percentage of $CD4^+$ cells producing IFN-γ was seen in the 1 patient coinfected with HTLV-1 and Ss: 20% of the $CD4^+$ T cells from this patient made IFN-γ—5 times more than any other patient.

The frequency of spontaneous IFN-γ production by $CD4^+$ cells was lower in Ss-infected patients, compared with that in uninfected control subjects (GM, 0.01% vs. 0.06%; $P = .04$), as well as in uninfected control subjects compared with that in patients with HTLV-1 infection (GM, 0.06% vs. 0.15%), although this difference was not statistically significant ($P = .35$; figure 1*B*).

The frequency of spontaneous IFN-γ production by $CD4^+$ cells among the HTLV-1–infected patients was greater in the

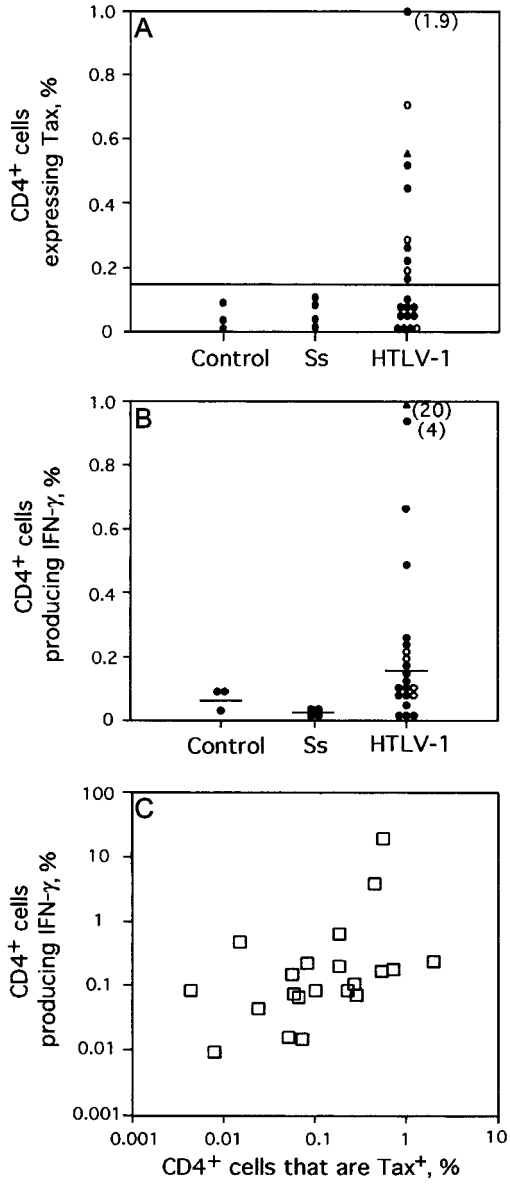

Figure 1. Tax, a human T cell lymphotropic virus type 1 (HTLV-1) transactivator, and interferon (IFN)–γ expression by $CD4^+$ cells in peripheral blood mononuclear cells (PBMCs) cultured in medium for 24 h. *A,* Percentage of $CD4^+$ cells expressing Tax in control subjects, *Strongyloides stercoralis* (Ss)–infected patients, and HTLV-1–infected patients, as determined by flow cytometry. The horizontal line demarcates 0.15%, the threshold for defining a patient as having detectable Tax. This threshold was determined by the maximum background measured by isotype staining. *B,* Percentage of $CD4^+$ cells producing interferon (IFN)–γ in control subjects, Ss-infected patients, and HTLV-1–infected patients, as determined by intracellular flow cytometry. *C,* Correlation between percentage of $CD4^+$ cells expressing Tax (Tax^+) and percentage of $CD4^+$ cells producing IFN-γ, as determined by intracellular flow cytometry. In the HTLV-1–infected groups in panels *A* and *B,* asymptomatic patients are represented by *closed circles,* patients with HTLV-1–associated myelopathy (HAM) are represented by *open circles,* and the 1 patient coinfected with HTLV-1 and Ss is represented by a *solid triangle.* In panels *A* and *B,* values in parentheses are outside the scale of the graph.

10 patients with detectable Tax than in those with undetectable Tax (GM, 0.38% vs. 0.06%; $P = .02$), and there was a significant correlation between the percentage of $CD4^+$ cells expressing Tax and the frequency of $CD4^+$ cells producing IFN-γ ($R = 0.55$; $P = .01$; figure 1*C*). This difference was not attributable to an increased potential to release IFN-γ, because there was no difference in the frequency of IFN-γ–producing $CD4^+$ cells after stimulation with phorbol myristate acetate–ionomycin in patients with detectable Tax versus those without detectable Tax (GM, 12.8% vs. 16.1%; $P = .62$). No difference was found in the frequency of $CD4^+$ cells producing IFN-γ in the 4 patients with HAM, compared to the 16 asymptomatic carriers (GM, 0.13% vs. 0.11%; $P = .78$).

