TCL1 Oncogene Activation in Preleukemic T Cells From a Case of Ataxia-Telangiectasia

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The TCL1 oncogene on human chromosome 14q32.1 is involved in chromosome translocations [t(14;14)(q11;q32.1) and t(7;14)(q35;q32.1)] and inversions [inv14(q11;q32.1)] with TCRα/β loci in T-cell leukemias, such as T-prolymphocytic (T-PLL). It is also involved in T-acute and -chronic leukemias arising in cases of ataxia-telangiectasia (AT), an immunodeficiency syndrome. Similar chromosomal rearrangements occur also in the clonally expanded T cells in AT patients before the appearance of the overt leukemia. We have analyzed the expression of TCL1 mRNA and protein in peripheral blood lymphocytes (PBLs) from four AT cases and from healthy controls. We found that the TCL1 gene was overexpressed in the PBLs of an AT patient with a large clonal T-cell population exhibiting the t (14;14) translocation but not in the lymphocytes of the other cases. Fluorescence in situ hybridization of the TCL1 genomic locus to lymphocyte metaphases from the AT patient with the T-cell clonal expansion showed that the breakpoint of the t(14;14) translocation lies within the TCL1 locus and is accompanied by an inverted duplication of the distal part of chromosome 14. These data indicate that TCL1 is activated in preleukemic clonal cells as a consequence of chromosome translocation involving sequences from the TCR locus at 14q11. Deregulation of TCL1 is the first event in the initiation of malignancy in these types of leukemias and represents a potential tool for clinical evaluation.

THE DEVELOPMENT OF human cancer is thought to be a multistep process that entails a progressively more aggressive phenotype through the evolution of cellular subsets with increasing numbers of genetic alterations. The identification of steps that are necessary for the full manifestation of the tumor is currently under study because of its impact on cancer diagnosis and treatment and for our understanding of the malignant process.[1]

In hematopoietic tumors, cytogenetic alterations such as chromosomal translocations and inversions are quite common. These chromosomal aberrations directly involve genes responsible for malignant transformation,[2,3] but development of leukemias and lymphomas is also a multistep process. It has been shown, for example, that follicular lymphoma (a low-grade malignancy) with BCL2 rearrangement can progress to a more aggressive form of tumor by acquiring a c-MYC gene rearrangement[4,5]; another example is the clinical transition from chronic to acute phase in chronic myelogenous leukemia, in which p53 mutations and deletions are observed in 20% to 30% of the cases of blastic crisis.[6]

An excellent model for studying tumor progression is represented by T-cell leukemias arising in patients with ataxia-telangiectasia (AT), an immunodeficiency syndrome associated with genomic instability, deficient DNA repair, cerebellar ataxia, and oculocutaneous telangiectasias. These patients are prone to cancer (10%), especially of the hematopoietic lineage, with a 70- to 250-fold increase in the probability of developing lymphocytic leukemia and lymphoma, particularly in childhood.[7] In contrast to the general population, in which B-cell leukemias are predominant, leukemias of T-cell phenotype are particularly frequent in the AT population. In these patients, the presence of clonal T-cell expansions has been observed before the onset of full-blown leukemia (reviewed by Kirsh[8]). These expansions are of two types. The first type of expansions is characterized by translocations and inversions [t(7;7)(p15;q35), inv 7(p15;q35), t(7;14)(q35;q11), and t(7;14)(p15;q11)] that usually involve rearrangements among T-cell receptor genes[9] and are not associated with neoplasia. The second type of expansions, which is observed in 7% of 10% of all AT cases,[7] involves the clonal expansion of cells carrying either the t(14;14)(q11;32.1) translocation or inv 14(q11;q32.1)inversion, or more rarely t(X;14)(q28;q11). In these cases, cells tend to expand in the peripheral blood with time until they represent 90% to 100% of all circulating lymphocytes and invariably progress to a frank leukemia.[10-12] Chromosomal translocations in the latter cases involve on one side the TCRα locus on chromosome 14q11 and, on the other side, either the TCL1 gene on 14q32.1[13] or the MTCP1/ 6.1b gene on Xq28.[12,14] These two genes have recently been cloned and, interestingly, show a high degree of homology to each other.[15] We have recently shown that the TCL1 gene is normally expressed in lymphoid cells as well as in cases of T-prolymphocytic leukemia (T-PLL), a very aggressive type of mature T-cell proliferation accompanied by rearrangements at 14q32 in 80% of the cases.[16] Interestingly, it has been reported that some of the full-blown leukemias

