Haematologica 1999; 84:988-995 original paper

An Epstein-Barr virus-infected lymphoblastoid cell line (D430B) that grows in SCID-mice with the morphologic features of a CD30+ anaplastic large cell lymphoma, and is sensitive to anti-CD30 immunotoxins

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ABSTRACT

Background and Objectives. **In this study we describe a newly established CD30+ Epstein Barr virus (EBV)-infected B cell line derived from an EBV-infected B cell culture (utilized, once irradiated, as a feeder) which showed a B clonal rearrangement and strong CD30 antigen expression.**

Design and Methods. **The cells injected into SCID mice were able to grow giving rise to CD30+ solid tumors with the morphologic features of an anaplastic large cell lymphoma (ALCL). Thus we tried to establish a model to investigate the potency of immunoconjugates containing a CD30 monoclonal antibody (Ber-H2) and ribosome-inactivating proteins (saporin, momordin and ricin A-chain) as toxic moieties.**

Results. **We observed a strong cytotoxic activity of the anti-CD30 immunotoxins on the *in vitro* growth of D430B cells. High levels of anti-tumor activity were also observed *in vivo*, in the SCID mouse model.**

Interpretation and Conclusions. **The antitumor immunotoxin therapy was sccessful in our chosen animal model, the effecacy seeming to be associated with strength of CD30 expression. Our data suggest that immunotoxins should be tested (before use) on the tumor cells of the subject to be treated and that immunotoxins should be directed to different tumor-associated antigens to avoid selection of cell populations with different antigenic mosaics.**

Key words: B cell-derived ALCL, CD30, RIPs, immunotherapy

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The CD30 antigen was originally identified as a typical cell surface antigen on primary and cultured Hodgkin's disease (HD) and Reed-Sternberg (RS) cells by the monoclonal antibody Ki-1, the first member of the CD30 cluster.[1] Subsequently the CD30-related antigen, first regarded as a peculiar feature of, and restricted to, HD and RS cells, was shown to have a broader expression on activated or virally transformed T and B cells,[2-4] as well as on natural killer cell clones and end-stage macrophages.[5,6] Moreover, a subset of normal T cells with the features of the Th2 subset (i.e. producing interleukin-4 (IL4) but not interleukin-2 (IL2) and interferon-γ (IFN-γ) following activation) may preferentially express the CD30 antigen.[7]

A series of cDNA cloning studies showed that CD30-related protein is a member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily as defined by sequence homology in the extracellular domain.[8] In addition the CD30 ligand (CD30L) belongs to the TNF ligand superfamily[9] and might mediate different biological phenomena, such as apoptosis or proliferation, on several CD30+ lymphoma types.[10]

Ki-1 (CD30)+ anaplastic large cell lymphoma (ALCL) was first described by Stein *et al.*[3] as a tumor in which all or nearly all neoplastic cells express the CD30 antigen. In its so called classic or common form[11] it shows an often bizarre, anaplastic cytomorphology with sinusoidal infiltration of lymph nodes and a pseudocohesive appearance. Nodal involvement is often non-contiguous, in contrast to HD.[12] This lymphoproliferative disorder is a morphologically and immunologically distinct subset of non-Hodgkin's lymphomas,[13-15] incorporated as a separate entity into the updated Kiel classification in 1988[16] and recently into the *Revised European American Lymphoma Classification*.[17] However the status of Ki-1 ALCL as a single entity has long been controversial. It has also been suggested that ALCL might

sometimes represent the final step of morphologic and biological progression of HD, since in a percentage of cases areas of ALCL transformation are found within HD.

Some features, such as CD30 expression and Epstein Barr virus (EBV) infection, the latter more significantly associated with HD (50% of cases) than with ALCL (10% of cases), might point to a common origin.[18,19] However, the recent discovery of the specific recurrent translocation t(2,5)(p23;q35) and of the chimeric protein NPM/ALK (p80), preferentially expressed by Ki-1 ALCL of classic morphology (T or null/indeterminate lineage) and negative in HD[20] suggests that similarities between HD and Ki-1 ALCL might be merely secondary features of two pathogenetically distinct entities.

