

Expression of the APO-1 Antigen in Burkitt Lymphoma Cell Lines Correlates With a Shift Towards a Lymphoblastoid Phenotype

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APO-1 is a cell surface molecule that induces apoptosis when ligated with the monoclonal antibody anti-APO-1. Expression of APO-1 and response to anti-APO-1 was investigated in a number of Epstein-Barr virus (EBV)-positive and -negative Burkitt lymphoma (BL) cell lines, in EBV-immortalized lymphoblastoid cell lines, and in cells from fresh BL biopsies. APO-1 was not expressed in EBV-negative cell lines and in EBV-positive BL cell lines with a phenotype corresponding to BL tumor biopsy cells (CD10⁺, CD21⁻, CD23⁻, CD30⁻, CD39⁻, CDw70⁻, CD77⁺). Accordingly, fresh BL cells obtained from three BL biopsies were APO-1 negative. EBV-positive BL cell lines that had acquired a lymphoblastoid phenotype (CD10⁻, CD21⁺, CD23⁺, CD30⁺, CD39⁺, CDw70⁺, CD77⁻) upon prolonged in vitro cultivation, as well as normal B-lymphoblas-

toid cell lines, expressed a high density of APO-1. APO-1 may, therefore, be regarded as a B-cell activation marker. APO-1 expression is not the only prerequisite for anti-APO-1-induced apoptosis because 6 of 7 APO-1-expressing EBV-positive BL cell lines were not sensitive to anti-APO-1, whereas all lymphoblastoid cell lines were killed by anti-APO-1. The sensitivity of lymphoblastoid cell lines to anti-APO-1-mediated apoptosis may open a new therapeutic approach for the treatment of EBV-induced lymphoproliferative lesions in immunocompromised individuals, because these are composed of cells with a lymphoblastoid phenotype.

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THE SIZE OF A GIVEN cell compartment, eg, the B-cell pool, is determined by the number of cells entering and leaving the system per unit time. In mice, about 10 million B cells, a number representing about one-tenth of the peripheral B-cell pool, are generated every day and migrate into the peripheral lymphoid organs.¹ To maintain a dynamic equilibrium, an equal number of cells has to be removed from the system. This elimination is so far only poorly understood. Recently, Liu et al² provided evidence that the elimination of B cells takes place in the germinal centers of lymphoid follicles. These investigators showed that germinal center B cells, in contrast to other B cells, undergo apoptosis, a physiologic form of programmed cell death, unless they are stimulated via their antigen receptor and the CD40 molecule. High-affinity binding of surface Ig to antigen in the germinal center can therefore rescue specific B-cell clones from undergoing programmed cell death. Because most clones do not receive such an

activation signal, apoptosis represents an important mechanism for the elimination of cells in vivo.

Another system has recently been described that shows that B cells enter apoptosis not only when a positive signal is missing but also when the APO-1 molecule on the surface is crosslinked by the monoclonal antibody (MoAb) anti-APO-1. The APO-1 antigen is a membrane antigen of approximately 50 Kd and is expressed on a number of cell lines and on activated T and B cells.^{3,4} Recent cloning and sequencing of a cDNA coding for APO-1 has shown its identity to the Fas antigen.^{4a,5,6} APO-1/Fas is thus a novel cell surface receptor belonging to the family of receptors that includes nerve growth factor receptor, the B-cell antigen CD40, and the tumor necrosis factor (TNF) receptors.⁶

Two models may account for APO-1-mediated apoptosis.⁴ APO-1 may represent a receptor that delivers an active apoptosis signal and may thus be involved in controlling the elimination of B cells and other APO-1-expressing cells. Alternatively, APO-1 may represent a growth factor receptor for a ligand that is essential for survival, and binding of the MoAb may prevent the ligand from binding to its receptor.

