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Differentiation-dependent sensitivity of human B-cell-derived lines to major histocompatibility complex-restricted T-cell cytotoxicity

(Epstein–Barr virus-induced differentiation/Burkitt lymphoma)

S. TORSTEINSDOTTIR*, M. G. MASUCCI*, B. EHLIN-HENRIKSSON*, C. BRAUTBAR†, H. BEN BASSAT‡, G. KLEIN*, AND E. KLEIN*

*Department of Tumor Biology, Karolinska Institute, S-104 01 Stockholm, Sweden; and †Laboratory of Immunohematology, Tissue Typing Unit, and ‡Department of Experimental Surgery, Hadassah University Hospital, 91 120 Jerusalem, Israel

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ABSTRACT **Sets of Burkitt lymphoma lines and Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) derived from the same individuals were compared for sensitivity to cytotoxic T-lymphocyte (CTL) clones. Major histocompatibility complex class I antigen-restricted CTL clones were generated by stimulating the lymphocytes of an EBV-seropositive individual with the autologous LCL. One clone (BK-20) lysed the autologous and allogeneic HLA-A11-expressing LCLs but not mitogen-induced B lymphoblasts. Thus the clone was selectively cytotoxic for LCLs. Allospecific CTL clones directed against the HLA-A11 antigen were generated from an EBV-seronegative individual. One clone (WP-36) was selectively cytotoxic for the appropriate allospecific LCL, whereas another clone (WP-21) lysed also T and B lymphoblasts. None of the four Burkitt lymphoma lines established in parallel with the CTL-sensitive LCLs were lysed. Two of the Burkitt lymphoma lines were EBV-negative, and EBV-positive sublines were derived from these by *in vitro* infection. One but not the other of the two convertants became sensitive to all three types of CTL clones. The CTL-sensitive converted line had also acquired some LCL characteristics: increased cell size, aggregation, and a shift in several of the B-cell-specific surface markers. The CTL-resistant convertant expressed EBV antigens but showed no phenotypic change. These findings suggest that the cellular phenotype plays a decisive role in the sensitivity of B-cell-derived lines to the lytic effect of LCL-selective autologous and allogeneic CTLs.**

Cytotoxic T lymphocytes (CTLs) that lyse autologous Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) in a major histocompatibility complex (MHC)-restricted fashion are believed to reflect the immunological surveillance that prevents the proliferation of EBV-infected B cells in healthy virus carriers (1). Rooney *et al.* (2) have recently shown that EBV-positive Burkitt lymphoma (BL) cells are resistant to CTLs generated by autologous LCL stimulation. They suggested that this reflects the escape of BL cells from immunological control.

The target recognized by the autologous CTLs has not been defined. The operational term LYDMA (lymphocyte-detected membrane antigen) has been coined on the basis of cytotoxic tests with the lymphocytes of acute mononucleosis patients (3). It received a more precise definition when Moss *et al.* (4) and Wallace *et al.* (5) showed that MHC class I antigen-restricted CTLs activated *in vitro* kill LCL cells but not mitogen-induced B lymphoblasts. This has led to the suggestion that lysis is due to the recognition of an EBV-encoded surface antigen. There is still no experimental evidence to prove this hypothesis. An alternative hypothesis is that CTLs selectively recognize the LCL-associated cellular phenotype. B lymphoblasts differ from LCL cells with regard to several differentiation-related surface markers.

BL lines differ from LCLs in cell morphology, growth properties, and several differentiation- and/or activation-related surface markers (6–8). LCLs of relatively recent origin are diploid, have a low agarose clonability, and do not grow in nude mice. BL cells carry one of the three immunoglobulin locus/*myc* oncogene (*MYC*) translocations, are clonable in agarose, and grow in nude mice (9–13). Nearly all BLs that arise in highly endemic regions carry EBV genomes (BLE^+) (14). The majority (80%) of the sporadic cases are EBV-negative (BLE^-) (15). Freshly isolated BLE^+ and BLE^- cells have similar phenotypic properties (16). BLE^- lines are relatively stable *in vitro*, whereas BLE^+ lines often acquire a number of LCL-associated markers during serial propagation, without assuming a full LCL phenotype. Similar changes were found to occur in some BLE^- lines after *in vitro* EBV conversion (16, 17). Independently converted sublines of the same BLE^- line with the same virus substrain showed similar phenotypic changes (17). The modulation of the phenotype is thus probably prompted by the conditions *in vitro* and appears to be dependent on the presence of EBV.