ALCL is usually responsive to aggressive chemotherapy, but 30% to 40% of patients eventually die of their disease. Therefore new or additional therapeutic approaches need to be evaluated. Previous studies have suggested that the CD30 molecule may represent an optimal target in HD. *In vitro* and *in vivo* studies have shown that anti-CD30 immunotoxins, obtained by linking the Ber-H2 mAb to ribosome-inactivating proteins (RIPs) saporin, momordin and PAP-S had a potent cytotoxic activity on $CD30^+$ neoplastic cells both *in vitro* and *in vivo*.[21-23]

In this study we describe the biological and antigenic characteristics of a newly established monoclonal EBV-infected B cell line which grows in SCID mice with the morphologic features of a $CD30^+$ ALCL and the *in vitro* and *in vivo* cytotoxic activity of Ber-H2-containing immunotoxins on this cell line.

Design and Methods

D430B cell line

D430B cells were derived by chance from the cells of an EBV-B lymphoblastoid cell line (LCL) (obtained by EBV infection of peripheral blood lymphocytes from healthy donors and named MK from the donor's initials) utilized as a feeder for T-cell cloning.

Briefly, cells from a twelve-day mixed lymphocyte culture (MLC) were cloned by limiting dilution using irradiated (100 Gy) EBV-infected MK cells as a feeder. Feeder cells were seeded in 96 U-bottomed well microtiter plates at the concentration of 2×10^4 cells/well in culture medium (RPMI 1640 + 10% FCS) (ICN Flow, Costa Mesa, CA, USA; Seromed, Biochrom KG, Berlin, Germany), containing 20 U/mL of IL2 (Endogen, MA, USA). After 7 days a few positive wells were picked up and expanded in 24-well plates. Surprisingly, the proliferating cell cultures showed a B-cell phenotype by immunofluorescence (see below) instead of a T-phenotype and were cloned at 0.3 cells/well. Positive wells were further expanded in culture medium (RPMI 1640 + 10% FCS), grown for 6 months and characterized as described below. The final cell culture used in this study was selected on the basis of the strong and homogeneous expression of the CD30 antigen. This cell line was named D430B.

Phenotype

D430B cells were harvested and checked for viability with the trypan blue dye exclusion test. Cells were stained only when viability exceeded 95%. Briefly, pelleted cells (10^5 cells/tube) were incubated for 30 min at 4°C with saturating amounts of the following monoclonal antibodies (mAbs): anti-CD19, -CD20, -CD21, -CD22, -DR, -CD10, anti-κ and anti-λ light chains (Simultest anti-κ/anti-λ) (all from Becton-Dickinson, San José, CA, USA), anti-CD25, -CD3, -CD2, -CD45RA (Dako, Copenhagen, Denmark); anti-CD30 (Ber-H2, from Prof. H. Stein laboratory), anti-CD23 (PL13, a kind gift from Dr B. Knowles, Raritan, NJ, USA). After two washes with cold phosphate-buffered saline (PBS) (Gibco, Paisley, Scotland) containing 2% FCS (Seromed), the cells were incubated for 30 min at 4°C with 5 ml of goat immunoglobulins ($F(ab')_2$ fragments) against mouse IgG, conjugated to fluorescein isothiocyanate (FITC) (Becton Dickinson). Appropriate Ig-matched controls were run for each sample. Single fluorescence histograms were generated by the Consort 30 program (Becton Dickinson).

Epstein-Barr virus analysis

The presence of EBV in the D430B cell line was determined by Western immunoblot using human antisera against latent antigens of the EBV. The B95.8 marmoset cell line was used as the positive control and the Ga-1 cell line as the negative control.[24] Lysates obtained from 2×10^6 cells of D430B cell line were sonicated in electrophoresis sample buffer (0.05 M tris-buffer, PH 6.8, 2% mercaptoethanol, 10% glycerol, 0.01% bromophenol blue), and boiled for 3 min. This sample was run with human serum detecting EBV nuclear antigens 1,2,3a,3b,3c. Human polyspecific antisera were probed overnight at 4°C, and positive samples detected by ^{125}I-Protein A (Amersham, Buckinghamshire, UK).

