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# Recognition of the Epstein–Barr virus-encoded nuclear antigens EBNA-4 and EBNA-6 by HLA-A11-restricted cytotoxic T lymphocytes: Implications for down-regulation of HLA-A11 in Burkitt lymphoma

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ABSTRACT **Evasion from cytotoxic T-lymphocyte (CTL) surveillance may be an important step in the pathogenesis of Epstein–Barr virus (EBV)-carrying Burkitt lymphoma (BL) as suggested by the consistent down-regulation of all transformation-associated viral antigens, except EBV nuclear antigen 1 (EBNA-1), and of certain HLA class I alleles in BL biopsies and cell lines that maintain the tumor cell phenotype *in vitro*. The most common HLA class I defect recorded in BL lines is a selective down-regulation of HLA-A11. To gain some insight into the role of HLA-A11 down-regulation in pathogenesis of BL, we have investigated the target specificity of HLA-A11-restricted CTLs derived by stimulation of lymphocytes from three EBV-seropositive individuals with autologous EBV-transformed lymphoblastoid cell lines. Recombinant vaccinia viruses carrying the coding sequences for EBNA-1, -2A, -2B, -5, -3, -4, and -6 (also known as EBNA-1, -2A, -2B, -LP, -3a, -3b, and -3c, respectively) and EBV latent membrane protein 1 were used to induce high levels of expression of the relevant EBV antigen in fibroblasts derived from HLA class I-matched individuals. EBNA-4-expressing fibroblasts were the predominant target of HLA-A11-restricted CTLs in all three donors. A less pronounced and less regular EBNA-6-specific cytotoxic component was found in two of the donors.**

Epstein–Barr virus (EBV), a human herpes virus that asymptomatically infects the majority of adult populations worldwide, causes infectious mononucleosis and is strongly linked with endemic Burkitt lymphoma (BL), nasopharyngeal carcinoma, and the large cell lymphomas arising in immunosuppressed patients. Underlying the association of EBV with lymphoid malignancies is the capacity of the virus to transform B lymphocytes into lymphoblastoid cell lines (LCLs), which constitutively express at least six EBV-encoded nuclear proteins, EBNA-1 to EBNA-6 (also known as EBNA-1, -2, -3a, -3b, -LP, and -3c) and two latent membrane proteins, LMP-1 and LMP-2 (reviewed in ref. 1).

Cytotoxic T lymphocytes (CTLs) are thought to play an important role in controlling the proliferative potential of EBV-infected B lymphocytes, which persist for life in healthy virus carriers. EBV-specific CTL precursors can be reactivated *in vitro* by stimulation of lymphocytes from EBV-seropositive individuals with the autologous EBV-transformed LCLs (2). The CTL target was operationally called LYDMA (lymphocyte-detected membrane antigen), but it was soon realized that more than one virally encoded protein could serve this function. The assumption has been recently confirmed by the finding that EBNA-2 (3, 4), -3 (5), and -6 (6) are recognized by EBV-specific effectors. The demonstration of selective interaction between peptides derived by processing of protein antigens and polymorphic residues within the antigen binding groove of major histocompatibility complex (MHC) molecules (7, 8) suggests that recognition of these proteins may depend on the repertoire of HLA class I alleles that serve as restriction elements in each individual.

The role of CTL surveillance in keeping EBV-transformed cells in check is substantiated by the finding that relaxation of immunosuppressive therapy can lead to regression of immunoblastic lymphomas that express a LCL-like phenotype (9, 10). In contrast, EBV-carrying BLs arise in patients with normal levels of EBV-specific CTL precursors (11). This suggests that BL cells escape EBV-specific immunity by virtue of cellular rather than systemic changes. The phenotypic differences between LCL and BL cells are consistent with this possibility. BL biopsies and representative cell lines express only EBNA-1 (12), have relatively low expression of adhesion molecules (13) and HLA class I (14), and show a selective down-regulation of certain HLA-A and -C alleles (15). The most common HLA class I defect recorded in BL lines is a selective down-regulation of HLA-A11, which has been found to occur in all seven BL lines derived from HLA-A11-positive individuals investigated so far (ref. 15; M.G.M., unpublished observations). These phenotypic characteristics are often lost on *in vitro* culture (16–18). Selection against immunogenic variants with up-regulated HLA class I and EBV antigen could play a role in the pathogenesis of BL. In this study, we have taken a step toward analysis of this hypothesis by identifying the viral antigens that are recognized by EBV-specific CTLs in association with HLA-A11. Recombinant vaccinia viruses were used as vectors for expression of the prototype B95-8 virus-derived EBNA-1 to -6 and LMP-1 in HLA-A11-positive fibroblasts. We show that EBNA-4 and, somewhat less regularly, EBNA-6 are recognized by HLA-A11-restricted EBV-specific CTLs.

