Suitability of Epstein–Barr virus-based episomal vectors for expression of cytokine genes in human lymphoma cells

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Plasmids carrying the Epstein–Barr virus (EBV) latent gene EBNA1 and the EBV latent origin of replication (oriP) stay in transfected human cells as autonomously replicating extrachromosomal genetic units. They thus might represent a suitable tool for cytokine gene introduction into human tumor cells with the prospect of therapeutic antitumor vaccination. The aim of this study was to analyze whether such plasmids permit stable and efficient expression of cytokine genes in human non-Hodgkin lymphoma cells. We tested physical stability and expression levels of plasmids carrying EBNA1 and oriP for episomal maintenance, immunoglobulin light chain enhancer elements for augmentation of expression, and cytokine or marker genes after introduction into human NHL cell lines in vitro and in vivo after inoculation into nude mice. Data

obtained with these EBV-based vectors were compared with another plasmid, not carrying EBNA1 and oriP. cDNAs coding for GM-CSF, IL6, TNF α , the chloramphenicolacetyltransferase (CAT) and the β -galactosidase (lacZ) gene were transfected into the EBV-positive Burkitt's lymphoma cell line BL60 and the EBV-negative B cell lymphoma cell line BJA-B. EBV-derived vectors permitted a high, host cell independent transfection efficiency and high and host cell independent levels of expression. After removal of the selection pressure (hygromycin B) cytokine expression could be detected for several weeks in vitro and in vivo but, however, declined continuously. These experiments suggest that episomal BC-derived vectors represent an effective tool for cytokine gene transfer in human lymphoma cells.

Keywords: cytokine gene transfection; episomally replicating plasmids; EBV latent origin of replication; malignant lymphoma

Introduction

Induction of an antitumor host response by vaccination with genetically modified, cytokine secreting autologous tumor cells has been demonstrated in several murine tumor models. In these studies mice were vaccinated, for instance, with interferon gamma (IFN γ)-transduced neuroblastoma cells,¹ interleukin 2 (IL2)-transduced fibrosarcoma cells² or IL2-transduced colon carcinoma cells.³ Other vaccination approaches included transduction of malignant cells with cDNA coding for granulocyte-macrophage colony-stimulating factor (GM-CSF), IL4, IL7, IL8 or tumor necrosis factor alpha (TNF α).⁴ Cytokine expression in tumor cells was shown to activate MHC-restricted cytotoxic T cells, lymphokine-activated killer (LAK) cells, natural killer (NK) cells and macrophages.⁵ These promising animal studies have now initiated several clinical phase I/II trials using solid

tumor cells transduced with retroviral constructs expressing human cytokine genes.⁶

Malignant lymphomas also represent attractive candidates for cytokine transfer-mediated immune therapy. Immunoglobulin (Ig) gene idiotypes expressed on B lymphoma cells are tumor-specific antigens and thus represent individual targets for specific immune therapy. In mice a humoral as well as a cellular immune response have been demonstrated against Ig idiotypes. Furthermore, idotype immunization led to tumor regression of murine B cell lymphoma.⁷ This effect could be augmented by systemic administration of IL2 and IFN γ .⁸ In the murine model the idiotypes themselves have only a weak immunogenic potential. A pronounced augmentation of idotype immunogenicity, was achieved by use of idotype/GM-CSF fusion proteins.⁹ Yet, no data exist with regard to augmentation of an immune response following cytokine gene transfection into human lymphoma cells.

The most widely used gene transfer systems are replication-incompetent retroviruses permitting a high transduction efficiency. Retroviral gene transfer, however, involves some still unsolved problems. Owing to integration of the vector into the host cell genome, expression

of transduced genes may vary with the integration site and cell type. In addition, insertional mutagenesis represents a possible hazard.¹⁰ Thus, alternative viral and nonviral gene delivery and expression systems are being explored.

Recently, infectious EBV-based vectors (mini-EBVs) were engineered to transfer genes into human B cells.¹¹ These infectious mini-EBVs carried *cis*-acting elements of EBV necessary for episomal maintenance, amplification and packaging of the vector, not however, any of the known latent EBV genes associated with transformation. Successful infection of B lymphoblastoid cells from a Fanconi anemia group C (FA-C) patient with a mini-EBV vector expressing the normal FACC cDNA could be demonstrated. Compared with retrovirus-mediated gene transfer, these mini-EBVs had the advantage of replicating episomally in the target cells, thus avoiding the possible hazards of integration into the host cell genome. However, there might still be concerns about the incalculable hazards of working with infectious particles. This concern might especially hold true for the cloning of cytokine genes into such vectors, which then might exert pleiotropic growth promoting and growth inhibiting effects.