Burkitt's lymphoma (BL) is a human B-cell malignancy with characteristic epidemiologic features. The tumor is frequent in children in equatorial Africa and New Guinea and occurs with a lesser incidence all over the world. Almost all tumors from high incidence areas are Epstein-Barr virus (EBV)-positive, whereas only about 15% of tumors from low-incidence areas are associated with EBV.⁷ EBV-positive and -negative tumors have an identical phenotype in vivo with respect to CD10⁺, CD21⁻, CD23⁻, CD30⁻, CD39⁻, CD70⁻, CD77⁺ (BL group I phenotype; see Table 1). Cells with this phenotype are also found in the germinal centers of normal lymphoid tissue, suggesting that BL cells represent the malignant counterpart of this cell population.⁸

From BL cases, cell lines can be established in vitro with a relatively high success rate. Usually EBV-negative cases give rise to cell lines that grow in single cell suspension and whose in vitro phenotype closely resembles that of the

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Table 1. Phenotype and Viral Gene Expression of BL Group I and Group III Cell Lines

	BL Group I	BL Group III
Cellular antigens		
CD10	+	—
CD21	—	+
CD23	—	+
CD30	—	+
CD39	—	+
CDw70	—	+
CD77	+	—
EBV antigens		
EBNA1	+	+
EBNA2	—	+
EBNA3a,b,c	—	+
EBNA-LP	—	+
LMP	—	+
TP1	—	+
TP2	—	+

BL tumor cells *in vivo* display the same phenotype and pattern of viral gene expression as BL group I cells. The phenotype of group III BL cell lines is identical to that of LCLs. Data are from Rowe et al¹¹ and Gregory et al.¹⁴

tumor cells *in vivo*. EBV-negative cell lines are normally quite fragile in the initial phase in culture, but may be maintained on a feeder layer of irradiated fibroblasts.⁹ On the contrary, EBV-positive BL cases often give rise to cell lines with a less stable phenotype and growth pattern. Initially, they also grow in single cell suspension and exhibit the *in vivo* phenotype (BL group I phenotype); however, after several passages the cells tend to grow in clumps and acquire the same phenotype as EBV-immortalized normal lymphocytes, so called lymphoblastoid cell lines (BL group III phenotype; see Table 1).^{10,11} The change in growth pattern and phenotype is caused by an activation of EBV latent genes.^{11,12} In cells with group III phenotype, at least six nuclear viral antigens (EBNA1, 2, 3a, 3b, 3c, EBNA-LP) and three membrane antigens are expressed (LMP, TP1, TP2), whereas group I cells express only the EBNA1 antigen.

EBV-negative and EBV-positive BL cell lines that have maintained the group I phenotype *in vitro* grow rapidly under optimal growth conditions. They are, however, very sensitive to the withdrawal of autocrine or exogenous growth factors and readily undergo apoptosis, whereas EBV-positive BL group III cell lines tend to be resistant¹³ (M. Falk, unpublished observation). We were interested to see whether the susceptibility versus resistance of BL cells to apoptosis is related to the presence or absence of the APO-1 antigen. Therefore, we investigated the expression of APO-1 in a number of representative BL cell lines, as well as on fresh BL cell biopsies and normal EBV-immortalized lymphoblastoid cell lines.

MATERIALS AND METHODS

Cell lines and cell culture. Most of the cell lines used in this study have been established in the laboratories of the investigators.^{9,12} Establishment of the EBV-positive MUTU-BL subclones

and of EBV-converted sublines from EBV-negative BL cell lines have been described.^{14,15} Phenotypic grouping of BL cell lines and lymphoblastoid cell lines (LCL) has been reported elsewhere.¹⁰ All cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 µg/mL penicillin, 50 µg/mL streptomycin, 1 mg/mL amphotericin B, and were free of mycoplasma as judged by hybridization with a probe specific for mycoplasma microsomal RNA. Cells were kept at 37°C in a humidified atmosphere with 5% CO₂.

Staining with anti-APO-1. For indirect immunofluorescence for APO-1, 10⁶ cells were washed twice, resuspended in 0.1 mL of 1 µg/mL anti-APO-1 (IgG_{3,κ}) or FII23 (an IgG_{3,κ} nonbinding control antibody), incubated in 0.1% NaN₃ for 30 minutes on ice, then washed twice, resuspended in 0.05 mL of 1:40 diluted fluorescein isothiocyanate (FITC)-conjugated goat-antimouse IgG (Dianova, Hamburg, Germany), incubated for 30 minutes on ice, washed again twice, and analyzed on a FACScan or a FACStar Plus II (Becton Dickinson, Heidelberg, Germany). A suspension of fresh tumor cells was obtained by mincing a piece of tissue through a sieve followed by two washes. Washing and incubation steps were performed in RPMI with 10% FCS. FACS staining was performed at least twice.

