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Abstract. After *in vitro* EBV infection of peripheral blood lymphocytes (PBL), we previously obtained IL-2-independent T-cell lines expressing EBNA1 and LMP1 viral latent genes. One tumorigenic clone, NC5, was further characterized for chromosomal abnormalities, rearrangement and expression of oncogenes, and constitutive or induced activation of cellular transduction pathways. NC5 as well as TC cells derived from an NC5-induced tumor exhibited the same few chromosomal abnormalities absent in normal PBL and B-cell lines (LCLs) from the same donor. No rearrangement or altered expression of C-MYC, BCL-2 and NF-KB2 oncogenes could be detected. In contrast, we found high levels of BCL-X and thioredoxin (TRX), as markers of EBV infection or T-cell activation/transformation status. No constitutive activation of NF-κB or STAT transcriptional complexes was observed in these cells. For NF-κB, this was in apparent contradiction with its reported inducibility mediated by LMP1, taking into account that NF-κB was still inducible by TNFα or PMA and ionomycin. Our results highlight independence of EBV protein-mediated transformation towards classical cellular pathways in T-lymphocytes.

Introduction

Besides its well established causative role in infectious mononucleosis, the Epstein-Barr virus (EBV) is traditionally associated with B-cell malignancies like Burkitt lymphomas (BL) but also with Hodgkin's disease (HD) and nasopharyngeal carcinomas (NPC) (1). The presence of EBV and expression are also detected in a growing number of T-cell neoplasms (2-5). More recently, cases of EBV association with non-Hodgkin's (6) and salivary gland (7) T-cell lymphomas were reported. In addition to these numerous cases of EBV expression in malignant T-cells *in vivo*, T-cell lines expressing EBV genes were recently established from peripheral blood of chronically infected donors (8).

However, one of the clear differences in the interpretation of the role of EBV in B- versus T-cell malignancies is based on the lack of an *in vitro* model of infection for the latter ones. Indeed, B-cell lines (LCLs) are readily established by EBV infection *in vitro* (1). For T-cells and despite the detection of the CR2 EBV receptor or of a related molecule at the surface of immature thymocytes or peripheral T-lymphocytes (9), iterative attempts to infect these cells were often frustrating. This was however achieved in few cases of already transformed thymocytes (10) or T-cell lines (11,12). Some reports also mention infection by EBV of normal thymocytes (13,14) and primary $CD4^+$ or $CD8^+$ lymphocytes (15). However, these latter studies only showed transient EBV expression and no cell lines could be established after infection. Thus so far, the only report of establishment of an immortalized T-cell line by EBV was obtained after selective transfection of the EBV genome (16).

We have recently been able to immortalize peripheral T-cells *in vitro* after EBV infection and a transfection/selection procedure (17). The different T-cell lines obtained express CD2, CD3 and CD4 or CD8 cell surface markers and more generally display a naive T-cell phenotype. All these cells show a complete and clonal rearrangement of their T-cell receptor. They grow independently of exogenously added IL-2 and do not secrete detectable amounts of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α or IFN-γ. However, IL-2 is produced by these cells after activation, pointing out the preservation of at least some of the inducible physiological pathways. In these cells, only EBNA-1 and LMP-1 type II

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latency genes are expressed from an EBV genome in an episomal configuration. Moreover we demonstrated the malignant status of these EBV transformed T-cells by showing for one clone (NC5) its ability to induce tumors in immunodeficient RAG-2 and nude mice.

Thus, one of the main differences between this EBV transformed T-cell line and B-LCLs is the tumorigenic status of the former. However, induction of tumoral features for LCLs can be achieved by overexpression of a transfected c-*myc* oncogene in these cells (18). Similarly, overexpression of the death-sparing BCL-2 oncogene results in growth enhancement of LCLs (19). In LCLs, the immortalisation process needs expression of the majority of expressed EBV latent genes (20), and the immortalized state of cells is characterized by activation of transduction pathways and target genes induced in physiological activation of B-cells (21). This is the case of the well documented LMP-1-mediated induction of the NF-κB transcription factor pathway (22-26).

In order to get insight into this EBV-initiated transformation process of T-cells *in vitro*, we have further characterized genetical and functional aspects of the NC5 clone. The rationale for this study was to test chromosomal abnormalities and/or putative rearrangements and expression of genes implicated in transforming process associated with EBV or lymphoid malignancies like C-MYC, BCL-2 or NF-KB2. We also tested constitutive and/or inducible pathways uncovered for EBV expression in LCLs such as NF-κB induction, or for T-cell activation as induction of the STAT factors. We particularly focused on pathways and target genes involved in the IL-2R-dependent mitogenic properties. This was done in comparison with cells from the same donor at different stages of EBV infection and transformation as well as with heterologous B- and T-cell lines characterized for these infection or transformation criteria. We demonstrate here that, despite chromosomal abnormalities, viral protein expression and tumorigenicity, cellular pathways classically altered in other B-EBV infected or LMP1 transfected cells remain surprisingly unaltered in the NC5 and TC T-cell lines.

Materials and methods

Cell culture conditions. The EBV-transformed NC5 and TC T-cell lines were described elsewhere (17). PBL25, NC5, B-EBV neo and TC cells from the same donor, KAS cells, an EBV-transformed LCL from a different donor, Jurkat cells (clone E6-1, human acute T-cell leukemia) and K562 cells (human chronic myelogenous leukemia) cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and gentamycin (8 μg/ml). For 19D cells, a Jurkat T-cell line transformed by the HTLV-I Tax protein (27), geneticin (400 μg/ml) was also added to maintain selection on the Tax expressing vector. Peripheral blood lymphocyte (PBL) cells were obtained directly after performing a Ficoll extraction (Pharmacia, Biotech) following the manufacturer's instructions.