## MATERIALS AND METHODS

**Cell Lines.** LCLs were obtained by transformation of B lymphocytes with the B95-8-derived EBV strain. Fibroblast lines were established from skin biopsies and were used within the first 20 *in vitro* passages.

**Vaccinia Virus Recombinants and Fibroblast Infection.** Generation of vaccinia virus recombinants carrying the coding sequences for EBNA-2A, -5, and -6 from the B95-8 virus, and EBNA-2B from the AG876 virus, has been described (4).

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

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Recombinants carrying the EBNA-3 and -4 and the LMP-1 genes were produced as described (19; unpublished data) by cloning the relevant B95-8-derived cDNAs into the pSC11 plasmid. In each case, the EBV sequences were positioned downstream of the vaccinia P7.5 early late promoter (20). In the recombinant vaccinia virus (Vacc)–EBNA-1 construct, the EBNA-1 coding sequence was cloned in front of the T7 polymerase promoter (21). Recombinant vaccinia viruses were generated by transfection of the plasmids described above into WR-strain wild-type vaccinia-infected thymidine kinase-negative $TK^-143$ cells. The viruses were expanded and titrated in CV-1 cells as described (4). Subconfluent monolayers of human fibroblasts were routinely exposed to the recombinant viruses at a multiplicity of infection (moi) of 5 for 12 hr at 37°C. Cells infected with the Vacc–EBNA-1 recombinant were coinfected with a vaccinia recombinant carrying the T7 polymerase gene in order to induce EBNA-1 expression (21).

**Detection of EBV Antigens.** Total cell extracts were separated in SDS/7.5% polyacrylamide gels, blotted on nitrocellulose filters, and probed with previously characterized human sera PG or HR containing high antibody titers to EBNA-1 to -6. Monospecific reagents included affinity purified anti-107 human antibodies and the absorbed human serum JAC specific for EBNA-1 (22), the monoclonal antibody (mAb) PE-2, which detects both the EBNA-2A and -2B isotypes (23), the mAbs JF186 (24) and S-12 (25), which are specific for EBNA-5 and LMP-1, respectively. EBNAs and LMP-1 staining were performed by indirect immunofluorescence with fluorescein-conjugated rabbit anti-human and rabbit anti-mouse IgG, respectively.

**Generation of CTLs.** HLA-A11-restricted EBV-specific CTLs were obtained by stimulation of lymphocytes from the EBV-seropositive donors BK (HLA-A2, -11 and HLA-B7, -35), SI (HLA-A11, -24 and HLA-B7, -27), and EA (HLA-A10, -11 and HLA-B35, -51) with the autologous B95-8 virus-transformed LCLs. HLA-A11 allospecific CTLs were generated by stimulation of lymphocytes from the HLA-A11-negative donors FB (HLA-A3, -25 and HLA-B8, -51) with the KK LCL (HLA-A11, -28 and HLA-B14, -40) (26). Polyclonal CTL cultures were expanded in complete medium supplemented with 30% (vol/vol) culture supernatant from the interleukin 2 producer gibbon lymphoma line MLA144 and 10 units of human recombinant interleukin 2 per ml.

**Cytotoxicity Assays.** Appropriate panels of EBV-transformed LCLs and recombinant vaccinia virus-infected fibroblasts were used as targets in standard 4-hr chromium-release assays (26). Under the conditions of infection described above, the spontaneous release of uninfected and recombinant vaccinia virus-infected fibroblasts did not, as a rule, exceed 20% of the total incorporation.