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arising in AT or, in one case, a preleukemic clonal expansion have morphologic features similar to those of T-PLLs.[17]

To examine the role of the TCL1 gene and gene product in clonal AT expansions, we have studied peripheral blood cells from an AT case exhibiting a clonal expansion of T cells (60% of peripheral blood lymphocytes [PBLs]) with t(14;14)(q11;q32.1) chromosome translocation. Comparison of these PBLs to PBLs derived from three other AT patients without cytogenetic evidence of clonal expansion in the peripheral blood and with a normal healthy control shows that TCL1 gene is deregulated only in the preleukemic cells carrying the chromosomal translocation.

MATERIALS AND METHODS

Patient samples and purification of T cells. Lymphocytes from four patients with AT and a healthy donor were analyzed in this study. AT94-1 is a 16-year-old girl with classical AT. The first karyotypic analysis of her lymphocytes was performed in 1986 and showed 4% clonal cells carrying a t(14;14) (q11;q32.1). This abnormality increased to 60% and has remained stable at this frequency in the last two semiannual observations.[17a] The white blood cell count was 6,220/mL, with lymphocytes ranging around 14.4%. The phenotype of her PBLs at the time of the last observation was the following: $CD45^+CD3^+$ (65%), $CD45^+CD20^+$ (11%), TCRα/β (61%), TCRγ/δ (3%), $CD4^+$ (49%), $CD8^+$ (17%).

T cells from AT94-1 and from a normal donor were enriched by adhering B cells and monocytes to a nylon-wool column at 37°C for 60 minutes and T-enriched cells were collected in the eluted fraction. After purification, T cells from normal control and AT94-1 represented approximately 77%, with the remaining cells being $CD3^-CD56^+$ (natural killer cells) and less than 1% being $CD19^+$ (B cells).

The other three patients were classical AT cases without cytogenetically evident clonal expansions.

Cytogenetics and fluorescence in situ hybridization (FISH). Chromosome preparations were obtained using standard methods from 72-hour lymphocyte phytohemagglutinin-stimulated cultures of AT patients. Analysis of spontaneous chromosome rearrangements was performed on at least 30 G-banded metaphases.

For FISH analysis, the cosmid clones pPLC-1 and pPLC-4 were isolated by screening a human placental genomic library using probes B0.9 and pE/S.[18] The pPLC-4 cosmid contains a genomic fragment corresponding to the region from pE/S to the region 40 kb centromeric to pE/S (Fig 1D). The TCL1 gene, and pPLC-1 cosmid are located about 120 and 250 kb centromeric to the end of the pPLC-4 cosmid, respectively.[18] For in situ hybridization, pPLC-1 or pPLC-4 was labeled with biotin-16-dUTP or digoxigenin-11-dUTP, respectively, using a nick-translation kit (Bethesda Research Laboratories, Gaithersburg, MD). The hybridization mixture contained 200 ng each of both labeled probes, pPLC-1 and pPLC-4, as well as 10 μg of sonicated salmon sperm DNA, 10 μg of *Escherichia coli* tRNA, and 350 ng of human Cot-1 DNA (Bethesda Research Laboratory) in 10 μL of 50% formamide, 50 mmol/L phosphate, 10% dextran sulfate, 2× SSC, pH 7.0. Before hybridization, the mixture was denatured at 75°C for 5 minutes, quickly chilled, and incubated at 37°C for 30 minutes to compete for repetitive sequences in the probe. After overnight hybridization, the slides were washed for 20 minutes in 50% formamide, 2× SSC, pH 7.0, at 37°C, followed by two 15-minute washes in 2× SSC, and finally rinsed in 4× SSC at room temperature. Fluorescein isothiocyanate (FITC)-conjugated avidin and antidigoxigenin tagged with rhodamine (Boehringer Mannheim, Mannheim, Germany) were used for the detection of biotinylated pPLC-1 and digoxigenin-labeled pPLC-4. The chromosomes were counter-stained with 0.1 μg/mL of Hoechst 33258 DNA dye.

