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Malignant Lymphomas

Late Epstein-Barr virus infection of a hepatosplenic γδ T-cell lymphoma arising in a kidney transplant recipient

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ABSTRACT

Background and Objectives. **γδ T-cell lymphomas are only exceptionally observed in transplanted patients. Aim of this study was the detailed characterization of one such case.**

Design and Methods. **The patient developed spontaneous splenic rupture six years after kidney transplantation. The splenic red pulp was infiltrated by medium-sized and large lymphoid cells with two or more nucleoli. At autopsy, similar lymphoid cells infiltrated the hepatic sinusoids. Histologic, immunologic and molecular studies were carried out.**

Results. By immunohistochemistry, the atypical lymphoid cells were found to express CD3, CD45 and CD43, indicating their T-lineage origin. Approximately 99% of spleen mononuclear cells (MNC) were CD3⁺, γδ TcR+, CD4-, CD8-, αβ TcR-. A clonal γδ TcR rearrangement (Vγ1-Jγ1.3/2.3-Cγ2; Vδ1-Dδ2-Jδ1) was detected. The final diagnosis was *peripheral T-cell lymphoma, hepato-splenic γδ-type*. EBV infection of spleen MNC was documented by molecular studies. However, in situ hybridization for EBER-1 (EBV-RNA) showed that only a minority of malignant lymphoid cells (5-7%) were EBV-infected.

Interpretation and conclusions. It is concluded that EBV infection was as a late event involving an already transformed γδ T-cell clone.

Key words: γδ T cell lymphoma, kidney transplant, Epstein-Barr virus

Human γδ T-cells account for 1 to 5% of peripheral blood lymphocytes and localize preferentially to certain peripheral lymphoid tissues, such as the red pulp of the spleen and the gastrointestinal tract.[1-4] In mice, γδ T-cells display special tropism for epidermal and epithelial linings.[5]

The function of γδ T-cells is still elusive, but it may be hypothesized that they serve as a first line defense mechanism at the epithelial surfaces against pathogens (bacteria, viruses, parasites) prior to the recruitment of αβ T-cells.[6]

Lymphoproliferative disorders of γδ T-cells include T-cell lymphoblastic lymphomas/lymphoblastic leukemias,[7] as well as peripheral T-cell lymphomas.[8-12] Among the latter disorders, hepatosplenic γδ T-cell lymphoma is a well defined entity that has been included in the *Revised European American Lymphoma* classification.[13] Hepatosplenic γδ T-cell lymphoma is characterized by the predominant involvement of the spleen, an aggressive course and a poor prognosis.[11] Non-hepatosplenic γδ T-cell lymphoma displays prominent involvement of the skin or the mucosal tissues and, in contrast to hepatosplenic γδ T cell lymphoma, is often infected by Epstein-Barr virus (EBV).[11,12]

Lymphoid neoplasms occurring in organ transplant recipients[14-16] are commonly referred to as post-transplantation lymphoproliferative disorders (PTLD). More than 85% PTLD are EBV-positive B-cell proliferations, whereas the remaining cases are reported to be αβ T-cell receptor (TcR) positive T-cell lymphomas.[17] Fewer than 20% of the latter tumors have been found to contain EBV genome or proteins.[17]

Expansion of CD3⁺, αβ TcR- cells possibly belonging to the γδ T-cell lineage has been reported in transplanted patients,[18] but only few cases of transplant-associated γδ T-cell lymphoma have been published so far.[19,20]

Here we report on a new case of hepatosplenic γδ T-cell lymphoma providing a detailed characterization of the tumor cells. Since only a minority of malignant lymphoid cells were EBV-infected, as assessed by different molecular techniques, it was concluded that EBV infection was a late event not directly responsible for γδ T-cell lymphomagenesis.

Case Report

A 23-year old Italian man suffering from dialysis-treated end-stage renal failure due to Alport's syndrome (hereditary nephritis) received a kidney transplant from his father in March 1990. The patient had no panel reactive antibodies before transplantation and was transplanted with a negative T- and B-cell cross-match. The HLA typings were the following: *Patient*: A69, A2; B17, B60; DR13, DR7. *Donor*: A69, A3; B51, B60; DR1, DR7.