The presence of EBV was also detected by polymerase chain reaction (PCR). One microgram of genomic DNA obtained from the D430B cell line was utilized according to the phenol-choloroform procedure. Specific primers BMRF1 were synthesized on the basis of published sequences[25] corresponding to a BAM M region of the EBV genome. The PCR reaction consisted of: 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, and 0.01% w/v of gelatin), 200 mM concentrations of each deoxynucleotide triphosphate, 25 pmol of each primer, 2.5 U of Ampli Taq polymerase (Cetus Corporation, Emeryville, CA, USA). The mixture was overlaid with mineral oil and then amplified in a thermal cycler (Cetus Corporation) for 35 cycles in the following conditions: 95°C for 1 min, 66°C for 2 min, 72°C for 1 min. Fifteen microliters of PCR product were electrophoresed in 2% agarose gel in Tris-borate/EDTA

buffer. The gel was stained with ethidium bromide and photographed. All buffers and pipettes were carefully isolated to prevent possible contamination of samples. DNA obtained from a B lymphoblastoid cell line (SL-1) was used as the positive control.

DNA analysis for Ig heavy chain gene rearrangement

DNA from D430B cells was extracted following a standard phenol-chloroform procedure, digested with restriction enzymes (Hind III and Eco RI) and electrophoresed on a 0.8% agarose gel. The DNA was then transferred to a nylon membrane and hybridized with a ^{32}P labeled probe. The probe used in this study was a 3.3 kb ECO RI/Hind III genomic fragment, containing the Ig heavy chain J region.[26].

Karyotype

Cytogenetic studies were performed on D430B cell cultures using standard techniques, without any stimulation. Samples were treated for 40 min. with 0.1 mg/mL of colcemid (Sigma, St. Louis, MO, USA) smeared on slides, and G-banding with Wright's stain was performed. Analysis was carried out with a Zeiss Axioscope microscope connected to a Genevision 121 system (Applied Imaging). Chromosomes were classified according to *International System for Human Cytogenetic Nomenclature.*[27]

In vitro sensitivity to anti-CD30 immunotoxins

Conjugates containing the RIPs saporin, momordin or ricin A-chain were prepared and purified as already described.[21] The immunotoxins obtained were tested against D430B cells in a standard [^{3}H]-leucine uptake assay.[21] Briefly, 2×10^4 D430B cells were seeded in U-bottomed microtiter plates in a 100 µL volume of RPMI 1640 complete medium. One hundred microliters of different dilutions of the anti-CD30 immunotoxins were added to final concentrations ranging from 10^{-13} M to 10^{-8} M. Appropriate control tests were run (RIPs alone, mAb alone, mixtures of unconjugated mAb and RIPs). The experiments were performed in triplicate and repeated three times. After 24 h of culture, cells were pulsed with 2 µCi of [^{3}H]- leucine (Amersham, UK) per well for an additional 18 h. The cells were then harvested onto glass fiber diskettes with a multiple-cell harvester (Skatron, Oslo, Norway) and the radioactivity was evaluated with a liquid scintillation β-counter.

In vivo experiments

Fox chase SCID mice were purchased from Nossan, Corezzana, Milan, Italy. Mice were housed in the animal facility of the Istituto Nazionale per la Ricerca sul Cancro (IST), Genoa, Italy, in a cabinet with filtered air. Manipulation was performed in sterile conditions. The experiments were performed in accordance with UKCCR guidelines[28] to minimize animal distress.

To assess the appropriate numbers of growing cells to be seeded in SCID mice, different amounts of D430B cells (10^7, 3×10^7, 5×10^7) were injected intraperitoneally (i.p.) to groups of three SCID mice, in order to determine the best growing conditions. When growth occurred, mice were euthanasized with carbon dioxide. The animals were autopsied and tumor masses were either regrown *in vitro* or treated for routine histology.

To evaluate the effects of the Ber-H2-saporin immunotoxin on the survival of SCID mice challanged with 3×10^7 human D430B cells, animals were randomly divided into two groups of six. The first group was treated with a single i.p. injection (0.5 mL) of a solution containing the immunotoxin diluted in PBS (0.1 mg/kg as linked saporin) 48 h after the D430B inoculum, while the second group received 0.5 mL of PBS only. The animals were observed daily and euthanasized when growth of abdominal masses occurred, or after 90 days, if no macroscopic signs of tumor growth were observed.