## RESULTS

**Expression of EBV Antigens in Recombinant Vaccinia Virus-Infected Fibroblasts.** The capacity of recombinant vaccinia viruses to induce expression of the relevant EBV antigen in human fibroblasts was confirmed by immunofluorescence and immunoblotting. As shown in Fig. 1, EBNA-1, -3, -4, and -6 and LMP-1 were expressed as the predicted 72-, 145-, 160-, 150-, and 62-kDa polypeptides, respectively, at levels comparable or higher than in the reference B95-8 virus-transformed LCLs. EBNA-2A and -2B appeared as doublets of 84–85 kDa and 74–75 kDa and EBNA-5 was expressed as a polypeptide ladder of 18–45 kDa. Fluorescence staining with the appropriate monoclonal and polyclonal reagents confirmed that the antigens expressed from vaccinia recombinants assumed the expected nuclear or cytoplasmic localization (Fig. 2). The only exception was EBNA-1, which consistently gave a diffuse cytoplasmic and membrane fluorescence. In spite of its abnormal localization, which may be due to interaction of EBNA-1 with vaccinia virus proteins or DNA, this fluorescence was specific, as confirmed by staining with the monospecific absorbed human serum JAC and with affinity-purified anti-107 antibodies.

Time course and titration experiments were carried out with each recombinant virus in order to optimize the conditions of infection. A representative experiment performed with the Vacc–EBNA-2A recombinant is shown in Table 1. Thirty-two percent of the fibroblasts expressed EBNA-2A after infection for 1 hr with the Vacc–EBNA-2A virus at a moi of 20. The number of positive cells increased with time and reached a plateau after 3 hr. Likewise, the intensity of the EBNA-2A-specific band detected in immunoblots reached levels much higher than in the B95-8-transformed reference LCL within 2–3 hr (data not shown). A significant cytopathic effect, characterized by cell rounding and detachment from the monolayer, became evident after 4–5 hr. Appearance of the cytopathic effect could be delayed by reducing the moi. Thus, the infections were routinely carried out at a moi of 5 for 12 hr. More than 90% of the fibroblasts infected under these conditions were viable as judged by trypan blue dye exclusion.

**Cytotoxic Sensitivity of Recombinant Vaccinia Virus-Infected Fibroblasts.** HLA-A11 allospecific CTLs were ob-

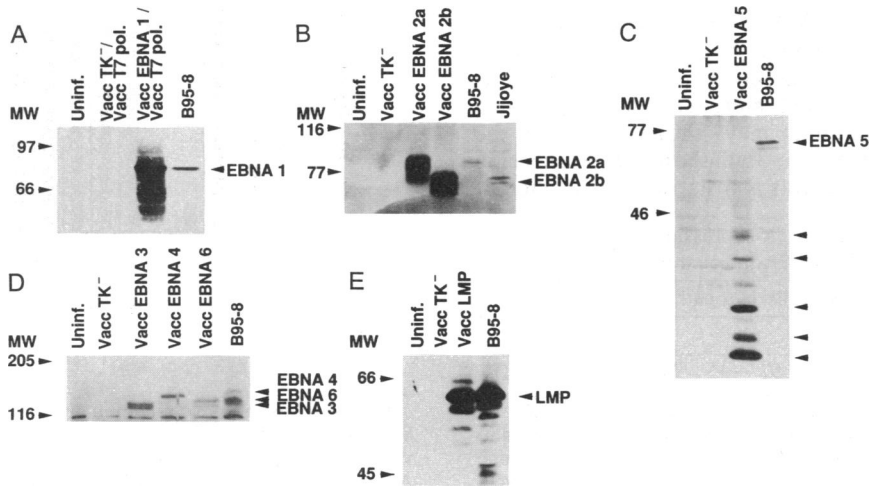


FIG. 1. Expression of EBV antigens in recombinant vaccinia virus-infected fibroblasts. Immunoblots of protein extracts from uninfected fibroblasts and from fibroblasts infected with Vacc–$TK^-$; Vacc–EBNA-1, -2A, -2B, -3, -4, -5, and -6; and LMP-1 were probed with the adsorbed human serum JAC (*A*), the PE-2 mAb (*B*), the JF186 mAb (*C*), the human serum PG (*D*), the S-12 mAb (*E*). Positions of molecular weight (MW) markers ($\times 10^{-3}$) are indicated.