Indirect immunofluorescence. Cells were fixed in paraformaldehyde (4% in PBS) and indirect immunofluorescence was performed as described (28). A fluorescein-coupled anti-mouse secondary antibody allowed detection of the LMP1 monoclonal mouse antibody (Novocastra Laboratories Ltd.). Cells were cytospun 2 min at 400 rpm and analyzed on a Leika Axioscop fluorescence microscope.

Flow cytometry analysis. Acquisition and analysis for the fluorescence procedures were carried out with a flow cytometer Epics Elite cell sorter (Coulter, Miami, FL).

Cytogenetic analysis

Karyotype. Metaphase chromosomes were obtained after hypotonic shock with diluted human serum (1:5) and fixation with ethanol and acetic acid. Slides were stored at -20°C until use. After thawing, slides were placed in distillated water for 5 min, denatured at 87°C in Earle's balance salt dilution (BSS) pH 5.3 for one hour and pH 6.5 for 30 min, and then immediately rinsed. After a 10 min Giemsa staining, a R-banding for identification of metaphase chromosome was obtained (RHG). Chromosome pictures were then cut, classified and stuck (29).

FISH analysis on metaphase chromosomes for detection of abnormalities. Standard cytogenetic preparations were used and mitotic cells were analyzed by metaphase-FISH (Fluorescent *in situ* hybridization). Preparations were painted with chromosome 2, 9, 10, 12 and 15 specific probes (Coatasome total chromosome probe labeling kit, Oncor) following the manufacturer's instructions.

Southern blot analysis. Cellular DNA of PBL, LCL, B-EBV neo, NC5, TC, Jurkat and 19D cells (20 μg) was obtained by standard methods (30), and digested with BamHI or HindIII restriction enzymes. DNA was size-fractionated by electrophoresis in a 0.8% agarose gel at 35 V and transferred to an Hybond C extra membrane (Amersham). A 726 bp fragment of the BCL-2 cDNA obtained by RT-PCR, a 2.1 kbp HindIII-Asp718 fragment of the NF-KB2 cDNA (provided by Dr J. Hiscott, McGill University, Montreal, Canada) and a 1.6 kbp ClaI-EcoRI fragment from the third exon of the C-MYC gene (31) were used as probes. For BCL-2, total RNA of NC5 cells (2.5 μg) was reverse transcribed for 1 h at 37°C using 200 units of M-MLV reverse transcriptase (Gibco BRL Life Technologies Inc.) according to the manufacturer's instructions. PCR assays were performed in a final volume of 100 μl using 1/10 of the reverse transcriptase reaction. After an initial denaturation step at 94°C for 2 min, 35 cycles of PCR were performed (1 min at 94°C, 1 min at 55°C, 1 min at 72°C) followed by a 10 min elongation step at 72°C. The following BCL-2 specific primers were used: 5': AGGATGG CGCACGCTGGGAGAAGTGGT, 3': TTCACTTGTGGCC CAGATAGGCA. After purification using the QIAquick gel extraction kit (Qiagen), these three fragments were labeled with α^{32}P-dCTP (Megaprime labeling kit, Amersham). Hybridizations were performed in stringent conditions as described (30). After washing, membranes were exposed to X-ray film. Of note, the same blot was used to perform consecutive hybridizations with the three probes.

Northern blot analysis. Total cellular RNA was extracted by lysing cells with Trizol (Gibco BRL Life Technologies Inc.) following the manufacturer's instructions. Twenty μg of RNA per lane were electrophoresed in 1% denaturing agarose gels.

Gels were transferred to Hybond C extra membranes. Blots were then hybridized to human DNA probes radiolabeled with α^{32}P-dCTP. C-MYC and NF-KB2 probes were described above. A 2 kbp BamHI fragment of C-JUN cDNA (32) was also labeled. The GAPDH, BCL-X and thioredoxin (TRX) probes were obtained by RT-PCR and subsequent labeling as for BCL-2. The following specific primers were used: GAPDH 5': ATCTCTGCCCCCTCTGCTGA, GAPDH 3': TGCCAGC CCCAGCGTCAAAG; BCL-X 5': GAGGCAGGCGAC GAGTTTGA, BCL-X 3': GGGGGTGGGAGGGTAGAGTG and TRX 5': AGACTCCAGCAGCCAAGATG, TRX 3': ATTCACCCACCTTTTGTCCC. The amplified products were 541 bp long for the GAPDH, 465 bp for the BCL-X and 282 bp for the TRX specific fragments. The fragments obtained by digestion or by RT-PCR were purified using the QIAquick gel extraction kit (Qiagen). Hybridizations were performed as above. After extensive washing, membranes were exposed to X-ray film.

Band shift assays

Preparation of whole cell and nuclear extracts. For whole cell extracts (WCE), suspension cells were harvested by centrifugation at 800 rpm, washed three times in 1X PBS, then pelleted again at 800 rpm for 5 min. WCE were prepared from 10^7 cells in 50 µl of a hypertonic buffer as described (33). Ten µg of WCE were used per bandshift reaction and the WCE were stored at -80°C.

For nuclear extracts, cells were left untreated or treated with tumor necrosis factor α (TNFα 10 ng/ml) for 4 h, or with phorbol 12-myristate 13-acetate (PMA 25 ng/ml) + ionomycin (3.5 µg/ml) for 16 h. Cells were then washed in phosphate-buffered saline (PBS), and nuclear extracts were purified as described (34).

