Interleukin-10 Gene Expression in Adult T-Cell Leukemia

By Naoki Mori, Parkash S. Gill, Tarsem Mougdil, Shuichi Murakami, Sumiya Eto, and Diane Prager

We studied the serum levels of interleukin-10 (IL-10), in patients with adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) infection. Elevated IL-10 levels were observed in 33 of 45 patients with ATL. Fresh leukemic cells from ATL patients as well as HTLV-I–infected T-cell lines MT-2, SLB-1, and C10/MJ expressed IL-10 mRNA by reverse transcription-polymerase chain reaction analysis, whereas IL-10 mRNA was not detected in normal peripheral mononuclear cells and an uninfected T-cell line Jurkat. IL-10 protein was also detected in the culture medium of leukemic cells from ATL patients as well as these HTLV-I–infected cell lines, and in the extracellular fluids of ATL patients. Interestingly, MT-4 cells, which did not express Tax although transformed by HTLV-I, did not express IL-10 at either the mRNA or protein level. To elucidate the role of the HTLV-I encoded transactivator Tax in IL-10 gene expression, Jurkat cells were transfected with a Tax expression plasmid. In transiently transfected Jurkat cells, endogenous IL-10 mRNA expression was induced by Tax. Stably transfected Jurkat cell lines expressed IL-10 mRNA and secreted IL-10 protein into the culture medium. The nuclear factor (NF)-κB pathway is a target for Tax transactivation. We treated MT-2 cells with phosphothioate antisense oligonucleotides to the p65 subunit of NF-κB. A reduction in the expression of p65 was accompanied by a reduction in IL-10 gene expression and IL-10 production. We showed that the IL-10 xB-like sites (κB1, −2,034 to −2,025; κB2, −1,961 to −1,952; κB3, −452 to −443) specifically formed a complex with NF-κB-containing nuclear extract from MT-2 cells and that NF-κB bound with the highest affinity to the κB2 element (κB2 > κB3 > κB1). These data suggest a general role for NF-κB activation in the induction of IL-10 gene transcription. Activation of IL-10 in HTLV-I–infected cells may contribute to the pathology associated with HTLV-I infection.

© 1996 by The American Society of Hematology.

A DULT T-CELL leukemia (ATL) is one of the peripheral T-cell malignant neoplasms strongly associated with a retrovirus, human T-cell leukemia virus type I (HTLV-I).  

ATL is characterized by high white blood cell counts with convoluted nuclei, hepatosplenomegaly, lymphadenopathy, skin lesions, frequently hypercalcemia, and a rapidly progressive terminal course.  

HTLV-I is also associated with chronic inflammatory disorders such as tropical spastic paraparesis/HTLV-I–associated myelopathy, HTLV-I–associated arthropathy, uveitis, alopecia, and dermatitis.  

HTLV-I has a transcriptional activator, Tax, that regulates viral replication at the transcriptional level.  

Tax is also able to transactivate a number of cellular genes that encode cytokines and their receptors.  

Some of these genes that can be transactivated by Tax are constitutively expressed in ATL cells, suggesting the possible existence of autocrine cytokine loops and a mechanism that might explain some symptoms observed in HTLV-I–associated diseases (ie, hypercalcemia by activation of the parathyroid hormone–related protein, arthropathy by activation of interleukin-6 [IL-6], etc.).  

A recently discovered member of the cytokine network is IL-10.  

Originally, IL-10 was described as a T-cell cytokine produced by Th-2 helper cells, which was capable of suppressing cytokine secretion by Th-1 helper T cells. Subsequent studies have shown that IL-10 is produced by various other cells, including B cells, mast cells, and monocytes, and that IL-10 activities are pleiotropic. For instance, IL-10 can induce proliferation and differentiation of B cells in humans, and proliferation of T cells in the mouse model, and differentiation of cytotoxic T cells.  

To date, IL-10 expression has been described in B cells derived from a variety of lymphoproliferative disorders including chronic lymphocytic leukemia, acute lymphocytic leukemia, Burkitt’s lymphoma and other non-Hodgkin’s lymphoma (NHL), and acquired immunodeficiency syndrome–related lymphomas, and in myeloma cells. The biologic properties of IL-10 have raised the question of the potential clinical relevance of this cytokine in lymphoproliferative disorders. Increased serum levels of IL-10 have been reported in patients with NHL and multiple myeloma.  