RNA extraction. A total of 7 to 10 mL of venous blood was obtained by standard venipuncture techniques using heparinazed tubes, mixed with an equal volume of RPMI medium, and then layered over 8 mL of Ficoll (Pharmacia, Uppsala, Sweden) in a 50-mL polystyrene tube.The gradient was centrifuged at 800*g* for 20 minutes. The lymphomonocyte layer was collected and washed twice with RPMI medium and then sedimented at 300*g* for 15 minutes. The supernatant was discarded and the pellet containing nucleated cells was used for RNA extraction using the RNAzol B method as described by the manufacturer (Tel-Test, Inc, Friendswood, TX).

Semiquantitave reverse transcriptase-polymerase chain reaction (RT-PCR). cDNA was made from total RNA using random primer oligonucleotides. Approximately 0.5 μg of RNA in a final volume of 35 μL was first denatured at 80°C for 5 minutes and then cooled on ice. First-strand DNA synthesis was performed by adding 1 μL of Superscript Reverse Transcriptase (Stratagene, La Jolla, CA), 100 mmol/L ditiothreitol (DTT), 10× RT buffer, 40 pmol/L of random primers, 40 pmol/L dRPN6 (Boheringer Mannheim), 10 mmol/L of dNTP, and 36 U of RNAse block to a final volume of 50 μL.

For monitoring the cDNA synthesis, 5 μL of this reaction was removed at the beginning of the synthesis and 5 μCi of a ^{32}Phosphorus dATP (800 mCi/mL) was added to the reaction. The two reactions (radiolabeled and not radiolabeled) were left at 37°C for 1 hour; the two cDNAs were then boiled for 5 minutes or kept on ice or at −70°C for further use. The radioactive fraction was purified through Sephadex Nick Spin Columns according to the manufacturer's instructions (Pharmacia) and then counted on a β scintillation counter (Packard, Downers Grove, IL) to evaluate the cDNA synthesis. The same amount of nonradioactive cDNAs was then used for PCR amplification. All the cDNA samples in Fig 2 were synthetized and used for PCR amplification in the same set of experiments to minimize the variations.

The primers used for amplification of a 306-bp product of the TCL1 transcript by PCR were TCL1-U1 (5′AGGCCTATGACCCCCACC3′), corresponding to the + strand from bp 232-249, and TCL1-R2 (5′CATTCCTCCCAGACCCCA3′), corresponding to the —strand from bp 511-529 of the TCL1.

Primers specific for amplification of a 154-bp product of the housekeeping β-actin gene were A1 (5′TCATCACCATTGGCAATGAG3′) and A2 (5′GTGTTGGCGTACAGGT3′). PCR and amplification were performed under the following conditions: denaturing for 1 minute at 94°C, annealing for 1 minute at 60°C, and elongation for 1 minute at 72°C for 25 cycles (TCL1) or 15 cycles (β-actin).

Immunostaining. Immunohistochemistry was performed on cytospins of T-cell-enriched PBLs. The slides were fixed in 100% acetone for 5 minutes, dried, postfixed in 4% paraformaldehyde for 10 minutes, washed in phosphate-buffered saline (PBS), and immunostained with optimal dilutions of anti-TCL1 polyclonal rabbit antiserum and mouse monoclonal anti-CD7, -CD19, and -CD68. Cytospins were preincubated with goat antiserum or rabbit serum and sequentially incubated with primary antibodies, goat antirabbit IgG, rabbit antimouse IgG, rabbit antiperoxidase (PAP), or mouse peroxidase antiperoxidase. Each incubation step lasted 30 minutes and was followed by 10 minutes of washing in PBS. The slides were then incubated with 0.03% H_2O_2 and 0.06% 3, 3′-diaminobenzidine for 2 to 5 minutes, washed in tap water, and counterstained with hematoxylin. The number of positive cells was determined by light microscopy at 400× magnification in five fields and expressed as the percentage of positive cells.

