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Immunology

Down-regulation of class I HLA antigens and of the Epstein–Barr virus-encoded latent membrane protein in Burkitt lymphoma lines

(B-cell phenotype/major histocompatibility complex antigens)

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ABSTRACT **Epstein–Barr virus (EBV)-carrying Burkitt lymphoma (BL) cells are relatively or completely resistant to the lytic effect of major histocompatibility complex class I HLA antigen-restricted cytotoxic T lymphocytes (CTLs) generated by stimulating lymphocytes of EBV-seropositive donors with the autologous EBV-transformed lymphoblastoid cell line (LCL). We previously found that EBV-negative and EBV-carrying BL lines derived from HLA-A11-positive donors were not only resistant to lysis by the HLA-A11-restricted CTL generated by stimulation with the autologous LCL, but also to HLA-A11-specific CTL derived from lymphocytes of an EBV-seronegative donor stimulated with an allogeneic LCL. Using the same and additional cell lines, we now show that the CTL resistance of the BL lines is probably due to a selective down-regulation of HLA-A11. We also show that the EBV-encoded latent membrane protein is expressed at a lower level in the EBV-carrying BL lines than in EBV-transformed LCLs. Only one of eight *in vitro* EBV-converted BL lines that shifted to a more LCL-like growth pattern expressed LMP at a high level. This line also reexpressed the HLA-A11 antigen that was undetectable in its EBV-negative progenitor. Our findings suggest that the typical BL cell phenotype is associated with low expression of both proteins.**

Reciprocal translocations juxtapose the cellular c-*myc* gene (*MYC* gene in humans) to an immunoglobulin locus in Epstein–Barr virus (EBV)-carrying and EBV-negative Burkitt lymphoma (BL) lines and in mouse and rat plasmacytomas (1, 2). It has been suggested that the translocations contribute to the tumorigenic process by bringing the c-*myc* gene under the regulatory influence of the adjacent, highly active immunoglobulin locus. This concept is supported by the high incidence of pre-B-cell and B-cell lymphomas in transgenic mice that carried immunoglobulin heavy chain *Igh* enhancer–*myc* constructs (3) and the appearance of translocation-free plasmacytomas in pristane oil-treated BALB/c mice infected with *myc*-expressing retroviral constructs (4). However, the monoclonality of the B-cell tumors that arose in the transgenic mice carrying the *Igh* enhancer–*myc* construct indicates that at least one additional step is needed for full tumorigenicity.

EBV-carrying BL cells are relatively or completely resistant to the lytic effect of major histocompatibility complex (MHC) class I HLA antigen-restricted cytotoxic T lymphomas (CTLs) generated by stimulating lymphocytes of EBV-seropositive donors with the autologous EBV-transformed lymphoblastoid cell line (LCL) (5, 6). This was taken to suggest that escape from immune surveillance may play a role in the pathogenesis of the EBV-positive BL lines.

We have found that EBV-negative and EBV-carrying BL lines derived from HLA-A11-positive donors were not only resistant to lysis by the HLA-A11-restricted CTLs generated by stimulation with the autologous LCL but also the HLA-A11-specific CTLs generated by stimulating the lymphocytes of an EBV-seronegative donor with an allogeneic LCL (6). We now report that this resistance, found in all five BL lines derived from HLA-A11-positive donors, is paralleled by and probably caused by a selective down-regulation of HLA-A11. We also show that in EBV-carrying BL lines, the down-regulation of HLA-A11 correlates with a low expression of the EBV-encoded latent membrane protein (LMP). The EBV-induced phenotypic shift of one BL line to a more LCL-like growth pattern resulted in the concomitant up-regulation of HLA-A11 and LMP, suggesting that the low expression of both proteins is a property of the BL phenotype.

MATERIALS AND METHODS

Cell Lines. BL lines were established from tumor biopsies; LCLs were established from blood B cells of tumor patients either spontaneously or by *in vitro* EBV-infection (7–10). EBV-converted sublines of the EBV-negative BL-28 and BL-41 lines were established by *in vitro* EBV infection with the B95-8 and P3HR1 strains of EBV, respectively.

Phenotypic Characterization and Expression of MHC Class I Antigens. The surface phenotype of the cell lines was assessed by their reactivity with a panel of mouse monoclonal antibodies (mAbs), including LB-1 (11) and AC2 (12) that react with activation antigens expressed on all LCLs, J5 (13) directed to the common acute lymphoblastic leukemia antigen (CALLA), and 38.13 (14) that binds to a BL-associated antigen (BLA). The phenotype was classified as described by Rowe *et al.* (15) in three groups: group I reacts with J5 and 38.13 only; group II reacts with J5, 38.13, LB-1, and AC-2; and group III reacts with LB-1 and AC2 only.