Thus, IL-10 expression has been extensively studied in malignant B cells, but only little information is available on IL-10 expression in malignant T cells. For these reasons, we investigated the possible involvement of IL-10 in the pathophysiology of T-cell malignancy, ATL, in vivo. Using an enzyme-linked immunosorbent assay (ELISA) recognizing human IL-10, we measured the serum concentrations of IL-10 in patients with ATL. Surprisingly, we found significantly high serum levels of IL-10 in ATL patients. We examined clinical specimens of patients with ATL as well as HTLV-I–infected cell lines for expression of IL-10 mRNA and protein, in order to identify the origin of production of IL-10 in ATL. Furthermore, we tested the regulation of its gene expression by HTLV-I Tax, which would provide a clue to understanding the molecular mechanism of IL-10 gene expression in ATL patients.

MATERIALS AND METHODS

Patients serum and cell samples.  

Forty-five sera from 41 patients with previously untreated, acute ATL and 4 patients with previously

From the Department of Medicine, Division of Endocrinology and Metabolism, Cedars-Sinai Medical Center; UCLA School of Medicine, Los Angeles; the Department of Medicine, University of Southern California School of Medicine, Los Angeles, CA; and the First Department of Internal Medicine, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan.  

Submitted January 10, 1996; accepted March 28, 1996.  

Supported in part by National Institutes of Health Grant R01 DK45484.

Address reprint requests to Naoki Mori, MD, Department of Medicine, Division of Endocrinology and Metabolism, Cedars-Sinai Medical Center, UCLA School of Medicine, Davis Bldg 3008, 8700 Beverly Blvd, Los Angeles, CA 90048.  

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8803-0021$3.00/0

1034-1045

Blood, Vol 88, No 3 (August 1), 1996; pp 1035-1045

1036
untreated, chronic ATL were collected and stored at −80°C. All ATL patients had a high white blood cell count with typical ATL lymphoblasts. Morphological diagnosis was independently confirmed by immunophenotyping of isolated cells from the peripheral blood (PB) and by the demonstration by Southern blot analysis of monoclonal integration of HTLV-I genome (data not shown). Clinical subtyping was performed according to the previously described criteria.29

PB mononuclear cells (PBMCs) from patients with ATL and normal blood donors were isolated by density gradient centrifugation with Ficoll-Hypaque (Pharmacia, Piscataway, NJ). RNA was immediately extracted from the cells. PBMCs were further incubated at a density of 1 × 10⁶ cells/mL in RPMI 1640 medium in plastic petri dishes for 2 hours at 37°C. Adherent cell-depleted PB subpopulation contained <2% monocytes when analyzed by fluorescence-activated cell sorting. In all cases, the percentage of leukemic cells determined by both CD4+ and CD25+ cells and used for each assay was greater than 90%. These suspensions were then introduced into the 24-well culture plates (2 mL/well) at cell densities of 1 × 10⁶ mL and cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Supernatants were collected after 48 hours and centrifuged to remove cell debris.

Cell lines. Jurkat is a human T-cell line established from T-cell acute lymphocytic leukemia (T-ALL) cells. MT-2, SLB-1, C10/MJ, and MT-4 are HTLV-I-infected T-cell lines. These cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin, and streptomycin. Jurkat cells were also stably transfected with human IL-10.2' Primer sequences for oligonucleotides 5' primer (sense strand) and 3' primer (antisense strand) for 0-actin, Tax, and p65 were as follows. The sequences for oligonucleotides 5' primer (sense strand) and 3' primer (antisense strand) for 0-actin, Tax, and p65 were as follows. The p65 primers 5'-GGGCGCAAGCTT-ACCAATGGCCACTTGGCG-3' define an amplicon of 150 bp. Antisense oligonucleotides. Phosphorothioate analog oligodeoxynucleotides for the human nuclear factor (NF)-κB p65 subunit correspond to the 5' end of the p65 mRNA and include one codon upstream from the translation initiation codon. Use of these oligodeoxynucleotides are as follows: p65 sense 5'-GCCATGGACGAACTGTCCCGCCAC-3' and p65 antisense 5'-GGGCGAAGCTTGGAC-3'. This sequence has been used previously to inhibit the translation of the respective mRNA.9

IL-10 assay. IL-10 was measured using a commercial solid-phase sandwich ELISA (Cytocreen; Biosource International, Camarillo, CA), following the manufacturer’s instructions. The lower limit of sensitivity of the assay is 5 pg/mL. All samples were studied in duplicate and results expressed as the mean of two determinations.