RESULTS

Cytogenetic analysis of the t (14;14) breakpoint. The cytogenetic analysis by chromosome banding performed on

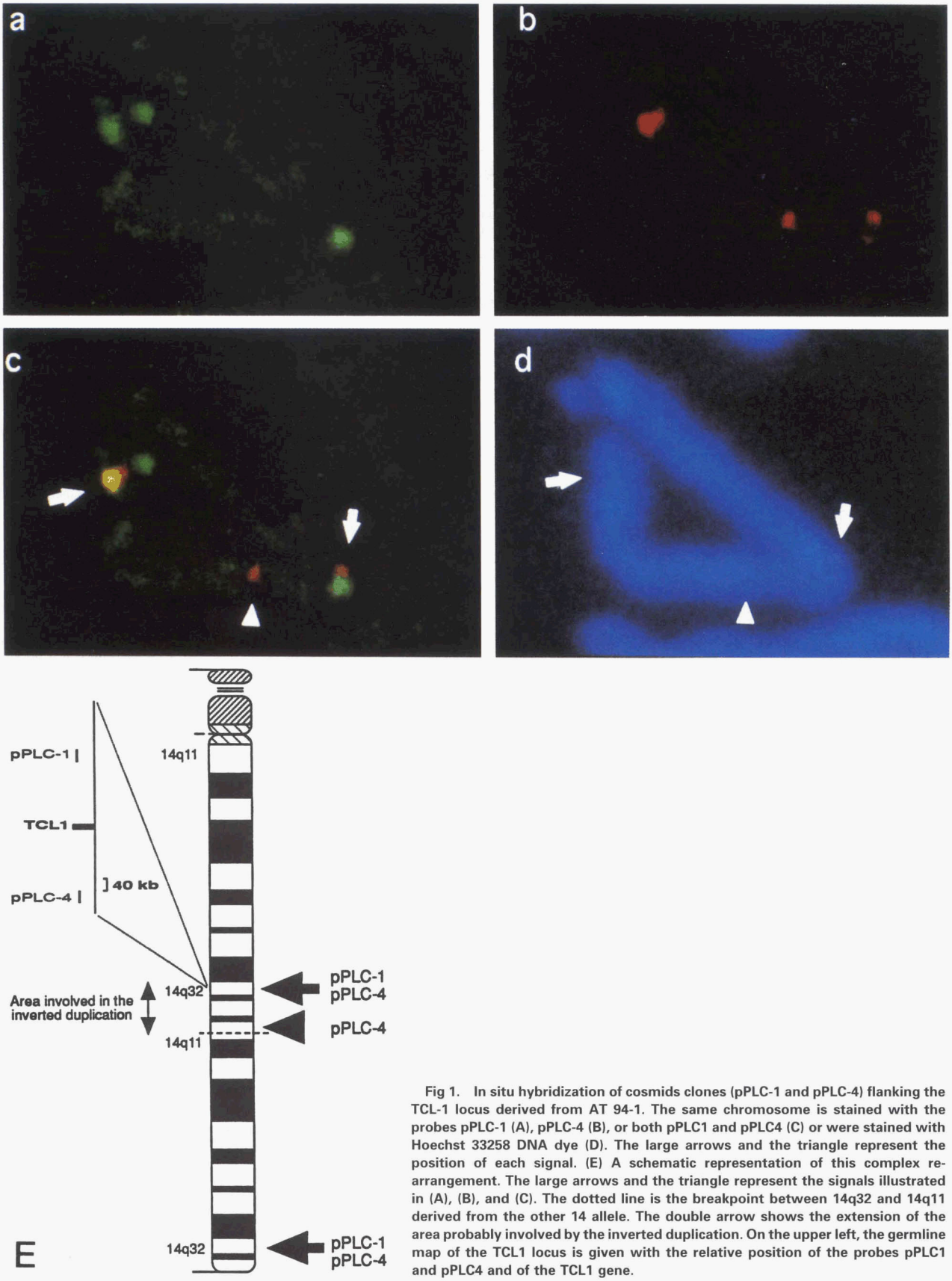

Fig 1. In situ hybridization of cosmids clones (pPLC-1 and pPLC-4) flanking the TCL-1 locus derived from AT 94-1. The same chromosome is stained with the probes pPLC-1 (A), pPLC-4 (B), or both pPLC1 and pPLC4 (C) or were stained with Hoechst 33258 DNA dye (D). The large arrows and the triangle represent the position of each signal. (E) A schematic representation of this complex rearrangement. The large arrows and the triangle represent the signals illustrated in (A), (B), and (C). The dotted line is the breakpoint between 14q32 and 14q11 derived from the other 14 allele. The double arrow shows the extension of the area probably involved by the inverted duplication. On the upper left, the germline map of the TCL1 locus is given with the relative position of the probes pPLC1 and pPLC4 and of the TCL1 gene.

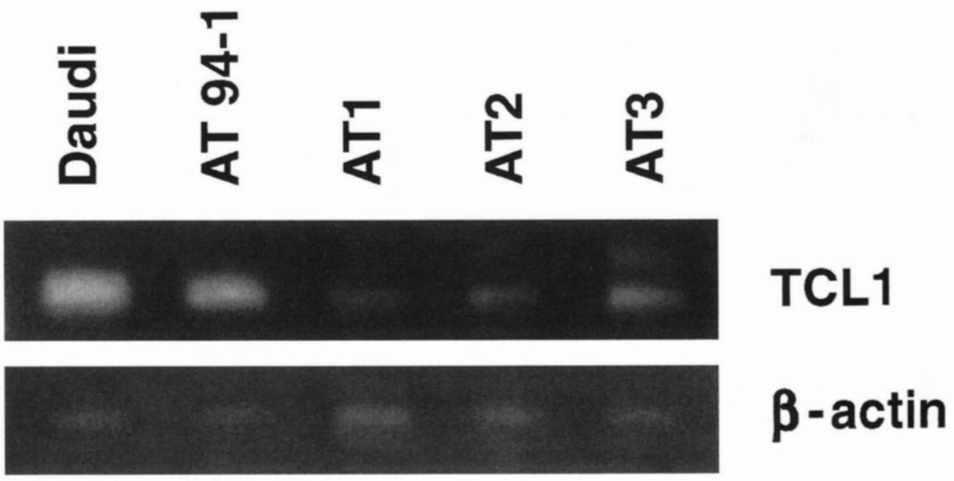

Fig 2. Semiquantitative RT-PCR.

the lymphocytes of case AT94-1 shows that 51 of 84 (60%) metaphases analyzed carried a t(14;14) chromosomal translocation. The majority of metaphases (34/51) showed that this translocation was the only evident cytogenetic abnormality; in the remaining 17 metaphases additional rearrangements were also observed: two subclones were defined by an iso(21q) chromosome (4/51) and by a supernumerary C-like chromosome (4/51), presumably derived from a rearranged chromosome 22.

No evident clonal populations with rearrangements at 14q32 were observed in lymphocytes of the other AT cases. To determine whether in AT 94-1 lymphocytes the DNA joined at the t(14;14) breakpoint involved the TCL1 locus, we conducted FISH analysis using probes close to the TCL1 breakpoint. Two cosmid clones, pPLC-1, localized about 120 kb centromeric to the TCL1 gene, and pPLC-4, localized about 250 kb telomeric to the TCL1 gene, were used (Fig 1). The biotinylated cosmid pPLC-1 and digoxigenin-labeled pPLC-4 were simultaneously hybridized to AT lymphocyte metaphases. The signals from pPLC-1 and pPLC-4 were visualized using FITC-conjugated streptavidin-and rhodamine-conjugated antidigoxigenin antibody, respectively.