The expression of MHC class I antigens was assessed by fluorescence-activated cell sorter (FACS) analysis (FACS 420 analyzer, Becton Dickinson) on cells stained with the mAbs W6/32, which is specific for a common framework determinant on all class I HLA molecules (16), and AUF 5.13, which is directed against a polymorphic determinant shared by the HLA-A3 and -A11 molecules (17). The difference mean fluorescence-to-volume ratio (Δ mean Fl/Vol) was calculated from the mean Fl/Vol ratios of cells exposed to the mAb and to fluorescein isothiocyanate-conjugated rabbit anti-mouse antibodies by subtracting the mean Fl/Vol

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Abbreviations: BL, Burkitt lymphoma; EBV, Epstein–Barr virus; LCL, EBV-transformed lymphoblastoid cell line; CTL, cytotoxic T lymphocyte; LMP, EBV-encoded latent membrane protein; EBNA, EBV-determined nuclear antigen; MHC, major histocompatibility complex; mAb, monoclonal antibody.

ratio of cells exposed to the fluorescein isothiocyanate-conjugated rabbit anti-mouse antibodies alone.

Establishment and Characterization of CTL Clones. Activation of CTL and cloning were performed as described (6). Clones WP-21, -38, and -5 were obtained by stimulating the lymphocytes of an EBV seronegative donor (WP) with the allogeneic LCL SJ-B-1 (HLA-A11/A24, -B7/B27). Clones 171-1 and 139-cult were obtained by stimulating the lymphocytes of healthy donors with the IARC-171 and IARC-139 LCLs. All clones expressed the T-cell differentiation markers CD3 and CD8. HLA specificity was demonstrated by the selective killing of allogeneic targets matched to a single HLA-A or -B antigen. The IE-B-1 LCL and IE-T-blasts were derived from a healthy donor (HLA-A3/A11, -B7/B35). T blasts were gene-rated by stimulating peripheral blood lymphocytes with 5 μg of concanavalin A per ml for 3 days. The specificity of the 139-cult could not be determined because of a lack of appropriate HLA matching targets. Saturating concentrations of the W6/32 mAb blocked cytotoxicity. K562, Daudi, and other natural killer cell-sensitive targets were not killed.

Immunoblotting and Immunodotting. Total cell extracts were prepared by resuspending 5×10^7 cells in 1 ml of 20 mM Tris buffer (pH 7.5) containing 1% Nonidet P-40, 1% Na deoxycholate, 0.1% $NaDodSO_4$, 2 mM phenylmethylsulfonyl fluoride, 4 mM EDTA, and 400 mM NaCl. The cell suspensions were sonicated and centrifuged for 20 min at 10,000 $\times$ *g*. Supernatants (20 μl) were resuspended in 80 μl of sample buffer and electrophoresed in $NaDodSO_4$/7.5% polyacrylamide gels by the method of Laemmli (18). Molecular weight determinations were made by running high and low molecular weight standards (Bio-Rad) in the same gels. Immunoblotting was performed as described (19). The blots were probed with the mAb S-12 directed to an antigenic determinant on the carboxyl-terminal part of the LMP molecule (20) or with previously characterized human sera containing antibodies to EBV-determined nuclear antigens (EBNAs) 1, 2, 3, and 4 (21).

Serial dilutions of total cell extracts were made from a starting concentration equivalent to 10^6 cells by double dilution in phosphate-buffered saline. The extracts were blotted on nitrocellulose filters prewetted in phosphate-buffered saline by using a dot-blot apparatus (Bio-Rad) under mild suction. After drying in air, the nitrocellulose sheets were stained with Ponceau-S to ascertain that equal amounts of proteins had been loaded for each cell line and were probed with the S-12 mAb.

RESULTS

Phenotypic Characteristics of the Cell Lines. These are listed in Table 1. All EBV-negative BL lines expressed a group I phenotype as expected (15). The two EBV-positive BL lines that originally belonged to group I had drifted to a more LCL-like group II phenotype during *in vitro* passage. The EBV-converted BL-28 sublines remained phenotypically similar to their progenitor, while the BL-41 convertants progressed to group II. This phenotypic switch was stable in lines converted with the transforming B95-8 substrain of EBV, whereas two lines that converted with the transformation-defective P3HR1 substrain lost part of their activation markers.