We have compared the sensitivity of LCLs and BLs to three types of CTL clones. We have used "trios" composed of an *in vitro*-transformed LCL, an EBV-negative BL line, and an EBV-converted subline of the latter, derived from the same patient, and "duos" consisting of a LCL and an EBV-carrying BL (18–20). The CTL clones were generated by stimulation with autologous and allogeneic EBV-infected B cells.

MATERIALS AND METHODS

Generation of Cytotoxic Cells. Mononuclear cells were obtained from heparin-treated blood of one healthy EBV-seropositive (BK) and one healthy EBV-seronegative individual (WP) (21). BK cells were infected with B95-8 virus as described (21). After 10 days of culture at 37°C in a 5% CO_2 atmosphere, T cells were isolated by sheep erythrocyte rosetting (21), restimulated with irradiated autologous LCL cells at a responder/stimulator ratio of 10:1, and cultured for 1 week. The cells of WP ($HLA\text{-}A11^-$) were stimulated with an allogeneic ($HLA\text{-}A11^+$) LCL (Fig. 1). Responder cells (2×10^7) were mixed with irradiated (6000 rads; 1 rad = 0.01 Gy) stimulator cells (7×10^6) and resuspended in 20 ml of complete medium. After 10 days in culture, the cells were washed and restimulated under the same conditions.

Cloning and Expansion of CTLs. Twice-restimulated T cells were cloned by limiting dilution (21). The culture medium was

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Abbreviations: CTL, cytotoxic T lymphocyte; LCL, lymphoblastoid cell line; BL, Burkitt lymphoma; EBV, Epstein–Barr virus; BLE^+, EBV-genome-positive BL; BLE^-, EBV-genome-negative BL; mAb, monoclonal antibody; IL-2, interleukin 2; MHC, major histocompatibility complex.

supplemented with 30% filtered culture supernatant from the gibbon leukemia line MLA-144 as a source of interleukin 2 (IL-2) (22). The cultures were fed weekly by replacing half of the medium. Growing cultures were transferred into 24-well Linbro plates and tested for cytotoxic activity against the stimulator LCL. Cytotoxic cultures were recloned under the same conditions and expanded in medium supplemented with IL-2.

LCLs and BLE$^+$ and BLE$^-$ Lines. The cell lines (Table 1) were maintained at 37°C in a 5% CO_2, water-saturated atmosphere in complete RPMI 1640 medium supplemented with 10% fetal bovine serum. EBV-transformed LCLs were obtained by exposing purified B cells from MHC-typed donors to B95-8 supernatants (21). MHC typing was performed by the standard microcytotoxicity test (23).

Cytotoxicity Tests. Short-term (4-hr) ^{51}Cr-release assays were performed as described (3). The effectors were washed twice in IL-2-free medium before the test. T and B lymphoblasts were generated by treatment with Con A and pokeweed mitogen, respectively (21).

Surface Marker Analysis. T-cell markers were detected by the Leu-4 (CD3), Leu-2a (CD8), and Leu-3a (CD4) monoclonal antibodies (mAbs) (Becton Dickinson). MHC components were detected by a mAb specific for the framework (monomorphic) region of class I antigens (mAb W6/32, kindly provided by W. Bodmer, Imperial Cancer Foundation, London, England) and a mAb specific for common class II antigen (mAb D1-12, kindly provided by R. Acolla, Ludwig Institute for Cancer Research, Lausanne, Switzerland). B-cell-specific marker expression was assessed by the mAbs J5 (anti-CALLA), 38.13 (anti-BLA), LB-1, AC-2, BB-1, and B2 (24–28). Surface antigen expression was estimated by indirect immunofluorescence, either by visual examination or by a fluorescence-activated cell-sorting (FACS) analyzer (model 420, Becton Dickinson) (21).