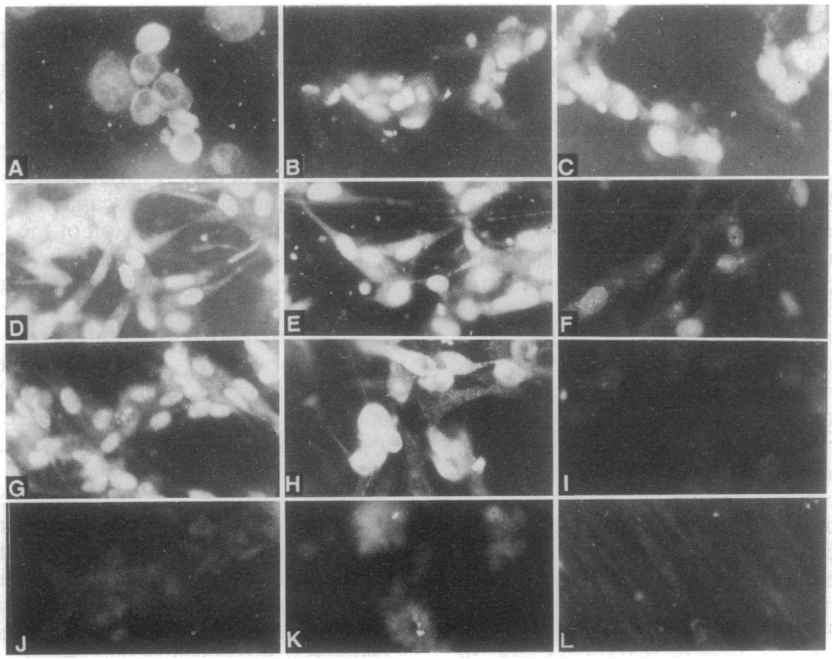


FIG. 2. EBV antigen expression detected by immunofluorescence. Human fibroblasts were grown on glass coverslips. Monolayers were fixed in acetone/methanol (2:1) for EBNA or in methanol for LMP staining. EBNA-2, EBNA-5, and LMP-1 expression was assessed by incubating monolayers with the PE-2, JF186, and S-12 mAbs, respectively, followed by incubation with fluorescein-conjugated rabbit anti-mouse IgG. For EBNA-1, -3, -4, and -6 staining, the monolayers were incubated with the PG serum, followed by fluorescein-conjugated rabbit anti-human and swine anti-rabbit IgG antibodies. The conjugate antibodies were preadsorbed with 10% serum from an EBV-seronegative donor. (*A–H*) Fibroblasts infected with Vacc–EBNA-1, -2A, -2B, -3, -4, -5, and -6 and LMP-1, respectively. (*I–K*) Fibroblasts infected with Vacc–TK$^-$ stained with PG serum, an EBV-negative human serum (IE), and BSS, respectively. (*L*) Uninfected fibroblasts stained with PG serum.

tained by stimulation of lymphocytes from FB with the HLA-A11-positive KK LCL. As shown in Fig. 3, uninfected KK fibroblasts were lysed by these effectors. Lysis was inhibited by preincubation of the targets with the anti-HLA class I framework mAb W6/32 (data not shown). Uninfected and recombinant vaccinia virus-infected fibroblasts were equally sensitive to these effectors.

Uninfected and recombinant vaccinia virus-infected fibroblasts were tested for sensitivity to EBV-specific, HLA-A11-restricted CTLs. Polyclonal CTL populations were obtained from three HLA-A11-positive donors—BK, EA, and SI—known to generate EBV-specific effectors predominantly restricted through HLA-A11. Each effector population lysed the autologous B95-8-transformed LCL and HLA-A11-matched LCLs and did not lyse HLA-A11-positive T blasts, HLA-A11-negative LCLs, and the prototype NK-sensitive target K562 (data not shown). Effector/target combinations sharing HLA-A11 only were chosen to avoid interference by CTLs acting through other restrictions that may still be present in the polyclonal cultures. The mean ± SE of the percentage specific lysis recorded in 12 experiments and statistical evaluation of the difference between uninfected and infected cells are shown in Fig. 4. EBNA-4-expressing fibroblasts were more sensitive than the uninfected and Vacc–TK$^-$-infected fibroblasts in each experiment. The difference was statistically significant ($P < 0.001$). Fibroblasts infected with Vacc–TK$^-$ and with vaccinia recombinants carrying the EBNA-1, -2A, -2B, -3, and -5 and LMP-1 genes showed no or only a slight increase of CTL sensitivity. As shown in Fig. 5, the effector populations