Sensitivity of LCL and BL Lines to Allospecific Cytotoxicity. Three EBV-negative (BL-28, BL-41, and DG-75) and the EBV-positive (WW-1-BL and BL-72) BL lines derived from HLA-A11-positive patients were resistant to HLA-A11-specific CTLs that lysed the LCLs derived from the same donors and HLA-A11-positive T and B blasts (Fig. 1). Two of the EBV-negative lines, BL-28 and BL-41, were converted to EBV-positive lines by exposing them to the B95-8 and P3HR1 viral substrains. Both independently converted BL-28 sublines and five of the six converted BL-41 sublines were as resistant to HLA-A11-specific lysis as were their EBV-negative progenitors. The sixth convertant, BL 41/95, became sensitive, but at a lower level than the LCL derived from the same donor (Fig. 2).

BL lines are not generally resistant to cell-mediated cytotoxicity. They are as sensitive to natural killer cells and to interferon- and mixed lymphocyte culture-activated nonspecific killer cells as were the corresponding LCLs (9, 22). Moreover, as shown in Fig. 1, at least three of the anti-HLA-A11 CTL-resistant BL lines were equally sensitive to CTLs directed against the HLA class I specificities B27, A24, and B35 as were the corresponding LCLs.

Down-Regulation of HLA-A11 in BL Cells. Previous studies (5, 6) detected no systematic difference in the MHC class I antigen expression of BL and LCL lines, as judged by their

Table 1. Characteristics of the cell lines

Name*	Cell type	EBV	HLA-A,B type†	Phenotype‡	Ref.
IARC-139	LCL	+	A1/A11, B8/B16	III	7, 8
BL-28	BL (8:14)	−	A1/−, B8/B16	I	7, 8
E95A-BL-28	B95-8 conv.	+	A1/−, B8/B16	I	This work
E95B-BL-28	B95-8 conv.	+	A1/−, B8/B16	I	This work
IARC-171	LCL	+	A11/A32, B35/Bw49	III	7, 8
BL-41	BL (8:14)	−	−/A32, B35/Bw49	I	7, 8
BL-41/95	B95-8 conv.	+	A11/A32, B35/Bw49	II	7, 8
E95A-BL-41	B95-8 conv.	+	−/A32, B35/Bw49	II	This work
E95B-BL-41	B95-8 conv.	+	−/A32, B35/Bw49	II	This work
E95C-BL-41	B95-8 conv.	+	−/A32, B35/Bw49	II	This work
EHRA-BL-41	P3HR1 conv.	+	−/A32, B35/Bw49	I/II	This work
EHRB-BL-41	P3HR1 conv.	+	−/A32, B35/Bw49	I/II	This work
WW-1-LCL	LCL	+	A11/A24, B27/B62	III	9
WW-1-BL	BL (8:14)	+	A11/A24, B27/B62	II	9
IARC-307	LCL	+	A11/Aw28, B27/Bw45	III	7, 8
BL-72	BL (8:14)	+	A11/Aw28, B27/Bw45	I/II	7, 8
DG-75	BL (8:14)	−	A2/A11, Bw41/Bw39	I	10

Conv., converted, obtained by *in vitro* infection of the EBV$^-$ BLS with the B958 or P3HR1 strains of EBV.
*All lines within each group have been derived from the same individual.
†The HLA-A11 antigen was not detected in the BL-28 and BL-41 lines by complement-mediated lysis using two anti-HLA-A11-specific typing antisera. The binding of these sera to HLA-A11-positive LCLs and peripheral blood lymphocytes was not diminished by absorption with up to 40 $\times$ 10^6 cells per ml of BL-28 and BL-41 (not shown).
‡As described in *Material and Methods*.