Electrophoretic mobility shift and supershift assays. Stat5 band shift assays were performed as previously described (33). The Stat5-binding site of the bovine ß-casein gene promoter was used as a radioactive probe (5'-AGATTTCTAGGAATTCAA ATC-3'). Sense and antisense oligonucleotides were annealed, end-labeled using polynucleotide kinase and γ^{32}P-ATP to a specific activity of 8,000 cpm/fmol, and purified through a Sephadex G-25 column. Sixteen fmol of probe were incubated on ice for 30 min with 10 µg of WCE in migration buffer as described (33). Reactions were electrophoresed at 200 V on a 5% polyacrylamide gel in 0.25X TBE for 2 h.

NF-κB band shift assays were performed according to Lacoste *et al* (34). The HIV enhancer κB site was used as a probe (5'-GGGACTTTCCGGGACTTTCC-3') (34). The probe was labeled as above and gel purified. Seventy fmol of probe were incubated at room temperature for 20 min with 10 µg of nuclear extracts. Reactions were electrophoresed at 170 V on a 5% polyacrylamide gel in 1X Tris glycine for 2 h. Supershift experiments were performed with anti-p50, p52, p65 and c-Rel antibodies (a generous gift from Dr J. Hiscott, McGill University, Canada) incubated for 10 min with nuclear extracts prior to probe addition.

Results

Detection of the LMP1 protein in the NC5 and TC EBV-transformed T-cell line. We have studied the expression of LMP1 in B-EBV neo, NC5 and TC by immunofluorescence quantified by flow cytometry (Fig. 1). An EBV-lymphoblastoid cell line (LCL) was previously obtained after isolation of peripheral blood lymphocytes (PBL) from a specific donor (PBL25). This EBV-LCL was electroporated with a PSV2-neoR vector in conditions which preferentially eliminate B cells and isolate T-cells. B-EBV neo cells hence obtained before G418 (geneticin) selection were then cultured in a medium containing G418. After G418 selection, an NC5 T-cell line was isolated. Injection of NC5 cells into nude mice or RAG2-deficient mice led to the formation of solid tumors. Cells from one of these tumors (TC cells) were isolated (17).

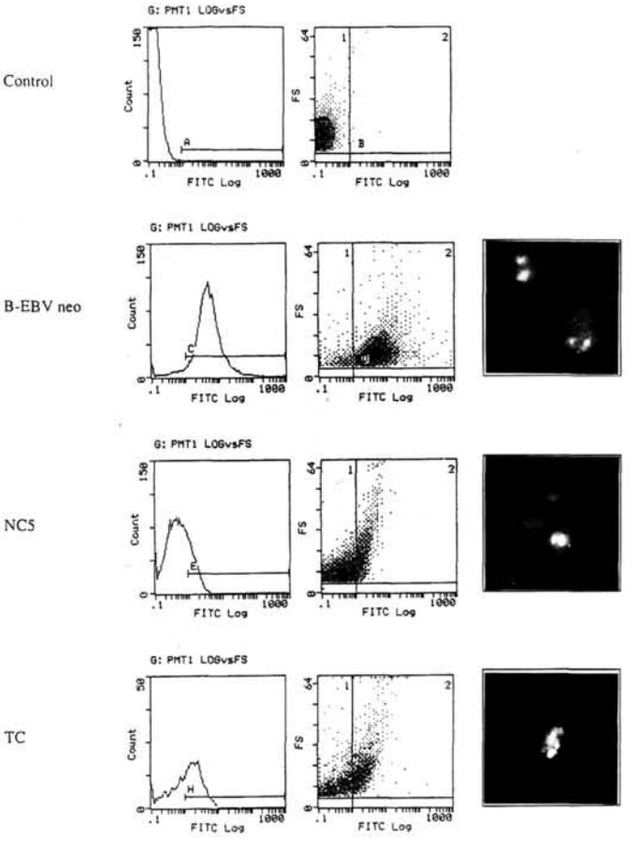

Figure 1. LMP1 immunodetection in EBV-transformed cells. Fluorescence histogram (left) and two-parameter flow cytometry histogram (middle) obtained with control, B-EBV neo, NC5 and TC cell lines. Labeling of the B-EBV neo cell line with an IgG1 secondary antibody was used as a control. LMP1 detection was analyzed by microscopy (right) in these cell types.

The percentage of positive cells above the baseline level was: 93.9%, 21.9%, 61% for the B-EBV neo, NC5 and TC cell lines respectively. Therefore, the NC5 and TC EBV-transformed T-cell line express LMP1 as expected. However, the level of LMP1 is higher in cells recovered from a tumor.

Chromosomal rearrangements in NC5 and TC cells. In order to identify chromosomal rearrangements after EBV transformation, we compared the karyotypes of the different cells or cell lines from the same donor: PBL25 normal cells, B-EBV neo, NC5 and TC cells. As controls, 3 karyotypes for PBL25 and B-EBV neo cells were performed (Fig. 2A). They did not