Nuclear extracts preparation and electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from cell lysates as described previously.10,11 The double-stranded oligonucleotides used as probes in EMSA, corresponding to the NF-κB1 site, NF-κB2 site, and NF-κB3 site, are shown in Fig 5B. HIV κB (5'-GATCCAAGGGACCTTCCGCG-3') is an oligonucleotide containing the human immunodeficiency virus type 1 (HIV-1) enhancer. The EMSA was performed as follows. Equal amounts of nuclear extracts (5 μg) were incubated in a reaction mixture consisting of 20 μL buffer containing 10 mM/1 Tris-HCl, pH 7.5, 50 mM/1 NaCl, 1 mM/1 EDTA, 1 mM/1 DTT, 5% glycerol, and 1 μg poly(dI-dC) (Pharmacia, Piscataway, NJ) for 15 minutes at room temperature. One microliter of [α-32P] labeled probe (0.2 ng, 50,000 cpn) was then added with or without a 500-fold excess of unlabeled competitor wild type or mutant oligonucleotides. The reactions were incubated at room temperature for 15 minutes and run on a 4% polyacrylamide gel in 0.5 X TBE (1 X TBE is 50 mM/1 Tris, 50 mM/1 boric acid, and 1 mM/1 EDTA) for 3 hours at 180 V at 4°C. All experiments were performed three times, and results of a representative experiment are shown.

RESULTS

Elevated serum levels of IL-10 in patients with ATL. Serum IL-10 levels were measured in 45 patients with ATL, 10 asymptomatic carriers, and 13 healthy blood donors (Fig 1). IL-10 was detected in 33 of the 41 patients (80.5%) with acute ATL compared with none of the 4 chronic ATL patients, 2 of 10 asymptomatic carriers, and none of the 13 healthy blood donors. In the 33 patients with ATL and detectable IL-10, the mean concentrations of IL-10 were 93.9 pg/mL with a range of 5.1 to 523.8 pg/mL. Serum IL-10 levels were determined sequentially in two patients with acute ATL: in both patients with detectable serum IL-10 at diagnosis, IL-10 decreased below the detection limit when the patients were in remission, but was detectable at relapse. The other patient had chronic ATL that progressed to the acute stage after the diagnosis. Concomitant to the progression of the malignancy, the serum level of IL-10 in this patient rose from undetectable to the detectable level. These results suggest that detectable serum IL-10 is associated with the presence of active disease.

IL-10 mRNA expression and protein production by ATL samples. We examined PBMCs obtained at primary diagnosis from patients with acute ATL for mRNA expression and protein production for IL-10, to identify the origin of production of IL-10 in ATL. These samples consistently contained more than 90% leukemic cells. RT-PCR analysis
was performed using primers specific for human IL-10, which does not amplify transcripts of BCRF1. All five samples from acute ATL (ATL 1-5) were strongly positive for IL-10 expression. In contrast, both normal PBMCs tested were negative for IL-10 mRNA. Integrity of mRNA was confirmed by the amplification of β-actin in all samples included in the analyses (Fig 2).

We examined IL-10 protein by PBMCs from patients with ATL. Freshly collected PBMCs were cultured in RPMI 1640 containing 10% FBS, without any mitogen for a period of 48 hours. The supernatants were analyzed for IL-10 by ELISA. Elevated levels of IL-10 were observed in all three ATL patients ranging from 376 to 1,185 pg/mL (Table 1). IL-10 protein was detected in only the 3 cell lines expressing IL-10 mRNA by RT-PCR. The T-cell control cell line, Jurkat, did not secrete IL-10. These results show that expression of Tax in T cells is correlated with the expression of IL-10. This correlation suggested that Tax is involved in the induction of IL-10 gene expression, as it is with other cellular genes.

**Activation of the endogenous IL-10 gene and protein production in Tax transfected Jurkat cells.** To determine whether Tax can activate IL-10 gene expression, we examined the effect of transient transfection either with the Tax expression plasmid (BC3.9 Sph) or a control plasmid (BC12) on IL-10 regulation. The Jurkat cell line was used because this cell line does not express endogenous IL-10 mRNA. Total RNA was extracted from these cells after 48 hours, and IL-10 gene expression was investigated by RT-PCR. Cells transfected with BC3.9 Sph but not BC12 expressed IL-10 mRNA expression and protein production by HTLV-I—infected T-cell lines. Four HTLV-I—infected T-cell lines were evaluated for the expression and secretion of IL-10. RT-PCR analysis of these cell lines showed expression of IL-10 mRNA in three HTLV-I—infected cell lines (MT-2, SLB-1, and C10/MJ; Fig 3). One HTLV-I—infected cell line (MT-4) did not express detectable levels of IL-10 mRNA. MT-2, SLB-1, and C10/MJ cells expressed the viral trans-activator, Tax mRNA, whereas, interestingly, MT-4 cells, although transformed by HTLV-I, did not express Tax mRNA (Fig 3). In the control cell line, HTLV-I—uninfected Jurkat, neither expression of IL-10 nor Tax was detected (Fig 3). The supernatants from cell lines were then tested for secretion of IL-10 protein by ELISA (Table 1). IL-10 protein was detected in only the 3 cell lines expressing IL-10 mRNA by RT-PCR. The T-cell control cell line, Jurkat, did not secrete IL-10. These results show that expression of Tax in T cells is correlated with the expression of IL-10. This correlation suggested that Tax is involved in the induction of IL-10 gene expression, as it is with other cellular genes.