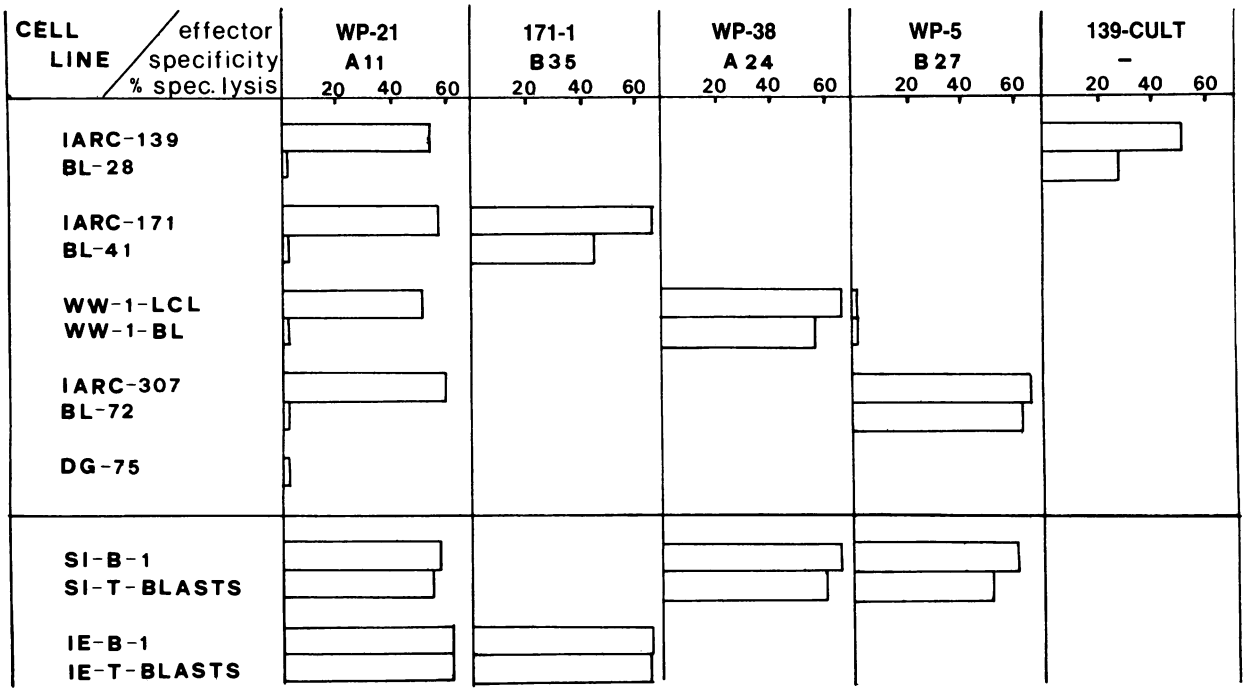

FIG. 1. Sensitivity of LCL and BL lines to allospecific cytotoxicity. The mean percentage of specific ^{51}Cr release at a 5:1 effector/target ratio from three to four assays for each effector/target combination is shown. The WW-1-LCL, WW-1-BL pair expresses a variant of the HLA-B27 antigen.

reactivity with an anti-MHC class I framework mAb. We now have compared LCL and BL lines derived from HLA-A11-positive donors with regard to their ability to bind the same antibody (W6/32), in comparison with the AUF 5.13 mAb directed against a polymorphic determinant shared by HLA-A3 and -A11 (17). There was no major difference in the binding of W6/32, while AUF 5.13 binding was strongly reduced in all five BL lines, suggesting that their resistance to anti-HLA-A11 CTLs is due to a selective decrease of HLA-A11 expression (Fig. 3). Only BL-41/95 expressed the antigenic determinant recognized by the AUF 5.13 mAb at a similar level to that expressed by LCLs and the mitogen-induced T blasts.

EBV Antigen Expression. Whole-cell extracts of LCLs, EBV-carrying BL lines, and EBV-converted BL lines were probed with human sera containing antibodies to EBNAs 1, 2, 3, and 4 and with the S-12 mAb directed against the carboxyl-terminal part of the LMP molecule. Bands for EBNAs 1–4 with characteristic size variations were detected in all of the cell lines (not shown). LMP was detected as a

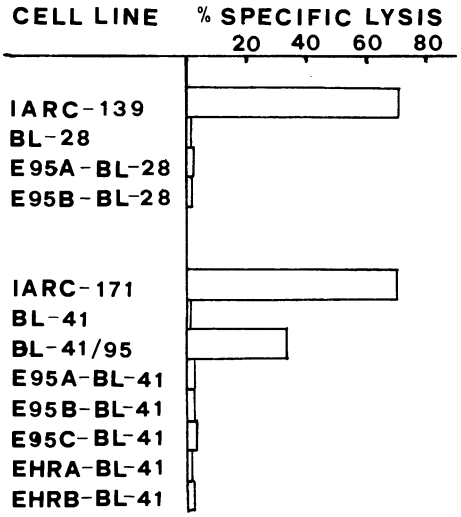

FIG. 2. Sensitivity of LCLs, EBV-negative, and EBV-converted BL lines to HLA-A11-specific CTL lysis. The WP-21 CTL clone was used as effector. Specific ^{51}Cr release (mean of three experiments) at a 5:1 effector/target ratio in 4-hr assays is shown.