The results of in situ hybridization experiments are shown in Fig 1. The hybridization of the pPLC-1 probe showed the presence of two peaks of hybridization on the t(14;14) chromosome [one located slightly centromeric to the translocation breakpoint and the other located on the tip of long arm of the t(14;14); Fig 1A], whereas the signal of pPLC-4 showed three peaks of hybridization (one slightly centromeric to the breakpoint, one at the breakpoint site, and the third at the tip of the der 14; Fig 1B).

These results show that this rearrengement involves the TCL1 locus splitting the two probes, pPLC-1 and pPLC4, at the breakpoint site; furthermore, the orientation of the pPLC1 and pPLC4 at the junction is inverted with respect to the germline position (Fig 1E), because pPLC-4 localized telomeric to both TCL1 and pPLC-1. This result indicates that an inversion duplication event had also occurred in this chromosome.

As we have already desribed,[18] this translocation, accompanied by an inverted duplication, will behave, in terms of juxtaposition of TCR elements (14q11) to the TCL1 gene, in a manner similar to that of the inv(14)(q11;q32.1) chromosome inversions. This inverted duplication event seems to be quite frequent on T-cell preleukemic and malignant clones, with a t(14;14) translocation in AT cases,[11,19-21] and could result from chromosomal instabilty underlying the primary defect of AT.

TCL1 expression in AT and normal lymphocytes. Because the 14q32.1 breakpoint of case AT94-1 was, as expected, in the TCL1 locus, we investigated TCL1 expression in cells from this case to determine whether the expression was altered from that seen in normal PBLs. For this purpose, we performed semiquantitative PCR amplification of reverse-transcribed mRNAs derived from lymphocytes of AT 94-1, from three other AT cases without apparent clonal cells harboring a t(14;14) translocation, and from the Daudi cell line that expresses high levels of TCL1 protein.[13]

Reverse transcripts derived from RNAs of these cells were normalized, as described in the Materials and Methods, and were amplified by PCR with TCL1 and β-actin primers.

As shown in Fig 2, the level of TCL1 expression in AT 94-1 cells is much higher that the level of expression of TCL1 mRNA observed in the other three cases of AT. cDNAs derived from three healthy donors expressed levels of TCL1 comparable to those of the latter three AT cases (data not shown). The level of β-actin was also used for further control. β-Actin was amplified from the same amount of cDNA template used for TCL1 amplification independently for 10, 15, or 20 cycles to determine whether the amplification is in the linear phase, because the level of amplification of this gene reaches a plateau effect under the conditions used for TCL-1. Results obtained at 15 cycles (linear phase) for the housekeeping β-actin gene also confirmed the data of amplification observed for TCL1 primers (Fig 2).

We then conducted immunostaining of T-cell-enriched PBLs from AT94-1 and from a normal donor using a rabbit polyclonal antiserum raised against TCL1 recombinant protein.[15] After enrichment on a nylon-wool column, 77% of the cells were of T-cell origin, as demonstrated by staining with an anti-CD7 monoclonal antibody (data not shown).