The frequencies of $CD4^+$ cells spontaneously making IFN-γ, as measured by flow cytometry, correlated strongly with IFN-γ concentrations measured by ELISA in supernatants harvested from 24-h–unstimulated cell cultures at 2×10^6 PBMCs/mL ($P = .005$, Spearman's rank correlation; data not shown),

The frequency of IFN- production as a function of Tax expression in patients with detectable Tax expression demonstrated that, although 2.67% (GM) of all Tax^+ $CD4^+$ cells made IFN-γ, only 0.34% (GM) of Tax^- $CD4^+$ cells made IFN-γ ($P = .03$; figure 2 and table 1); however, because of the vastly greater numbers of Tax^- $CD4^+$ cells, the majority of $CD4^+$ cells producing IFN-γ were found not to express Tax (GM, 342 Tax^- $CD4^+$ IFN-γ–positive cells/100,000 $CD4^+$ cells vs. 16 Tax^+ $CD4^+$ IFN-γ–positive cells/100,000 $CD4^+$ cells; $P = .0004$). The percentage of IFN-γ–positive $CD4^+$ cells that did not express Tax ranged from 63% to 99% (GM, 89.7%). These findings strongly suggest that the bulk of IFN-γ production in patients with HTLV-1 infections comes from cells not expressing HTLV-1 Tax protein.

Discussion. Of the 21 patients tested who were seropositive for HTLV-1, 10 had detectable Tax, as measured by flow cytometry. The majority of Tax^+-expressing cells were $CD4^+$ in all patients studied, and detectable Tax was found more frequently in symptomatic patients (3/4 patients) than in asymptomatic carriers (6/16 asymptomatic carriers).

HTLV-1–infected patients had greater frequencies of IFN-γ–producing $CD4^+$ cells than either uninfected control subjects or patients infected with Ss, and patients infected with Ss had fewer IFN-γ–producing $CD4^+$ cells than either uninfected control subjects or patients infected with HTLV-1, mirroring the findings of previous studies that measured the production of IFN-γ from PBMC cultures of these different patient groups [3, 4].

The observation that frequencies of $CD4^+$ cells producing IFN-γ were higher in the 10 patients with detectable Tax compared with those in the 11 in whom Tax was not detected suggests that active expression of HTLV-1 viral protein is associated with a more vigorous Th1 response. Notably, we showed that the majority of IFN-γ–producing $CD4^+$ cells in patients with detectable levels of Tax expression were cells that did not express Tax. Because a large proportion of cells infected with HTLV-1 express Tax [10], these findings suggest that the majority of IFN-γ in HTLV-1–infected patients comes from uninfected cells. Consequently, we can conclude that the elevated IFN-γ levels seen in HTLV-1–infected patients are not the result of a direct effect of HTLV-1 Tax protein in up-regulating IFN-γ transcription.

There are 2 possible explanations for this finding. One explanation is that the increased IFN-γ production seen in HTLV-1–infected patients is caused by a specific action of the HTLV-1 virus on the host immune response rather than direct Tax-mediated up-regulation of IFN-γ. For example, Tax or an-