Table 1. Time course of EBNA-2A expression and induction of cytopathic effect in Vacc–EBNA-2A-infected fibroblasts

| | % positive cells/cytopathic effect | | | | | |
|---|---|---|---|---|---|---|
| moi | 1 hr | 2 hr | 3 hr | 4 hr | 5 hr | 12 hr |
| 20 | 32/− | 70/± | 92/+ | 97/++ | 100/+++ | ND |
| 5 | 0/− | 15/− | 61/− | 89/± | 88/+ | 91/++ |

Semiconfluent monolayers of human fibroblasts were exposed to Vacc–EBNA-2A preparations. At different times from the beginning of infection the cells were stained with the PE-2 mAb by indirect immunofluorescence as described (24). Cytopathic effect was scored as follows: −, no apparent cytopathic effect; ±, cell rounding in some parts of the monolayer; +, diffuse cell rounding; ++, diffuse cell rounding with detachment of up to 10% of the cells; +++, detachment of up to 30% of the cells. ND, not determined.

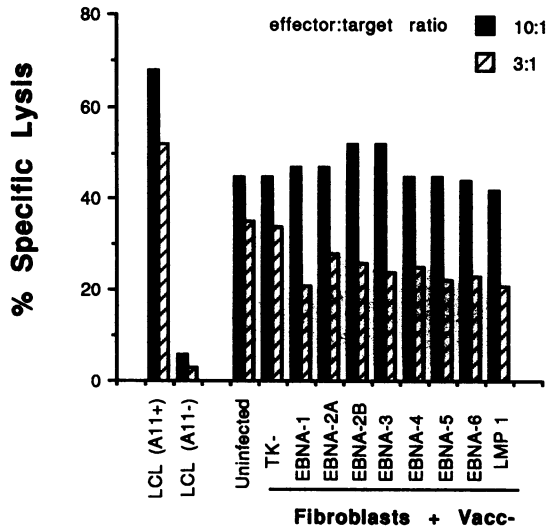


FIG. 3. Sensitivity of recombinant vaccinia virus-infected fibroblasts to HLA-A11 allospecific CTLs. Uninfected and recombinant vaccinia virus-infected fibroblasts from KK were exposed to HLA-A11 allospecific CTLs. More than 75% and up to 100% of the infected cells expressed the relevant EBV antigen by immunofluorescence. Effectors were shown to be HLA-A11 allospecific by their capacity to lyse allogeneic LCLs sharing the HLA-A11 but not the HLA-A28, -B14, -B40 alleles [LCL (A11−)]. One of three representative experiments is shown.

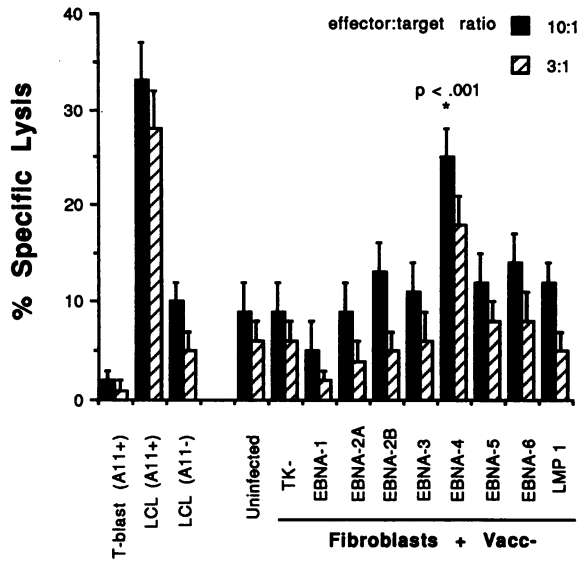


FIG. 4. Sensitivity of recombinant vaccinia virus-infected fibroblasts to HLA-A11-restricted EBV-specific CTLs. Uninfected and recombinant vaccinia virus-infected fibroblasts from the HLA-A11-positive donors BK and KK were tested for sensitivity to lysis by HLA-A11-restricted EBV-specific CTLs from the EBV-seropositive donors BK, EA, and SI. Specificity of effectors was in each case confirmed by the capacity to lyse autologous and allogeneic HLA-A11-positive LCLs. HLA-A11-positive T blasts, HLA-A11-negative LCLs, and the prototype NK-sensitive target K562 (data not shown) were not lysed. The mean ± SE of percentage specific lysis recorded in 12 experiments is shown.

