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Retroviral Interference on STAT Activation in Individuals Coinfected with Human T Cell Leukemia Virus Type 2 and HIV-1

Chiara Bovolenta,*2 Elisabetta Pilotti, † Massimiliano Mauri,* Barbara Panzeri,* Monica Sassi, † PierPaolo Dall’Aglio, † Umberto Bertazzoni,* Guido Poli,* and Claudio Casoli †

Human T cell leukemia virus (HTLV) type-2 is a human retrovirus whose infection has not been tightly linked to human diseases. However, the fairly high prevalence of this infection among HIV-1-positive individuals indicates the importance of better understanding the potential interference of HTLV-2 infection on HIV-1 infection and AIDS. We previously demonstrated that one signature of PBMC freshly derived from HIV-1-infected individuals is the constitutive activation of a C-terminal truncated STAT5 (STAT5Δ). Therefore, we analyzed the potential activation of STATs in HTLV-2 monoinfected and HTLV-2/HIV-1 dually infected individuals. We observed that PBMC of HTLV-2-infected individuals do not show STAT activation unless they are cultivated ex vivo, in the absence of any mitogenic stimuli, for at least 8 h. The emergence of STAT activation, namely of STAT1, in culture was mostly related to the secretion of IFN-γ. Of note, this phenomenon is not only a characteristic feature of HTLV-2-infected individuals but also occurred with PBMC of HIV-1+ individuals. Surprisingly, HTLV-2/HIV-1 coinfection resulted in low/absent STAT activation in vivo that paralleled a diminished secretion of IFN-γ after ex vivo cultivation. Our findings indicate that both HTLV-2 and HIV-1 infection prime T lymphocytes for STAT1 activation, but they also highlight an interference exerted by HTLV-2 on HIV-1-induced STAT1 activation. Although the nature of such a phenomenon is unclear at the present, these findings support the hypothesis that HTLV-2 may interfere with HIV-1 infection at multiple levels. The Journal of Immunology, 2002, 169: 4443–4449.

H uman T cell leukemia virus (HTLV)3 type 2 and HIV-1 are retroviruses belonging to the oncovirinae and lentivirinae subfamilies, respectively (1). HIV-1 is the causative agent of AIDS, whereas a definitive etiologic role for HTLV-2 in human malignancy or in other human diseases has not been clearly established. Although HTLV-2 was initially derived from a patient affected with hairy cell leukemia (2), only some subjects suffering from neurodegenerative and lymphoproliferative disorders have been shown to harbor the virus (3–5). The worldwide incidence and the pathogenicity of HTLV-2 infection are considerably lower than are those of HIV-1 infection. Furthermore, the distribution of HTLV-2 tends to be more localized to certain population groups compared with the pandemic spreading of HIV-1. Indeed, HTLV-2 is endemic in some native American Indian and African tribes. Epidemiologic surveys, based on serologic and molecular assays, have indicated that the virus is also prevalent among frequently transfused individuals and i.v. drug users (6–9). This latter group is also frequently coinfected with HIV-1 both in Europe (10–15%) and in up to 30% in urban areas of North America (10, 11). Spontaneous lymphocyte proliferation in vitro as well as a higher DNA proviral load are characteristic features of PBMC and CD8+ lymphocytes, respectively, derived from HTLV-2-infected individuals (12, 13). Nevertheless, HTLV-2 provirus has been detected also in CD4+ T and CD19+ B cells of patients with very high viral load (14–17).

Regarding the pathophysiological consequences occurring upon HTLV-2 infection in a subject concomitantly infected with HIV-1, it is a matter of controversy whether HTLV-2 infection has a protective role, an enhancing role, or no role in the progression of AIDS (8, 18–20). Part of the controversy is due to the fact that conventional serological assays, especially those used in the past, do not distinguish between HTLV-1 and HTLV-2, and consequently HTLV-2/HIV-1 coinfection has been often considered analogous to HTLV-1/HIV-1 dual infection, a condition that some studies have linked to an accelerated progression of the HIV-1 disease toward AIDS (21). Although HTLV-1 and HTLV-2 are strictly related viruses, their effects on AIDS progression should not be confused. In this regard, most seroprevalence studies have reported that HTLV-2 infection does not adversely affect the course of HIV-1 disease (8, 19, 20). Indeed, we and others have previously shown that HTLV-2 infection can potentially interfere with the replicative capacity of HIV-1 by up-regulating the secretion of HIV-1 suppressive chemokines, in particular C-C chemokine ligand 3/macrophage-inflammatory protein-1α (22, 23).

