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Human cytotoxic T-cell responses against Epstein–Barr virus nuclear antigens demonstrated by using recombinant vaccinia viruses

(immunosurveillance/herpesvirus/antigen recognition)

R. J. MURRAY*, M. G. KURILLA†, H. M. GRIFFIN*, J. M. BROOKS*, M. MACKETT‡, J. R. ARRAND‡, M. ROWE*, S. R. BURROWS§, D. J. MOSS§, E. KIEFF†, AND A. B. RICKINSON*¶

*Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham, Birmingham, B15 2TJ, United Kingdom; †Departments of Medicine and of Microbiology and Molecular Genetics, Harvard Medical School, 75 Francis Street, Boston, MA 02115; ‡Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Wilmslow Road, Manchester, M20 9BX, United Kingdom; and §Queensland Institute for Medical Research, Herston, Brisbane, Australia 4006

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ABSTRACT **The potentially pathogenic effects of infection with Epstein–Barr virus (EBV), a B-lymphotropic agent with cell growth-transforming potential, are contained in healthy virus carriers by virus-specific cytotoxic T-lymphocyte (CTL) surveillance. The target antigens against which such CTL responses are directed are yet undefined, but the antigens probably derive from one or more of the EBV "latent" proteins constitutively expressed in virus-transformed B cells. We have analyzed target specificity of CTL responses from two EBV-immune donors that are preferentially reactive against autologous cells transformed with type A but not with type B virus isolates. Coding sequences for four EBV latent proteins with allelic polymorphism between A and B virus types—namely, the EBV nuclear antigens (EBNAs) EBNA 2, EBNA 3a, EBNA 3c, and EBNA leader protein—have been introduced into vaccinia virus vectors under control of vaccinia promoter *P7.5* and used to express relevant EBNA proteins in appropriate target cells. Thus the CTL response from one donor has been mapped to type A EBNA 2 protein and from a second donor to type A EBNA 3a protein. Thereafter, a series of recombinant vaccinia viruses were constructed that carried specific internal deletions within the EBNA 2 type A coding sequence; by using these vectors, the above EBNA 2 type A-specific CTL response was shown to be directed against an epitope within a 100-amino acid fragment near the N terminus of the protein. This work clearly shows human CTL recognition of virus-coded nuclear antigens in the EBV system; moreover, it establishes an experimental approach that can be extended to all EBV latent proteins and to the more common CTL responses that cross-react against type A and type B virus isolates.**

Epstein–Barr virus (EBV), a human herpesvirus with potent growth-transforming activity in B lymphocytes, causes infectious mononucleosis and is strongly linked with two B-cell malignancies, endemic Burkitt lymphoma and the oligoclonal lymphomas to which immunologically compromised patients are especially prone (1). In healthy virus carriers, EBV-specific HLA antigen-restricted cytotoxic T lymphocytes (CTLs) appear important in controlling the virus infection in the B-cell system (2), and therefore determining the target antigens against which such CTL responses are directed is important. In this context EBV-transformed B cells, growing *in vitro* as lymphoblastoid cell lines (LCLs), constitutively express a limited number of virus gene products, the so-called virus latent proteins. These include six EBV nuclear antigens (EBNAs), EBNA 1, 2, 3a, 3b, 3c, and leader protein (EBNA-LP); a latent membrane protein (LMP), and possibly a second membrane-associated product, LMP-2 or terminal protein (3, 4). Any one of these proteins could potentially provide peptide epitopes for EBV-specific CTL recognition.

There are two distinct families of EBV isolate, types A and B, the existence of which was first recognized by allelic polymorphisms in the EBNA 2 coding sequence, producing EBNA 2 type A (EBNA 2A) and type B (EBNA 2B) proteins that share only 50% amino acid homology (5). However, more recent work showed that type A and type B virus genomes also differ in the exons of EBNA-LP (5, 6) and in the EBNA 3a, EBNA 3b, and EBNA 3c coding sequences. Allelic polymorphism in the EBNA 3 family of nuclear antigens was first suggested by serological studies (7), and sequence data have confirmed that the type B EBNA 3a, 3b, and 3c proteins show 86%, 80%, and 80% amino acid homology, respectively, with their type A counterparts (J. Sample and E.K., unpublished work). These findings were particularly relevant to recent work from one of our laboratories that identified CTL clones from two virus-immune donors that specifically recognized target cells transformed with type A but not with type B virus isolates (8). This result implied that in such cases one EBV latent protein that showed allelic polymorphism between the two virus types was providing the target epitope for type A-specific CTL recognition. In this study, we directly tested this hypothesis by using recombinant vaccinia viruses as vectors for expression of the type A EBNA 3a, EBNA 3c, EBNA-LP, and EBNA 2A proteins.