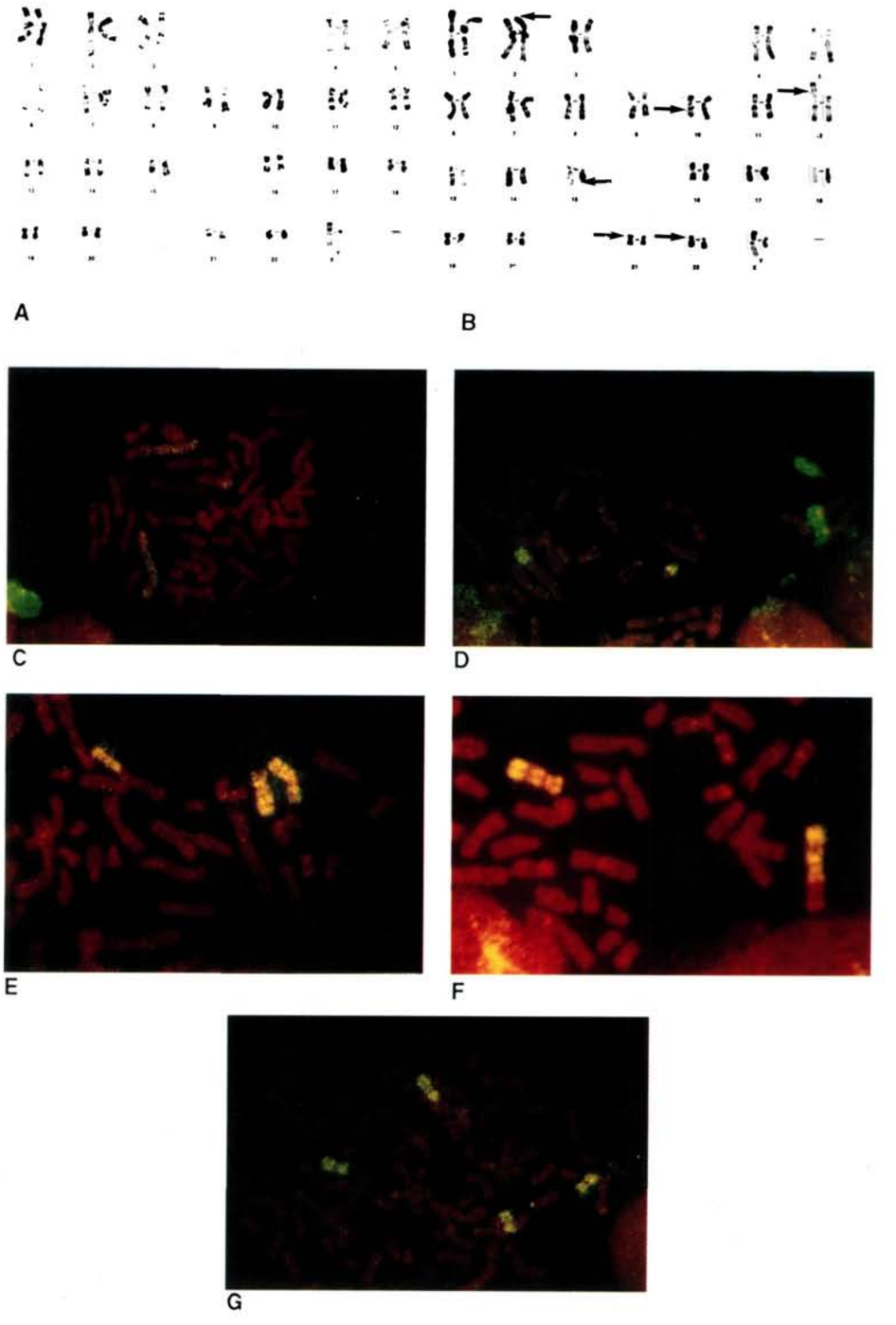

Figure 2. A, Normal R-banded karyotype of a B-EBV neo cell. B, R-banded karyotype of an NC5 cell showing rearrangements. C, D, E, F, G, Metaphase spreads of NC5 cells after hybridization with a painting probe for chromosome 2 (C), 15 (D), 9 (E), 12 (F) and 10 (G). A signal is detected on the distal q arm of rearranged chromosome 15 (C) and on the distal p arm of rearranged chromosome 2 (D). The chromosome 9 signal detected on chromosome 12p reveals a partial 9q trisomy (E). Painting on chromosome 12 confirms the t(9;12) translocation (F). The signal corresponding to the interstitial deletion fragment of chromosome 10 was not found on another metaphase chromosome (E).

show any chromosomal rearrangement. Eleven different karyotypes of NC5 cells were achieved on the basis of R banding patterns with the following result (Fig. 2B): 46,XY with abnormalities on one 2p, 10q24, 12p (with a possible translocation with a 9q), 15q, 21p and 22p chromosomes. In order to clarify the karyotypic rearrangements of the NC5 cells, unicolor chromosome painting with chromosomes 2, 9, 10, 12 and 15 specific probes were used. A short fragment of chromosome 2 was found in the distal part of the long arm of rearranged chromosome 15 (Fig. 2C) and, conversely, a fragment of chromosome 15 was found in the distal part of the short arm of rearranged chromosome 2 (Fig. 2D). With chromosome 9 painting specific probes, we found two normal chromosomes 9 and a supernumerary chromosome 9q