For *ex vivo* cytokine transfer in lymphoma (and other tumor) cells it would be desirable to use potent expression vectors, which have the advantage of episomal replication, but do not represent infectious particles. In this study we have tested cytokine gene introduction and expression in human lymphoma cell lines using plasmids containing the Epstein-Barr virus nuclear antigen 1 (EBNA1) gene and the viral latent origin of replication (oriP) permitting the episomal state of these plasmids and in addition immunoglobulin kappa light chain enhancer elements for augmentation of expression. Results with these episomal vectors were systematically compared with results obtained with a vector not carrying these EBV and Ig kappa light chain gene-derived genomic elements.

Results

Construction of expression vectors

The expression vectors pKEX-CAT, pKEX-TNF α and pKEX-IL6 were obtained by cloning cDNAs for the CAT gene, TNF α and IL6 into the pKEX-2XR-plasmid. The expression vectors BC219-IL6, BC219-TNF α and BC219-GM-CSF were obtained by cloning the cDNAs into the plasmid BC219, which replicates episomally after transfection due to the presence of the EBV nuclear antigen 1 (EBNA1) and the EBV latent origin of replication (oriP). Table 1 summarizes the vectors constructed. Figure 1

shows the maps of the basic vectors. For detailed cloning strategy see Materials and methods.

Transfection of BL60-P7 and BJA-B cells with pKEX-2XR- and BC219-derived expression vectors

pKEX-CAT, pKEX-IL6 and pKEX-TNF α were transfected into the Burkitt's lymphoma cell line BL60-P7 and the EBV-negative B cell lymphoma cell line BJA-B using electroporation. The number of growing, hygromycin B-resistant clones was determined 3 weeks after electroporation. The stable transfection efficiency was defined as the ratio of hygromycin B-resistant clones to viable cells after electroporation. For each cell line and vector type transfection efficiencies were rather similar, regardless of the given insert. Using the pKEX constructs, average transfection efficiency was approximately 1 to 7.1×10^4 for BJA-B cells and 1 to 2.5×10^6 for BL60-P7 cells.

The BC219-IL6, BC219-TNF α , BC219-GM-CSF, BC230a (=BC219-CAT) and BC-DZ17 (=BC219-*lacZ*) were transfected into BL60-P7 and BJA-B cells by the same procedure. For these vectors the average transfection efficiency was approximately 1 to 2.4×10^4 for BJA-B cells and 1 to 2.7×10^4 for BL60-P7 cells. Thus, by comparison with pKEX-2XR, BC219-derived vectors show a 3.2-fold higher transfection efficiency in BJA-B cells and a 92-fold higher transfection efficiency in BL60-P7 cells (see also Table 2).

Southern blotting was performed to determine the physical state of the transfected pKEX-plasmids in hygromycin B-resistant BL60-P7 and BJA-B transfectants. Cellular DNA from four representative BL60-pKEX-CAT transfectants was digested with *Bam*HI and hybridized with the ³²P-labeled pKEX-CAT DNA (Figure 2a). *Bam*HI digestion of pKEX-CAT plasmid results in two fragments of 6.0 and 1.6 kb length, respectively. Thus, after integration of the pKEX-CAT plasmid into the BL60 genome in Southern blot analysis, three *Bam*HI fragments are expected. The total length of these fragments should be at least 7.6 kb (plus the length of adjacent cellular fragments). In all four BL60-pKEX-CAT clones three fragments were detected. The upper band of 5.8 kb size is due to hybridization of the pKEX-CAT plasmid to the pSV2neo-plasmid¹² which had been transfected into this cell line previously.¹³ The additional two bands which differ in size in all transfectants are interpreted to represent integrated pKEX-CAT sequences joined to BL60-P7 sequences. In all four clones the detected pKEX-CAT fragments are shorter than the total length of 7.6 kb of the pKEX-CAT plasmid. This may be due to a partial deletion of the plasmid during the integration process into the cellular genome. This might underlie the non-expression of CAT protein of these transfectants (see below) since promoter-CAT gene sequences could be affected by the deletion.

Southern blot analysis was also performed to determine the physical state of the transfected vectors in the BJA-B and BL60-P7-BC transfectants. Figure 2b shows DNA of three BJA-B-BC230a and three BL60-BC230a transfectants which were *Bam*HI digested and hybridized with the ³²P-labeled BC230a plasmid. In all BJA-B and BL60 clones exclusively the expected two fragments of 12.9 and 2.0 kb, respectively, were found to be generated by digestion of the BC230a plasmid with *Bam*HI demonstrating the episomal state of the vector. The additional band in all BL60-P7 clones of 5.8 kb is generated from

Table 1 Vectors constructed for CAT, *lacZ* and cytokine expression

Nonepisomal vectors (pKEX-2XR-derived) ^a	Episomal vectors (BC219-derived) ^a
pKEX-CAT	BC230a (=BC219-CAT)
pKEX-IL6	BC219-IL6
pKEX-TNF α	BC219-TNF α
	BC219-GM-CSF
	BC219-DZ17 (=lacZ)

^aFor detailed map see Figure 1a and b.