derived from the EA and BK donors (Fig. 5 *A* and *C*) lysed the EBNA-6-expressing fibroblasts in some cases. Significant killing was observed in one of four and two of four experiments, respectively, and was in each case lower than the killing recorded against EBNA-4-positive fibroblasts. Expression of EBNA-1 to -6 and LMP-1 did not render HLA-A11-negative fibroblasts sensitive to HLA-A11-restricted CTLs and did not alter their sensitivity to non-MHC-restricted LAK cells (data not shown).

## DISCUSSION

Down-regulation of MHC class I antigens in tumors of different types may provide a common escape route from immune surveillance. The down-regulation may affect the entire MHC class I cluster, as in adenovirus-induced and $\beta_2$-microglobulin-deficient tumors; one of the HLA-A, -B, or -C loci, as in colon carcinoma and melanoma; or specific alleles, as in our studies on BL, with HLA-A11 being the prototype representative (for reviews, see ref. 27). Selective down-regulation of certain HLA class I alleles may favor neoplastic proliferation by preventing the recognition of strong rejection targets. To critically evaluate this hypothesis, we have endeavored to identify the virally encoded antigen or antigens that elicit HLA-A11-restricted responses against EBV-carrying immunoblasts.

Fibroblasts infected with recombinant vaccinia viruses carrying the EBNAs and LMP-1 genes were suitable targets for this analysis. The relevant EBV antigen could be detected in these cells prior to the appearance of vaccinia virus-induced cytopathic effects and the level of expression was comparable or higher than in regular EBV-transformed LCLs. Moreover, vaccinia infection did not alter their sensitivity to allospecific and non-MHC-restricted lysis. Among the EBV antigens tested, EBNA-4 was outstanding in its capacity to induce sensitivity to HLA-A11-restricted CTLs. Expression of both HLA-A11 and EBNA-4 was needed because infection with Vacc–EBNA-4 did not induce lysis of

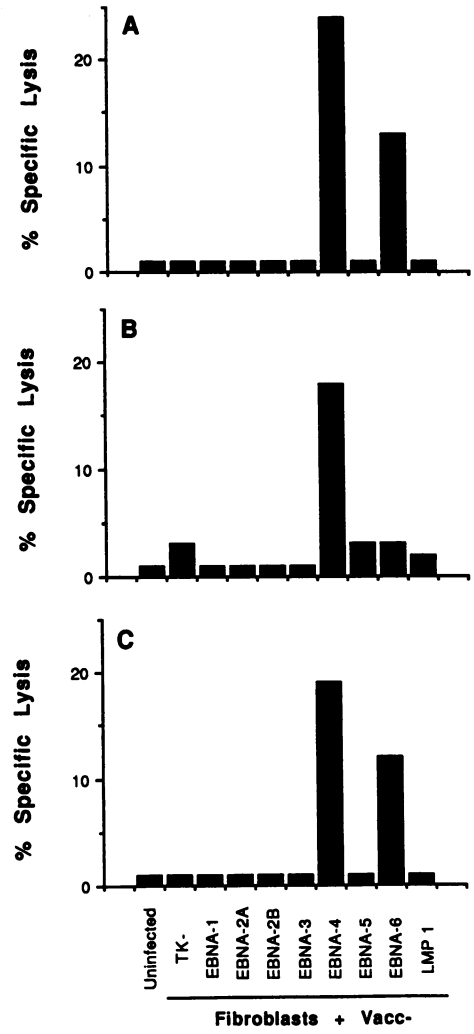


FIG. 5. Cytotoxicity of EA, SI, and BK CTLs against EBV antigen-expressing fibroblasts. Three experiments in which the cytotoxic activity of HLA-A11-restricted EBV-specific CTLs from EA (*A*), SI (*B*), and BK (*C*) donors was tested against allogeneic vaccinia virus-infected fibroblasts sharing HLA-A11. Percentage specific lysis recorded at an effector/target ratio of 10:1 is shown.

