Hairy Cell Leukemia Associated With Large Granular Lymphocyte Leukemia: Immunologic and Genomic Study, Effect of Interferon Treatment

By Jean-Pierre Marolleau, Tawfiq Henni, Philippe Gaulard, Jean-Pierre Le Couedic, Marie-Francoise Gourdin, Marine Divine, Andre Katz, Michel Tulliez, Michel Goossens, Felix Reyes, and Jean-Pierre Facet

The authors describe a patient who presented an association of hairy cell leukemia (HCL) and large granular lymphocyte (LGL) leukemia. An eventual relationship between these two rare entities is analyzed. Hairy cells (HCs) were present in the blood, bone marrow, and spleen. An excess of LGLs was found only in the blood and bone marrow. After splenectomy the patient received an a2-interferon (a2-IFN) treatment. The HC surface phenotype was \( \mu \times \delta \times \lambda_5 \), CD20, and CD226. The LGLs consisted in CD3, CD8, HNK1, and WT31 T lymphocytes. These were absent in the spleen. a2-IFN treatment resulted in the disappearance of the HCs in the blood and bone marrow, whereas the LGLs remained unchanged. Before a2-IFN treatment, peripheral blood cells, predominantly LGLs, exerted low cytotoxicity that increased up to a normal level after treatment. Using Southern blotting the authors studied the rearrangements of the T-cell receptor \( \beta \)-chain (CB) and \( \gamma \)-chain (JY) genes and immunoglobulin heavy (JH)- and light (Cx, CA)-chain genes. An unique JH and Cx gene rearrangement was found in the blood and spleen, whereas CB and JY gene rearrangements were present in the blood, not in the spleen. Under a2-IFN treatment, the JH gene rearrangement fattened dramatically, in contrast to that of the CB gene. The study of messenger RNA (mRNA) of the T-cell receptor \( \alpha \) and \( \beta \) chains evidenced the 1.3-kilobase (kb) and 1.6-kb bands in the blood and their absence in the spleen. The patient was human T-cell leukemia virus (HTLV)-II negative by Southern analysis of blood and spleen cells. It is concluded that the LGL expansion was clonal and not reactive to the HCL. Although the authors cannot definitely exclude that both HC and LGL proliferations stem in a common leukemic precursor, their findings support an association of the two entities.

MATERIALS AND METHODS

Case report. The patient was a 55-year-old white man admitted for a splenomegaly and pancytopenia. He had never traveled out of France and did not belong to any known risk group for retroviral infection. The WBC count was 4.8 x 10^9/L, hemoglobin level 10.7 g/dL, platelet count 75 x 10^9/L. On peripheral blood smear the presence of HCs (19%) and of LGLs (47%) was noted. The diagnosis of HCL was confirmed by the bone marrow biopsy. Histologic findings included a cellularity of 75%, a diffuse infiltration by medium-sized mononuclear cells having irregular, bean-shaped nuclei with irregularly clumped chromatin surrounded by a large pale cytoplasm. HCs represented 70% of the bone marrow cells. In addition to the splenomegaly, an abdominal computer tomography (CT) scan disclosed colic and retroperitoneal enlarged lymph nodes. The patient underwent a splenectomy. The spleen weighed 3.750 g, and the cords and red pulp were infiltrated by HCs with sinusoidal dilatation and formation of small blood lakes. After splenectomy the WBC count was 10.8 x 10^9/L, which comprised 3% neutrophils, 10% HCs, 37% lymphocytes, and 50% LGLs. Hemoglobin level was 12.9 g/dL and platelet count 215 x 10^9/L. Thereafter the patient received an interferon treatment (a2-IFN, Boehringer Inst, A-120 Vienna) at a dose of 5 x 10^6 U/dL. After a 3-month treatment the lymph nodes on the CT scan had disappeared, the WBC count was 6.9 x 10^9/L (33% neutrophils, 28% lymphocytes, 30% LGLs, 6% monocytes, and 1% HCs), hemoglobin level 15 g/dL, and platelet count 321 x 10^9/L. The bone marrow biopsy showed a cellularity of 50% and a reduction to less than 5% in the infiltration by HCs. a2-IFN was then continued twice weekly. After 1-year treatment the WBC count was 7 x 10^9/L, with 45% LGLs and no HCs. Serum samples obtained at intervals were screened for human immunodeficiency virus (HIV)-1 and human T-cell leukemia virus (HTLV)-1 antibodies. Specimens were negative in both the enzyme-linked immunosorbent assay (ELISA) and western blot assays.