MATERIALS AND METHODS

Generation of Vaccinia Virus Recombinants. All EBNA coding sequences used to generate vaccinia virus recombinants were of B95.8 virus (type A) origin except the EBNA 2B sequence, which lay within a 2.1-kilobase (kb) fragment of the AG876 virus genome obtained from a partial *Rsa* I–*Sma* I digest (5). EBNA-LP sequence was derived from cDNA clone T65 (6), and EBNA 3c sequence was derived from cDNA clones T36 and T27, as described (9). A partial EBNA 3a sequence (which could encode a truncated protein beginning at internal methionine residue 133) lay within a genomic clone comprising nucleotides (nt) 92703–95239 obtained from a partial *Bam*HI–*Eco*RI digest, and the EBNA 2A sequence lay within a genomic clone comprising nt 48475–50305 (10). EBNA 2A deletion mutants (except EBNA 2Δ) were produced by cloning the above EBNA 2A sequence into vector

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Abbreviations: EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocyte; EBNA, EBV nuclear antigen; EBNA 2A and EBNA 2B, type A and type B EBNA 2, respectively; EBNA-LP, EBNA leader protein; LCL, lymphoblastoid cell line; mAb, monoclonal antibody; nt, nucleotide(s); moi, multiplicity of infection; TK, thymidine kinase; Vacc–, vaccinia (recombinant).

¶To whom reprint requests should be addressed.

Bluescript pBS at *Kpn* I and *Xba* I sites and were designed to keep the initiation and termination codons intact. Thus, pEBNA2HB was derived by cutting at the *Bam*HI site (48848), repairing the ends, cutting at the *Hin*cII site (48555), and subsequent recircularization; pEBNA2BB was derived by digestion of the *Bst*EII site (48939), repairing the ends, cutting at the *Bal* I site (49473), and recircularization; pEBNA2St was derived by digestion at three internal *Stu* I sites (49098, 49149, and 49203), gel purification of the vector, and recircularization; pEBNA2Sp was derived by digestion with *Sph* I (49240, 49645) and recircularization; pEBNA2PP was cloned by digestion with *Ppu*MI (49931) and partial digestion with *Pfl*MI (49704), gel purification of the vector band, repair of ends, and recircularization (note that the pEBNA2PP construct created an additional arginine codon at the deletion site). The remaining deletion mutant, EBNA 2Δ, was expressed from a B95.8 virus genomic fragment (48848–50303) generated by *Bam*HI/*Dra* I double digestion.

The above EBNA coding sequences, except for EBNA 3a and EBNA 2Δ, were cloned into the pSC11 plasmid (11); the partial EBNA 3a coding sequence was cloned into a related plasmid pGS62 (12), whereas EBNA 2Δ DNA was inserted into p62M, a transfer vector made by inserting a polylinker into the *Bam*HI–*Eco*RI site of plasmid pGS62, such that the inserted coding sequence was in frame with an initiating ATG. In each case, the EBV sequences were positioned downstream of the vaccinia *P*7.5 early-late promoter, which is the promoter of choice for expressing target proteins for CTL recognition (13). Vaccinia virus recombinants were generated according to Chakrabati *et al.* (11) by insertion of the above vectors into the vaccinia thymidine kinase (TK) gene, thus allowing negative selection for recombinants in bromodeoxyuridine.

Screening for Expression of EBV Latent Proteins. Monolayer cultures of TK$^-$143 cells and cell pellets of type B virus-transformed LCLs were exposed for 2 hr to recombinant vaccinia viruses at a multiplicity of infection (moi) of 10:1. Protein extracts were prepared from infected cells at regular intervals to 24 hr postinfection, separated by SDS/PAGE, and probed with appropriate monospecific antibodies by using published methods (14). Expression of EBNA 2A and 2B proteins was assayed by using mouse monoclonal antibody (mAb) PE2 (15), EBNA-LP was assayed by using mAb JF186 (16), and EBNA 3a and EBNA 3c were assayed by using affinity-purified antibodies from human serum RS22 (7).

Source and Generation of CTL Populations. Two EBV-immune donors were used, JS (HLA A1, A2, B8, B51) and LC (HLA A1, B8, B18). The dominant EBV-specific CTL response of both donors has been shown to be selectively directed against autologous type A virus-transformed cells (8, 17). Polyclonal CTL lines and CTL clones generated by seeding in semisolid agarose were established from these donors and maintained as described (8). All CTL clones were CD3$^+$ CD4$^-$ CD8$^+$.