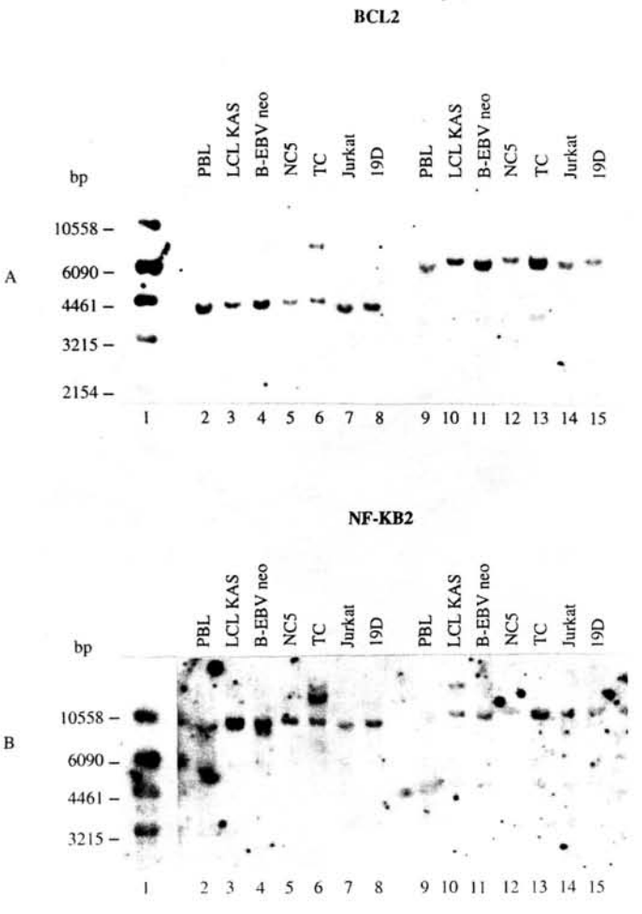

Figure 3. Southern blot analysis of BCL-2 (A) and NF-KB2 (B) genes. DNAs were digested either with BamHI (lanes 2-8) or with HindIII (lanes 9-15). The same blot was used in A and B. The upper band in lane 6 (A and B) results from partial digestion and the upper band in lane 10 (B) seems to result from a polymorphism. Lane 1, molecular weight marker.

translocated to rearranged chromosome 12 (in the distal part of the short arm) (Fig. 2E). With chromosome 12 painting specific probes, we found two signals: one corresponding to normal chromosome 12 and the second one corresponding to translocated chromosome 9q-12p (Fig. 2F). With chromosome 10 painting specific probes, we did not find the interstitial deletion fragment of chromosome 10 in another place on the metaphase (Fig. 2G). In conclusion, after FISH studies, the particularities of the karyotypic cell line NC5 seem to be a 46,XY,t(2;15)(15qter→15q21.1::2p22→qter;15pter→15q21.2::2p24→pter),del(10)(q24),der12t(9;12)(9qter→9q12::12p13→12qter),-12,add(21)(p12),add(22)(p12). So, the main abnormalities concerned 2 translocation events, t(2;15) and t(9;12), and a 10q24 deletion. For TC cells, 3 karyotypes were performed, and they showed the same rearrangements as in NC5 cells (data not shown).

In the NC5 T-cell line, we identified chromosomal rearrangements that cannot be observed in uninfected or EBV-infected B cells of the same donor. These results suggest that EBV infection led to T-cell specific disorders. The clonality of the NC5 cells was demonstrated previously (17) by Southern blotting using a TCR-Cß1 specific probe. Cytogenetic studies of these cells confirmed this clonality since the abnormalities were observed without exception in all the karyotypes performed. These karyotypic studies were performed on NC5 cells after a few or many passages (data not shown). We did not detect further karyotypic abnormalities even after more than 30 passages. Also, these rearrangements did not evolve with time. We were not able to detect any significant phenotypic difference between NC5 and TC cells. This observation suggests that no clonal selection of an additional abnormality detectable at the karyotypic level was achieved *in vivo*.

The BCL-2, NF-KB2 and C-MYC genes are not rearranged in NC5 and TC cell lines. BCL-2, NF-KB2 and C-MYC genes were chosen for this study because of their rearrangement in different lymphomas (EBV-associated B lymphomas or not EBV-associated B or T lymphomas). EBV is known to be etiologically associated with Burkitt's lymphoma (BL) (35). The consistency with which t(8;14), t(2;8), and t(8;22) translocations (36) occur in all known forms of BL clearly indicates that juxtaposition of the C-MYC and immunoglobulin loci is an essential step in the lymphomagenic process. In a B lymphoma, BCL-2 also has been found translocated to an immunoglobulin gene locus. In addition, we also studied the NF-KB2 gene because its translocation is associated with T neoplasms (37). Furthermore, after cytogenetic studies, we noted the lack of one allele of NF-KB2 (located in the 10q24 region which is missing on one chromosome 10) possibly translocated to another chromosomal region. We studied these three potential rearrangements by Southern blotting of PBL, LCL, B-EBV neo, NC5, TC, Jurkat and 19D cell DNA (Fig. 3).

The results showed no rearrangement of BCL-2, NF-KB2 (Fig. 3) and C-MYC (data not shown) genes in NC5 and TC cells. The pattern obtained after TC DNA digestion with BamHI and hybridization with BCL-2 or NF-KB2 specific probe likely results from partial digestion. The pattern obtained after LCL KAS digestion with HindIII and hybridization with NF-KB2 seems to be the result of a polymorphism.

Expression analysis of specific EBV- and T-activation targeted genes. The lack of rearrangement for the three genes tested above did not preclude a transcriptional activating event, either following a long distance reshuffling of the locus or consecutive to a constitutive activation of cellular pathways. Thus we completed our study by performing Northern analyses on these genes and others which could be associated with EBV-dependent and/or T-cell activations. NF-KB2 mRNA expression seemed to be lower in transformed T-cells than in PBL25 or B-EBV cell lines. C-MYC was also less expressed in NC5 and TC than in B-EBV neo or Jurkat and 19D lines which showed a high expression level (Fig. 4A). BCL-2 was hardly detectable in all the cell lines (data not shown). Owing to the IL-2-independent growth of NC5 and TC cells, we also decided to focus on target genes of the IL-2 receptor-triggered transduction pathways. Two of these lead to the C-MYC and BCL-2 genes induction mentioned above, whereas a third one leads to C-FOS/C-JUN (AP-1) induction (38). As shown in Fig. 4A, C-JUN mRNA was expressed at low levels in all cell lines. Among the numerous other genes which could be regarded as specific of T-cell activation or transformation states we also tested BCL-X and thioredoxin (TRX) (Fig. 4B).