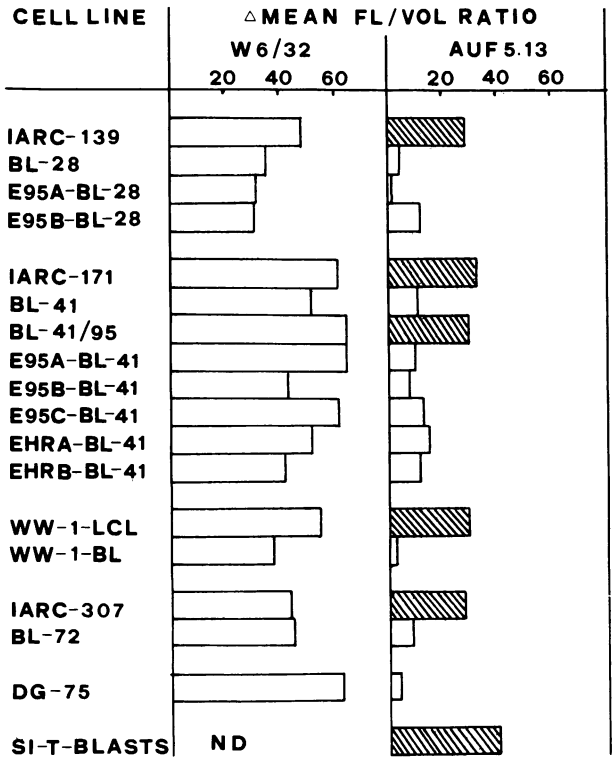

FIG. 3. Expression of HLA class I (framework) and HLA-A11 antigens in B-cell lines and T blasts. One representative experiment is shown out of three performed. The Δ mean Fl/Vol ratio of the cells sensitive to HLA-A11 specific cytotoxicity is indicated by the hatched bars.

major band of ≈62 kDa in all four LCLs (Fig. 4) as expected (20, 23). In the two EBV-positive BL lines WW-1-BL and BL-72, LMP appeared as a weak band of ≈58 and ≈62 kDa, respectively. BL 41/95 was unique among the EBV-converted lines in expressing a readily detectable 62-kDa LMP band. A weak band was detected in E95B-BL-28 and E95B-BL-41 in some of the blots.

The relatively low expression of LMP in the EBV-carrying BL cells was confirmed by immunodotting analysis of serially diluted whole-cell extracts (Fig. 5). With the exception of BL-41/95, all originally EBV-positive and *in vitro* EBV-converted BL lines were either negative or expressed LMP levels 1/5th to 1/10th that of the LCLs derived from the same donors. In contrast, the LMP expression of BL-41/95 was similar to the corresponding LCL, IARC-171. Analogous results have been recently obtained with a highly sensitive LMP-specific radioimmunoassay (24).

DISCUSSION

Down-regulation of certain HLA class I antigens and the parallel resistance of BL cells to CTL-mediated responses, whether triggered by EBV antigens or, in the case of the EBV-negative BL lines, by other stimuli, may contribute to the successful neoplastic growth of the cells *in vivo*. The preferential down-regulation of HLA-A11 in BL lines raises the interesting possibility of functional differences among various members of the HLA complex. This has been suggested previously by family studies showing that cytotoxic responses to autologous EBV-infected cells are usually restricted to certain HLA specificities (25).

LMP is the major EBV-encoded membrane antigen in growth-transformed cells (20, 26) and, therefore, is regarded as a probable target for EBV-specific CTLs (26, 27). Its decreased expression in BL cells might be viewed as the consequence of immunoselection *in vivo*. Our finding that, independently of their EBV-carrying status, BL cells show a selective down-regulation of HLA-A11 would imply that both EBV-negative and EBV-positive BL lines have been subjected to immunoselection. However, this possibility is inconsistent with the fact that LMP expression was equally low in the *in vitro* EBV-converted BL lines as in the lines derived from the EBV-carrying tumors. Therefore, a low expression of LMP may be a phenotypic property of the BL cells.