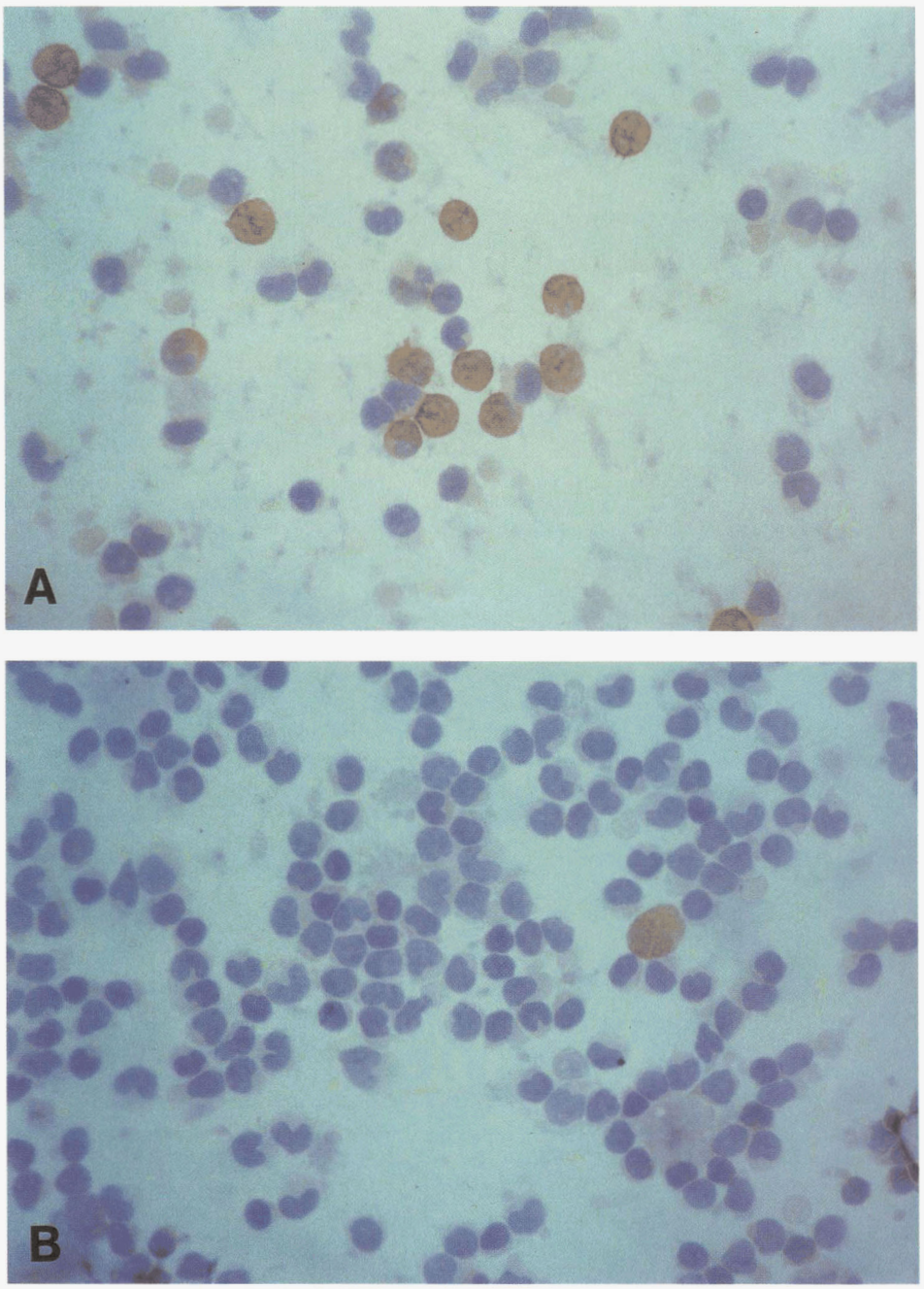

Fig 3. Cytospins of T-cell-enriched PBLs from AT94-1 (A) and from a normal healthy control (B) immunostained with polyclonal anti-TCL1 antiserum.

Cytospins of the two samples were then stained with anti-TCL1 antiserum; 27% of the AT94-1 (Fig 3A) cells showed a strong cytoplasmic signal for the TCL1 protein, whereas less than 1% of the T-cell-enriched population from the normal donor (Fig 3B) showed positive signals. We also analyzed cytospins of unfractioned PBLs from another AT patient; only 0.8% of the cells stained positive (data not shown).