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Six days after transplantation the patient underwent a biopsy-proven acute cellular rejection that was treated with three corticosteroid boluses. Because of no response to steroids, the patient received a subsequent eight day treatment with antilymphocyte globulin (ALG) that produced complete regression of the rejection episode. The patient was placed on triple immunosuppressive therapy (cyclosporin A, azathioprine, corticosteroids) and was eventually discharged one month after transplantation with good renal function (serum creatinine 1.2 mg/dL).

The patient did very well with stable renal function until March 1995, when severe leukopenia (WBC 1300/mm^3; neutropenia with relative lymphocytosis and monocytosis), sideropenic anemia and mild thrombocytopenia were detected in association with fever and generalized arthralgias. An upper urinary tract infection was also diagnosed. Despite azathioprine withdrawal and antibiotic treatment no improvement of leukopenia occurred. No abnormalities were found following bone marrow aspiration. CT scans of lungs and abdominal organs did not show alterations. Repeated immunophenotype analyses of peripheral blood mononuclear cells (MNC) gave normal results.

In December 1995, treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) on alternate days was started because of persistence of neutropenia, anemia and fever. This treatment was continued for three months and allowed, in association with recombinant erythropoietin administration, temporary management of the patient (mean WBC count: 2,700/mm^3). During this time interval, a mild decrease of kidney graft function (mean serum creatinine: 2.3 mg/dL), high lactic dehydrogenase serum values (mean values: 1,500 U/L) and thrombocytopenia (mean: 35,000/mm^3) were observed. A bone marrow biopsy performed at this time did not reveal any abnormality. No serologic evidence of acute viral infections (cytomegalovirus, EBV, hepatitis B virus) was detected.

The patient's conditions remained stable until February 1996, when he was admitted to the Hospital with the suspicion of hemolytic-uremic-like syndrome because of: persistently high lactic dehydrogenase serum levels, thrombocytopenia (25,000/mm^3) and anemia, presence of fragmented red blood cells (schistocytes), high reticulocyte count (up to 22%) and a further mild decrease of the graft function. CSA was withdrawn with a parallel increase of steroid dosage (60 mg/kg prednisone) and the patient underwent several cycles of plasmapheresis in association with platelet concentrate administration. Following the above treatment, there was only a slight improvement of the patient's clinical conditions and no significant modification of his laboratory parameters. In April 1996, abdominal ultrasound and CT scans showed a remarkable increase in spleen size. The spleen rapidly enlarged and the patient underwent a surgical splenectomy because of rupture of the spleen on April the 20th. The histologic diagnosis at that time was splenic T-cell lymphoma. After splenectomy, a further decrease of kidney graft function occurred that required hemodialysis treatment. Multiple spontaneous episodes of epistaxis occurred as a consequence of the profound thrombocytopenia and a positive direct anti-globulin test was detected. The patient eventually died on May the 13th, 1996 of multi-organ failure syndrome.

Results

Morphologic and immunohistochemical investigations

Formalin-fixed and paraffin-embedded tissue sections were stained with hematoxylin-eosin or Giemsa for histologic studies. Paraffin sections were incubated with CD3, CD43, CD45RO and MIB-1 (Ki-67) monoclonal antibodies (mAbs) (all from Dako, Glostrup, Denmark) and stained using the alkaline-phosphatase/anti-alkaline phosphatase (APAAP) method.[21]

Histologic examination revealed that the red pulp of the spleen was diffusely infiltrated by medium-sized and large lymphoid cells showing a high nuclear to cytoplasmic ratio, pleomorphic nuclei and two or more nucleoli (Figure 1A, panel 1). The sinuses and pulp cords were diffusely permeated by the lymphoproliferative process. At autopsy, similar lymphoid cells infiltrated the hepatic sinusoids (Figure 1A, panel 2) and were also found in the lung, whereas no lymphoid infiltration was detected in lymph nodes, in the skin, in the intestine or in the brain (not shown).

Malignant lymphoid cells displayed strong reactivity with CD3, CD45RO and CD43 mAbs, thus confirming their T-lineage derivation (Figure 1B; boxes 1, 2 and 3, respectively). Approximately half of the lymphoid cells were actively proliferating, as assessed by staining for Ki67 (Figure 1B; box 4).