**Activation of the endogenous IL-10 gene and protein production in Tax transfected Jurkat cells.** To determine whether Tax can activate IL-10 gene expression, we examined the effect of transient transfection either with the Tax expression plasmid (BC3.9 Sph) or a control plasmid (BC12) on IL-10 regulation. The Jurkat cell line was used because this cell line does not express endogenous IL-10 mRNA. Total RNA was extracted from these cells after 48 hours, and IL-10 gene expression was investigated by RT-PCR. Cells transfected with BC3.9 Sph but not BC12 expressed IL-10 mRNA expression and protein production by HTLV-I—infected T-cell lines. Four HTLV-I—infected T-cell lines were evaluated for the expression and secretion of IL-10. RT-PCR analysis of these cell lines showed expression of IL-10 mRNA in three HTLV-I—infected cell lines (MT-2, SLB-1, and C10/MJ; Fig 3). One HTLV-I—infected cell line (MT-4) did not express detectable levels of IL-10 mRNA. MT-2, SLB-1, and C10/MJ cells expressed the viral trans-activator, Tax mRNA, whereas, interestingly, MT-4 cells, although transformed by HTLV-I, did not express Tax mRNA (Fig 3). In the control cell line, HTLV-I—uninfected Jurkat, neither expression of IL-10 nor Tax was detected (Fig 3). The supernatants from cell lines were then tested for secretion of IL-10 protein by ELISA (Table 1). IL-10 protein was detected in only the 3 cell lines expressing IL-10 mRNA by RT-PCR. The T-cell control cell line, Jurkat, did not secrete IL-10. These results show that expression of Tax in T cells is correlated with the expression of IL-10. This correlation suggested that Tax is involved in the induction of IL-10 gene expression, as it is with other cellular genes.

**Activation of the endogenous IL-10 gene and protein production in Tax transfected Jurkat cells.** To determine whether Tax can activate IL-10 gene expression, we examined the effect of transient transfection either with the Tax expression plasmid (BC3.9 Sph) or a control plasmid (BC12) on IL-10 regulation. The Jurkat cell line was used because this cell line does not express endogenous IL-10 mRNA. Total RNA was extracted from these cells after 48 hours, and IL-10 gene expression was investigated by RT-PCR. Cells transfected with BC3.9 Sph but not BC12 expressed IL-10 mRNA expression and protein production by HTLV-I—infected T-cell lines. Four HTLV-I—infected T-cell lines were evaluated for the expression and secretion of IL-10. RT-PCR analysis of these cell lines showed expression of IL-10 mRNA in three HTLV-I—infected cell lines (MT-2, SLB-1, and C10/MJ; Fig 3). One HTLV-I—infected cell line (MT-4) did not express detectable levels of IL-10 mRNA. MT-2, SLB-1, and C10/MJ cells expressed the viral trans-activator, Tax mRNA, whereas, interestingly, MT-4 cells, although transformed by HTLV-I, did not express Tax mRNA (Fig 3). In the control cell line, HTLV-I—uninfected Jurkat, neither expression of IL-10 nor Tax was detected (Fig 3). The supernatants from cell lines were then tested for secretion of IL-10 protein by ELISA (Table 1). IL-10 protein was detected in only the 3 cell lines expressing IL-10 mRNA by RT-PCR. The T-cell control cell line, Jurkat, did not secrete IL-10. These results show that expression of Tax in T cells is correlated with the expression of IL-10. This correlation suggested that Tax is involved in the induction of IL-10 gene expression, as it is with other cellular genes.
IL-10 mRNA (Fig 4). Untransfected Jurkat cells did not give any significant bands of the mRNA. As control, β-actin primers produced almost constant amounts of amplified DNA in all samples. This indicated that transient expression of Tax can indeed stimulate endogenous IL-10 gene expression.