Figure 1 (a) Map of pKEX-2XR. CMV: human cytomegalovirus immediate-early promoter-enhancer element; splice and PA: simian virus 40 (SV40)-derived small T-antigen splice and late gene polyadenylation signal; TKp: thymidine kinase promoter; hygR: hygromycin B resistance gene; ampR: ampicillin resistance gene.

cross-hybridization of the BC230a probe to the pSV2neo-plasmid (see above). Figure 2c shows Southern blot analysis of four BJA-B and BL60-BC-TNF α transfectants which were hybridized with a 32 P-labeled BC219 probe. Again an identical hybridization pattern is found in all clones, namely two bands of 12.9 and 2.0 kb which represent the *Bam*HI digested BC-TNF α plasmid. The 5.8 kb fragment in the BL60-clones is again generated by cross-hybridization with the pSV2neo-plasmid.

Expression of cytokines and CAT protein in BJA-B and BL60-P7 transfectants

For each pKEX-2XR-derived expression vector, namely pKEX-CAT, pKEX-IL6 and pKEX-TNF α , 10 stable BL60-P7 and 10 stable BJA-B transfectants were isolated. After 24 h culture in fresh medium, the cytokine concentration of 1×10^6 exponentially growing cells/ml culture medium and CAT protein in 50 μ g of total extracted cellular protein were measured. CAT expression of the BJA-B-pKEX-CAT clones reached from 90 to 2100 pg/ μ l. Cytokine concentration was measured from 8 to 700

pg/ml for the BJA-B-pKEX-IL6 and from 40 to 2050 pg/ml for the BJA-B-pKEX-TNF α clones. In contrast, no expression could be found in the BL60-P7 cells stably transfected with pKEX-2XR-derived expression vectors (Table 3). Cytokine and CAT expression was also measured for BL60-P7 and BJA-B clones stably transfected with BC219-derived vectors. In 10 out of 10 BJA-B and BL60-P7 clones, respectively, high expression levels for each transfected gene were found. IL6 expression ranged between 900 and 1210 pg/ml, TNF α expression between 1350 and 1900 pg/ml, GM-CSF expression between 650 and 655 pg/ml and CAT protein expression between 3800 and 5100 pg/ml (Table 3).

Taken together these data demonstrate that transfection with pKEX-2XR-derived expression vectors leads to quite variable expression patterns differing considerably between transfected clones of the same cell line as well as in between the cell lines BJA-B and BL60-P7, with no detectable expression in the latter. In contrast, transfection with the BC219 vector leads to transfectants with quite similar expression levels in all BJA-B and BL60-P7

b

Figure 1 (b) Map of BC219. MAR: matrix attachment region; Ei: intron enhancer element; E3: 3' enhancer element of the human Ig kappa light chain gene locus; EBNA1: Epstein-Barr virus nuclear antigen 1 gene; oriP: Epstein-Barr virus latent origin of replication.

clones. In addition, the absolute amounts of cytokines or CAT protein were higher in all BJA-B and BL60-P7 clones transfected with BC219-derived vectors compared to pKEX-2XR-derived transfectants with the exception of BJA-B-pKEX-TNF α clones with expression levels comparable to those of BJA-B-BC-TNF α clones.

LacZ expression in BL60-P7 and BJA-B transfectants

To analyze the expression status of the episomal vectors in single cells, one clone of each BL60-P7 and BJA-B transfected with BC-DZ17, containing the *lacZ* gene, were stained for *lacZ* expression in a β -gal-*in situ* assay. Blue staining of the cells indicates expression of the *lacZ* gene. As shown in Figure 3a, about 50% of the BL60-BC-DZ17 clone 1.3.5. expressed the *lacZ* gene with a varying intensity. In BJA-B-BC-DZ17 clone 4.3.5. about 80% of the cells were positive for *lacZ* expression (Figure 3b). The number of positive cells was constant in both clones over a cultivation period of 6 months after transfection using hygromycin B selection.

No changes of endogenous cytokine expression in BL60-P7 cells after transfection with BC219-derived vectors

Introduction of foreign DNA into eukaryotic cells may influence their endogenous gene expression pattern. To

analyze whether transfection of BL60-P7 cells with BC219 vectors leads to altered cytokine expression, BL60-BC219 transfectants were examined for their endogenous cytokine expression using ELISA. Untransfected BL60-P7 cells did not express the cytokines IL6, IL8, IL10 and TNF α . BL60-BC219 transfectant clones also did not express these cytokines (data not shown). Thus, transfection of BL60-P7 cells with BC219 vectors seems not to induce endogenous cytokine expression and the IL6 expression of BL60-BC-IL6 and TNF α expression of BL60-BC-TNF α transfectants, respectively, is due to expression of the transfected BC219-derived vector.