Western blotting. For each lane, 10⁶ cells were resuspended in 16 µL 50 mmol/L Tris, pH 6.8, 20 mmol/L EDTA, 0.5% Triton-X-100, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), incubated 30 minutes on ice, and centrifuged for 15 minutes at 6,000g. The supernatant was adjusted to a final concentration of 10% glycerol, 2% sodium dodecyl sulfate (SDS), and separated on a nonreducing discontinuous 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE and immunoblotting on Immobilon membrane (Millipore, Eschborn, Germany) were performed following standard procedures.¹¹ For detection of APO-1, blots were incubated for 16 hours at 4°C with anti-APO-1 hybridoma supernatant or isotype-matched control antibody, washed, and incubated for 2 hours at 18°C with peroxidase-conjugated rabbit-antimouse IgG antibody (Dakopatts, Copenhagen, Denmark). Staining was performed using a chemiluminescence system (ECL-system; Amersham, Braunschweig, Germany) following the instructions of the manufacturer.

Anti-APO-1-induced inhibition of cell proliferation. Cells (6 × 10⁴/per 200 µL) were incubated in flat-bottomed 96-well plates (Falcon; Becton Dickinson, Heidelberg, Germany) in duplicates in either the presence of anti-APO-1 (1 ng/mL to 1 µg/mL) or in RPMI with FII23 control antibody (1 µg/mL) for 2 days. Cells were then pulsed with 0.5 µCi/well ³H-thymidine (Amersham) for 16 hours, harvested on a Titertek cell harvester on filter mates (Flow, Meckenheim, Germany), and counted in a liquid scintillation counter (Beckman, München, Germany). All functional assays were performed at least twice. Because crosslinking by protein A increases the sensitivity of anti-APO-1-mediated apoptosis,⁴ all assays were performed in the presence of 5 µg/mL protein A (Sigma, München, Germany). Medium controls as well as control antibody showed no effect on ³H-thymidine incorporation in all cell lines. Results were expressed as mean ³H-thymidine incorporation compared with control antibody.

RESULTS

APO-1 is not expressed on EBV-negative BL cell lines, on EBV-positive BL cell lines with group I phenotype, or on BL tumor cells *in vivo*. EBV-negative BL cell lines and EBV-positive BL cell lines with group I phenotype most closely resemble BL cells *in vivo*. Therefore, APO-1 expression was studied first by flow cytometry on 6 EBV-negative BL cell lines (BL2, BL30, BL31, BL40, BL41, and BL70) and on 5

EBV-positive BL lines that have maintained the phenotype of BL cells *in vivo* (group I) (BL29, BL37, ELI-BL, MUK-BL, and MUTU-BL). Figure 1 shows five representative examples of each and illustrates that APO-1 staining is within background on each of these cell lines (two left panels of Fig 1). To see whether the cell lines *in vitro* are indeed representing the situation *in vivo*, tumor cells were isolated from BL biopsies of three German pediatric cases and stained for APO-1 by indirect immunofluorescence. The cells of all three biopsies were APO-1-negative (data not shown). This finding indicates that the selected panel of cell lines is indeed representative for the *in vivo* situation of BL with regard to the absence of APO-1 expression.

APO-1 is expressed on BL cells with group III phenotype and on EBV-immortalized normal B-lymphoblastoid cell lines. EBV-positive BL cells change their growth pattern and phenotype upon prolonged cultivation *in vitro* as a conse-

quence of activation of latent viral genes, particularly EBNA2 and LMP.^{11,12} To see whether APO-1 is expressed on cells with an activated latent viral genome, APO-1 expression was studied on 8 EBV-positive BL cell lines with group III phenotype and on 7 EBV-immortalized lymphoblastoid cell lines. As shown for 5 representative cell lines of each class, APO-1 is highly expressed on EBV-positive BL cells with group III phenotype as well as on EBV-immortalized normal lymphocytes (two right panels of Fig 1).