As the results of the transient transfection experiments are not always applicable to endogenous genes, we further examined the effect of Tax on the expression of endogenous IL-10 using the Jurkat clones, Jurkat-Tax-1, -9, and -12, stably transfected with BC3.9 Sph. IL-10 mRNA was detectable in all three Jurkat Tax clones (Fig 4). This induction did not occur in the BC12-transfected Jurkat clone (data not shown). Therefore, transactivation of the IL-10 gene by Tax is independent of the physical location of Tax, occurring whether Tax is integrated in the genome or transiently expressed. Supernatants from Jurkat stably expressing Tax (Jurkat-Tax-1, -9, and -12) were also assayed for IL-10 levels. All three Jurkat Tax clones constitutively produce low levels of IL-10 (Table 1).

### Inhibition of IL-10 mRNA expression and secretion by p65 antisense oligonucleotides

The activation of cellular and viral genes by Tax is mediated through at least two cis-acting DNA sites: cyclic AMP-responsive elements (CRE; eg, those present in the HTLV-I long terminal repeat and in the c-fos promoter) and NF-κB elements (eg, those present in the IL-2 and IL-2 receptor α chain promoters and in the HIV-1 long terminal repeat). To determine through which cis-acting elements Tax may act, the 5’-flanking region of the human IL-10 sequence was examined for the presence of sequence motif that have been associated with the regulation of gene transcription. Interestingly, the promoter does not contain a CRE but has three NF-κB-like elements (Fig 5A). The NF-κB complex, consisting of p50 and p65 subunits, is a cytoplasmic transcription factor activated by a number of intracellular pathways including stimulation of cytokine receptors, direct activation of second messenger pathways, shear stress, and oxidative processes and exerts rapid pleiotrophic activation of a variety of genes. We have used antisense oligonucleotides to p65 in an initial attempt to explore the role of NF-κB in the activation of the IL-10 gene in HTLV-I–infected cells.

Sense or antisense to p65 had no significant effect on cell viability. It was important to establish that p65 antisense oligonucleotides inhibited expression of p65 mRNA. We first estimated the level of p65 mRNA in MT-2 cells after treatment with the p65 antisense oligonucleotides by RT-PCR. The level of p65 mRNA was not detectable after treatment with the p65 antisense oligonucleotides compared with

| Table 1. Concentrations of IL-10 in Conditioned Medium of Established Cell Lines and Leukemic Cells and in the Extracellular Fluids |
|-----------------|------------------|
| Samples         | IL-10 (pg/mL)    |
| Cell lines      |                 |
| Jurkat          | <5               |
| MT-2            | 82,732           |
| SLB-1           | 4,719            |
| MT-4            | <5               |
| C10/MJ          | 1,335            |
| Jurkat-Tax-1    | 21               |
| Jurkat-Tax-9    | 15               |
| Jurkat-Tax-12   | 11               |
| Leukemia cells  |                 |
| ATL case 1      | 438              |
| case 2          | 376              |
| case 3          | 1,185            |
| Normal peripheral mononuclear cells |           |
| Ascitic fluid   | 36               |
| Pleural fluid   | 83               |

Cells were plated at 1 × 10⁶/mL and cultured for 48 hours in RPMI 1640 supplemented with 10% FBS. Levels of IL-10 release/mL in the supernatants of cells were measured by ELISA. Data of cell lines represent the mean of three independent experiments performed in duplicate.
that of the untreated control or treatment with the p65 sense oligonucleotides (Fig 6). Therefore the treatment with 10 μmol/L of the p65 antisense oligonucleotides could have inhibited expression of p65 mRNA in MT-2 cells.

To evaluate a possible role of the NF-κB in regulation of IL-10 gene expression of MT-2 cells, we examined the direct effect of p65 antisense oligonucleotides treatment on expression of IL-10 mRNA. Figure 6 shows that antisense treated MT-2 cells display no IL-10 mRNA as visualized by PCR with gene-specific primers. Control cells and p65 sense oligonucleotides treated cells show no abrogation of the IL-10 mRNA. p65 antisense oligonucleotides do not affect expression of the housekeeping gene β-actin. These results indicate that antisense to p65 does inhibit IL-10 mRNA expression, and suggest that NF-κB could contribute essentially to expression of the IL-10 gene in MT-2 cells.

To further assess the role of NF-κB in regulation of IL-10 gene expression, sense and antisense oligonucleotides to p65 were tested for their ability to inhibit IL-10 production in MT-2 cells. Exposure to p65 antisense, but not sense, oligonucleotides led to marked decreases in IL-10 secretion (8.3 ± 0.5 ng of IL-10 per mL in medium-control cultures, 2.4 ± 1.3 ng/mL in p65 antisense oligonucleotides-treated cultures, and 9.6 ± 0.2 ng/mL in p65 sense oligonucleotides-treated cultures, n = 3) by MT-2 cells (Fig 7). These results provide further evidence for the involvement of NF-κB in IL-10 gene expression and suggest that modulation of the level of NF-κB can dramatically affect IL-10 production.