RESULTS

Characterization of the CTL Clones. The HLA-A11-restricted CD3,CD8 clone BK-20 was established from the lymphocyte culture of an EBV-seropositive donor, stimulated with autologous LCL. It lysed the autologous and HLA-A11-positive allogeneic LCLs. It did not kill autologous mitogen-induced B and T lymphoblasts (Fig. 1). We shall refer to this pattern of killing as an LCL-selective cytotoxic pattern.

An allospecific anti-HLA-A11 CD8 clone, WP-21, was established by stimulating lymphocytes of an HLA-A11-negative, EBV-seronegative donor with SI-B-1, an HLA-A11-positive LCL. It lysed a panel of HLA-A11-positive allogeneic LCLs and T and B lymphoblasts (Fig. 1). Another CD8 clone, WP-36, derived from the same T-cell culture, lysed the stimulator line and other allogeneic A11-positive LCLs but not A11-positive T lymphoblasts (Fig. 1). This clone was thus LCL-selective and required the HLA-A11 antigen on the target. It did not lyse WP-B-1, the autologous LCL (Fig. 1).

The lytic effect of the CTL clones was abrogated by preincubation of target cells with mAb against the monomorphic part of the HLA class I antigens (W6/32) (Fig. 1). Anti-MHC class II mAb D1-12 had no effect (data not shown). None of the CTL clones lysed K562 cells.

Sensitivity of LCLs and BL Lines to CTL-Mediated Lysis. To explore the possibility that the "auto-LCL-generated" clone BK-20 recognizes an EBV-encoded cell surface antigen, we have compared the cytotoxic sensitivity of "trios" and "duos" derived from BL patients. The trios consisted of a LCL, an EBV-negative BL line, and an *in vitro*-converted subline of the latter. The duos consisted of an EBV-positive BL line and a LCL from the same patient. The LCLs were sensitive, but neither the two EBV-negative BL lines (BL-28 and BL-41) nor the two EBV-positive BL lines (WW-1-BL and BL-72) were lysed (Fig. 2*A*). The two EBV-converted sublines differed in their sensitivity. E95A-BL-28 was resistant, whereas BL-41/95 was slightly but definitely sensitive. The highest ^{51}Cr release obtained with 25:1 effector/target ratios was 12%. *In vitro* EBV conversion of an originally EBV-negative BL line was thus paralleled by the induction of sensitivity to the lytic effect of HLA-A11-restricted, autologous-LCL-generated CTLs in one case but not the other.

The HLA-A11-allospecific clone WP-21 and the allorestricted, LCL-selective clone WP-36 (Fig. 2 *B* and *C*) showed a similar lytic pattern. Line E95A-BL-28 was resist-

Table 1. Characteristics of the cell lines used

Donor	Cell type	Name	HLA phenotype		
Goujon	LCL	IARC-139	A1,A11	B8,B16	Cw2,Cw7
	BLE$^-$	BL-28			
	BLE^{+*}	E95A-BL-28			
Bon	LCL	IARC-171	A11,Aw32	B35,B49	Cw3,Cw4
	BLE$^-$	BL-41			
	BLE^{+*}	BL-41/95			
Wewak-I	LCL	WW-1-LCL	A11,Aw24	B27,Bw62	Cw2,Cw4
	BLE$^+$	WW-1-BL			
Ous	LCL	IARC-307	A11,Aw28	B27,Bw45	
	BLE$^+$	BL-72			
BK	LCL	BK-B-1	A2,A11	B7,Bw62	Cw7
IE	LCL	IE-B-1	A3,A11	B7,B35	Cw4
KK	LCL	KK-B-1	A11,A28	B14,–	Cw3
SI	LCL	SI-B-1	A11,A24	B7,B27	Cw1,Cw7
LS	LCL	LS-B-1	A3,A32	B7,B44	Cw5,Cw6
ST	LCL	ST-B-1	A2,A28	Bw40,Bw62	Cw3
OR	LCL	OR-B-1	A2,Aw33	B14,B44	Cw8
GK	LCL	GK-B-1	A2,Aw24	B13,B35	Cw4
WP	LCL	WP-B-1	A2,A3	Bw38,–	Cw4
—	—	K562†			

All LCLs were derived from B cells by *in vitro* EBV transformation. LCLs and BLE$^-$ lines from donors Goujon and Bon, as well as the LCL and the BLE$^+$ line from donor Ous, were kindly provided by G. M. Lenoir (International Agency for Research on Cancer, Lyon, France). The LCL and the BLE$^+$ line from donor Wewak-I were kindly provided by D. Moss (Queensland Institute of Medical Research, Brisbane, Australia).