Stability of expression of pKEX-2XR- and BC219-derived expression vectors in vitro and in vivo

To analyze whether release of selection pressure leads to loss of the vectors transfected, one BJA-B-pKEX-TNF α clone and one BJA-B-pKEX-IL6 clone were cultivated without hygromycin B selection. TNF α and IL6 expression were measured over a period of 8 weeks in the supernatant of continuously proliferating cells. The BJA-B-pKEX-IL6 clone 1.8.6. expressed 250 pg/ml/24 h IL6 4 weeks after transfection. No significant decrease of expression could be found in the culture without hygromycin B after 36 weeks when IL6 expression was still 210 pg/ml/24 h. Similarly, TNF α expression of BJA-B-pKEX-

Table 2 Transfection efficiencies of pKEX-2XR and BC219 plasmids after electroporation of BL60 P7 and BJA-B cells

Transfected cell line	Viable cells seeded after electroporation	Number of stable transfected clones	Transfection efficiency
BJA-B-pKEX-2XR	1.2×10^7	196	$1/6 \times 10^4$
BJA-B-pKEX-IL6	9.5×10^6	101	$1/9.3 \times 10^3$
BJA-B-pKEX-TNF	1.1×10^7	178	$1/6.1 \times 10^4$
BJA-B-pKEXCAT	7.5×10^6	107	$1/7.0 \times 10^4$
BL60-pKEX-2XR	9.8×10^6	3	$1/3.3 \times 10^6$
BL60-pKEX-IL6	5×10^6	2	$1/2.5 \times 10^6$
BL60-pKE X-TNF	7.5×10^6	3	$1/2.5 \times 10^6$
BL60-pKEX-CAT	9.6×10^6	5	$1/1.9 \times 10^6$
BJA-B-BC219	1×10^7	800	$1/1.3 \times 10^4$
BJA-B-BC-IL6	4.5×10^6	225	$1/2.0 \times 10^4$
BJA-B-BC-TNF α	8.4×10^6	210	$1/1.0 \times 10^4$
BJA-B-BC-GM-CSF	3.5×10^6	163	$1/2.1 \times 10^4$
BJA-B-BC230a	1.1×10^6	135	$1/8.1 \times 10^3$
BJA-B-BC-DZ17	5.4×10^6	432	$1/1.3 \times 10^4$
BL60-BC219	6.8×10^6	338	$1/2 \times 10^4$
BL60-BC-IL6	6.8×10^6	270	$1/2.5 \times 10^4$
BL60-BC-TNF α	1.3×10^7	990	$1/1.3 \times 10^4$
BL60-BC-GM-CSF	3×10^6	121	$1/2.4 \times 10^4$
BL60-BC230a	1.5×10^7	273	$1/5.5 \times 10^4$
BL60-BC-DZ17	6.5×10^6	258	$1/2.5 \times 10^4$

TNF α clone 2.2.5 did not change significantly after release of selection pressure. Taken together these data demonstrate a stable cytokine expression reflecting the stable integration of pKEX-2XR-derived expression vectors in BJA-B transfectants without hygromycin B selection.

The stability of cytokine expression of BL60-BC-IL6 transfectants was examined. One BL60-BC-IL6 clone was cultivated *in vitro* over a period of 9 weeks with and without hygromycin B selection and IL6 expression was measured weekly in the supernatant of continuously proliferating cells using ELISA. While IL6 expression remained stable in BL60-BC-IL6 cells cultivated with hygromycin B selection over the whole period, BL60-BC-IL6 transfectants cultivated without hygromycin B showed a decrease of IL6 expression. After 9 weeks cells expressed only 12.5 pg/ml IL6 in comparison to 456.5 pg/ml in the first week (Table 4). A similar IL6 expression pattern was found for BL60-BC-IL6 transfectants *in vivo*. Four nu/nu mice were inoculated with 1×10^7 BL60-BC-IL6 cells in each flank. After 2, 4 and 6 weeks, mice were killed, tumor cells were recultivated and IL6 expression was measured after 24 h. IL6 expression decreased from 212.6 pg/ml after 2 weeks to 94.3 pg/ml after 5½ weeks (Table 5). These data demonstrate that under *in vivo* conditions, without selection pressure, cytokine expression can be achieved only transiently with BC-derived expression vectors.