Pathology

For the histologic analysis, tumor samples, and samples from normal organs used as controls, were fixed in 10% buffered formalin and embedded in paraplast at 56°C. Five micron-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and stained with Giemsa and hematoxylin-eosin stain for light microscopy examination. The expression of the CD30 antigen was analyzed by the Ber-H2 mAb which recognizes a fixative-resistant epitope of the molecule[29] and the alkaline phosphatase (APAAP) technique as previously described.[30] Controls were run without the primary antibody and with unrelated mAbs.

Results

Generation and characterization of D430B cell line

The D430B cell line was generated from an irradiated EBV-infected B-cell line (MK) used as feeder cells for T-cell cloning by the limiting dilution technique. A rapidly growing microculture was expanded several times in a period of six months. These cells appeared as a typical lymphoblastoid cell line (LCL), growing in cell clumps and with a doubling time of approximately 24h.

As shown in Table 1, D430B cells were stained with CD19, CD21, CD23 and CD45RA mAbs, thus demonstrating the B origin of this cell line and a phenotype which is compatible with an EBV-LCL derivation. Figure 1 clearly demonstrates the strong expression of the CD30 antigen and it is worth noting that this expression was quite stable along the six months of *in vitro* culture (results not shown). Staining with CD30-saporin conjugate showed no difference with the anti-CD30 mAb only. In addition, D430B cells showed a λ chain restriction, as demonstrated by the use of anti-κ and anti-λ mAbs. The monoclonality of the D430B cell line was further confirmed through the analysis of heavy chain Ig rearrangements, which

Table 1. Phenotype of D430B cells.

mAb	*Positive cells*
CD3	< 2%
CD2	< 2%
CD19	98%
CD20	99%
CD21	95%
CD22	97%
CD10	3%
CD45RA	89%
CD25	8%
CD23	99%
CD30	98%
κ/λ	negative/positive

Table 2. IC_{50} of immunotoxins (molar concentration as RIP) and controls evaluated by protein synthesis on D430B cells.

CD30-saporin	2.3×10^{-13}
CD30-momordin	2.6×10^{-13}
CD30-ricin A-chain	3.0×10^{-13}
CD30 + saporin	3.9×10^{-9}
CD30 + momordin	$> 10^{-8}$
CD30 + ricin A-chain	$> 10^{-8}$
CD30	$> 10^{-8}$
saporin	7.7×10^{-9}
momordin	$> 10^{-8}$
ricin-A chain	$> 10^{-8}$

showed a monoclonal rearrangement for heavy chain genes (data not shown).

D430B cell line karyotype

An abnormal karyotype was observed in the D430B cell line, analyzed after six months of culture. The interpretation of 15 representative metaphases was as follows: 11 cells showed a consistently hyperdiploid karyotype (49,XY), and the additional chromosomes were 5, 12 and 19. In one cell the metaphase was similar (48,XY,+5,+12) Two metaphases showed a karyotype 46XY,-16,+der(16). The last one was normal. Tumor masses obtained from the growth of D430B cells in SCID mice also showed an abnormal karyotype closely resembling that of the parental cell line. Thirteen out of the 15 metaphases analyzed showed a clonal karyotype 46XY,-16, +der(16), while 2 metaphases were hyperdiploid: 48XY, +5,+12. Der(16) was interpretated as a translocation between 11q13 and 16p13 bands: der(16)t(11;16) (q13;p13), with a trisomy of 11q13-> 11qter chromosomal segment.

Analysis of EBV

The results of the western blot showed that D430B cells express LMP and all the EBNA antigens (EBNA1, EBNA2, EBNA-LP, EBNA3a, EBNA3b and EBNA3c). In addition the *EBNA fingerprint* (i.e. the characteristic pattern of EBNA sizes which differs from one virus to another) closely resembled that of the marmoset cell line B95.8 used as positive control, the culture supernatant of which is routinely used for the *in vitro* generation of EBV-infected B-LCLs (data not shown). PCR detection of EBV showed a positive reaction, with a pattern closely resembling that obtained from the SL–1 cell line (Figure 2).