Biopsies and early *in vitro* cultures of EBV-negative and EBV-positive BL cells have a similar phenotype. Their expression of the common acute lymphoblastic leukemia antigen, BL-associated antigen, and other markers that are usually associated with resting B cells and the lack of activation markers is particularly noteworthy (15, 28). EBV-carrying BL lines tend to drift towards more "LCL-like" phenotypes after prolonged *in vitro* culture (11). Similar changes could be induced in some EBV-negative BL lines by *in vitro* EBV conversion (15, 28). This phenotypic shift was particularly pronounced in the BL-41/95 line. In addition to the expression of several activation markers, BL-41/95 was unique among the EBV-converted BL-41 sublines in showing a decreased agarose clonability (10–20%)—intermediate between the highly clonable BL-41 (60–80%) and the lowly clonable (<1%) LCL IARC-171 (S.T., unpublished data).

The lack of activation markers in cells expressing the "genuine" BL phenotype is in line with our suggestion that BL lines may originate from deregulated memory B cells (29). If T cells normally limit the overproliferation of activated B blasts, memory cells would have to be resistant to this regulation, otherwise there would be no memory. It is tempting to speculate that the juxtaposition of the c-*myc* gene to immunoglobulin gene sequences may play a double role in the tumorigenic process. Constitutive expression of c-*myc* may favor proliferation at the expense of maturation and differentiation, as demonstrated in other systems (30–32). In addition, the phenotype-associated down-regulation of cer-

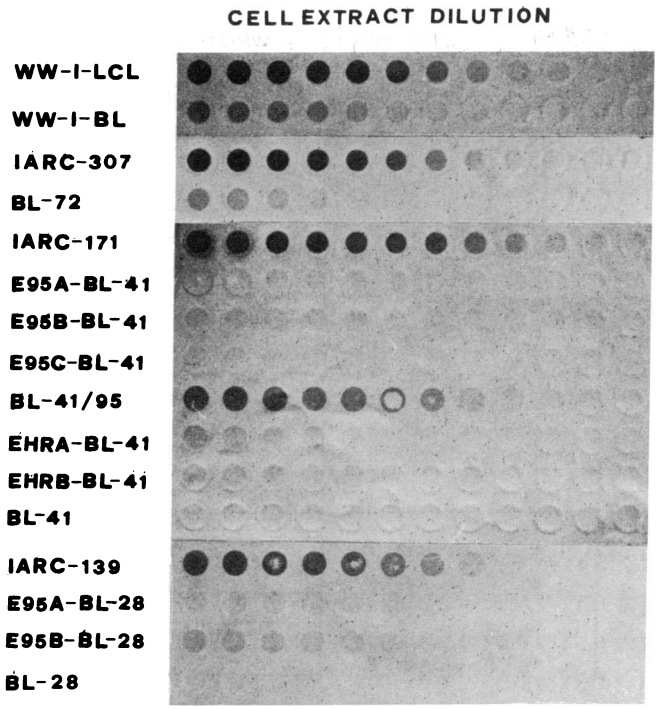

FIG. 5. Quantitative analysis of LMP expression in EBV-positive B-cell lines. Serial dilutions of total cell extracts were blotted on nitrocellulose paper and probed with the S-12 mAb.

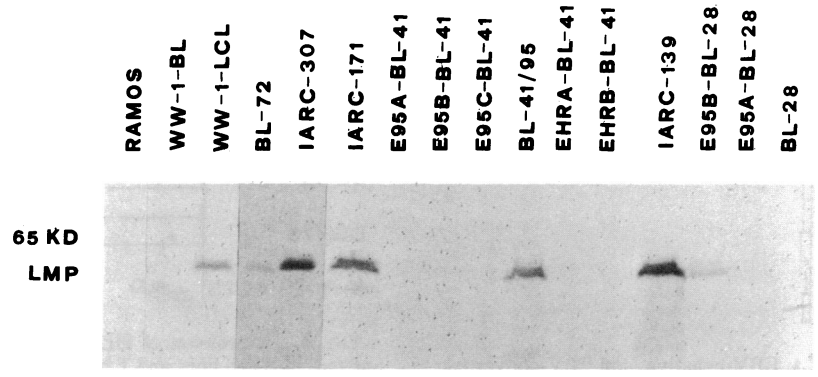

FIG. 4. Detection of LMP in LCLs, EBV-carrying BL lines, and EBV-converted BL lines. Total cell extracts (10^6 cells per lane) were electrophoresed on 7.5% polyacrylamide gels. The proteins were blotted on nitrocellulose papers and probed with the S-12 mAb.

tain HLA specificities and, in the virus-carrying BL lines, also of EBV LMP, may facilitate the escape of the cells from CTL-mediated controls.

The frequency of the immunoglobulin gene/*MYC* translocation in BL cells approaches 100% (33). It would not be surprising if an event of such high penetrance would favor progressive tumor growth by more than one mechanism.

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