The sequences from −2,034 to −2,025, −1,961 to −1,952, and −452 to −443 in the 5′-flanking region of the IL-10 gene form DNA-nuclear protein complexes. We identified three sequences corresponding to 5′-GGGTTAGACC-3′, in positions −2,034 to −2,025, 5′-GGGGAAACCC-3′, in positions −1,961 to −1,952, and 5′-GGGGGGACCC-3′, in positions −452 to −443 in the 5′-regulatory region of the IL-10 gene and remarkably similar to the consensus sequence for NF-κB transcription factors. The possibility that the sequence is actually a binding site for NF-κB proteins and is operative in the HTLV-I-infected cells was investigated. Double-stranded oligonucleotides comprising the sequence and designated as KBI (−2,039 to −2,020), KB2 (−1,966 to −1,947), and KB3 (−457 to −438) were tested, in gel shift analysis, for binding of nuclear proteins in extracts from the HTLV-I-infected cell line MT-2 and from uninfected Jurkat cells. As shown in Fig 8, three oligonucleotides detected complexes with nuclear extract from MT-2 cells,
whereas in Jurkat cells, the levels of complexes were not detectable. NF-κB specificity was shown by elimination of complexes following the addition of excess unlabeled κB1, κB2, and κB3 oligonucleotides. It should be noted that complexes were also eliminated by the addition of excess of unlabeled oligonucleotide that corresponds to the NF-κB site within the HIV-1 enhancer and not by mutant oligonucleotides to the IL-10 κB sites (Fig 9). According to these competitions, the fastest-migrating complex was nonspecific. Supershift assays with NF-κB-specific antibodies demonstrated that p50, p65, and c-Rel were the components of the protein-DNA complex as previously reported (data not shown). Thus, NF-κB from MT-2 cells, in which we have shown IL-10 production, recognizes three IL-10 κB sites.

As shown in Figs 8 and 9, κB2 site binds NF-κB more efficiently than the κB1 or κB3 site (κB2 > κB3 > κB1). We compared the three NF-κB sites also by competition with increasing amounts of the unlabeled κB2 (Fig 10; lanes 2 through 4), κB1 (lanes 5 through 7), or κB3 oligonucleotide (lanes 8 through 10) using the κB2 oligonucleotide as a probe. κB1 and κB3 oligonucleotides competed significantly less efficiently than κB2 oligonucleotide, suggesting that binding of NF-κB to κB2 site has the highest affinity (κB2 > κB3 > κB1).

Finally, we tested nuclear extracts from other IL-10-producing cells to determine whether they were also able to bind to the κB2 oligonucleotide, because the κB2 site binds NF-κB the most efficiently. Nuclear extracts from all other cells tested (SLB-1 and C10/M1) also retarded the migration of oligonucleotide κB2, which was prevented by addition of excess unlabeled probe κB2 or HIV κB, but not by an unlabeled mutant κB2 probe. In contrast, the nuclear extract from MT-4 cells, which did not express the HTLV-I Tax and IL-

**Fig 6.** Inhibition of p65 and IL-10 mRNA expression by p65 antisense oligonucleotides. MT-2 cells were cultured in the presence of 10 μmol/L sense or antisense p65 phosphorothioate oligonucleotides for 48 hours. Total RNA was isolated from untreated (lane 1), sense (lane 2) and antisense (lane 3) treated MT-2 cells, and 1 μg was reverse transcribed and PCR amplified using p65, IL-10, and β-actin primers as described in Materials and Methods. One fifth of the reaction was run on a 1.5% agarose gel, ethidium-bromide stained, and photographed. The expected sizes of the products of the p65, IL-10, and β-actin are 150 bp, 350 bp, and 548 bp, respectively.

**Fig 7.** Exposure of MT-2 cells to p65 antisense oligonucleotides inhibited IL-10 production. MT-2 cells were cultured with 10 μmol/L p65 antisense or sense oligonucleotides. Bars represent IL-10 levels in culture supernatants, expressed as the percentage of values seen in medium-control cultures (cultures not exposed to either sense or antisense oligonucleotides). These results represent the mean ± SD of three experiments.
IL-10 IN ATL

Fig 8. NF-κB-binding activity in Jurkat and MT-2 cells. Nuclear extract (5 μg) from Jurkat (lanes 1, 3, and 5) or MT-2 (lanes 2, 4, and 6) cells were incubated with [32P]labeled oligonucleotide probes containing wild type NF-κB (lanes 1 and 2), NF-κB (lanes 3 and 4), or NF-κB (lanes 5 and 6) and analyzed by EMSA. The arrow points to the NF-κB specific complex. N.S., nonspecific complex.