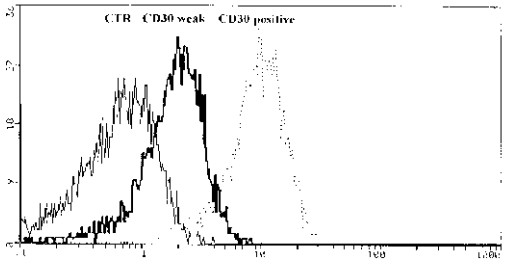

Figure 1. Different expression of CD30-related antigen in *in vitro* growing D430B cells (CD30 positive) and in D430B cells obtained from mice which were resistant to anti-CD30-saporin treatment (CD30 weak).

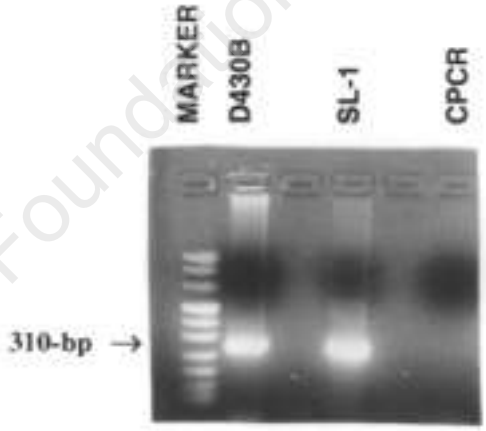

Figure 2. PCR detection of EBV genome in the DNA of D430B cell line. The figure represents the ethidium bromide-stained gel of amplification products. Lane 2 and 4 (left to right) show the amplified EBV BAM M genomic region obtained from D430B and SL-1 cell lines, respectively. Lane 6 (CPCR) is a negative control (mixture of PCR reaction without DNA). Lane 1 contains weight markers.

In vitro sensitivity of D430B cells to CD30 immunotoxins

D430B cells were tested with different immunotoxins containing the Ber-H2 (anti-CD30) antibody conjugated to different toxic moieties (Ber-H2-sapor-

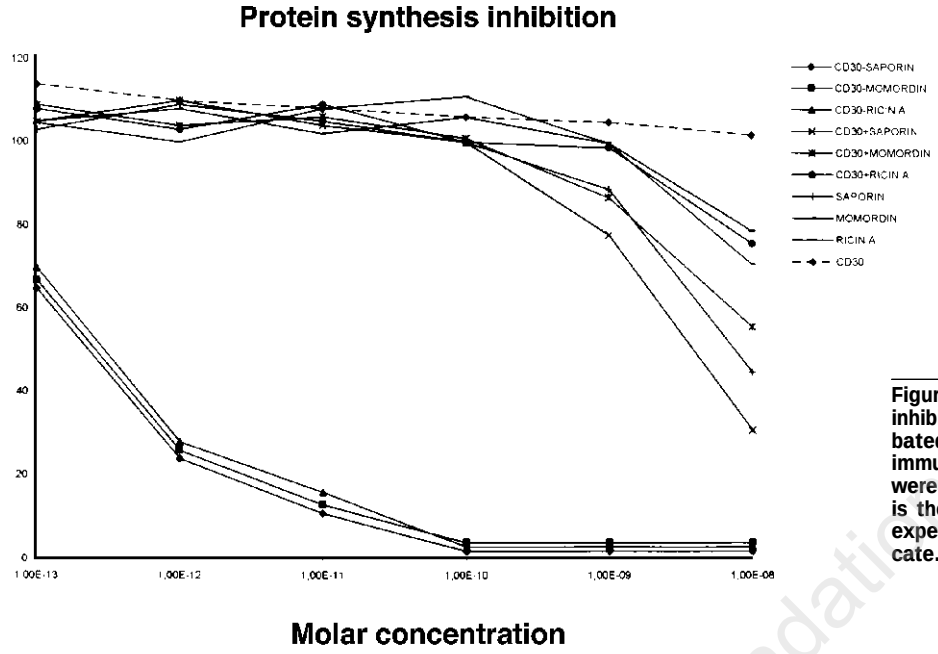

Figure 3. Protein synthesis inhibition in D430B cells incubated with Ber-H2 containing immunotoxins. Control values were 23,456 cpm. Each value is the mean of three different experiments, seeded in triplicate.

in, Ber-H2-momordin, Ber-H2-ricin A chain). All the immunotoxins were tested at final concentrations ranging from 10^{-13} M to 10^{-8} M, as shown in Figure 3. A high specific activity in inhibiting the protein synthesis by D430B cells was detected for all the immunotoxins used with IC_{50} (Table 2) in the range of 10^{-12} M (as RIPs or ricin A-chain). No toxicity was observed in control experiments run as described in the *Design and Methods* section (Figure 3 and Table 2).