The above data were consistent with the diagnosis of peripheral T-cell lymphoma *hepato-splenic γδ type*.[13]

Immunologic characterization of malignant lymphoid cells

Spleen and peripheral blood MNC were isolated by Ficoll-Hypaque density gradients, stained with a battery of murine mAbs and analyzed by flow cytometry. The following mAbs were used: CD1, CD2, CD3, CD4, CD7, CD8, CD11b, CD16, CD19, CD21, CD25, CD38, CD56, CD57, CD122, anti-HLA-DR, all from Becton-Dickinson (San Josè, CA, USA).

Most splenic MNC were CD3$^+$, CD2$^+$, CD7$^+$ T-cells that did not express either CD4 or CD8. The CD19 and CD21 B-cell-associated markers were present on less than 1% and 53% MNC, respectively. The discrepancy between the number of CD19$^+$ and CD21$^+$ cells was clearly attributable to T-cell expression of the CD21 marker. The CD11b, CD16, and CD56 large granular lymphocyte (LGL)-associated markers were expressed by 74-98% MNC; 40% MNC were CD57$^+$. Most MNC were CD122$^+$, whereas only a minor proportion expressed CD38 (Figure 2). Lymphocytes with the same immunophenotypic and morphologic features as splenic lymphoid cells were detected on two occasions in the peripheral blood following splenectomy (not shown).

To characterize the TcR expressed by malignant lymphoid cells, spleen MNC were first stained with two different anti-γδ TcR mAbs (A13 and BB3) or

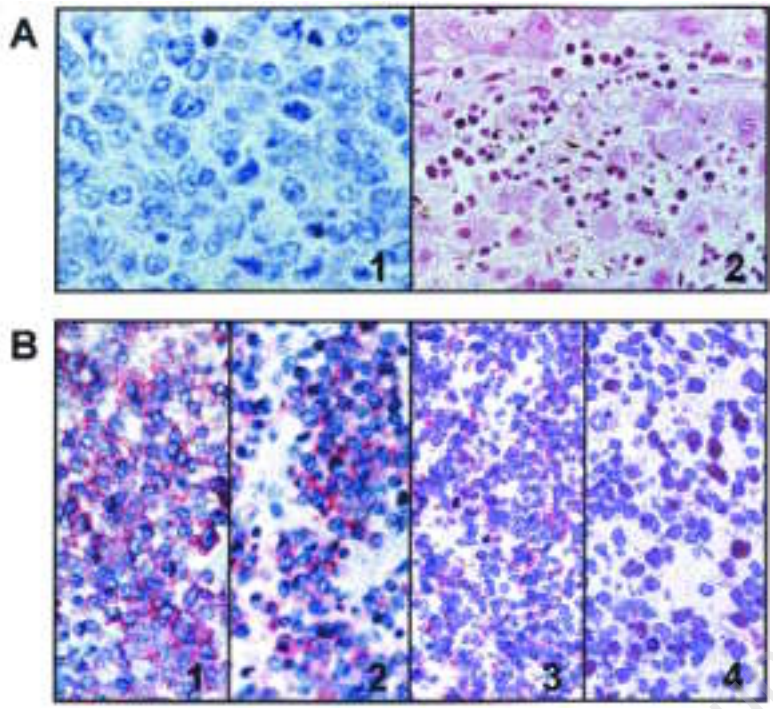

Figure 1. Morphologic (A) and immunohistochemical (B) investigations. A. Panel 1: Giemsa-stained spleen tissue section showing the morphologic features of malignant lymphoma cells (x60). Panel 2: hematoxylin-eosin stained liver section (x10) in which neoplastic cells appear to infiltrate the hepatic sinusoids. B. Immunohistochemical staining of spleen sections with CD3 (1), CD45RO (2), CD43 (3) and Ki67 (4). Staining was carried out by the APAAP technique. Gill's hematoxylin nuclear counterstaining (x40).