Discussion

The attraction of modified viruses for gene therapy is mainly based on their capacity to infect a high percentage of cells *in vitro* and *in vivo*. Thus, retroviruses, adenoviruses, adeno-associated viruses (AAV), herpesviruses and others have been modified as vectors for introduc-

tion of cDNAs in human tumor cells.^{14–19} However, the use of infectious viruses in gene therapy has also been described to be associated with disadvantages, eg insertional mutagenesis inducing secondary cancers,²⁰ recombination events leading to pathogenic infectious agents,²¹ induction of an immune response against the viral vectors²² and, last but not least, the complexity of the manufacturing procedures.

Noninfectious, plasmid-based expression vectors may circumvent several of these problems. These vectors can be introduced into the target cells, for instance by direct inoculation of the DNA (naked DNA approach), after mixture with polycationic lipids (liposome-mediated gene delivery) or by complexing with polycations such as poly-(L-lysine).^{23–27}

As a rule such plasmid vectors persist in the majority as episomal elements and permit short-term gene expression. A minority of the transfected plasmids, however, become integrated by chance into the host cell genome. In contrast, plasmids containing the EBV latent protein EBNA1 and in addition the viral latent origin of replication (oriP) are stably maintained after transfection as autonomously replicating episomes.²⁸ However, no data have been raised with regard to their long-term stability *in vivo*.

We were interested, whether such EBV-derived vectors might be suitable for cytokine gene expression in lymphoma cells with the prospect of vaccination therapy using cytokine-transfected lymphoma cells. Thus, we cloned the cDNAs for different human cytokines and for the marker genes CAT and *lacZ* into expression vectors carrying EBNA1, oriP and in addition human immunoglobulin kappa light chain enhancer elements (BC219-derived plasmids). After transfection into a human EBV-positive and an EBV-negative lymphoma cell line we tested stable transfection efficiency, expression level and

long-term expression *in vitro* and *in vivo* in a nude mouse model. The results were systematically compared with results obtained after transfection of plasmid vectors not carrying the EBV elements for episomal maintenance (pKEX-derived vectors)²⁹ into the same cell lines.

The episomal EBNA1/oriP vectors permitted in general a higher transfection efficiency compared with the pKEX-derived vectors, ie about a three-fold elevated number of stably transfected clones for cell line BJA-B and about 90-fold augmented for the BL60 cell line. These results reflected the stable, cell line-independent episomal maintenance of the EBNA1/oriP vectors during selection of hygromycin B-resistant clones after transfection. This was confirmed by Southern blot analysis which demonstrated the episomal state of the vectors without any evidence of integration into the host cell genome. By comparison, the lower rate of stable transfected cells using the pKEX-derived vectors reflected loss of episomal plasmid DNA shortly after transfection and the long-term persistence exclusively of vector DNA integrated into the host cell genome, as also demonstrated by Southern blot analysis.

Expression analysis after transfection of the EBNA1/oriP vectors revealed high cytokine concentrations in the supernatant of proliferating cells and high levels of intracellular CAT protein, respectively, in BL60 as well as in BJA-B cells. With the exception of TNF α , expression levels were higher with EBNA1/oriP vectors compared with pKEX-derived vectors. Moreover, and also in contrast to pKEX-derived vectors, with the EBNA1/oriP vectors expression levels of a given cytokine differed only slightly between different transfected clones of the same cell line as well as between the two cell lines tested. This observation confirmed the absence of position effects on gene expression due to vector integration when using the EBNA1/oriP vectors.

In contrast to the BJA-B cell line, BL60 cells transfected with the pKEX vectors did not express cytokines or the CAT gene at all. This observation was surprising, since integration of the pKEX vectors in the genome of BJA-B was demonstrated by Southern blot analysis. In addition, expression of transfected genes in BL60 cells using a pKEX vector has already been described.^{30,31} Possibly the integration process led to destruction of the vector. This was suggested by the results of Southern blot analysis, where the addition of the length of the integrated vector fragments did not result in the length of the whole plasmid.

Maintenance of expression of the EBNA1/oriP vectors was also analyzed without hygromycin B selection pressure. *In vitro* as well as in nude mouse tumors, cytokine expression could be measured over several weeks but, however, declined continuously. In the 4th week after removing hygromycin B the expression level of IL6 was approximately 50% of the initial level, and after about 2 more weeks approximately 25%. Thus, these vectors cannot be used for gene therapeutic strategies where long-term gene expression is essential, as for instance introduction of a multidrug resistance gene in hematopoietic stem cells or substitution of gene defects as in Gaucher's disease. However, for inducing an antitumor immune response by cytokine transfection of tumor cells they represent a suitable tool.