10, formed no complex with the κB2 probe (Fig 11). Taken together, these results show that the specific complexes formed with the IL-10 κB sequences recognized in extracts from several cell lines are correlated with the production of IL-10.

DISCUSSION

Serum IL-10 was detected in 80% of the patients with acute ATL, whereas it was mostly undetectable in patients with chronic ATL, asymptomatic carriers of HTLV-I as well as in all healthy blood donors tested. Noteworthy is the correlation between the IL-10 concentration and the evolution of ATL from the active phase to remission or from the chronic to acute phase. These results suggest that detectable serum IL-10 is associated with the presence of active disease. These observations are consistent with a recent report showing that serum IL-10 is of prognostic value in intermediate or high-grade NHL.

It will be of interest to determine the source of the IL-10, especially whether it originates from ATL cells, because reactive T cells can produce this cytokine. In this study, we have confirmed high levels of IL-10 mRNA expression and protein not only in HTLV-I-infected cell lines, but also in freshly isolated cells from ATL patients. This finding suggests that ATL cells in vivo may be the source of serum IL-10. However, serum IL-10 was not correlated with serum LDH and the number of ATL cells in the PB (data not shown). Alternatively, IL-10 may originate from normal monocytes/macrophages or T cells under deregulated stimulation by malignant ATL cells. To exclude positive signals for IL-10 mRNA due to contamination with non-ATL cells, we purified ATL cell preparations using the CD4 monoclonal antibody bound to magnetic beads, and assessed for the presence of contaminating non-ATL cells by RT-PCR detection for CD8 and CD14 mRNAs, respectively. These preparations that were negative for CD8 and CD14 mRNAs showed IL-10 mRNA signals (data not shown). These results strongly argue against the possibility of contamination.

The HTLV-I–encoded Tax protein activates transcription. This deregulation of gene expression is mediated through several distinct classes of cellular DNA binding proteins, as Tax does not bind DNA in a sequence-specific manner. Previous observations showing that Tax was capable of inducing the expression of genes encoding cytokines and their receptors led us to examine the biological effect of expression of the HTLV-I Tax on the gene activity of IL-10. We have presented an analysis of the mechanism of IL-10 activation in HTLV-I–infected cells. Our results showed that Jurkat cells expressing functional Tax displayed markedly increased IL-10 mRNA expression and protein production. The level of IL-10 protein in culture supernatants of Tax-transfected Jurkat was much lower than that of HTLV-I–infected cell lines in repeated experiments. We and others previously reported similar observations on the IL-2 or IL-6 production by Tax-transfected Jurkat, although these genes were transactivated by Tax in transient transfection studies. These findings suggest that factors of either viral or cellular origin other than Tax are also involved in the constitutive production of cytokines in HTLV-I–infected
cells. On the other hand, tumor necrosis factor-α (TNF-α) is known to induce IL-10 in carcinoma cell lines. As TNF-α gene is transactivated by Tax, we assessed for the possibility that Tax-mediated production of IL-10 in Jurkat is due to a Tax-induced autocrine stimulation by TNF-α. However, IL-10 production of Tax-transfected Jurkat could not be further upregulated by addition of TNF-α. Furthermore, Tax-expressing Jurkat cells did not produce detectable amounts of TNF-α, suggesting that additional cytokines do not trigger the expression of IL-10 (data not shown).

The IL-10 promoter contains numerous potential recognition sequences for known transcriptional factors. However, none of these regulatory regions has been implicated directly in the control of the IL-10 gene expression. Treatment of p65 antisense oligonucleotides revealed that NF-κB appears to be involved in IL-10 gene expression of HTLV-I–infected cells. We report the identification of three identical sequences in the 5′-regulatory region of the IL-10 gene which are specific binding sites for regulatory members of the NF-κB transcription factors. The three nuclear factor binding domains, which we referred to as κB1 site (−2,034 to −2,025), κB2 site (−1,961 to −1,952), and κB3 site (−452 to −443), show high affinity for NF-κB proteins, which appear to be constitutively expressed in the IL-10 producing cells. Furthermore, IL-10 κB binding activity correlates with the IL-10 gene expression in various cell lines. EMSA suggested that NF-κB binds with the highest affinity to the NF-κB2 site (κB2 > κB3 > κB1). These data indicate that Tax may upregulate expression of the IL-10 gene, resulting in induction of IL-10 expression in HTLV-I–infected T cells, and that this activation is dependent on the NF-κB binding sites in the IL-10 promoter. However, further functional studies on transient transfection of IL-10-chloramphenicol acetyltransferase reporter constructs will be required to prove this hypothesis.