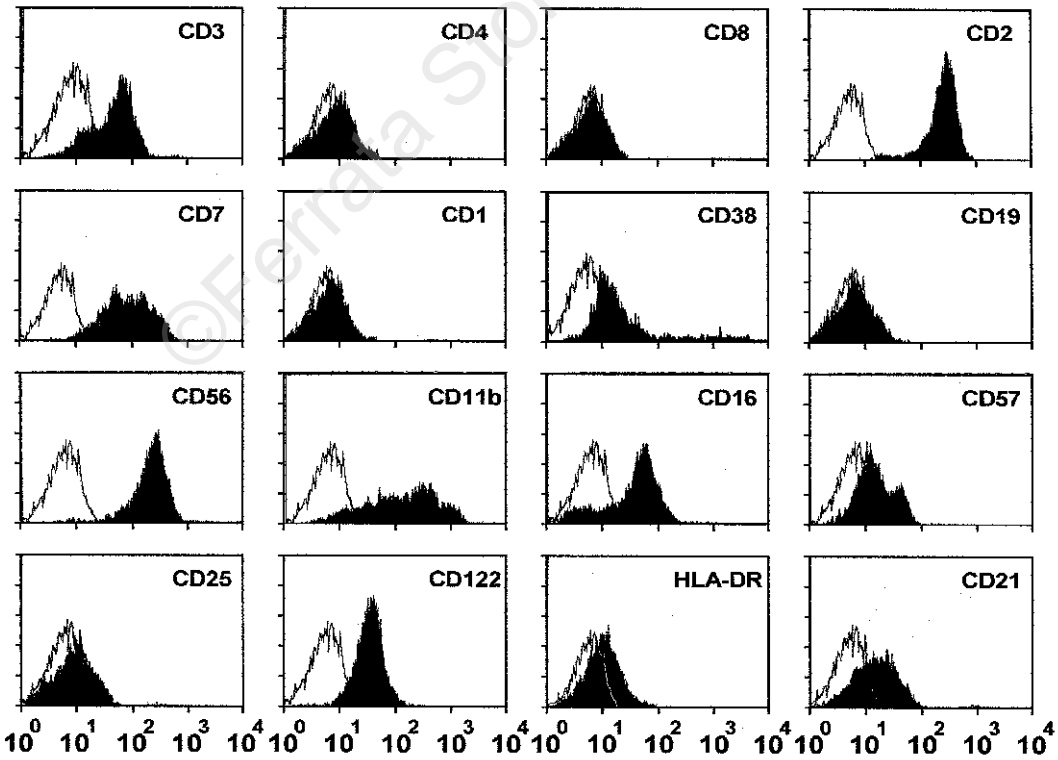

Figure 2. Immunophenotypic analysis of spleen MNC suspension. Purified MNC were stained with a battery of mAbs and analyzed by flow cytometry as indicated in the *Design and Methods* section.

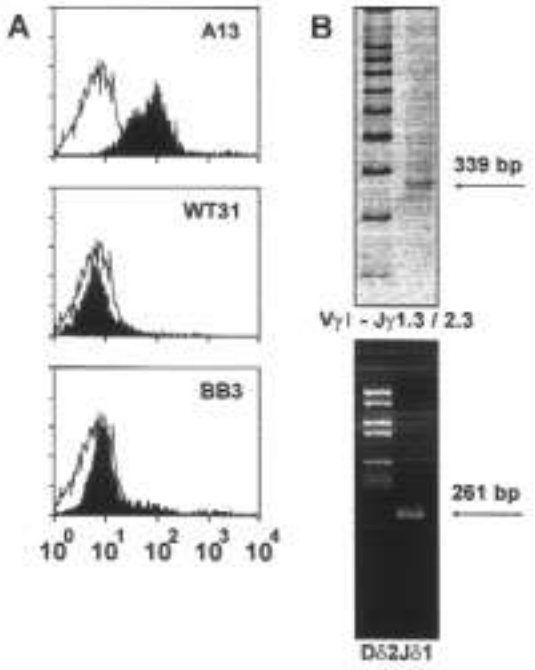

Figure 3. Serologic (panel A) and molecular (panel B) analyses of the TcR expressed by splenic lymphoma cells. In panel A, MNC were stained with anti-αβ or anti-γδ TcR mAbs and analyzed by flow cytometry. Panel B shows the results of TcR gene rearrangement studies by PCR. Left lanes show m.w. markers, right lanes show rearranged γ (upper panel) and δ (lower panel) TcR bands with the indication (arrows) of the expected m.w. To allow a better visualization of the γ TcR rearranged band, the latter band was eluted and silver stained.