*BLE$^+$ sublines were produced from the BLE$^-$ lines of donors Goujon and Bon by conversion *in vitro* with EBV.

†An erythroleukemia line derived from a chronic myeloid leukemia blast crisis.

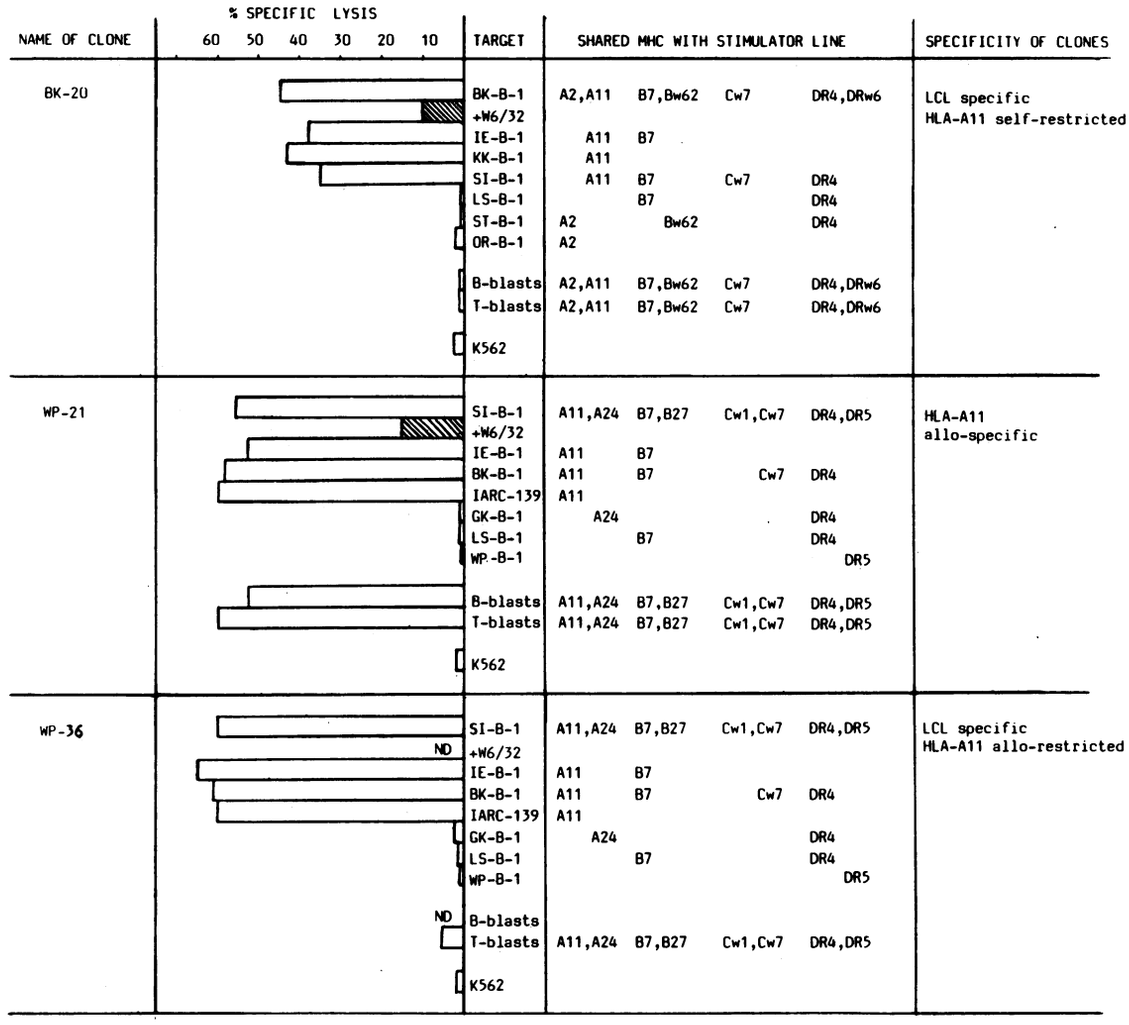

FIG. 1. Cytotoxic activity of the T-cell clones BK-20, WP-21, and WP-36. Effector/target-cell ratio was 5:1 (BK-20) or 10:1 (WP-21, WP-36). Data are shown as mean % specific lysis from six experiments with each target tested at least twice. Hatched bars show cytotoxicity against targets preincubated with anti-HLA class I framework mAb W6/32 (0.1 μg per microwell) for 20 min before addition of the effectors. ND, not done.

ant, but the EBV-converted line BL-41/95 was highly sensitive, in contrast to its EBV-negative BL-41 progenitor.

The similar target selectivity of the autologous-LCL-generated clone BK-20 and the allospecific clones derived from the EBV-negative donor WP challenges the idea that an EBV-encoded antigen serves as the cytotoxic target.

MHC Class I Antigen Expression of the LCLs and BL Lines. Both trios were tested for MHC class I antigen expression by fluorescence-activated cell-sorting analysis, using mAb W6/32. The LCLs gave stronger fluorescence signals than the BL lines, but they were also larger. The mean fluorescence/volume ratios were closely similar (for IARC-139, the ratio was 70; for BL-28, 63; for E95A-BL-28, 55; for IARC-171, 56; for BL-41, 65; and for BL-41/95, 59). However, the antigen densities cannot be defined exactly, due to the differences in the surface topography of the two cell types (6).