Concerning HTLV-2, it was early reported that STAT proteins were not activated in chronically HTLV-2-infected cells (24). In contrast, we recently demonstrated that the ability of the human CD34+ IL-3-dependent TF-1 cell line of proliferating after HTLV-2 exposure in conditions of IL-3 deprivation is the consequence of the production of the GM-CSF and IFN-γ, both signaling through the activation of the Janus kinase JAK/STAT pathway (25). In addition, we have recently shown that most (75%) HIV-1 seropositive patients display a constitutive activation of STAT5A and STAT1 in their freshly isolated and unstimulated PBMC (26). Interestingly, this abnormality...
was transiently suppressed by zidovudine monotherapy (27). However, no information on the activation state of STATs in HTLV-2-infected individuals is currently available.

In this study, we analyzed the potential activation of the JAK/STAT pathway in HTLV-2 monoinfected and HTLV-2/HIV-1 dually infected individuals. In particular, we investigated 1) whether PBMC of HTLV-2-infected individuals show a constitutive STAT activation, and, eventually 2) which T lymphocyte subsets (CD4+ or CD8+) are involved in STAT activation either in HTLV-2+ individuals or after in vitro infection and, finally, 3) the possible influence of HTLV-2 infection on the HIV-1-associated activation of STATs in individuals coinfected with both viruses.

Materials and Methods

Study cohort

Three HTLV-2 monoinfected, five HIV-1 monoinfected, and 15 HTLV-2/ HIV-1 dually infected individuals were studied after their informed consent was obtained. The clinical and virologic features of the cohort are summarized in Table I. HIV-1 and HTLV-2 proviral DNA content in their PBMC was quantified by competitive PCR, as previously described (28). The HTLV-2b subtype was characterized by sequencing the long terminal repeat region of the viral isolates. The stage of HIV-1 infection was classified according to the Centers for Disease Control and Prevention criteria (29). The clinical history of the patients, including the CD4+ and CD8+ T cell counts, was available through medical records. HIV-1 RNA quantification in the plasma was measured by Amplicor Monitor (sensitivity, 50 copies/ml; Chiron kit; Chiron, Emeryville, CA) and the subsequent binding of the magnetically labeled cells to high gradient immunomagnetic columns (StemCell Technologies). The enriched CD4+ and CD8+ T cell subpopulations were >95% pure by flow cytometric analysis.

In vitro HIV-1 infection of unstimulated PBMC isolated from HTLV-2+ individuals was performed by incubating 1 × 10^6 cells with three different strains of HIV-1, the CXCR4-dependent IIIB and MN, and the CCR5-dependent BaL strain at 0.5 multiplicity of infection for 2 h at 37°C. After three washes, the cells were maintained for 24 h in RPMI complete medium in the absence of IL-2 or any other stimuli.

Table I. Virological and clinical features of patients infected with HIV-1 and HTLV-2

<table>
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<tr>
<th>Patient</th>
<th>HIV-1 DNA (copies/10^6 cell)</th>
<th>HTLV-2b DNA (copies/10^6 cell)</th>
<th>HIV-1:HTLV-2 (DNA ratio)</th>
<th>Clinical Stage</th>
<th>CD4 (cells/μl)</th>
<th>HIV-1 RNA (copies/ml)</th>
<th>Therapy</th>
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* Based on criteria of the Centers for Disease Control and Prevention (29).
| 3TC, Lamivudine; AZT, zidovudine; d4T, stavudine; EFV, efavirenz; IDV, indinavir; ddI, didanosine.