For one cell line, MUTU-BL, isogenic cell clones have been developed that differ in their growth pattern and phenotype as well as in their pattern of latent viral gene expression.¹⁴ These clones show either a group I or a group III phenotype and thus allow study of phenotypic differences on an identical genetic background. APO-1 is expressed in group III clones of MUTU-BL and is not

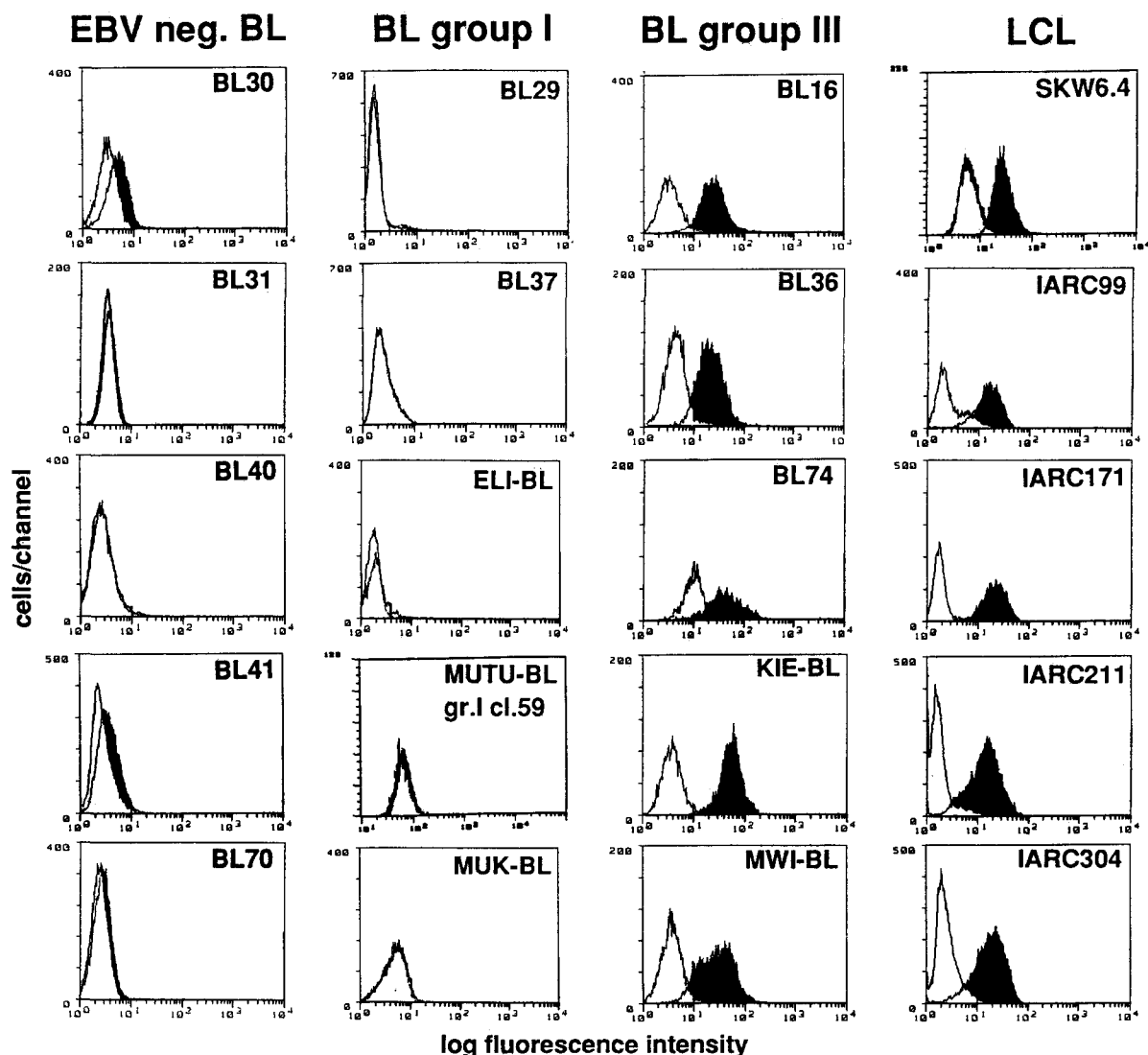


Fig 1. Expression of APO-1 in EBV-negative, in EBV-positive BL cell lines with group I and group III phenotype, and in LCLs. Cells were stained with anti-APO-1 and an isotype-matched control antibody and analyzed by flow cytometry as described. Results are plotted on a logarithmic scale. APO-1-negative cells yielded identical staining with both antibodies. Positive cell populations are indicated by the solid curves.

expressed in group I clones, thus confirming the correlation between APO-1 expression and EBV latent viral gene expression in an isogenic cell system (Fig 2).