Target Expression of B-Cell-Specific Surface Markers. Monoclonal antibodies were selected on the basis of their ability to define different stages of B-cell maturation or activation and to distinguish between BL lines and LCLs. Both LCLs expressed the blast-cell-associated LB-1, AC-2, and BB-1 antigens (26, 27) and the complement receptor (CR2)-related B2 moiety (28, 29) (Table 2). They did not react with CALLA and BLA (24, 25), known to be expressed on the majority of the BL lines. The two EBV-negative BL lines reacted weakly with LB-1, AC-2, and BB-1 but were highly positive for CALLA. Line BL-41 expressed BLA, but line BL-28 did not. The phenotype of the EBV-converted line E95A-BL-28 was similar to that of the parental line, whereas the EBV-converted line BL-41/95 showed considerable changes. Its LB-1, AC-2, and BB-1 expression was increased in comparison with the original BL-41 line, while the BLA expression has diminished. Parallel changes in cellular morphology and growth pattern were observed. The converted cells were larger and more irregularly shaped than the EBV-negative BL-41 cells and showed an increased tendency to grow in aggregates. The BL-41/95 cells have thus become more LCL-like, but the persistence of high CALLA expression still distinguished them from genuine LCL cells.

DISCUSSION

We have confirmed the results of Rooney *et al.* (2) by showing that LCLs, but not BL lines, are sensitive to the cytotoxic activity of CTLs generated from blood lymphocytes by stimulation with autologous, EBV-transformed B cells. It has been postulated that such CTLs recognize EBV-encoded antigens, presented in the context of autologous MHC determinants (30, 31). It was proposed that the resistance might be relevant for the escape of the BL cell from the immune surveillance of the host.