Antibodies

Rabbit antiserum raised against C-terminal epitopes of STAT5A (PA-75A) and STAT5B (PA-75B) were obtained from R&D Systems (Minneapolis, MN); affinity-purified rabbit polyclonal Abs raised against an N-terminal (residues 5–24) epitope of STAT5 (sc-836), and anti-STAT1β rabbit polyclonal Ab (E-23, sc-346) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Whole-cell extracts (WCE) and EMSA

WCE were prepared by repeated cycles of cell freezing and thawing as previously described in detail (25). EMSA was performed by incubating WCEs with [γ-32P]ATP end-labeled double-stranded oligonucleotide corresponding to the prolactin-responsive element (PRE), located within the promoter of the β-casein promoter (31). The DNA-protein complexes were resolved as previously described (25, 32).

ELISA

Cell-free culture medium was assayed for IFN-γ content by the ELISA kit (sensitivity, <2 pg/ml) from Endogen (Woburn, MA), recognizing only the bioactive protein, according to manufacturer’s instructions.

Results

Analysis of STAT activation in monoinfected and HTLV-2/HIV-1 coinfected individuals

We previously reported that most HIV-infected individuals present a constitutive pattern of STAT activation in their PBMC (26). To
analyze the status of activation of the STAT proteins in HTLV-2- and HTLV-2/HIV-1-infected individuals we performed EMSA experiments incubating WCE of unstimulated PBMC derived from three HTLV-2 monoinfected, two HIV-1 monoinfected, and 15 HIV-1/HTLV-2 coinfected patients with a radiolabeled probe encompassing a STAT-binding specific DNA element. As shown in Fig. 1, STATs were indeed activated, in agreement with our previous results (26), in both HIV-1-infected individuals, whereas the three HTLV-2 monoinfected patients were negative. A minority of HIV-1/HTLV-2 coinfected patients was weakly positive (patients 8RE, 2PC, 3PC, 2RA, and 12PR), whereas the remainders were negative for STAT activation. These findings suggest that in vivo HTLV-2 infection per se, unlike HIV-1 infection, does not lead to an evident activation of STAT and, furthermore, that the concomitant infection of HTLV-2 in HIV-1 patients either prevents or turns off the activation of STATs associated with HIV-1 infection.

**Emergence of STAT activation after ex vivo culture of cells derived from HTLV-2-infected individuals**

We next cultivated for different periods of time PBMC derived from two different individuals representative of each type of infection (single vs dual infection) and from two healthy donors as controls. The WCEs obtained from cells cultivated in the absence of any stimuli at different times were analyzed by EMSA for STAT activation (Fig. 2). In contrast to the analysis conducted immediately after PBMC isolation (time 0), the WCE of both HTLV-2-infected individuals (Fig. 2A, PR#3 and PR#1) showed a strong DNA binding activity after 8 h of culture that was maintained up to 24 h with a slight decrease in intensity at the last time point for PR#3. The onset and persistence of STAT-DNA binding activity was variable among the three HTLV-2 individuals. We then analyzed WCE from one STAT-positive individual (HPR#6) and one STAT-negative at time 0 (HPR#7) HIV-1-infected individual (Fig. 2B). In subject HPR#6, the basal DNA binding activity disappeared after 30 min and a lower migrating band appeared 2–4 h afterward. In subject HPR#7 the lower migrating band was visible after 2 h of culture (Fig. 2B). These findings indicate also that the PBMC derived from the minority of HIV-1-infected individuals not showing constitutive STAT phosphorylation in freshly isolated cells are indeed “primed” for the activation of STATs.

We then examined two HIV-1/HTLV-2 coinfected individuals, 2RA and 12PR, who were either strongly or weakly positive after PBMC isolation for STAT activation, respectively. Of note, their positivity or negativity for STAT activation was associated, on the one hand, with a higher and a lower HIV-1 DNA load, and, on the
other hand, with a low and a very high HTLV-2b proviral DNA level, respectively (Table I). The basal DNA binding activity of 2RA did not disappear after 30 min, as observed with cells of both HIV-1-infected individuals, but only after 2 h, whereas the slower migrating band became visible after 4 h of cultivation. In contrast, 12PR showed a kinetic similar to those of HTLV-2 monoinfected individuals, but with an overall weaker intensity (Fig. 2C). As expected, the WCE from the two healthy donors chosen as controls were negative during the entire time course (Fig. 2D). To verify that equal amount of proteins were loaded on the gel the constitutive YY1 binding activity was assessed by adding the YY1 probe together with the STAT probe (data not shown), as published (26).