APO-1 expression was also studied by Western blotting. Using membrane-enriched cell lysates, a band of approximately 48 Kd was visualized in all BL group III lines and LCLs were studied after incubation with anti-APO-1 antibodies (Fig 3A), but not with isotype-matched control antibody (data not shown). Slight size variations might be due to differential glycosylation or to different amounts of contaminating protein in the extracts. The different intensities of the bands indicate different amounts of APO-1 in the extracts. The weak bands of lower molecular weight probably represent degradation products of APO-1.

APO-1 is induced in B95-8 virus-converted cell lines. B95-8 virus, but not the immortalization-defective P3HR1 strain of EBV, is capable of inducing B-cell activation markers upon in vitro infection of EBV-negative BL cell lines.¹⁵ To see whether APO-1 follows the same expression pattern, triplets of EBV-negative-, P3HR1 virus-, and B95-8 virus-converted BL lines were analyzed for APO-1 expression. Conversion with B95-8, but not with P3HR1 virus, led to the induction of APO-1 expression in BL30, BL31, BL40, and BL41 cells. APO-1 expression varied in different B95-8 virus-converted cell lines. APO-1 was highly expressed in BL41-B95-8 cells (Figs 3B and 4) and moderately in five other B95-8 convertants (data not shown).

BL group III cell lines are resistant to anti-APO-1-mediated apoptosis. Binding of anti-APO-1 antibody to the APO-1 antigen induces apoptosis in SKW6.4 cells, the EBV-immortalized lymphoblastoid cell line that had been used for immunization to raise anti-APO-1. As shown before, anti-APO-1-mediated inhibition of cell proliferation is due to induction of apoptosis.³ We therefore used this assay to analyze the relationship between APO-1 expression and sensitivity to apoptosis in response to anti-APO-1 in the various cell lines. Cells were incubated with or without MoAb or control antibody in concentrations ranging from 1 ng/mL to 1 µg/mL and proliferation was measured in a standard ³H-thymidine incorporation assay.

The growth of APO-1-negative cell lines (BL group I lines and EBV-negative BL lines) was not inhibited in the presence of the MoAb (data not shown). Ten APO-1-

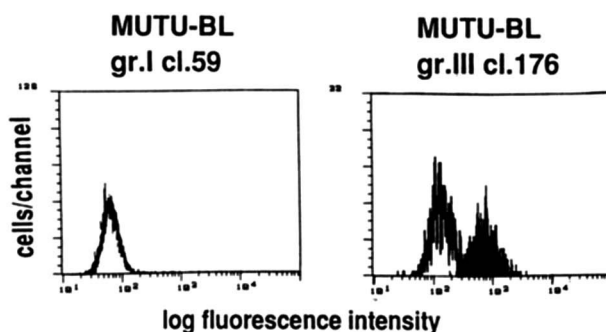


Fig 2. Expression of APO-1 in two representative MUTU-BL subclones. The MUTU-BL cl.59 cell line displays a group I phenotype and is negative for APO-1, whereas the MUTU-BL cl.176 (group III) is APO-1-positive.