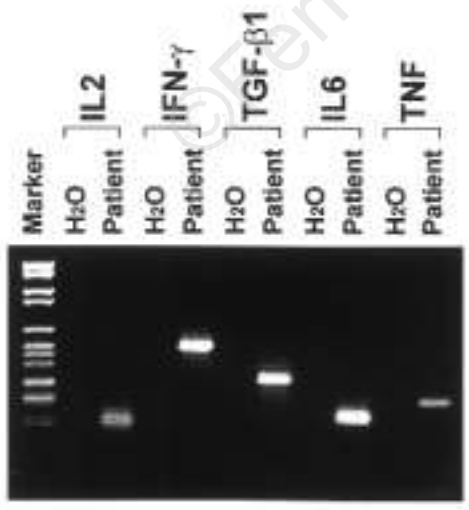

Figure 4. RT-PCR analysis analysis of IL2, IFN-γ, TGF-β1, IL6 and TNF gene expression in spleen MNC. Negative control was water included in the reaction mixture in the place of cDNA.

with an anti-αβ TcR mAb (WT31). The BB3 and A13 mAbs were a kind gift from Dr. Silvano Ferrini, Laboratory of Immunopharmacology, IST, Genoa, Italy. The BB3 mAb defines a Vγ2+ γδ TcR+ cell subset expressing the Cγ1-encoded δ chain,[22,23] whereas the A13 mAb reacts with Vδ1+ γδ TcR+ cells equipped with Cγ2-encoded γ chains.[23,24] The sum of the percentage of BB3+ and A13+ cells accounts for more than 90% of peripheral blood γδ TcR+ cells.[23] The WT31 mAb was from Becton-Dickinson.

As shown in Figure 3A, lymphoid cells were found to express γδ (A13), but not αβ (WT31) TcR. Reactivity to anti- γδ TcR mAbs was restricted to the A13 mAb since cells did not stain for BB3 (Figure 3A). These findings indicate that the malignant lymphoid cells expressed Vδ1 and Cγ2 protein chains.[22-24]

The rearrangements of γδ TcR encoding genes in spleen MNC were next investigated by PCR.[25,26] DNA was extracted from spleen MNC by conventional techniques and subsequently amplified with a set of oligonucleotide primers specific for TcR γ and TcR δ genes according to the sequences published by Breit *et al.*[25,26] A clonal band corresponding to a Vγ1-Jγ1.3/2.3 rearrangement was detected (Figure 3B, upper panel). Likewise, a Dδ2-Jδ1 clonal rearrangement was identified (Figure 3B, lower panel).

The above studies demonstrate that lymphoma cells were comprised of a clonal γδ TcR+ population displaying Vγ1-Jγ1.3/2.3-Cγ2 and Vδ1-Dδ2-Jδ1 rearranged genes.

Cytokine gene expression (IL2, IL4, IL5, IL6, IL10, IFNγ, TGFβ1 and TNF) was next investigated in T-lymphoma cells by RT-PCR as previously reported.[27,28]

As shown in Figure 4, the transcripts of IL2, IL6, IFNγ, TGFβ1 and TNF were detected. In contrast, IL4, IL5 and IL10 transcripts were absent (not shown). These findings indicate that T-lymphoma cells expressed Th1-type (IL2, IFNγ) as well as pro-inflammatory (TNF, IL6) and immunosuppressive (TGFβ1) cytokines.

EBV infection of γδ TcR+ lymphoma cells

Although most EBV-related PTLD are of B-cell origin,[17,29] EBV-positive T-cell or NK-cell lymphomas have been reported.[30] We therefore investigated whether malignant lymphoid cells from our patient were EBV-infected using different approaches.

EBV latent antigens were detected by Western blotting as described elsewhere.[31,32] Splenic MNC were found to express the EBNA 1, 2, 3a, 3b, 3c and LMP1 EBV antigens (Figure 5, panel A). The corresponding bands were faint compared to those detected in control cell lines (Figure 5, panel A), suggesting that only a minority of T-lymphoma cells were EBV-infected.