**STAT1 is activated in ex vivo cultures of PBMC of both HIV-1- and HTLV-2-infected individuals**

To demonstrate which member(s) of the STAT family were activated after cultivation of the patient cells ex vivo, Ab-mediated supershift experiments were performed using the PRE probe and WCEs obtained from either HIV-1- or HTLV-2-infected individuals. As shown in Fig. 3A, when HTLV-2-infected cells derived from PR#3 were cultivated for 8 h STAT1 was the only protein activated, because the addition of the specific anti-STAT1 Ab completely eliminated the retarded band (Fig. 3A, lane 2). In agreement with our previous findings (26), the HIV-1-infected cells derived from HPR#5 showed a preferential activation of the truncated isoform of STAT5 (STAT5Δ) and only minor activation of STAT1 (Fig. 3B, time 0) immediately after isolation, whereas after 12 h of culture (similarly to the 12-h cultivated HTLV-2-infected cells), STAT1 was the only activated STAT protein. We chose subject HPR#5 for this experiment because the intensity of the retarded bands was equal in the two time points analyzed, unlike what observed with WCE from HPR#6 and HPR#7 (Fig. 2). In conclusion, these results indicate that constitutive activation of STAT5Δ, typical of PBMC from HIV-1-infected individuals, is rapidly lost when the cells are cultivated ex vivo, whereas STAT1 activation emerges after at least 2 and 8 h of cultivation of PBMC from HIV-1- and HTLV-2-infected individuals, respectively.

**Spontaneous IFN-γ production from PBMC derived from monoinfected and dually infected individuals correlates with the level of STAT1 activation**

To investigate whether the spontaneous activation of STAT1 was the result of the secretion of IFN-γ, we determined its levels by ELISA in the culture supernatants of PBMC derived from the same individuals described in Fig. 2. Among the candidates, IFN-γ was the cytokine of choice to explain the pattern of STAT1 activation. Indeed, the cumulative IFN-γ production (Fig. 4) paralleled the pattern of STAT1 activation shown in Fig. 2. The peak of IFN-γ secretion was at 12 h for HPR#6 and 8 h for HPR#7, respectively (Fig. 4B), corresponding to the maximal intensity of STAT1-DNA binding activity of each individual (see Fig. 2). Of interest is the fact that the coinfected individuals (Fig. 4C) showed very low levels of IFN-γ production consistent with the low intensity or absence of STAT1-DNA binding activity (see Fig. 2). Their levels were just above the undetectable cytokine production of healthy uninfected controls (Fig. 4D). To statistically corroborate this finding we examined the level of IFN-γ produced from PBMC of an additional eight HIV-1+ and six dually infected individuals after cultivation in the absence of stimuli for 24 h. The median value of IFN-γ secreted was 66 ± 27 pg/ml for monoinfected and 19 ± 8 pg/ml for dually infected individuals, respectively (data not shown). Furthermore, we infected in vitro PBMC derived from the three HTLV-2+ individuals with three different strains of HIV-1, the IIIB (X4), MN (X4), and BaL (R5), at 0.5 multiplicity of infection, and the levels of IFN-γ produced was measured 24 h postinfection. The levels of IFN-γ secreted by PR#1 were 3, 2, and 2 pg/ml after BaL, IIIB, and MN infection, respectively; for PR#2 the levels were 5, 6, and 5 pg/ml after BaL, IIIB, and MN infection, respectively, whereas for PR#3 the concentrations were 14, 8, and 11 pg/ml after BaL, IIIB, and MN infection, respectively.

Taken together these observations suggest that the spontaneous activation of STAT1 observed in both HIV-1- and HTLV-2-infected individuals is very likely explained by the constitutive secretion of IFN-γ.