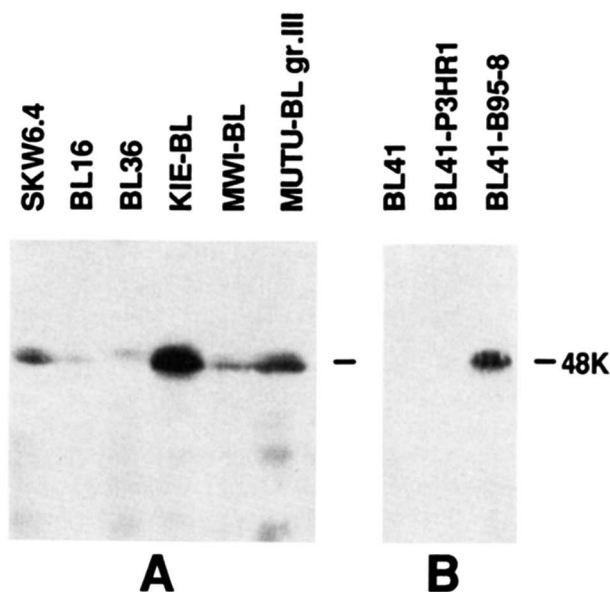


Fig 3. Western blots of BL group III cell lines and LCLs (A), and of BL41 triplet cells (B) incubated with anti-APO-1. Each lane contains membrane-enriched extracts from 10⁶ cells. Bands were visualized by chemiluminescence. APO-1 was detected in all BL group III cell lines, LCLs (A), and in BL41-B95-8 cells, but not in BL41 and BL41-P3HR1 cells (B).

positive LCLs tested responded to the growth inhibitory effect of the MoAb, although to a lesser extent than SKW6.4 cells. For most lymphoblastoid cell lines, a 10-fold higher concentration of anti-APO-1 antibody was required to block proliferation completely compared with SKW6.4 cells (lower panel of Fig 5). The BL group III lines were an exception. Although they expressed the APO-1 antigen at a level comparable with LCLs (Figs 1 and 3A), 6 of 7 cell lines tested showed no reduction of proliferation when exposed to anti-APO-1 at concentrations up to 1 µg/mL. Four representative cell lines are shown in Fig 5. To rule out the possibility that BL group III lines showed a delayed response to anti-APO-1, proliferation was measured up to day 5 after addition of the antibody. Prolonged exposure to anti-APO-1 did not result in inhibition of proliferation in BL group III lines (data not shown).

Only one BL group III line (BL 74) showed moderate inhibition of proliferation in response to anti-APO-1. Another exception with regard to anti-APO-1 sensitivity was the B95-8 virus-convertant line of BL41 cells (Fig 4). This cell line proved to be highly susceptible to APO-1-mediated apoptosis, in contrast to 5 other B95-8 virus-convertant lines (data not shown).

DISCUSSION

EBV-positive BL lines with the phenotype of BL cells in vivo (group I), as well as many EBV-negative BL cells, are highly susceptible to apoptosis upon withdrawal of growth factors¹³ (M. Falk, unpublished observation). To see whether a correlation exists between expression of the APO-1 antigen and susceptibility to apoptosis, we have studied expression of this antigen in BL cell lines, in fresh biopsies,

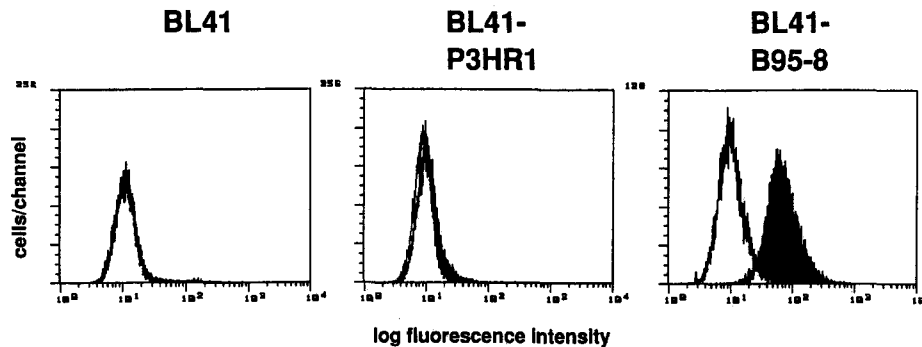


Fig 4. Expression of APO-1 in BL41 cells and their P3HR1-(EBNA2-defective EBV) and B95-8-(prototype EBV) converted lines. Anti-APO-1 and control antibody yielded identical staining in BL41 and BL41-P3HR1 cells.

and EBV-immortalized lymphoblastoid cell lines. BL cell lines with a phenotype similar to that of the tumor cells in vivo (EBV-negative and EBV-positive BL group I lines) were APO-1-negative. Consistent with this finding, tumor cells obtained from three biopsies of German pediatric BL cases were also negative for APO-1 expression.