In contrast with the latter results, attempts at identifying LMP-1+ cells in spleen tissue sections by immunohistochemistry failed, possibly because the expression of the viral protein on the cell surface was below the threshold of detection. However, in view of the results of Western blot experiments and of the high degree of purity of spleen MNC, it is conceivable that the EBV-infected malignant T-cells displayed a type III latency.[32,33]

The *in situ* hybridization procedure for the detection of EBER-1 transcripts was carried out by a fluo-

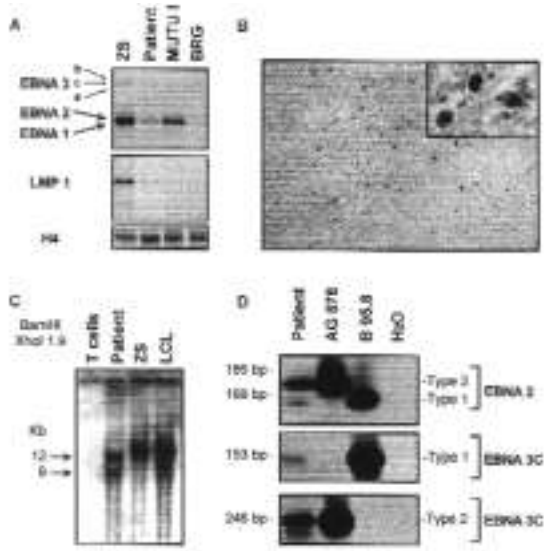

Figure 5. EBV infection of spleen lymphoma cells. Panel A. Western blot analysis. From left to right: ZS EBV-positive B cell lymphoma, patient sample, MUTU I EBV-positive B cell lymphoma, BRG EBV-negative B cell lymphoma. Panel B. *In situ* hybridization on patient spleen tissue section (x10) using EBER-1 specific probe (APAAP). The inset shows EBER-1-positive T lymphoma cells at higher magnification (x60). Panel C. Southern blot analysis of EBV fused termini. From left to right: normal peripheral blood cells (negative control), patient's sample, EBV-positive ZS B lymphoma cells, polyclonal LCL. Panel D. EBV strain typing by PCR. From left to right: patient's sample, AG876 LCL, B95.8 marmoset cell line and H_2O as a negative control.

rescein-conjugated oligonucleotide (Dako) complementary to a nuclear RNA sequence of the EBER-1 gene that is actively transcribed in cells latently infected by EBV. These experiments showed that few spleen MNC (approximately 5-7%) expressed EBV-specific RNA sequences (Figure 5, panel B). In contrast, the percentage of EBER-1 positive cells in a lymph node tissue section from an EBV-positive non-Hodgkin's B-cell lymphoma tested as control was higher than 70% (not shown). Morphologic analysis of serial spleen sections from our patient provided unambiguous evidence that EBER-1^+ cells were indeed the malignant lymphoid cells (Figure 5, panel B, inset). Scattered EBER-1^+ neoplastic lymphocytes were also detected in the liver (data not shown).

High molecular weight DNA was subsequently extracted from spleen MNC by a standard procedure and subjected to Southern blot analysis for the EBV fused termini using the XhoI 1.9 cDNA probe, which detects a DNA sequence adjacent to right terminal repeat sequences of the viral genome.[34] Two discrete bands of viral episome formation were detected in splenic MNC (Figure 5, panel C), suggesting the presence of two clonal cell populations.

Finally, EBV DNA was detected and typed by polymerase chain reaction (PCR) with primers specific for the EBNA-2 and EBNA-3C genes, as reported elsewhere.[35] Spleen MNC were found to be infected by EBV strains type 1 and 2. In particular, PCR with EBNA 2 specific primers showed the presence of the EBV type 2-associated 185 bp band and the EBV type 1-associated 169 bp band (Figure 5, panel D). Likewise, experiments with EBNA 3c specific primers demonstrated the EBV type 1-associated 153 bp band and the EBV type 2-associated 246 bp band (Figure 5, panel D).[36]

Taken together, the Southern blot and PCR experiments point to the heterogeneity of EBV in malignant lymphoid cells, thus implying multiple infections by different viral strains.

Additional studies carried out by PCR failed to demonstrate the presence of HIV-1, HLTV-I, HTLV-II or HHV6 in spleen MNC.

Discussion

Most T-cell lymphomas occurring in transplanted patients are $\alpha\beta$ TcR^+. Expansion of $CD3^+$, $\alpha\beta$ TcR- lymphocytes has been detected in renal allograft recipients,[18] but only few cases of monoclonal $\gamma\delta$ TcR^+ T-cell lymphoma affecting transplanted patients have been published.[19,20]

In a recent review on post-transplant T-cell lymphoma, only 4 out of 25 cases were reported to be associated with EBV,[30] suggesting that the virus plays a minor role in T-cell, as opposed to B-cell, lymphomagenesis in this setting.