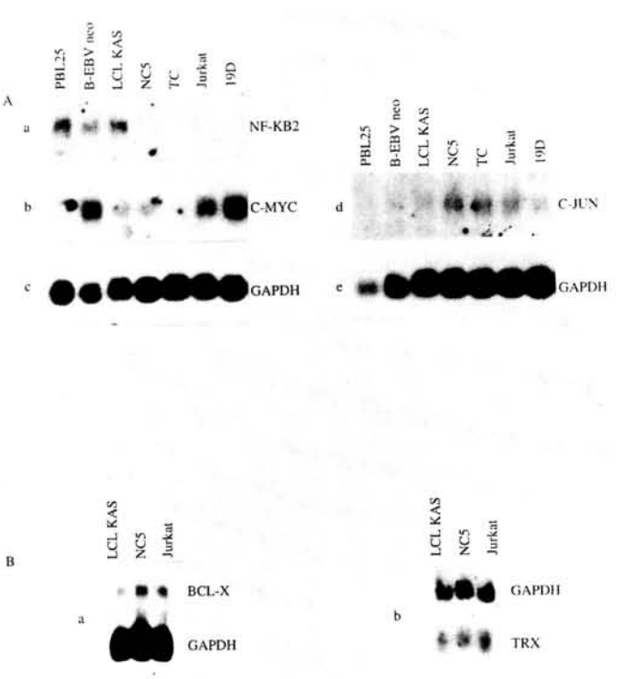

Figure 4. Northern blot analysis. RNA was extracted from PBL25, B-EBV neo, LCL KAS, EBV-transformed T cells NC5, TC, Jurkat and 19D cells. A, Northern blots were performed on total RNA (20 µg per lane). Membranes were hybridized with indicated gene probes: a, NF-KB2; b, C-MYC; c, GAPDH; d, C-JUN and e, GAPDH. B, Northern blots performed with total RNA (20 µg per lane) extracted from LCL KAS, EBV-transformed T cells NC5 and Jurkat cells were probed with a, BCL-X (upper band) and GAPDH (lower band) and b, TRX (lower band) and GAPDH (upper band).

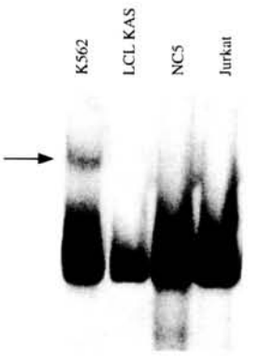

Figure 5. Whole cell extracts were analyzed by band shift experiments with a STAT5-binding site of the bovine ß casein gene promoter probe to study the activation state of STAT5. The arrow indicates the position of migration of the specific DNA-protein complex.

BCL-X which is the main inducible member of the anti-apoptotic BCL-2 family in T-cells was expressed in NC5 and Jurkat cells. Similarly, TRX was found transcribed in the same cells, but also in LCL KAS. This is in accordance with previous reports of its high expression in transformed and EBV-infected cells or tissues (39). Thus the gene expression pattern in EBV-transformed T cells did not fit some expected results for EBV-induced transcription of the BCL-2 gene or for transcription of C-MYC or C-JUN targeted by T-cell activation pathways. In contrast, we could detect expression of two other genes (BCL-X, TRX) associated with EBV etiology or activation/transformation state of T-cells.

Activation status of the Stat pathway. One of the four different signaling pathways known to mediate IL-2 receptor activation leads to the activation of the Jak/Stat pathway via the Stat5 protein. For these reasons and since our cells were IL-2 independent, we decided to study the constitutive status of this pathway in different cell lines. In a gel retardation assay, Stat5-specific ß-casein probe permits detection of Stat5. Stat5 was not constitutively activated in the NC5 and LCL KAS cell lines (Fig. 5), suggesting that constitutive activation of IL-2 by Stat5 is not involved in LMP1-mediated transformation, as opposed to HTLV-I Tax-mediated transformation (40). As expected, Stat5 is constitutively activated in the K562 cell line (41) and not in the Jurkat cell line. Binding specificity was confirmed by supershift experiments using an anti-STAT5 antiserum (data not shown).

Activation status of the NF-κB pathway. The NF-κB pathway is known to be constitutively activated by viral proteins such as LMP1 (22) and HTLV-I Tax protein (42). In order to get insights into the activation status of the NF-κB pathway in EBV-transformed T-cells, we tested the NF-κB DNA-binding activity by band-shift experiments. In the absence of stimulation (Fig. 6A), the NF-κB pathway is activated in B-EBV neo cells and in 19D cells. In both cases, the major complex is a dimer of p50/c-Rel as shown by antibody-specific supershifting (dots in Fig. 6) accompanied by lower detection of the NF-κB complex in the same lane. This is consistent with previous results reported for the 19D cell line (43). In 19D and B-EBV neo cells, the smear detectable with the anti-p50 antibody, raises the possibility of p50 homodimers in these cells. This assumption is consistent with previous results obtained with B-JAB cells transfected with LMP-1 (25). In contrast, this pathway is not constitutively activated in Jurkat, NC5, TC and KAS cells. However, the NF-κB pathway can still be activated by TNFα (Fig. 6B) and PMA + ionomycin (Fig. 6C) in Jurkat, NC5, and KAS cells, where the major complexes are respectively p50/p65 and p50/c-Rel as expected (43).

These results indicate that constitutive NF-κB activation is not necessarily the consequence of LMP-1-mediated transformation since, in the donor tested, only EBV-infected B cells (B-EBV neo) exhibit constitutive NF-κB activation, in contrast to EBV-infected T-cells (NC5 and TC). However, in EBV-infected B cells of a different origin (LCL KAS), the NF-κB pathway is not constitutively activated, suggesting that activation of this pathway is not instrumental in the maintenance of the transformed state.