**STATs are activated in both CD4+ and CD8+ T cell subsets after HTLV-2 infection in vivo and in vitro**

Because HTLV-2 infection is reported to be preferential for CD8+ T lymphocytes (14), we investigated whether STAT activation was restricted to this cell subset. Thus, we analyzed by EMSA WCE derived from PBMC of the third HTLV-2 monoinfected individual of our cohort, PR#2, at time 0 and after 0.5, 8, and 24 h of culture. Consistent with what we observed for HPR3 and PR5 (Fig. 2), STAT1 was spontaneously activated after 8 h of culture and its intensity increased sharply at 24 h (Fig. 5A). In parallel, we separated CD4+ and CD8+ T lymphocytes by immunomagnetic fractionation and cultivated them separately for 8 and 24 h (Fig. 5A, lanes 5–10). STAT1 was spontaneously phosphorylated in both subpopulations after 8 h of culture, but only in CD8+ cells was the activation sustained up to 24 h.

Next, we verified whether the same phenomenon could occur after HTLV-2 infection in vitro. To address this question we either incubated unfractionated PBMC of uninfected individuals with HTLV-2 or we first separated the CD4+ and CD8+ T cell subsets...
by immunomagnetic fractionation of PBMC and subsequently incubated them in vitro with HTLV-2 Mo strain for 24 h. As shown in Fig. 5B, both total PBMC and CD4\(^+\)/H11001 and CD8\(^+\)/H11001 T lymphocytes showed STAT activation after HTLV-2 incubation.

**Discussion**

In the present study, we demonstrated that STATs are not constitutively activated in unstimulated PBMC freshly isolated from HTLV-2-infected individuals. Nevertheless, when their PBMC or their PBMC-derived CD4\(^+\) and CD8\(^+\) T lymphocytes were cultivated ex vivo in the absence of exogenous stimuli for up to 24 h, they showed a spontaneous activation of STAT1. This phenomenon, mostly or totally related to the secretion of IFN-γ, was a common feature of PBMC from both HIV-1- and HTLV-2-infected individuals. Surprisingly, coinfection by HIV-1 and HTLV-2 resulted in a low/absent STAT activation in vivo, which correlated with a diminished secretion of IFN-γ in ex vivo cultivated cells.

The JAK/STAT signaling pathway is a common transducing system used by several cytokines whose role is crucial in the homeostatic regulation of the immune system (33–35). Of note, we previously demonstrated that a constitutive activation of STAT5 in HIV-1-infected individuals was mostly sustained by an N-terminal truncated species (26), known in other systems to play a role as a trans-dominant negative form possessing DNA binding capacity but devoid of transcriptional activity (36–39).

To our knowledge, this is the first documentation of STAT activation after infection by HTLV-2, a human retrovirus with undefined consequences for human health. Nonetheless, the fairly high prevalence of this infection among polytransfused individuals and i.v. drug users, as well as its relatedness to the pathogenic HTLV-1, underscores the relevance of better understanding its interaction with the human immune system. The lack of a basal STAT activation in freshly isolated PBMC of HTLV-2-infected individuals, containing a fraction of chronically infected cells, is in line with the data previously published by Mulloy et al. (24), who demonstrated that several cell lines chronically infected with HTLV-2 underwent cellular transformation independently from the activation of the JAK/STAT pathway. Conversely, the demonstration that the incubation of either unfraccionated PBMC or
separated CD4⁺ and CD8⁺ T cells with HTLV-2 up to 24 h triggers STAT activation in agreement with our recent observation that incubation of CD34⁺ TF-1 cells with HTLV-2 induced the secretion of IFN-γ and GM-CSF, which in turn activated STAT1 and STAT5, respectively (25).