This conclusion appears to hold true also for the $\gamma\delta$ TcR^+ lymphoma herein reported. EBV genome and proteins were detected in malignant lymphoid cells, but *in situ* hybridization experiments with an EBER-1 specific probe demonstrated that only a minority of cells were EBV-infected. These findings suggest that EBV infection was a late event taking place in already transformed cells.

Two discrete bands of viral episome formation were found by Southern blot hybridization with a cDNA probe specific for the EBV fused termini, indicating the presence of two subclones of malignant lymphoid cells infected by EBV. Furthermore, lymphoma cells carried the genomes of both type 1 and type 2 EBV strains. These strains differ by the sequence of EBNA 2 ad EBNA 3C genes and can co-infect the same patient, particularly in the setting of immune suppression.[35]

The simultaneous presence of the two viral strains in the malignant $\gamma\delta$ T-cells may suggest that the bands detected by the EBV fused termini-specific cDNA correspond to two distinct cell subpopulations, clonally infected by type I and type II EBV strains, respectively. An alternative, but less likely, explanation is that the two viral strains infected a single cell subclone giving rise to two different bands of viral episome formation. A final (and not mutually exclusive to the others) hypothesis is that when EBV infection of T-lymphoma cells took place, various subclones were infected but a subsequent selection process led to the emergence of two major subclones.

Whatever the explanation, it should be stressed that a large fraction of the malignant cells expressed the CD21 marker. Since CD21 is the receptor for EBV,[37] the presence of CD21 at the cell surface helps us to

understand how EBV entered into and subsequently infected a subpopulation of malignant lymphoid cells.

The pattern of cytokine gene expression in tumor cells was of Th1-type, with IL2 and IFNγ, but not IL4 or IL5, transcripts detected. Furthermore, tumor cells expressed TNF and IL6, which act as pro-inflammatory cytokines, and TGFβ1, that may have contributed to local immune suppression favoring the growth of lymphoma cells. Although Th1- and Th2-type γδ TcR+ cells have been identified,[38] the Th1-type profile is more frequently detected in γδ-T-cells.[39]

A final point that deserves discussion is how this lymphoproliferative disorder developed (see also recent pertinent papers in this journal[43,44]). Transplanted patients receiving immunosuppressive regimens may have an impaired clearance of pathogens that, in turn, would result in excessive antigenic stimulation of T-cells, including the γδ TcR+ subset. Since γδ TcR+ cells reactive with various pathogens have been identified,[40-42] excessive antigenic stimulation might represent a promoter event for the outgrowth and expansion of a malignant clone like that reported in this study.

In conclusion, we have reported on a patient who developed a hepato-splenic γδ TcR+ T-cell lymphoma, 6 years after a kidney transplant, with a monoclonally rearranged γδ TcR. EBV infection was restricted to a minor proportion of malignant lymphocytes, leading to the conclusion that EBV infection was a late event taking place in an already transformed γδ TcR+ cell clone.

Contributions and Acknowledgments

SR took care of cell separation and immunophenotypic studies; GC was responsible for Western blot studies and contributed to the immunophenotypic cell characterization; MT performed and reviewed histologic studies; IA was responsible for cytokine gene expression studies; AP and MU performed the Southern blot experiments; AV and DDM carried out the experiments for TcR gene rearrangements; PD was responsible for immunohistochemical and in situ *hybridization studies; ADR carried out PCR experiments for EBV strain characterization; IF and AN followed the patient after kidney transplant and wrote the case report; UV, MF and VP planned and designed the study and critically reviewed the drafts of the manuscript; MF and VP prepared the final manuscript. All authors approved the final version to be submitted.*

The order of authorship was decided jointly taking into account the relevance of the contribution offered by each investigator. In particular Dr. Roncella, who is first author co-ordinated all the experimental work, whereas Dr. Pistoia, who is the last author, was responsible for assembling the manuscript in its final version.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

- EBV infection may be a late event in some malignant lymphoms that develop in immunodeficient individuals.

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