In vivo growth of D430B cell line

All mice injected i.p. with 3×10^7 and 5×10^7 D430B cells developed tumor masses within 15 days. Abdominal distension was the first sign indicating tumor growth. The *post-mortem* examination revealed firm patchy white masses throughout the omentum and on the outer surface of the small bowel and of the colon. Spleen, liver and pancreas were often macroscopically infiltrated.

Histology and immunohistology of the tumors

At light microscopy, all samples from the pancreas, spleen and liver showed diffuse infiltration by mononuclear or multinucleated large cells with a prominent nucleolus and a moderate rim of cytoplasm, which turned grey-violet at Giemsa staining. Mitotic figures were numerous. Immunohistochemistry revealed strong reactivity of the large cells with the mAb Ber-H2 (CD30) in the form of either diffuse cytoplasmic staining or membrane-bound positivity (Figure 4), thus demonstrating that $CD30^+$ tumors with the morphology of an ALCL could be obtained from the D430B cells.

In vivo antitumor activity of Ber-H2-saporin immunotoxin

The untreated six mice which represented the control group developed tumor masses within 15 days and died within the 18th day. The post-mortem examination of these untreated SCID mice revealed a picture similar to that described above.

Treatment with a single dose of Ber-H2-saporin 48 hours after injection of D430B cells, completely inhibited the development of tumors in 4 out of 6 SCID mice. Ninety days after tumor challenge these mice were euthanasized and subjected to *post-mortem* exam-

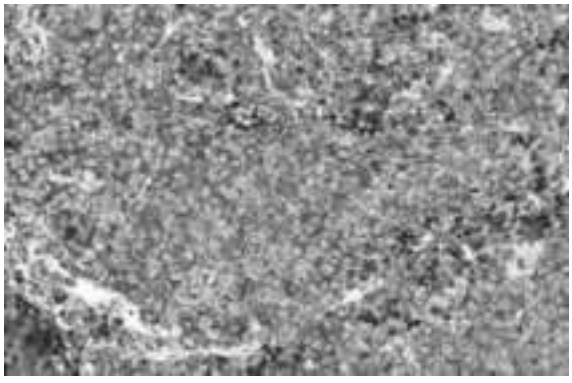

Figure 4. D430B cells injected into SCID mice gave rise to lymphoid tumors with the appearance of ALCL. The immunohistochemical staining by Ber-H2 mAb shows strong expression of the CD30 molecule of the tumor, infiltrating mouse pancreatic tissue.

ination. No neoplastic lesions were observed, thus providing a demonstration of the *in vivo* efficacy of the treatment with the CD30-saporin immunotoxin.

Two treated mice showed only a delay in the growth of the tumor, developing visible abdominal tumor masses at day +68 and +73 respectively, and after 10 more days they were euthanasized. At autopsy, the picture closely matched that of the untreated mice with firm and patchy white masses throughout the abdomen and omentum. It is worth noting that in these last two cases the cells obtained from the tumors and regrown in culture showed weaker expression of the CD30 antigen in comparison to both the D430B cells injected and those regrown from the tumors obtained in the untreated mice (Figure 1). This may suggest that a few cells with a lower expression of CD30 might be resistant to the immunotoxin treatment.

Discussion

In this study we describe a monoclonal EBV-infected B cell line (D430B) expressing the CD30 antigen and capable of growing in SCID mice developing solid tumors with the morphology of CD30$^+$ ALCL lymphoma. Through the use of D430B cell line we could also establish an animal model to assess the *in vivo* anti-CD30 (Ber-H2) immunotoxin treatment of human CD30$^+$ EBV-positive tumors with a B-cell phenotype.