Cell specimen preparation. Peripheral blood mononuclear cells (PBMN) were obtained by blood sedimentation on a Ficoll-Hypaque (FH) gradient (Pharmacia, Upsala, Sweden; density 1.077 kg/L), stored frozen in liquid nitrogen, and thawed when necessary. Part of

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the spleen was teased through a sieve to make a single-cell suspension that was enriched in HCs by sedimentation on a FH gradient and stored frozen. Another part of the spleen and the bone marrow biopsies were snap frozen in liquid nitrogen after one-hour incubation in a gum-sucrose solution for immunohistologic and genomic studies.

**MoAbs.** The MoAbs used in this study are listed in Table 1. In addition, the μ, δ, γ heavy chains and κ, λ light chains of immunoglobulins were also detected by specific MoAbs (Coulter Immunology, Hialeah, FL).

**Cell phenotyping.** Cell surface phenotyping was performed on a spleen-cell suspension and on PBMCN before and after α2-IFN treatment. The cells were studied for their ability to react with the various MoAbs using the indirect technique with fluorescein isothiocyanate (FITC)-labeled goat antiserum F(ab')2 fragments (Cappel, West Chester, PA). For double staining the procedure of first-antigen capping was used20 with FITC-labeled and tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat antiserum F(ab')2 fragments as the two color reagents.

Cell phenotyping was also performed on cryostat sections of spleen and on bone marrow biopsy samples before and after α2-IFN treatment. The indirect immunoalkalin phosphatase (APAAP) method21 was used. Rabbit antiserum immunoglobulins and alkaline phosphatase antiphosphatase complexes were obtained from DAKO (Dakopatts, Denmark). For double staining, the indirect two-color immunofluorescence assay22 was carried out using the Leu-4 and Leu-2a MoAbs of IgG1 isotype and the Leu-7 MoAb of IgM isotype; accordingly, FITC-labeled goat antiserum μ chain antibodies (Nordic Immunologic, The Netherlands) were used.

**Cell fractionation.** PBMCN obtained after 1 year of α2-IFN treatment were fractionated into HNK1-positive and negative fractions by cell sorting using a fluorescence-activated cell sorter (Odum ATC 3000, Wissembourg, France). Cell fractions (≥95% purity) were used for genomic studies.

**Lectin-dependent cellular cytotoxicity.** The lectin-dependent cellular cytotoxicity (LDCC) procedure23 was performed to determine the cytotoxic potential in PBMCN before and after α2-IFN treatment. The cytotoxicity assay was carried out in microtiter plates with round-bottomed wells (Nunc, Denmark). The effector cells were added to 10^7 Cr-labeled target cells (K562 cell line) with an effector/target ratio of 50:1, 25:1, 12:1 in Iscove's medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 2 μg phytohemagglutinin (PHA)-P (Difco, Detroit, MI) in a volume of 200 μL. The plates were incubated for four hours at 37°C in a 6.5% CO_2 and 100% humidity, then centrifuged (200 g for 5 min). Seventy-five microliters of supernatant were removed for the measurement of 51Cr release in a gamma counter. All tests were carried out in triplicate. The percentage of 51Cr release was calculated according to the formula:

\[ \text{cpm in sample} - \text{cpm in medium} \times 100 \]

**Molecular genetic study.** DNA analysis was performed using standard methods detailed elsewhere.12,24 DNA was extracted from spleen cells and from PBMCN samples obtained before and after α2-IFN treatment. Ten micrograms of DNA from each sample were digested with the appropriate restriction endonucleases, subjected to electrophoresis in 0.6% to 1% agarose gel, transferred onto a nitrocellulose filter by the method of Southern, and hybridized to 32P-labeled DNA probes.