These results show overexpression of the TCL1 protein in cells carrying translocated TCL1. The discrepancy between the data obtained by immunostaining (27% of cells overexpressing TCL1) and the cytogenetic data [60% of metaphases with the t(14;14) translocation] could reflect either the lower sensitivity of the antibody or, alternatively, a more mitogenic responsiveness of the clonal cell population to PHA. It is well known that in this disease T cells may not respond very well to PHA[22]; for this reason it could be possible that t(14;14) cells divide more frequent after a mitogenic stimulus. Therefore, it is possible that both explanations could account for protein expression results in AT94-1 lymphocytes.

DISCUSSION

AT represents a human model of a genetic disease leading to T-cell leukemia; because of the underlying genetic defect in these patients, there is a tendency for VDJ joining mis-

takes and, consequently, chromosomal translocations with the TCR loci to the TCL1 locus[11,19,23,24] or to the MTCP-1/C6.1b locus.[13,25,26] Because these patients are routinely checked for chromosomal abnormalities, various examples of clonal expansions with a premalignant phenotype have been widely reported in the literature.[8] These clones are invariably associated with t(14;14), t(X;14), or inv (14) chromosome translocations or inversions and expand over time to represent up to 100% of the T-cell population. These preleukemic cells consistently progress to a overt leukemic stage that usually resembles the one observed in T-PLL.[17,27] Taking advantage of the recent molecular cloning of the TCL1 gene, we have shown that TCL1 is overexpressed at the RNA and protein level in one case of AT bearing 60% of clonal T cells with a t(14;14)(q11.q32.1) with the breakpoint in the TCL1 locus compared with other AT cases and normal healthy individuals without apparent clonal populations of T cells. These results indicate a pivotal role for TCL1 in the initiation of malignant transformation of these T-cell leukemias.

As a consequence of the translocation event, regulatory elements, such as enhancers of the TCRα gene, move in proximity of the TCL1 gene, probably dysregulating its expression. It seems that TCL1 deregulation confers a proliferative advantage to T cells; whether this is due to an increase in cell survival or to a mitogenic stimulus is currently under investigation and will remain unknown until the function of this gene is ascertained. However, these results suggest that the TCL1 product acts as an inducer of T-cell expansion. This situation can last for many years, and additional changes may be required for a full malignant transformation. This last situation is apparent in vivo in T-PLLs in which the t(14;14)translocation or the inv (14) inversions are involved in 75% of the cases and in which other cytogenetically abnormalities, such as iso 8q or iso 20, are also consistently present.[16] These other rearrangements or other genetic events could play a secondary role in tumor progression.

Sherrington et al[12] have recently studied similar cases of clonal expansion in two cases carrying a t(14;14) and a t(X;14) translocation. By means of densitometric studies on serial samples using probes detecting a rearrangement band at the TCL1 locus and the TCR β-locus, they have shown that cells containing the translocation are oligoclonal with respect to TCRβ in the preleukemic stage and β-clonality was established at the time of the leukemia. In the present report, we have not analyzed this aspect, but the presence of several other small clones at cytogenetic analysis could suggest the possibility of different oligoclonal populations within the t(14;14) clone, indicating, in agreement with these investigators, that rearrangements at the TCL1 locus can occur at a very early stage during ontogeny of the immune system. This hypothesis is also substained by the presence, near the breakpoint of molecularly cloned cases,[2,8,11,19] of elements such as heptamer-nonamer sequences and of N-region, indicating that such events may take place during VDJ rearrengement of T-cell receptor genes.

It has been reported in the literature that at least 10% of these patients will, over time, develop a clonal T-cell population in their peripheral blood that can comprise 100% of the peripheral T-cell metaphases karyotypically analyzed. Availability of antibodies and molecular diagnostics based on TCL1 RT-PCR could help to detect and to monitor these leukemias from their early stages.

These data also raise the possibility of alternative approaches for treating these early stages in AT patients that, as is widely known, are particularly difficult to treat using conventional cytostatic therapy. Innovative therapies aimed at the TCL1 gene or at its product may result in the purging of leukemic cells.

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