Cytotoxicity Assays on Recombinant Vaccinia Virus-Infected Targets. Type B virus-transformed LCLs were infected with recombinant vaccinia viruses at a moi of 10:1 as above. Four hours after infection, cells were pelleted and incubated with $^{51}CrO_4$ for 1 hr, then washed three times, and used as targets in a standard 5-hr chromium release assay; the assay period was usually timed to run from 6 to 11 hr after infection. Effector CTLs were added to the assay at effector/target ratios between 2:1 and 20:1. For each assay, expression of the relevant EBV protein in target cells was confirmed from protein extracts of the same recombinant vaccinia virus-infected cells made 6 hr after infection.

RESULTS

Individual vaccinia virus recombinants were generated carrying the coding sequences for EBNA 2A; type A EBNA 3a, EBNA 3c, and EBNA-LP proteins; and for EBNA 2B protein. The capacity of these recombinant viruses to express the relevant EBNA species was, in each case, confirmed by infecting EBV-negative TK$^-$ 143 cells and, where appropriate, type B EBV-transformed LCLs of the kind used as targets in later cytotoxicity assays. By using a moi of 10, maximal levels of EBNA expression were achieved by 4 to 6 hr after infection for each recombinant virus and remained stable for at least 12 hr thereafter. Fig. 1 presents a composite

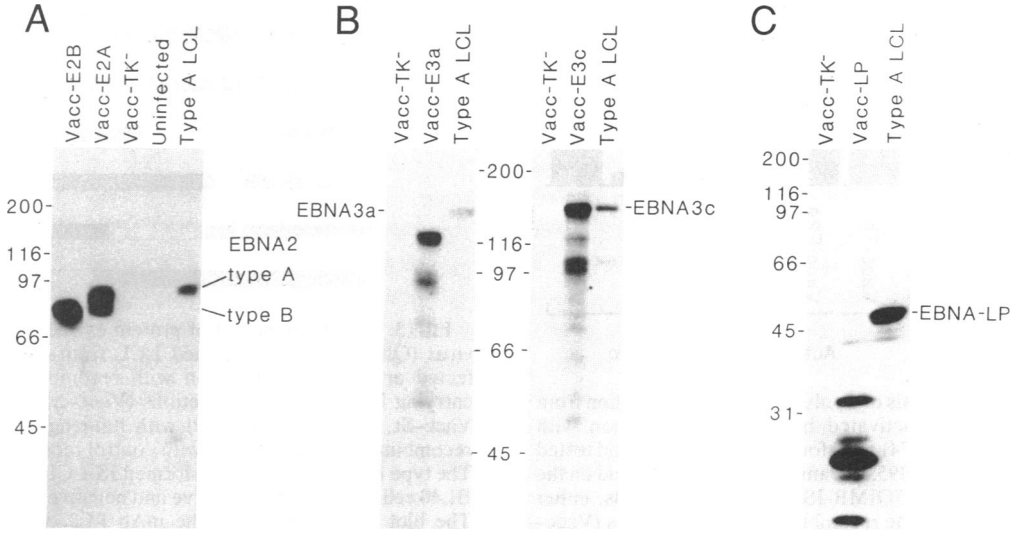

FIG. 1. Immunoblots of protein extracts from TK$^-$143 cells 6 hr after infection with recombinant vaccinia viruses carrying coding sequences for EBNA 2A (Vacc–E2A), EBNA 2B (Vacc–E2B), EBNA 3a type A (Vacc–E3a), EBNA 3c type A (Vacc–E3c), and EBNA-LP type A (Vacc–LP). TK$^-$143 cells either uninfected or infected with a control vaccinia recombinant (Vacc–TK$^-$) served as negative controls, and the type A virus (B95.8)-transformed IB4 cell line served as positive control. Blots were probed with mAb PE2 for EBNA 2 expression (*A*), with affinity-purified monospecific human antibody preparations for EBNA 3a and EBNA 3c expression (*B*), and with mAb JF186 for EBNA-LP expression (*C*). Each EBNA protein expressed in recombinant vaccinia virus-infected cells was of predicted size except EBNA-LP, which was expressed as a protein ladder in the 18- to 45-kDa gel region. Such variability of EBNA-LP may be from heterogeneity in the Vacc–LP virus preparation generated by recombination within the repeated *Bam*HI W exons of the EBNA-LP coding sequence (6).