Rearrangements of the T-cell receptor β chain genes were analyzed using a probe25 specific for the constant regions (Cβ1 and Cβ2) after DNA digestion with EcoRI, BamHI, and Hind-III endonucleases; rearrangements of the T-cell γ chain genes were analyzed using a Jγ1 probe specific for the joining region26 after EcoRI digestion. Analysis of immunoglobulin gene rearrangements was performed by using a JH probe specific for the heavy-chain joining region27 after BamHI-HindIII double digestion and a Cγ and a CH probe28 specific for the light-chain constant regions after BamHI and EcoRI digestion, respectively (all above probes gifted by Th. Rabbits). The detection of internal proviral sequences was performed by using the pMoIA probe29 specific for the HTLV-II retrovirus (gift from F. Wong Staal, National Institutes of Health) after DNA digestion with BamHI; the HTLV-II-transformed Mo cell line was used as a positive control (gift from D.W. Golde). RNA was extracted from PBMCN before α2-IFN and from spleen cells using the lithium chloride procedure.30 Ten micrograms total RNA were electrophoresed in 1.2% agarose gel and 37% formaldehyde, transferred onto a nitrocellulose filter, and hybridized to the Cβ probe described above and to a DNA probe specific for the variable and constant region genes of the α chain of the T cell receptor (gift from O. Acuto).

**RESULTS**

Under α2-IFN treatment, the HCs with a B cell phenotype disappeared in PBMCN in contrast to the excess of LGLs with a T-cell phenotype. After splenectomy and before the onset of the α2-IFN treatment, the peripheral blood smear showed the presence of 10% HCs and 50% LGLs, as detailed in the case report. Cell-surface phenotyping with MoAbs confirmed the coexistence of two homogenous cell populations (Table 2). HCs expressed the B lineage CD20 marker, the CD25 marker (i.e., IL-2 receptor), and a monotypic μδ immunoglobulin on their membrane. A T-cell population was also identified as having a CD3CD8 membrane phenotype; this population comprised a large proportion of CD3CD8HNK1 positive cells, as assessed by double staining. The 3-month α2-IFN treatment resulted in a decrease in CD25 and CD20 positive cells from 21% down to 1% and from 29% down to 5%, respectively. In good correlation, a decrease from 10% down to 1% of HCs was detected on the peripheral blood smear. In contrast, the number of CD3CD8HNK1 positive cells remained unchanged. After 1 year of α2-IFN treatment, the data were similar (Table 2). In addition, the number of WT31 positive cells could be determined at that time. WT31 positive cells (i.e., cells

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*Coulter Immunology, Hialeah, FL.
†Becton-Dickinson, Mountain View, CA.
§Sanbio bv, Uden, The Netherlands.
‡Gift from Th. Waldmann at National Institutes of Health, Bethesda, MD.
expressing the α/β heterodimer of the T cell receptor) paralleled in percentage the CD3 positive T lymphocytes, hence including the CD8 subset.

The spleen consisted of HCs with a B-cell phenotype, whereas the bone marrow consisted of a mixture of HCs and T cells on immunohistology. The spleen and initial bone marrow specimens showed a diffuse infiltration by the CD20, CD25-positive HCs, which also expressed a monotypic μκ surface immunoglobulin. In the spleen, rare CD3-positive cells were scattered among the HCs with no CD8 or HNK1 labeling by the MoAbs. In contrast, the bone marrow demonstrated numerous CD3-positive cells that formed small nodules that could not be identified on the regular histologic examination. An excess of CD3-HNK1 and CD8-HNK1 double-stained cells was evidenced in this patient and not in six other HLC patients used as controls (Table 3).

The 3-month α2-IFN treatment resulted in a marked reduction of the bone marrow infiltration by the HCs with the characteristic phenotype. Thus a decrease was noted of μκ-positive cells that paralleled a decrease from more than 300 CD25− and CD20− down to 26 and 23 positive cells per field respectively. On the other hand, the presence of CD3CD8HNK1 positive cells remained unchanged (Table 3).