Discussion

Our previous results showed isolation of a transformed T cell line infected by the Epstein Barr virus (17). Here, we have

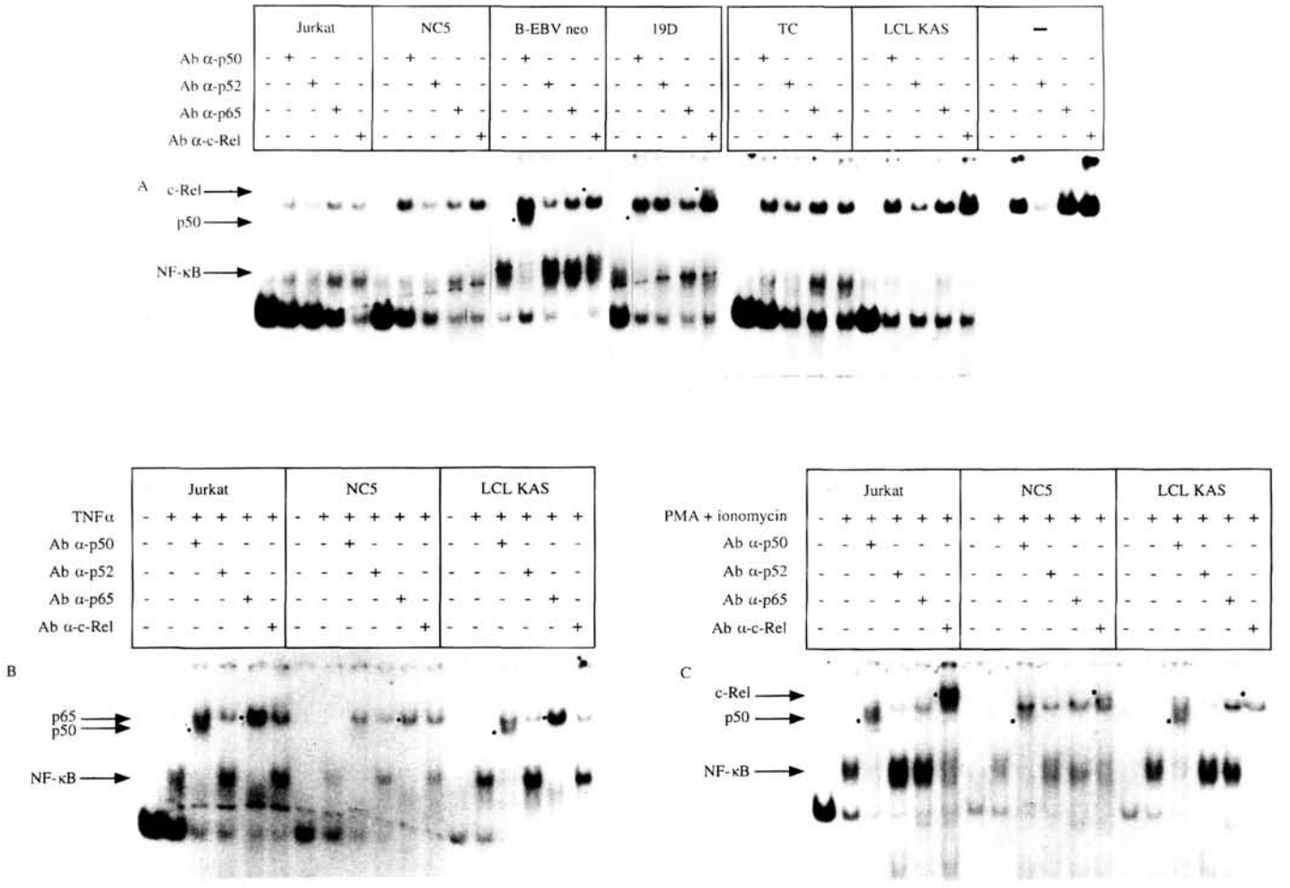

Figure 6. Nuclear extracts were analyzed by band shift experiments with an HIV enhancer κB site probe to study the activation state of NF-κB. Cells were left untreated (A) or activated by TNFα (B) or by PMA + ionomycin (C). Binding of the different antibodies (Ab) to the probe is shown on the right of panel A. The arrows indicate the position of migration of specific DNA-protein complexes. Position of supershifted complexes is indicated with dots.

focused on the genetical characteristics of this cell line, compared to *in vitro* EBV-transformed B lymphocytes. First, we studied the chromosomal rearrangements of these cells. Then, we examined the expression of genes involved in T cell activation, in activation by EBV or in activation of the IL-2 receptor transduction pathway.

Immunofluorescence analysis shows LMP1 expression in the B-EBV neo, NC5 and TC cell lines. However, this expression appears heterogeneous as the percentage of labeled cells above the baseline level is higher in TC cells than in NC5 cells. This variable LMP1 expression seems to be due to the number of passages, since the NC5 cells underwent more passages than the TC cells, and not to an upregulation of LMP1 expression in the tumor from which TC cells have been obtained.

The NC5 cell line exhibits some chromosomal rearrangements, as shown by cytogenetic analysis. Region 12p13 implicated in a translocation with 9q11 is particularly interesting given the amount of genes present in this region that could potentially be involved in immortalization and transformation of the NC5 cell line. One of these is TEL, a transcription factor of the ETS family, which is found rearranged in some myeloid and lymphoid leukemias (44-47). Translocation t(12;21) which fuses TEL to AML1 has been extensively characterized as the major translocation event involving the TEL gene in acute lymphoblastic leukemia (ALL) (48). Preliminary RT-PCR results indicate that this translocation does not occur in the NC5 cell line (C. Preudhomme, unpublished data). It is worthy noting that the four genes (AML1, ABL, PDGFr and MN1) implicated so far in translocations with TEL are not located in region q11 of chromosome 9 translocated to 12p13 in NC5 and TC cells. However, TEL may be involved in a cryptic rearrangement with one of the three other genes not yet tested or in a different type of rearrangement. These possibilities are being investigated. In this region, other candidates may be involved in ultimately transforming translocations, such as cyclin D2 or the cyclin kinase inhibitor p27/KIP1. This latter gene is of particular interest because its deletion together with TEL has been frequently reported in ALLs with or without TEL-AML1 translocation on the other chromosome 12 (49). Another interesting feature of p27/KIP1 is its identification as a target for IL-2-mediated degradation upon T-cell proliferation induced by this cytokine (50).