Recently, a vector has been described which also carries the EBV genomic elements EBNA1 and oriP to allow

stable, non-integrative maintenance. In addition, this vector carried EBV elements necessary for packaging, thus representing an infectious particle (mini-EBV).¹¹ The use of infectious particles includes risks difficult to estimate. Naturally, EBV infects B lymphocytes³² and epithelial cells.³³ It has also been found in T lymphocytes of patients with chronic EBV infection,³⁴ in parotid gland ducts,³⁵ in hairy leukoplakia of the tongue³⁶ and in smooth muscle tumors of immunosuppressed children.³⁷ Thus, a wide spectrum of cells might be infected when using such mini-EBVs for gene therapy. In addition, since over 90% of the general population harbor the virus, recombination events might occur generating a recombinant EBV carrying the 'therapeutic' genes. Considering the pleiotropic growth stimulating and activating effects of cytokines the use of such mini-EBVs for cytokine-mediated gene therapy might be dangerous.

In contrast, the vectors presented in this work, permit the introduction of cytokine genes in lymphoma cells as non-integrative, self-replicating, extrachromosomal genetic units. Thus, they avoid the hazards of integration into the host cell genome as well as those of using infectious particles. In addition, they are easy to handle and possess a high cloning capacity. Owing to their structural and functional similarity to the EBV genome these vectors are expected to be functional also, when they contain multiple inserts. Thus, bi- or polycistronic vectors can be constructed, eg vectors containing the cytokine cDNA together with a suicide gene (HSVtk) and/or the cDNA encoding for the second signal molecule B7. To restrict the expression of these vectors after transfection the CMV promoter could be replaced through cell-type-specific promoters. The p1-myc promoter for example would lead to expression exclusively in B cells.³⁸ Taken together, our experiments suggest that these BC219-derived vectors may represent an effective tool for cytokine gene transfer in human lymphoma cells.

Materials and methods

Basic expression vectors

Vector pKEX-2XR (Figure 1a) was described by Rittner *et al.*²⁹ The 6.8 kb plasmid carries the human cytomegalovirus immediate-early (HCMV-IE) promoter-enhancer element, the simian virus 40 (SV40)-derived small T-antigen splice and late region polyadenylation signal and the hygromycin B resistance gene. Vector BC219 (Figure 1b) was described by Polack *et al.*³⁹ Briefly, the 13.9 kb plasmid carries the EBV latent origin of replication (oriP), and EBNA1 leading to episomal persistence of the plasmid in eucaryotic cells. Beside the HCMV-IE promoter-enhancer it carries additional enhancer elements for increase of expression, namely the matrix attachment region (MAR), the intron and the 3' enhancer elements of the human Ig kappa light chain gene locus.³⁹ BC230a contains the chloramphenicolacetyltransferase (CAT) gene as a *Sfi*I fragment and BC-DZ17 contains the *lacZ* gene, cloned into the *Nae*I site of BC219.

Construction of expression vectors

cDNAs of the human cytokines IL6 and TNF α were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The CAT gene was kindly provided by M von Knebel-Döberitz (DKFZ Heidelberg, Germany).

Figure 2 (a) Southern blot analysis of BL60-pKEX-CAT-transfectants. Lane 1: BL60-pKEX-CAT clone 3.7.2.; lane 2: BL60-pKEX-CAT clone 3.6.7.; lane 3: BL60-pKEX-CAT clone 3.6.7.; lane 4: BL60-pKEX-CAT clone 3.6.2.; lane 5: BL60-P7. The genomic DNA of four BL60-pKEX-CAT clones was BamHI digested, blotted and hybridized with a 32 P-labeled pKEX-CAT-plasmid. The fragment of 5.8 kb size is due to cross-hybridization of pKEX-CAT to pSV2neo plasmid. The additional bands of different size in each BL60-pKEX-CAT clone represent integrated pKEX-CAT sequences joined to genomic BL60-P7 sequences. (b) Southern blot analysis of BJA-B and BL60-BC230a transfectants. Lane 1: BJA-B-BC230a clone 6.6.7.; lane 2: BJA-B-BC230a clone 6.10.2.; lane 3: BJA-B-BC230a clone 7.2.6.; lane 4: BL60-BC230a clone 6.9.4.; lane 5: BL60-BC230a clone 6.7.2.; lane 6: BL60-BC230a clone 6.2.2.; lane 7: BL60-P7. The genomic DNA of BJA-B and BL60-P7-BC230a clones was BamHI digested, blotted and hybridized with the 32 P-labeled BC230a plasmid. The two bands visible in all clones refer to the 12.7 and 2.0 kb fragments resulting after BamHI digestion of the episomal BC230a DNA. The 5.8 kb fragment results from cross-hybridization of BC230a plasmid with the pSV2neo-plasmid.