A shift towards an activated phenotype is frequently observed upon establishment of EBV-positive BL cells in culture as a consequence of EBV latent gene activation. This shift to a group III phenotype (Table 1) is associated with the induction of the APO-1 antigen on the cell surface. Thus, all EBV-positive BL lines with group III phenotype express APO-1. The correlation between induction of APO-1 expression and activation of latent viral gene

expression was further shown in cell clones of the BL line MUTU-BL, which differ in phenotype (group I v group III) and pattern of latent viral gene expression. APO-1 expression was observed on MUTU-BL group III, but not on isogenic group I cells. EBV-immortalized LCLs show essentially the same phenotype and growth pattern as group III BL lines and are also APO-1-positive. APO-1 is, in fact, induced in EBV-negative BL cells, concomitantly with the other activation markers CD21, CD23, CD30, and CD39, upon infection with the immortalizing strain B95-8, but not with the immortalization-defective P3HR1 strain. APO-1 may therefore be regarded as an EBV-inducible activation marker in the B-cell system. It will now be interesting to see which viral latent gene product is involved in inducing

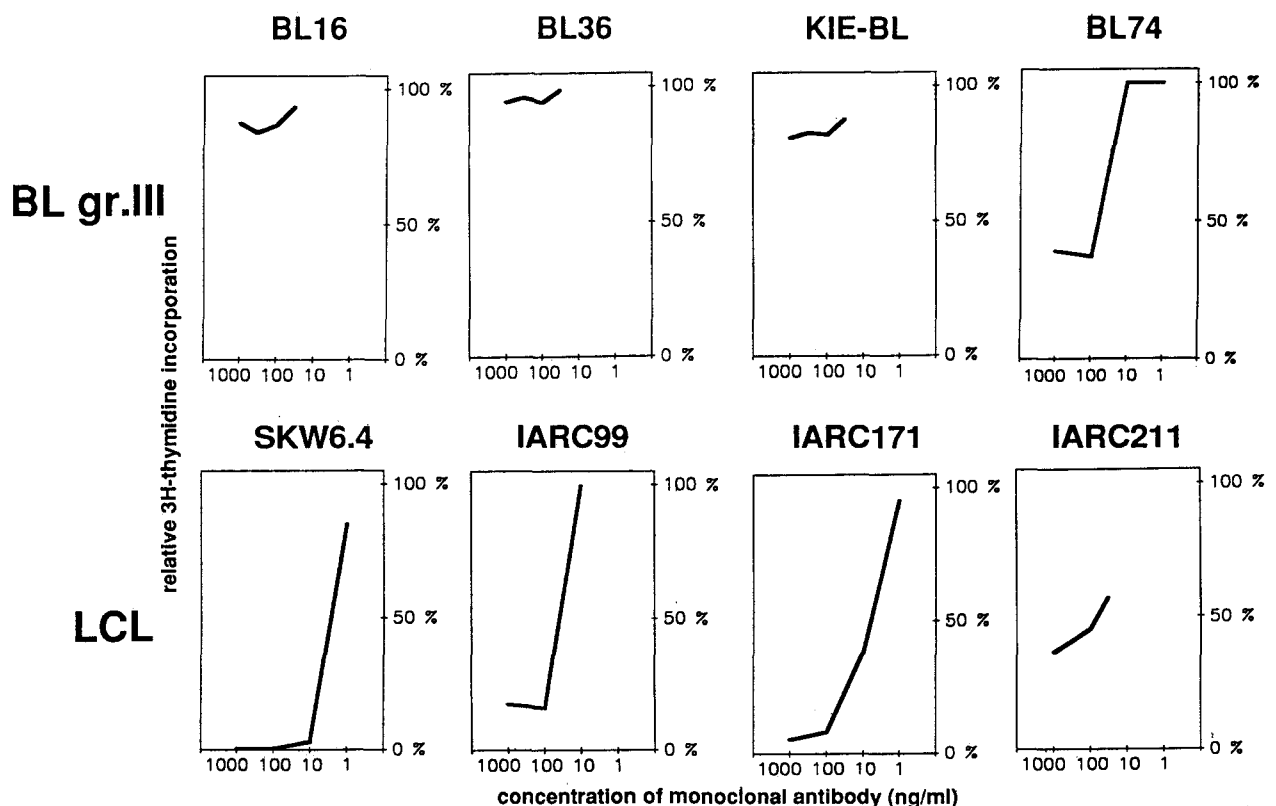


Fig 5. Anti-APO-1-induced inhibition of proliferation. BL group III (upper panels) or lymphoblastoid cell lines (lower panels) were grown in the presence of the indicated concentrations of anti-APO-1 antibody, and proliferation was measured in a ^3H -thymidine assay after 3 days. Data were compared with control samples grown either in the presence of a control antibody or in medium only. Results are expressed as relative ^3H -thymidine incorporation compared with cells grown in control antibody.

APO-1 expression. EBNA2 is a prime candidate because it appears to activate a number of different viral and cellular genes. EBNA2 transactivates the LMP and TP promoters,¹⁶⁻¹⁸ and was shown to increase the CD21 and CD23 messenger RNA (mRNA) levels upon transfection of the EBNA2 gene into P3HR1 virus-converted cells.^{19,20} LMP, however, does also increase CD23 expression in EBV-negative cells,²¹ suggesting that EBNA2 and LMP may cooperate in switching on the activation markers. The recent finding that EBNA3C also upregulates CD23 expression points, in fact, to a more complex regulation in which many of the latent viral gene products might be involved.¹⁷ It will therefore be important to understand how the various viral gene products interact at a molecular level with respect to regulation of expression of the cellular activation markers.