The molecular genetic analysis demonstrated the coexistence of B-cell and T-cell clonal populations and the absence of HTLV-II provirus. When hybridized to the JH probe, the DNA from spleen and PBMN before α2-IFN treatment showed two additional bands located below germ-line one (Fig 1). These additional bands resulted from a clonal rearrangement of immunoglobulin heavy-chain genes involving two alleles. In spleen-cell DNA the germ-line band was faint, showing that cells other than clonal were few in proportion, as expected from immunohistologic findings. The observation of a germ-line pattern with the CA probe and of the germ-line configuration and eliminate DNA polymorphism in addition to the 24-kb germ-line one, in contrast to the germ-line pattern observed after HindIII digestion (not illustrated). These findings demonstrate a clonal rearrangement of the T-cell receptor Cβ1 gene with Cβ2 gene in germ-line configuration and eliminate DNA polymorphism or partial digestion. The two bands observed presumably correspond to a single clone rearranging both productive and nonproductive allele rather than a biclonal T-cell process. After a 3-month α2-IFN treatment, analysis of DNA from PBMN showed that the intensity of Cβ1 rearrangements remained unchanged (Fig 2). To determine the membrane

When hybridized to the Cβ probe the DNA from PBMN before α2-IFN treatment displayed rearranged fragments. After EcoRI digestion two bands were found in addition to the 11- and 4-kb germ-line configurations (Fig 2); these additional fragments were absent from spleen cell DNA, which displayed only the germ-line bands. BamHI digestion of DNA from PBMN confirmed the presence of two bands in addition to the 24-kb germ-line one, in contrast to the germ-line pattern observed after HindIII digestion (not illustrated). These findings demonstrate a clonal rearrangement of the T-cell receptor Cβ1 gene with Cβ2 gene in germ-line configuration and eliminate DNA polymorphism or partial digestion. The two bands observed presumably correspond to a single clone rearranging both productive and nonproductive allele rather than a biclonal T-cell process. After a 3-month α2-IFN treatment, analysis of DNA from PBMN showed that the intensity of Cβ1 rearrangements remained unchanged (Fig 2). To determine the membrane

![Fig 1. Southern analysis of JH gene rearrangement with BamHI-HindIII digestion. (——) indicates the position of the germ-line band as defined by the study of DNA from a normal control. This band corresponds to a DNA fragment of 8 kb. (——) indicates the position of rearranged bands. (a, b) Blood cells before and after α2-IFN treatment. Note that after 3-month α2-IFN treatment, the rearrangement of the JH gene had fainted dramatically in the blood sample.](image-url)
phenotype of cells containing the Cβ1 rearrangements, HNK1 positive and negative fractions were isolated by cell sorting of PBMN. The rearranged Cβ1 fragments were present in both the HNK1 positive and negative fractions. In addition, in the HNK1 positive fraction the Cβ1 germ-line configuration was deleted. Therefore most clonal T cells were HNK1 positive and expressed the CD3CD8HNK1 phenotype (Table 2), with a subset being HNK1 negative. EcoR1-digested DNA was hybridized to the Jγ1 probe (not illustrated). Spleen cells displayed a germ-line pattern that is two bands of 3.2 and 1.5 kb. In contrast, PBMN both before and after a 3-month α2-IFN treatment displayed two additional bands of 4.2 and 5.4 kb. Again this excluded the possibility of a pattern being caused by DNA polymorphism and demonstrated the presence in PBMN of a clonal rearrangement of the T-cell γ chain genes.

A messenger RNA (mRNA) study of the T-cell α and β transcripts performed before α2-IFN treatment. The expected 1.6-kb and 1.3-kb bands respectively were evidenced in the peripheral blood cells, not in the spleen cells (data not shown). Finally, a search for an integrated internal HTLV-II fragment was negative. The probe did not hybridize with BamH1-digested DNA from spleen and peripheral blood cells (Fig 3).

The excess of LGLs did not correlate with an increase in the cytotoxic potential of PBMN. Using the LDCC assay, the cytotoxic capability of PBMN was studied before and after a 3-month α2-IFN treatment (Fig 4). Before treatment a defective cytotoxicity was observed compared to normal PBMN. After treatment the cytotoxicity was restored to normal but remained far below the cytotoxic capability of the cells from a patient with a natural killer (NK) cell leukemia, included as a positive control.