Standard cloning techniques were used as described.⁴⁰ For construction of pKEX-CAT the CAT coding sequence was isolated as a *HincII* fragment and cloned into the *SmaI* site of pKEX-2XR. For construction of pKEX-IL6 the IL6-cDNA was isolated as an *EcoRI*-*HindIII* fragment from the vector pGEMII-2.⁴¹ After blunt-end formation with the Klenow fragment of DNA polymerase I the fragment was inserted into the *SmaI* site of pKEX-2XR. For construction of pKEX-TNF α the TNF α cDNA was isolated as a *PstI* fragment of the vector pE4,⁴² cloned into the Bluescript vector pBS M13 KS (Stratagene, La Jolla, CA, USA), cut out as a *XhoI*-*XbaI* fragment and ligated to the *XhoI*-*XbaI* sites of pKEX-2XR. For cloning cytokine cDNAs into BC219, they were first cloned into pBS*, a variant of pBS M13 KS with a different polylinker site, namely *SacI*-*SfiI*-*PstI*-*EcoRI*-*XbaI*-*HindIII*-*XhoI*-*BamHI*-*SfiI*-*KpnI*. It was constructed to provide cytokine cDNAs with a *SfiI* site before cloning into BC219. BC-IL6 was constructed from the *EcoRI*-*HindIII*-IL6 cDNA

fragment, BC-GM-CSF from the *EcoRI*-cDNA⁴³ and BC-TNF α from the *PstI*-TNF α cDNA.⁴²

Plasmid preparation

For transfection, plasmids were amplified in *E. coli* strain Sure cells (Stratagene), isolated using the Ish Horowitz method⁴⁰ and purified by cesium chloride gradient centrifugation. The DNA was dialysed against 10 mM Tris, 1 mM EDTA, pH 8 (TE), ethanol-precipitated and resuspended in sterile water.

Cell lines

BL60-P7 is a neomycin-resistant and hypoxanthine-guanine-phosphoribosyltransferase (HGPRT)-deficient subline of the Burkitt's lymphoma cell line BL60.¹³ BJA-B is an EBV-negative B cell lymphoma cell line.⁴⁴ Cells were cultured in RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% heat-

Figure 2 (c) Southern blot analysis of BJA-B and BL60-BC-TNF α transfectants. Lane 1: BJA-B-BC-TNF α clone 6.7.8.; lane 2: BJA-B-BC-TNF α clone 7.1.2.; lane 3: BJA-B-BC-TNF α clone 6.2.6.; lane 4: BJA-B-BC-TNF α clone 7.2.1.; lane 5: BL60-BC-TNF α clone 6.9.2.; lane 6: BL60-BC-TNF α clone 7.11.3.; lane 7: BL60-BC-TNF α clone 6.2.7.; lane 8: BL60-BC-TNF α clone 6.7.5.; lane 9: BL60-P7; lane 10: BJA-B. The genomic DNA of BJA-B and BL60-P7-BC-TNF α clones was BamHI digested, blotted and hybridized with the 32 P-labeled BC219 plasmid. The two fragments detected of 12.9 and 2.0 kb size represent the BamHI digested BC-TNF α plasmid. The 5.8 kb fragment in the BL60-BC-TNF α clones is due to cross-hybridization of the BC219 plasmid with pSV2neo-plasmid.

Table 3 Secretion of cytokines and expression of CAT protein in BJA-B and BL60 P7 transfectants

Cytokine/marker gene	Target cell	Vector	Numbers of clones	Gene expression
CAT	BJA-B	mock	1	negative
		pKEX-CAT	10	90–2100 pg/ml
		BC230a	10	3800–5200 pg/ml
TNF α	BJA-B	mock	1	negative
		pKEX-TNF	9	40–2050 pg/ml
		BC219-TNF	10	500–2100 pg/ml
IL6	BJA-B	mock	1	negative
		pKEX-IL6	10	8–700 pg/ml
		BC219-IL6	10	900–1250 pg/ml
GM-CSF	BJA-B	mock	1	negative
		BC219-GM-CSF	10	655–680 pg/ml
CAT	BL60	mock	1	negative
		pKEX-CAT	5	negative
		BC230a	10	3800–5100 pg/ml
TNF α	BL60	mock	1	negative
		pKEX-TNF	9	negative
		BC219-TNF	10	1350–1900 pg/ml
IL6	BL60	mock	1	negative
		pKEX-IL6	5	negative
		BC219-IL6	10	900–1210 pg/ml
GM-CSF	BL60	mock	1	negative
		BC219-GM-CSF	10	650–655 pg/ml

The values given represent cytokine concentrations in the supernatant of the most productive and the less productive clone. Each value is a mean of two independent measured values. Measurements were performed using ELISA (Quantikine, Biermann, Germany).