In ATL cases, a significantly lower expression of Tax mRNA is observed, suggesting that high expression of IL-10 gene in fresh ATL cells does not result from continuous high expression of Tax protein. This finding implies the presence of a different mechanism independent of HTLV-I Tax underlying the production of IL-10 in fresh ATL cells. However, this conclusion may not necessarily exclude a role...
for Tax in the expression of IL-10 in fresh leukemic cells. The same notion could be applied to other Tax-responsive genes encoding cytokines and their receptors that are overexpressed in ATL cells. Transactivation of these cellular genes could occur early in the course of HTLV-I infection, priming the T cells for a second event, resulting in leukemogenesis. The circulating ATL cells would be in a terminally differentiated, autonomous state and may no longer require Tax production for expression of these cellular genes.

This study raises the question of the role of this cytokine in leukemogenesis. The effect of HTLV-I-infected T cell-derived IL-10 in ATL remains to be determined. Although the presence of IL-10 in the serum of these patients with ATL could be an epiphenomenon, previous reports suggest at least two different mechanisms of action for this cytokine. IL-10 may rescue ATL cells from spontaneous apoptosis. Supporting this hypothesis, IL-10 was recently shown to rescue human T cells from spontaneous apoptosis.\(^5,47\) IL-10 does not induce proliferation of human activated T cells.\(^48,49\) In fact, exogenous added IL-10 did not stimulate proliferation of MT-2 cells and ATL cells (data not shown), suggesting that IL-10 may not be directly involved in an autocrine loop of HTLV-I-infected T cells growth. By RT-PCR using primers specific for the IL-10 receptor-encoding sequences, we found that ATL samples expressed IL-10 receptor mRNA (data not shown). Furthermore, we have preliminary data showing that IL-10 inhibits apoptotic cell death in ATL cells from some cases.

An alternative interpretation could rely on the immunosuppressive properties of IL-10.\(^50\) In particular, IL-10 possesses several properties that may be inhibitory to the generation of antitumor immunity. For the malignant growth of infected cells in ATL cases, an alteration in host cell function may be required. IL-10 inhibits antigen-presentation function and lymphokine-activated killer cell function, and blocks cytokine production by macrophages in vitro.\(^23,31,34\) Pretreatment with IL-10 protects tumor cells from lysis by tumor-specific cytotoxic T cells.\(^52\) Tumor cells transfected with the IL-10 gene produce immunosuppression and prevent the induction of tumor-specific cytotoxic T lymphocytes in vivo.\(^56\) The excessive production of IL-10 by ATL cells may offer a selective advantage. The high concentrations of IL-10 found in patients with ATL could conceivably exert an inhibitory effect on macrophage and antigen-specific T-cell response and thus contribute to leukemia progression in vivo. These two mechanisms of action for IL-10 are a result of survivals of leukemic cells under the host immune surveillance. Additionally, cellular and humoral immune responses are markedly impaired in patients with ATL.\(^57,55\) Therefore, it is relevant to speculate that IL-10, produced by ATL cells, may contribute to disturbed cellular immunity, frequently associated with ATL.

As mentioned before, HTLV-I is also associated with a wide spectrum of chronic inflammatory disorders of various organs. Tax is considered to play a central role in the pathogenesis of these HTLV-I-related diseases.\(^7,12\) IL-10 is a potent cytokine capable of regulating immune reactions at the tissue level.\(^59\) In conclusion, although additional studies are required to further elucidate the biological and clinical significance of IL-10 production during infection with HTLV-I, our results indicate that aberrant expression of IL-10 through Tax-mediated transactivation may contribute to HTLV-I-associated pathologies.

REFERENCES


roles of interleukin-2 and interleukin-10 in the generation of lympho-
J, Pisa P, Zhang Q-J, Masucci MG, Kiessling R: Interleukin 10
pretreatment protects target cells from tumor- and allo-specific cyto-
180:2371, 1994
56. Wang L, Goillot E, Tepper R: IL-10 inhibits alloreactive cyto-
57. Morimoto C, Matsuyama T, Oshige C, Tanaka H, Hercend
T, Reinhez EL, Schlossman SF: Functional and phenotypic studies
leukemia virus; its discovery and role in leukemogenesis and immu-
59. Katsikis PD, Chu C-Q, Brennan FM, Maini RN, Feldmann
M: Immunoregulatory role of interleukin 10 in rheumatoid arthritis.
Interleukin-10 gene expression in adult T-cell leukemia

N Mori, PS Gill, T Mougdil, S Murakami, S Eto and D Prager