The observation that PBMC obtained from both HIV-1- and HTLV-2-infected individuals activate STAT1 as consequence of the spontaneous release of IFN-γ is supported by previous findings indicating an up-regulation of this cytokine as a consequence of either HIV-1 or HTLV-2 infection (40, 41). In this regard, the HTLV-2 Tax trans-activator has been shown to enhance transcription of IFN-γ (42, 43). In addition, a number of studies have demonstrated that PBMCs derived from HTLV-2-infected individuals undergo spontaneous proliferation in short-term cultures in association with the secretion of several cytokines including TNF-α, IL-5, IL-6, and IFN-γ (40). However, why the release of IFN-γ by PBMC was evident only after ex vivo cultivation and not immediately after PBMC isolation (in contrast to what observed with PBMC of HIV-1-infected individuals) is currently unclear. The IFN-γ gene could be silenced in vivo or, alternatively, IFN-γ could be secreted, but complexed by soluble inhibitor(s). In this respect, it has been demonstrated that the production of IFN-γ by IL-12-activated macrophages was inhibited by IL-4, a cytokine responsible for STAT6 activation (44). We explored this latter hypothesis both in terms of direct determination of IL-4 mRNA by real-time PCR analysis on freshly isolated PBMC from HTLV-2-infected individuals and by probing WCEs from these cells for STAT6 binding activity in both cases with negative results (data not shown). In conclusion, PBMC from HTLV-2-infected individuals are indeed primed for IFN-γ production, and the minimal activation provided by ex vivo cultivation (otherwise never resulting in STAT activation in control seronegative healthy individuals) allows either Tax 2-dependent or independent expression of IFN-γ.

A large body of evidence accumulated over the time describes an increased level of either IFN-γ or its correlates (such as neopterin or IP-10) in the plasma/serum of HIV-1-infected individuals (45), likely explaining the low but detectable constitutive STAT1 activation observed in HIV-1-infected, but not HTLV-2-infected, individuals (26). Therefore, T cells from both HIV-1- and HTLV-2-infected individuals share a constitutive priming for IFN-γ secretion and, consequently, for STAT1 activation; in contrast, only HIV-1 infection in vivo is characterized by activation of STAT5Δ in vivo (26). HIV-1-induced STAT5Δ activation is rapidly lost after few hours of in vitro cultivation. This observation indicates that the factor(s) leading to both cleavage and preferential activation of STAT5 is present in vivo and is lost by PBMC isolation. We previously published that an increased activation of STAT5Δ was observed in PBMC derived from HIV-1-infected subjects enrolled in a phase II clinical trial who received intermittent IL-2 administration, but not in normal PBMC infected in vitro with HIV-1 and subsequently stimulated with IL-2 (27). Because these factors are absent in both HTLV-2- and HTLV-2/HIV-1 coinfected individuals, it is tempting to speculate that this may be a direct reflection of the higher pathogenic potential of HIV-1 vs. HTLV-2, but also highlights a sort of dominance of HTLV-2 over HIV-1 in terms of maintaining T cells in a primed but not absolutely activated state. In this regard, there is anecdotal evidence that HTLV-2 coinfection is frequently associated with a state of long-term nonprogression of HIV-1 disease (19, 46). Although this hypothesis remains to be fully demonstrated, we recently observed that CD8⁺ T cells from HTLV-2 monoinfected and coinfected individuals are potent inhibitors of HIV-1 infection and replication consequent to their enhanced synthesis of CCR5-interacting chemokines. In particular, C-C chemokine ligand 3/macrophage-inflammatory protein-1α appeared as the most relevant anti-HIV-1 chemokine produced ex vivo, and its secretion was significantly correlated to the HTLV-2 proviral load in these individuals (22).

Therefore, HTLV-2 infection and coinfection represent an important model for better understanding the interaction between human exogenous retroviruses and the immune system. IFN-γ-related priming for STAT1 activation may be a signal of “danger” that biases the immune response toward a Th1-model of containment of HTLV-2 infection overwhelmed by an anomalous STAT5Δ activation in HIV-1-infected individuals. In addition, IFN-γ is the pivotal cytokine triggering a phagocyte-dependent Th1 response leading to CTL response against invading pathogens (47). In this regard, a poor Th1 response and a dominant Th2 response have been implicated in the pathogenesis and progression of HIV infection (48, 49). Therefore, HTLV-2 priming for a Th1 response via up-regulation of IFN-γ expression may contribute to the “protective” effect of HTLV-2 infection on HIV-1 disease progression.

In conclusion, analysis of STAT activation may be useful in monitoring the immune reconstitution of HIV-1-infected individuals after conventional and immune-based therapies.
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