Although BL group III lines showed the same APO-1 staining pattern as EBV-immortalized LCLs, they behaved quite differently when the biologic activity of the antibody was studied. Growth inhibition by anti-APO-1 antibody is easily measurable in a quantitative fashion and was therefore taken as an indicator of induction of apoptosis by the antibody. As expected, cell lines that were negative for APO-1 staining did not respond to anti-APO-1 antibody. The reverse, however, was not true. Of the EBV-positive, APO-1-expressing group III BL lines, only BL74 responded to anti-APO-1, and only to a lesser extent than EBV-immortalized LCLs. Of 10 LCLs tested, all were sensitive to the action of the antibody.

The possibility of dissociating APO-1 expression from APO-1 function has also been described for T cells.⁴ This may turn out to be important for dissecting the various steps in the signal transduction pathway. An interesting possibility, which might explain the different sensitivity of EBV-immortalized LCLs and group III BL lines to APO-1 antibody, is that *c-myc* overexpression may interfere intracellularly with the delivery of the APO-1 signal in BL cells. Alternatively, following the model that APO-1 is a growth

factor receptor, the *c-myc* translocation may have rendered the cells independent from a growth or survival factor that is absolutely required for lymphoblastoid cells. By introducing an activated *c-myc* gene into lymphoblastoid cells such as SKW6.4, the possible influence of this oncogene can now be tested.

EBV-immortalized lymphoblastoid cells were invariably found to express APO-1 and to respond to anti-APO-1 antibodies with induction of apoptosis. This sensitivity of EBV-immortalized cells to anti-APO-1 might eventually be exploited for the treatment of human lymphomas induced by EBV in immunocompromised individuals. Part of the immunoblastic lymphomas in acquired immunodeficiency syndrome (AIDS) patients are presumably caused by EBV as a consequence of the severe immune dysbalance^{22,23} (Delecluse et al, manuscript submitted). It would be important to see whether APO-1 is indeed expressed in such lesions in AIDS patients. The lymphomas developing in SCID mice upon transplantation of human blood or bone marrow cells are also clearly lymphoblastoid in phenotype, so that an animal model is readily available to study the effect of anti-APO-1 antibodies on the growth of lymphoblastoid tumor cells *in vivo*.²⁴⁻²⁶

The data in this report show that the inherent susceptibility of BL cells to apoptosis is not correlated to the expression of the APO-1 antigen. APO-1 is not found in BL cells that undergo apoptosis upon removal of a positive signal and is induced in LCLs that have lost spontaneous susceptibility to apoptosis upon removal of growth factors.

Further insight into the biology of APO-1 and its role in the B-cell system is necessary to answer the crucial question of whether natural binding of a putative ligand to APO-1 induces cell death or cell survival.

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