of immunoblots of protein extracts from cells infected with vaccinia (Vacc)–EBNA 2A and Vacc–EBNA 2B (Fig. 1*A*), Vacc–EBNA 3a and Vacc–EBNA 3c (Fig. 1*B*), and Vacc–EBNA-LP (Fig. 1*C*) recombinants, probed with the relevant monoclonal or monospecific antibody. EBNA 2A, EBNA 2B, and EBNA 3c were expressed as the predicted 85-kDa, 75-kDa, and 149-kDa proteins, respectively, at levels significantly higher than those found for the corresponding virus-coded proteins in reference LCLs. The Vacc–EBNA 3a recombinant (carrying the large BERF1 exon of the EBNA 3a sequence) encoded a major 120-kDa molecule, which was slightly smaller than the full-length 142-kDa protein (BLRF3/BERF1-encoded) in LCLs. EBNA-LP was strongly expressed as a ladder of proteins in the 18- to 45-kDa region (see also Fig. 1 legend); such a ladder is also frequently seen in LCLs (16), although the X50-7 type A LCL used as control in Fig. 1*C* gives unusually strong expression of one of the high-molecular-weight EBNA-LP species.

In initial cytotoxicity experiments, a polyclonal CTL population from donor JS with a predominant type A virus-specific cytotoxic component was assayed on autologous type B virus-transformed LCL cells infected with the above recombinant vaccinias and with a control vaccinia TK$^-$ recombinant. Fig. 2 illustrates the clear pattern of results reproducibly obtained with this particular CTL population and also with a second polyclonal CTL line prepared from the same donor. Expression of EBNA 2A in the type B LCL led to lysis at levels at least as high as that seen with autologous type A virus-transformed target cells, whereas these same targets infected with the other recombinant vaccinias were never recognized. Other effector/target combinations included as controls in the same experiments confirmed that sensitization through EBNA 2A was specific to the autologous JS effector/target combination (Fig. 2 legend).

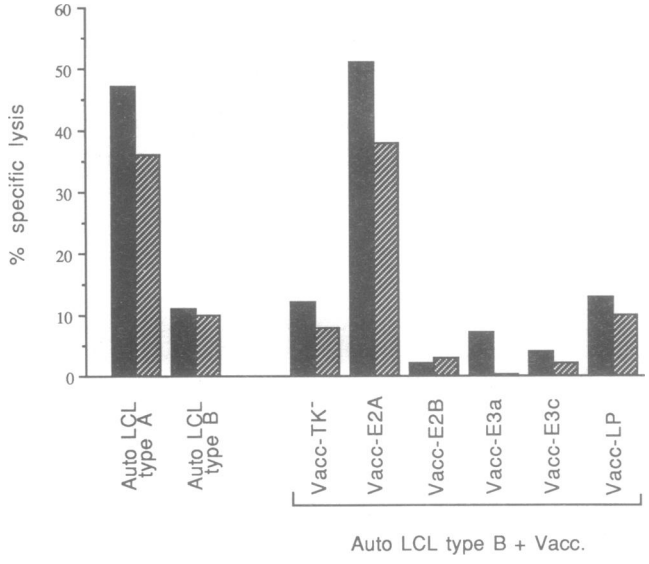

FIG. 2. Functional analysis of a polyclonal CTL preparation from the blood of donor JS reactivated by *in vitro* stimulation with autologous type A virus (BL74)-transformed LCL cells (8) and tested on autologous type A virus (B95.8)-transformed LCL cells and on the corresponding type B virus (QIMR-JSM)-transformed cells, either uninfected or infected with the recombinant vaccinia viruses (Vacc–TK$^-$, Vacc–E2A, Vacc–E2B, Vacc–E3a, Vacc–E3c, and Vacc–LP). Results are expressed as percentage specific lysis seen in a standard 5-hr chromium release assay; the two columns represent results obtained at effector/target ratios of 14:1 (■) and 6:1 (▩). In the same experiment EBV-specific CTLs from the HLA-mismatched donor CG (HLA A25, A28, B39, B62) gave 70% specific lysis of the autologous CG LCL but <12% specific lysis of the JS LCL targets (with or without recombinant vaccinia virus infection) (data not shown).