The four BLs tested by us were also resistant to allospecific CTL clones directed against the HLA-A11 antigen. This is

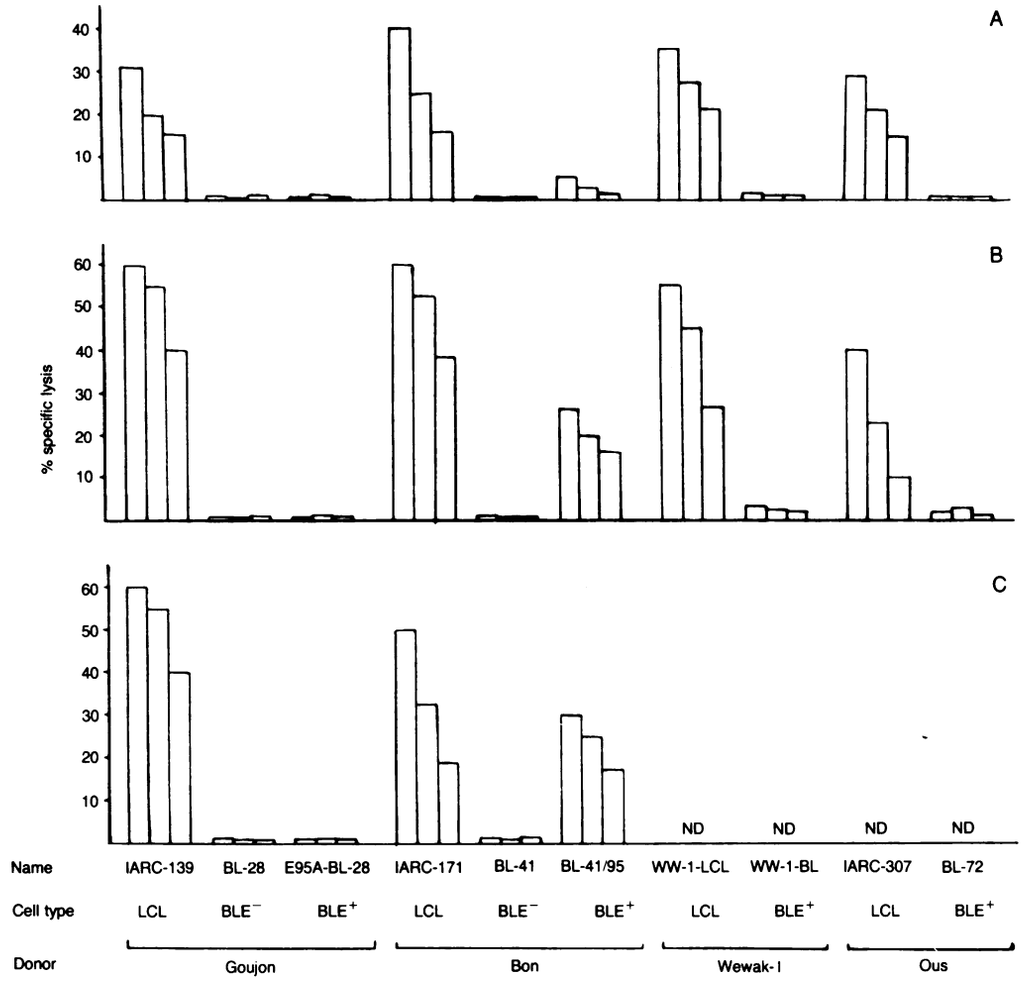

FIG. 2. Cytotoxic activity of the BK-20 clone, generated by auto-LCL stimulation, and of the WP-21 and WP-36 clones, generated by allo-LCL stimulation, against HLA-A11-positive LCLs and BL lines. Target cells are described below the histograms. (*A*) BK-20. Percent specific ^{51}Cr release at 5:1, 1.6:1, and 0.5:1 effector/target ratios (bars from left to right in each group) is shown for one representative experiment out of five. (*B* and *C*) WP-21 and WP-36, respectively. Percent specific ^{51}Cr release at 10:1, 3:1, 1:1 effector/target ratios is shown for one representative experiment out of three. ND, not done.

not due to a general resistance to cell-mediated cytotoxicity. BL/LCL pairs derived from the same donor and kept in culture during similar periods were equally sensitive to natural and interferon-activated killer cells (20, 32). This was also true for the cell lines used in the present study (data not shown).

Our results suggest that differences in the density of MHC class I molecules do not account for the resistance of the BL lines. However the resistance of BL cells to HLA-A11-restricted lysis may be due to down-regulation or changed presentation of the A11 epitope.