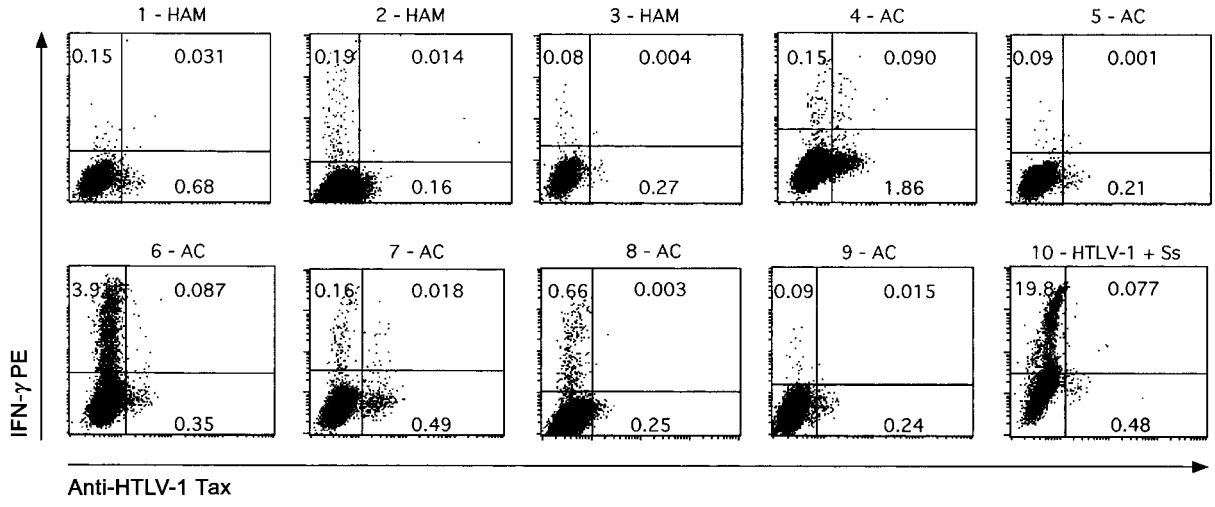

Figure 2. Interferon (IFN)-γ production by $CD4^+$ cells expressing and not expressing Tax, a human T cell lymphotropic virus type 1 (HTLV-1) transactivator. Dot plots of intracellular flow cytometry for Tax expression and IFN-γ production of all 10 patients infected with HTLV-1 with detectable Tax. AC, asymptomatic HTLV-1 carrier; HAM, HTLV-1–associated myelopathy; PE, phycoerythrin; Ss, *Strongyloides stercoralis.*

Table 1. Interferon (IFN)–γ production by CD4⁺ cells expressing and not expressing Tax, a human T cell lymphotropic virus type 1 (HTLV-1) transactivator (Tax⁺ and Tax⁻, respectively) after culture of peripheral blood mononuclear cells (PBMCs) for 24 h, as determined by intracellular flow cytometry.

Patient	Disease	Tax^- $CD4^+$ producing IFN-γ, %	Tax^+ $CD4^+$ producing IFN-γ, %	Tax^- $CD4^+$ producing IFN-γ, no./10^6 $CD4^+$ cells	Tax^+ $CD4^+$ producing IFN-γ, no./10^5 $CD4^+$ cells	All $CD4^+$ cells producing IFN-γ that are Tax^-, %
1	HAM	0.15	4.48	151	32	83
2	HAM	0.19	8.00	189	15	93
3	HAM	0.08	1.63	78	5	94
4	AC	0.15	4.64	151	90	63
5	AC	0.09	0.01	87	1	99
6	AC	3.93	19.46	3913	87	98
7	AC	0.15	3.50	155	18	90
8	AC	0.66	1.25	661	3	99
9	AC	0.09	5.77	93	15	86
10	HTLV-1 + Ss	19.91	13.72	19,796	76	99

NOTE. AC, asymptomatic HTLV-1 carrier; HAM, HTLV-1–associated myelopathy; HTLV-1, human T cell lymphotropic virus type 1; Ss, *Strongyloides stercoralis*.

other HTLV-1 factor may up-regulate some cytokine in infected cells that, after release, causes neighboring uninfected cells to up-regulate IFN-γ production. The other possibility is that the greatly increased IFN-γ levels seen in HTLV-1 infections are simply caused by the normal immune response to infection by a virus. If this is the case, it suggests that the failure of the immune system to establish an effective Th2 response in patients coinfected with HTLV-1 and Ss may occur in other situations.

Because IFN-γ, the dominant Th1 cytokine, and IL-4, the dominant Th2 cytokine, can counter-regulate each other [11], it is plausible that the immune system may have difficulties establishing strong Th1 and Th2 responses simultaneously, in which case, a person infected with 1 organism requiring a Th1 response and another requiring a Th2 response could have problems mounting an effective response against either.

Clinical findings that lend credence to this possibility of an immune system in limbo come from data on people coinfected with HTLV-1 and Ss. Not only are Ss infections more severe in patients coinfected with HTLV-1, but there also is evidence that HTLV-1 replication is poorly controlled in these patients. Indeed, HTLV-1 carriers coinfected with Ss have higher provirus loads [12] and develop adult T cell leukemia earlier [13] than those infected with HTLV-1 alone.

Patients with leprosy coinfected with helminths are another clinical example of poor immune control of simultaneous infections by organisms requiring different arms of the cellular immune response. Leprosy, which requires a strong Th1 response for control, is more frequently multibacillary in areas where onchocerciasis (a chronic filarial infection that promotes a strong Th2 response) is prevalent [14] and in patients with intestinal helminths [15].

In conclusion, the majority of $CD4^+$ cells producing IFN-γ in HTLV-1–infected patients do not express Tax protein, suggesting that the elevated IFN-γ levels of patients infected with HTLV-1 are not directly due to Tax expression but, rather, are due to either a bystander effect that HTLV-1–infected cells have on noninfected cells or simply the normal Th1 response to HTLV-1. If the latter is true, it would suggest that the inability of patients coinfected with HTLV-1 and Ss to mount a strong Th2 response against Ss is a nonspecific phenomenon of immune dysregulation that may potentially occur in any patient coinfected with organisms requiring different effector arms of the T cell–mediated immune response.

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