HLA-A11-negative fibroblasts. It is noteworthy that the LFA-1 and LFA-3 adhesion molecules are expressed at very low levels on fibroblasts (28, 29). Thus, down-regulation of adhesion molecules might not be sufficient to protect BL cells from antiviral immune surveillance.

EBNA-6-expressing fibroblasts were also killed in some of the experiments performed with CTLs from two of the donors, although the cytotoxic sensitivity was in each case lower than that induced by EBNA-4. The dual recognition is likely to reflect the presence of effector populations with different specificities in our polyclonal cultures. This possibility is supported by the observation that only two of three donors recognized EBNA-6. Moreover, the level of EBNA-6-induced lysis varied among experiments performed with the same culture, probably reflecting fluctuations in the predominance of single CTL clones. The recognition of more than one EBV antigen by HLA-A11-restricted CTLs may explain the early observation that effectors restricted through this allele often dominate in polyclonal EBV-specific CTL cultures from HLA-A11-positive donors (30, 31). The regularity of this response implies that the HLA-A11-restricted epitopes contained in the EBNA-4 and -6 antigens are conserved among different virus strains and play a predominant

role in the rejection of EBV-carrying B blasts. Our findings add to earlier reports demonstrating CTL recognition of EBNA-2, -3, and -6 (4–6) in showing that several of the EBV antigens expressed in LCLs may serve as rejection targets against EBV-transformed immunoblasts. The large choice of antigens, and the presumably even larger selection of antigenic peptides, may have a positive selective value because it will ensure recognition in the context of a wide variety of polymorphic HLA determinants.

How can we interpret these findings in view of the allele-selective down-regulation of HLA class I in BL cells? EBV-carrying BL cells are phenotypically different from EBV-transformed immunoblasts. All transformation-associated viral antigens are down-regulated in the tumors except EBNA-1 (16). EBNA-1-specific CTLs have not been demonstrated so far (this paper and unpublished data). This, and our observation that EBNA-1 expression cannot induce rejection of nonimmunogenic mammary carcinomas in syngeneic mice, while LMP-1 does (32), support the assumption that this viral antigen may not be recognized as broadly as other viral gene products. It is noteworthy that the recognition of EBNA-4 and EBNA-6 by our effectors is strong, although not conclusive, evidence that the requirements for EBNA recognition in EBV-infected B lymphocytes are not sufficiently different from those found in recombinant vaccinia virus-infected fibroblasts to account for the failure to detect EBNA-1-specific CTLs. A lower immunogenicity of EBNA-1, which is required for maintenance of the viral episomes in proliferating cells, and its expression in the absence of other highly immunogenic EBNAs in particular stages of B-cell activation/differentiation could be part of a viral strategy aiming to guarantee the lifelong persistence of EBV in immunocompetent hosts.

Our findings imply that selective down-regulation of HLA-A11 would not favor the escape of EBV-positive BLs from anti-viral immune surveillance since EBNA-1 is the sole EBV antigen characteristically expressed in the tumor. It is noteworthy that allele-selective defects of HLA class I were detected in phenotypically representative cell lines derived from both EBV-carrying and EBV-negative tumors (15). There are two possible interpretations for this finding. A low expression of HLA class I, which may be more pronounced for certain alleles, could reflect the mode of HLA gene regulation operating at the particular stage of normal B-cell activation/differentiation represented by BL. We have indeed shown that germinal center centroblasts express significantly lower levels of HLA class I compared to B blasts and memory B cells (18). This explanation, however, does not account for the heterogeneity of the HLA class I defects in BLs and the different capacity to restore allele-selective defects after interferon treatment (15). It is also inconsistent with the observation that HLA-A11 expression can be restored by transfection of plasmids carrying the gene under control of the autologous promoter (M.G.M., unpublished data). Alternatively, cellular antigens that are highly expressed or mutated in BL cells may serve as tumor-specific rejection targets in association with certain HLA class I specificities. Identification of the HLA-A11-restricted epitopes contained in EBNA-4 and -6, and definition of an HLA-A11 peptide binding motif, as already done for HLA-A2 and -B27 (7, 8), should provide the means to experimentally test this possibility.

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