**DISCUSSION**

Altogether the results demonstrate the presence in the patient of two separate clones. One consists of HCs with B-cell characteristics, the other of LGLs with T-cell characteristics. The identification of these two entities is based on morphological criteria, membrane markers, and genomic patterns. Their distribution in the tissues studied and their course under α2-IFN treatment are also discriminative. Thus HCs with the CD20 and CD25 markers, μδε membrane immunoglobulin, and immunoglobulin gene rearrangements were found in the spleen, bone marrow, and peripheral blood. The LGLs with CD3-, CD8-, HNK1-, WT31- positive phenotype and T-cell receptor gene rearrangements were present in the bone marrow and peripheral blood but not in the spleen. In addition, the α2-IFN treatment resulted in a drastic reduction in HCs, which contrasted with the persistence of an excess in LGLs. The LGL percentage remained consistent with that of CD8HNK1-positive cells along 1 year follow-up.

Consequently the finding of two separate clones in the
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patient eliminates the possibility of a single proliferative disease with a hybrid phenotype and/or genotype. Also, the present setting is distinct from eventual fluctuations in the T- and B-membrane characteristics of HCs as previously reported. In the patient, if the cells with a T-cell phenotype were HCs in origin they would rearrange the JH not the Cβ genes. Nevertheless, an infdel Cβ gene rearrangement can be observed in up to 10% clonal B-cell malignancies. In such cases the inconstant gene rearrangement has been found to be nonproductive. In the patient the HCs and LGLs belong to two clear-cut clones, the former with an immunoglobulin gene rearrangement and μκ membrane immunoglobulin, the latter with a T-cell receptor gene rearrangement and expression of the CD3–T-cell receptor complex. To note, the authors’ finding of a germ-line configuration of the T-cell γ-chain genes in HCs is in keeping with data showing that T-cell γ-chain genes do not rearrange in cells from chronic B-cell proliferations in contrast to immature B cells from acute leukemia. The presence of a T-cell clone in the context of HCs should be distinguished from the other settings reported in HCL. First, a T-cell variant of HCL can be eliminated, although the CD3, CD8 membrane phenotype is similar to that in a case reported recently. In this variant the HCs infiltrate the spleen and have a characteristic hairy morphology, whereas here the T cells were absent in the spleen and displayed a large granular morphology. In addition, the T-HCL variant has been associated with the HTLV-II retrovirus. In the author’s patient the serologic detection of a HTLV-related virus was negative, and the Southern blot analysis showed that HTLV-II was absent in the DNA extracted from the PBMN and spleen cells. Second, an expansion of the CD8 lymphocyte subset has been described in HCL patients with an active disease and/or after splenectomy. Also, an increase in HNK1-positive cells has been quoted with low or NK activity. In these cases a double staining has not been performed to determine whether the HNK1-positive cells expressed simultaneously the CD8 marker. The authors performed a study of the Cβ gene in a similar HCL patient that showed a polyclonal T lymphocyte pattern, hence discriminating from an association of HCs with a T-cell clone as reported here.

The frequency of HCL and LGL leukemia has been estimated to be 2% of all leukemias and 1.5% of chronic lymphocytic leukemias, respectively. A fortuitous association would be quite exceptional. Therefore an eventual relationship between these two rare entities has to be discussed. The large granular morphology of the T cells suggests a cytotoxic potential and their reactivity to the HCs. This is unlikely because (1) no peculiar cytotoxicity was evidenced in the PBMN, (2) the LGLs were absent from the spleen massively infiltrated by HCs, (3) the LGLs persisted after α2-IFN treatment, although a marked reduction of the HC infiltration occurred. Another hypothesis would be that HCs and LGLs share a common leukemic stem cell in the patient, as proposed in a recent case of lymphoma containing both B- and T-cell clones. This hypothesis cannot be formally excluded. Finally, the emergence of a second malignancy in HCL is a more likely explanation for the presence of a T-cell clone. A number of second malignancies have been recognized concomitantly with or post the diagnosis of HCL. Among these, B-cell lymphomas are not infrequent and can be of separate clonal origin. To the author’s knowledge, a T-cell disorder has not been reported in association with HCL, although a Sezary syndrome has been described by Zucker-Franklin et al as evolving from the HCs. Yet an immunogenomic study could not be performed at that time.

In conclusion, the authors demonstrate the possibility of an association of HCL with a clonal T-cell disorder. It has to be emphasized that the α2-IFN treatment was ineffective on the LGL leukemia and that the initial neutropenia was likely due to the HCL, since the neutrophil count normalized when the HCL went into remission.

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