The conditions through which the D430B cell line was raised are quite intriguing, since the starting cell culture was derived from irradiation-resistant cells used as a feeder layer. A similar cell line, EBV-infected and with chromosomal abnormalities, was previously described, but it was not able to give rise to tumors with the morphological features of ALCL and the presence of the CD30 antigen was not evaluated.[24]

The presence of EBV might be regarded as a possible peculiar characteristic of D430B cells. In fact a few ALCL cases, especially of B-cell origin, have been reported as EBV-positive[31] thus suggesting that EBV may contribute to the neoplastic transformation of at least a category of ALCLs.[32]

Another interesting feature of the D430B cell line is the monoclonal rearrangement of both the alleles of the JH genomic segment and the restriction for λ light chain in the final culture. Most LCLs, in fact, show a polyclonal rearrangement pattern.[24] It is worth noting that the monoclonal rearrangement of D430B cell line, together with the λ-chain restriction, was present in the starting bulk culture, shortly after the irradiation (7 days), as well as in the final culture, after the limiting dilution. Thus it is possible that D430B cells were derived from a limited monoclonal cell population and that the irradiation may have induced selection of a resistant subpopulation with peculiar features such as monoclonality and easier growth. Furthermore, in comparison to a large panel of conventional EBV-LCLs, the D430B cell line showed very strong and stable expression of the CD30 antigen, thus providing a good *in vitro* target for anti-CD30 immunotoxins.

No phenotypic differences were observed between the cells of the parental line and those recovered from the tumors obtained in untreated mice, once regrown *in vitro*: this finding could demonstrate the stability of the phenotypic features of these cells. The staining of paraffin-embedded tumor tissues also demonstrated strong expression of the CD30 antigen. High levels of CD30 membrane expression are required to allow the maximum effect in inhibiting tumor growth by targeting CD30-related protein.

A previous study by Pasqualucci *et al*.[33] showed a strong toxicity of an anti-CD30 immunotoxin (Ber-H2-saporin) on tumors derived in SCID mice from a human ALCL line which, at difference to the D430B cell line, show a T-cell phenotype (JB6).[33] It is worth noting that our B-cell line, D430B, was different from JB6 not only in terms of phenotype but also of karyotypic abnormalities. In contrast to JB6, showing the distinctive t(2,5)(p23;q35) translocation typical of ALCL of T-cell origin, D430B was characterized by trisomies 5 and 12, already reported in mutated karyotypes from CD30$^+$ lymphomas.[34] Trisomy 12 has also been reported as frequently associated with 30-60% of advanced atypical B-CLL cases.[35] An additional +12 chromosome has been observed in ALC lymphomas derived from the injection of lymphocyte suspension from HD patients' lymph nodes in SCID mice.[36] Moreover, the genetic instability of lymphocytic cells is a well-known phenomenon in HD34. The cytogenetic analysis of the *in vivo* tumoral D430B cells showed a clonal translocation t(11,16) in most of the metaphases studied and this mutation has been already described in a B-cell derived Ki-1 cell lymphoma.[37] The 11q13 represents the locus of the BCL-1 oncogene and is frequently involved in hematologic malignancies such as multiple myeloma[38] and mantle cell lymphomas.[39]

Based on the successful *in vivo* use of the Ber-H2-saporin in a pilot study conducted on patients with HD resistant to conventional radio/chemotherapy,[22] we designed a protocol to treat SCID mice developing CD30$^+$ EBV-positive B-cell tumors with the same immunotoxin. The immunotoxin displayed a potent antitumor activity: a single injection of Ber-H2-saporin in SCID mice xenografted with D430B cells led to the survival of four out of six mice and slowed down the growth of tumor masses in the other two mice. In these latter mice, the cells in the tumor masses, which developed 67 and 73 days after i.p. injection, showed weaker CD30 expression. These data suggest that (i) immunotoxins should be tested, prior to therapy, on the tumor cells of the patient to be treated, and (ii) confirm that an immunotoxin-based therapy, to be efficient, should be designed on the basis of two or more conjugates directed to different tumor-associated antigens to avoid the selection of cell populations with different antigenic mosaics.

Contribution and Acknowledgments

PLT, DdT and AB designed the experiments and drafted the article; NT, SR and GR performed the experiments; SP, HS, MG and FS supervised the work, revised the article and gave final approval.

Funding

This work was supported by the University of Bologna, funds for selected research topics, the Associazione Italiana per la Ricerca sul Cancro, Milan and by the Ministero dell'Università e della Ricerca Scientifica, Rome, Italy.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received March 23, 1999; accepted July 9, 1999.

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