Table 4 *In vitro* expression of BL60-BC-IL6 cells with and without hygromycin B selection

	W ₀	W ₁	W ₂	W ₃	W ₄	W ₅	W ₆	W ₇	W ₈	W ₉
IL6 + Hygro	456.5	456.5	399.9	454.3	470.2	448.8	459.6	461.3	469.7	440.0
IL6 – Hygro		444.3	358.6	367.7	222.0	52.6	71.5	69.8	17.6	12.5

W₀: IL6 concentration at the beginning of the experiment; W₁: IL6 concentration after 1 week; W₉: IL6 concentration after 9 weeks.

Table 5 IL6 expression of BL60-BC-IL6 cells after inoculation *in vivo*

Tumor size (mm)	t1: IL6 conc. before s.c. inoculation (pg/ml)	t2: IL6 conc. after 2 weeks (pg/ml)	t3: IL6 conc. after 4 weeks (pg/ml)	t4: IL6 conc. after 6 weeks (pg/ml)
0 × 0	456.5			
10 × 10 right		212.6		
0 × 0 left		0		
20 × 20 right			66.7	
20 × 20 left			133.0	
25 × 12 right				118.9
30 × 30 left				69.8

inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU penicilline and 100 µg streptomycin/ml.

Transfection

BL60-P7 and BJA-B cells were transfected using the electroporation method.⁴⁵ Briefly, 2×10^7 cells and 20 µg DNA in a total volume of 200 µl PBS were placed in an electroporation chamber with an electrode distance of 4 mm. A high voltage of 200 V (BJA-B), respectively 220 V (BL60-P7), was applied with an electropulsing device, capacitance 1050 microfarads (Easyject, Eurogentec, Belgium). After 5 min on ice, 15 ml of growth medium was added, and cells were incubated for 24 h. Then, cells were plated on 96-well microtiter plates at a concentration of 2×10^5 cells/µl for selection of hygromycin B-resistant clones (400 µg hygromycin/ml medium). Clones of transfected cells were identified 21 days after transfection and were transferred into 24-well microtiter plates.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of human cytokines IL6, TNFα and GM-CSF in cell culture supernatants were determined by a commercially available ELISA (Quantikine), distributed by Biermann, Bad Nauheim, Germany. Cells were plated 24 h before measurement in fresh medium at a concentration of 1×10^6 cells/µl without hygromycin B. ELISA was performed according to the manufacturer's instructions. The sensitivity thresholds were: 0.35 pg/ml (IL6), 0.17 pg/ml (TNFα) and 1.5 pg/ml (GM-CSF). CAT protein in cell lysates was measured with a CAT-ELISA (Boehringer-Mannheim, Mannheim, Germany) by using 50 µg of total protein extract.

Southern blotting

Extraction of total cellular DNA and restriction endonuclease digestion were performed using standard protocols.⁴⁰ After restriction enzyme digestion 10 µg of cleaved

a

b

Figure 3 β-Gal assay of BJAB-BC-DZ17 and BL60-BC-DZ17 transfectants. (a) BL60-BC-DZ17 clone 1.3.5.: 50% of the transfected cells show lacZ expression. (b) BJA-B-BC-DZ17 clone 4.3.5.: 80% of the transfected cells show lacZ expression.

cellular DNA were separated by agarose gel electrophoresis and transferred on to a nylon filter (Gene Screen Plus; NEN, Boston, MA, USA). Hybridization was performed in 50% formamide, $2 \times \text{SSC}$ at 42°C with ^{32}P -labeled DNA probes.⁴⁶ The following probes were used: the pKEX-CAT (Figure 2a), the BC230a plasmid (Figure 2b) and the BC219 plasmid (Figure 2c).

Tumorigenicity assays

Untreated, 4-week-old female nu/nu mice (Swiss background, purchased from Charles River, Wilmington, MA, USA) were inoculated with exponentially growing cells (with a viability of at least 90%). In 0.1 ml RPMI 1640 1×10^7 cells were suspended and subsequently inoculated under the skin of both flanks of untreated, 4-week-old female nu/nu mice. The animals were examined twice weekly and the maximal and minimal diameter of each tumor was measured.

β -Galactosidase (β -gal) assay

For the β -gal assay exponentially growing cells were centrifuged, washed once with PBS, resuspended to a concentration of 2×10^5 cells/ μl and transferred on to adhesion slides (Biorad, Munich, Germany). After 10 min, cells were fixed in formaldehyde (3.7%)-PBS, and incubated with an X-gal solution (0.1 M sodium phosphate buffer, pH 7.6, containing 2.5% (v/v) of 40 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Stratagene) solution in DMSO, 5 mM of both potassium ferri-cyanide and potassium ferrocyanide, 2 mM MgCl_2 at 37°C for 2–24 h. The β -gal reaction was stopped by washing with 0.1 M phosphate buffer.

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