Deletion of one of the 10q24 chromosomal region is also noteworthy. For example, the HOX11 homeotic gene, which is located in this region, is found translocated in several T-ALL close to TCRα/ß genes on chromosome 14 (51,52). NF-KB2 is another gene located in this region which was initially found translocated in a B lymphoma (53) but which

is more commonly rearranged in neoplasms derived from mature T-cells (37,54). This rearrangement results in disruption of the gene transcription unit. No rearranged NF-KB2 gene was detected at the DNA and RNA levels in NC5 and TC cells. Concerning the levels of expression of the NF-KB2 gene, we observed a very low level of the normal NF-KB2 mRNA in NC5 and TC cells when compared to the different B cell lines. However, a similar result is also obtained in 19D and Jurkat cells, indicating cell-specificity of the mRNA level, consistently to previous results on T transformed cells lacking NF-KB2 reshuffling (55). Band shift experiments performed with unstimulated EBV-transformed B and T cells indicated that the NF-κB pathway is not activated constitutively in NC5 and TC cells, whereas as expected the EBV-B cells from the same donor displayed this constitutive activation. Interestingly, this result is in striking contrast with previous data showing that LMP1 activated this pathway in other cell types such as LMP1-transfected Burkitt lymphoma cells (22,25), epithelial cells (26) and even T-cell lines (24,56). To our knowledge, this is the first report of uncoupling of viral LMP1 expression and NF-κB induction. Therefore, it appears that in NC5 and TC EBV-transformed cells the transformation pathway by LMP1 is transduced through other intracellular signals than the NF-κB pathway. Upon stimulation by TNFα or PMA + ionomycin, the NF-κB pathway is still inducible in the NC5 and TC cells. As in control cells, these induced complexes mainly engage p50/p65 and p50/c-Rel subunits for TNFα and PMA + ionomycin treatments, respectively. This NF-κB inducibility is on line with our previous results showing IL-2 production by these EBV-transformed T-cell lines upon activation (17).

The NC5 growth is IL2-independent (17). This observation prompted us to investigate whether the IL2-mediated activation and proliferation pathways were constitutively activated in these cells. To date, 4 pathways activated by the IL-2 receptor have been described, leading respectively to C-MYC, C-FOS/C-JUN, BCL-2 and STAT5 activation (38). Induction of two of any of these pathways seems necessary and sufficient to transduce the IL-2 mitogenic effect.

The reason to study expression of the C-MYC gene is that it is rearranged with the Ig chains in 100% of Burkitt lymphomas (36) and that transfection of a C-MYC oncogene can confer phenotypical and transforming features of a Burkitt lymphoma to LCLs (18). Cytogenetic and Southern blot analyses excluded the possibility of a rearrangement of the C-MYC gene. In addition, Northern blot analysis ruled out a long distance activating event. This result also suggests that MYC is not responsible for IL-2 independent growth, although we cannot exclude the possibility of an aberrant post-translational regulation. Similarly, our results did not show any modulation of C-JUN expression in the NC5 or TC cell lines either.

In several respects, examining the BCL-2 gene is also of crucial interest in this study. BCL-2 is not only activated by IL-2, but also by LMP1 (24). In addition, it is found rearranged in some B lymphomas (57). We show here that the integrity of this gene seems preserved and, apparently, it is not induced in the NC5 T-cell line (data not shown). However, some data indicate that BCL-2 is not always the only or relevant anti-apoptotic target of LMP1. Indeed, MCL-1 (58) and A20 (23) are two other target genes. In addition, there is lack of correlation between expression of LMP1 and BCL-2 *in vivo* (59). In our study, we tested BCL-X, another anti-apoptotic gene known as specifically induced in activated T-cells (60) and expressed at high levels in EBV-infected cells of Hodgkin's disease (61). BCL-XL cooperates with BCL-2 to counteract apoptosis, while the alternatively spliced species BCL-XS has the opposite effect (60). The Northern blot analysis presented here showed that BCL-X is induced in the NC5 cells. A RT-PCR analysis using primers allowing discrimination between the two BCL-X forms showed a balance largely in favor of BCL-XL expression (data not shown). These results suggest that BCL-X may be involved in the prolonged survival of the NC5 and TC cells.

STAT5, an instrumental component of another IL-2-triggered pathway, and which is also activated in the course of *in vitro* transformation of T-cells by HTLV-1 (40), is not constitutively activated in NC5 cells. This was also the case for STAT1, STAT3 and STAT6 (data not shown). Taken together all the results show that none of the four pathways triggered by IL-2R are constitutively activated. Therefore, these pathways, shared by other cytokine receptors are unlikely to be responsible for constitutive autonomous proliferation of the NC5 and TC cells.

Finally, we found expression of the thioredoxin gene (TRX) which is known as actively transcribed in numerous neoplams with viral etiology (including EBV) and in LCLs or BL lines (39). Its secreted product is reported as an autocrine growth-promoting factor for malignant cells. Of note, its role seems to potentiate mitogenic effects of various interleukins and it is sensitive to minimal amounts of interleukins. In this respect, it might be informative to test whether the potentialisation by TRX of suboptimal and undetected autocrine production of some interleukins takes place in NC5 and TC cells.

In conclusion, our data show that EBV-transformed T-cell lines can exhibit autonomous proliferative and tumorigenic properties without apparent constitutive activation of classical mitogenic pathways.

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