The involvement of an EBV-encoded antigen in the target structure responsible for the lytic effect of the CTLs generated in the autologous stimulation system cannot be proven unless a difference can be demonstrated between EBV-negative and EBV-carrying sublines of the same cell that are

Table 2. Surface marker expression and growth characteristics of the two cell trios Goujon and Bon

Trio	Cell line (type)	Morphology	Growth pattern	mAb binding, % positive cells*					
				Anti-CALLA	Anti-BLA	LB-1	AC-2	BB-1	B2
Goujon	IARC-139 (LCL)	Heterogeneous large cells	Large clumps	0	0	85 ± 6	99	84 ± 10	50 ± 12
	BL-28 (BLE⁻)	Homogeneous small cells	Single cells	84 ± 4	1 ± 1	0	0	57 ± 5	0
	E95A-BL-28 (BLE⁺)	Homogeneous small cells	Small clumps	90 ± 6	0	0	0	71 ± 15	20 ± 4
Bon	IARC-171 (LCL)	Heterogeneous large cells	Large clumps	3 ± 2	11	100	100	82 ± 7	35 ± 5
	BL-41 (BLE⁻)	Homogeneous small cells	Single cells	100	64 ± 12	6 ± 1	10 ± 4	10 ± 1	12 ± 3
	BL-41/95 (BLE⁺)	Heterogeneous	Large clumps	96 ± 3	8 ± 5	52 ± 10	84 ± 4	78 ± 1	72 ± 5

*Mean ± SEM of 3–6 experiments for each marker and cell line.

closely similar phenotypically. The EBV-negative BL lines and their EBV-converted sublines seemed to provide an appropriate system for this purpose. Our experiments failed to provide supportive evidence for the involvement of an EBV-encoded antigen, however. Conversion of the EBV-negative line BL-28 to a permanent EBV-carrying state did not impose cytotoxic sensitivity. The EBV-converted subline BL-41/95 did acquire some cytotoxic sensitivity, but this was accompanied by a phenotypic switch in the "LCL direction." The intermediate expression of LCL-like markers corresponded to the intermediate cytotoxic sensitivity of the converted subline. The parallel results with the allospecific CTLs suggest that this phenotypic change was decisive for the acquisition of lytic sensitivity.

Antigen-specific CTLs recognize the MHC class I antigen either as primary target or as a restrictive element for an antigen. Studies (33, 34) with LCLs have suggested that the MHC class I epitopes recognized by allospecific responses are closely related to the ones that restrict LCL-specific T cells generated by autologous stimulation. It remains to be seen whether our results reflect a difference between LCLs and BL lines in the expression or the accessibility of the A11 antigen.

Two important questions arise from these findings. The first concerns the nature of the LCL-associated antigen recognized by the LCL-selective auto- and allogenerated killer cells. It may be an EBV-encoded antigen or could reflect a constellation of differentiation markers, specific for the stimulating LCLs. Donor WP, whose cells repeatedly provided LCL-selective allorestricted CTL clones, was EBV-seronegative. If the target were an EBV-encoded surface antigen, this experiment would reflect a primary sensitization against it when presented together with an alloantigen. The role of the alloantigen seems to be decisive, because with cells of seronegative individuals, LCL-selective CTLs cannot be generated in autologous systems.

The second question concerns the biological significance of the CTL resistance of BL. EBV-carrying BLs were believed to be more immunogenic than the EBV-negative ones. The occurrence of spontaneous regressions and good response to chemotherapy suggested that EBV-positive BLs elicit an EBV-specific immune response and that the tumors grow from relatively immunoresistent clones (35). If this argument is valid, the *in vitro* experiments indicate that immunoselection has to act on the EBV-negative BLs as well.

All BLs, but not LCLs, carry one of three chromosomal translocations that brings the *myc* oncogene (*MYC*) into juxtaposition with one of the three immunoglobulin loci (12, 13, 36). Since the *myc* gene is expressed in proliferating but not in resting (G_0) cells, its constitutive activation through the translocation may alter the responsiveness of the cells to growth- and/or differentiation-inducing signals or programs (12). The difference in the immunological behavior of the LCL and BL cells related to the differentiation markers suggests that the phenotype of BL also contributes to its growth capacity *in vivo*.

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