Further analysis of the EBNA 2A-directed CTL response shown by donor JS was done by using cloned CTL preparations established from this donor and target cells infected with a panel of vaccinia virus recombinants carrying deleted forms of the EBNA 2A coding sequence. Fig. 3 shows the various EBNA 2A deletions in schematic form and presents an immunoblot of protein extracts from type B virus-transformed LCL cells (from donor JS) after infection with the Vacc–EBNA 2A recombinants. High expression of truncated EBNA 2A proteins was obtained from all recombinant viruses, the apparent size of the truncated product always being consistent with that predicted from deletion size. The Fig. 3*A* immunoblot was probed with EBNA 2-specific mAb

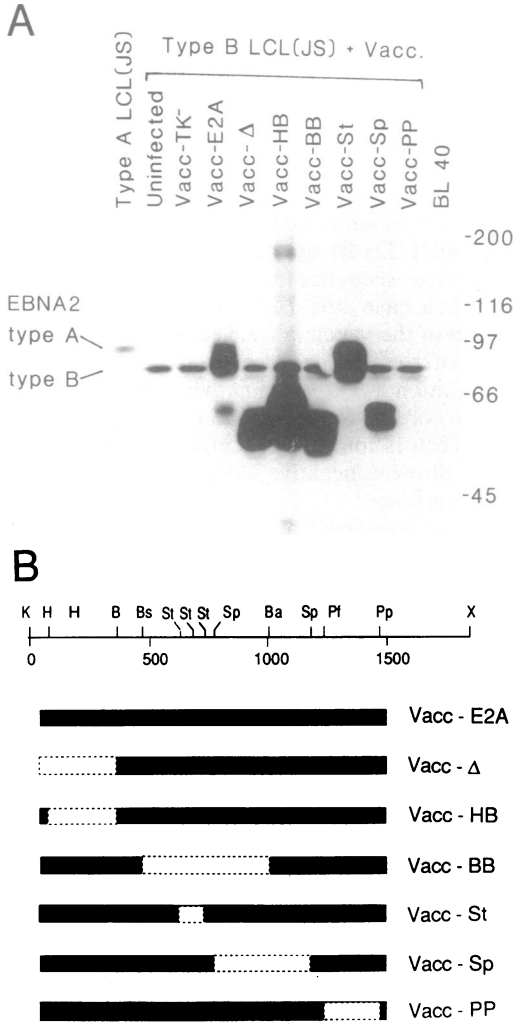

FIG. 3. (*A*) Immunoblot of protein extracts made from a type B virus (QIMR-JSM)-transformed LCL from donor JS, either uninfected or 7 hr after infection with recombinant vaccinia viruses carrying EBNA 2A gene deletions (Vacc–Δ, Vacc–HB, Vacc–BB, Vacc–St, Vacc–Sp, Vacc–PP) with full-length EBNA 2A vaccinia recombinant (Vacc–E2A) or with control recombinant (Vacc–TK$^-$). The type A virus (B95.8)-transformed JS-LCL and the EBV-negative BL40 cell line served as positive and negative controls, respectively. The blot was probed with the mAb PE2, which recognizes both EBNA 2A and 2B proteins. (*B*) Schematic diagram illustrating segments of B95.8 EBNA 2 coding sequences that were deleted (open areas) in constructing the vaccinia recombinants. Relevant restriction enzyme sites within the EBNA 2 coding sequence are shown as follows: K, *Kpn* I; H, *Hin*cII; B, *Bam*HI; Bs, *Bst*EII; St, *Stu* I; Sp, *Spn* I; Ba, *Bal* I; Pf, *Pfl*MI; Pp, *Ppu*MI; and X, *Xba* I). These deletion mutants have the capacity to encode truncated EBNA 2A proteins lacking the following amino acid residues: Δ, 1–118; HB, 19–118; BB, 151–327; St, 203–237; Sp, 251–384; and PP, 405–480.

PE2 and illustrates the high-level expression of the truncated proteins in this LCL when compared with the level of endogenous EBNA 2B protein. The apparent absence of an EBNA 2A species in cells infected with the Vacc–PP recombinant virus in Fig. 3*A* is because this deletion removes the epitope recognized by mAb PE2; presence of truncated EBNA 2A in these cells was confirmed by using a relevant polyclonal human serum (data not shown). In addition, immunofluorescence staining with mAb PE2 or with selected human sera showed that all truncated EBNA 2A proteins still localized to the nucleus of infected cells, although the nuclear staining patterns differed. EBNA 2-deletion (del) St and -del PP gave granular staining like that of the wild-type protein, EBNA 2-del BB and -del Sp produced larger more discrete aggregates (4–10 per nucleus) relative to wild type, whereas EBNA 2-del HB gave diffuse staining throughout the nucleus but spared the nucleolus (data not shown).

Three independent CTL clones derived from donor JS and showing type A-specific recognition were assayed on the autologous type B virus-transformed LCL after its infection with the above panel of recombinant vaccinia viruses. Fig. 4 illustrates the results obtained from one such clone; two other clones and the polyclonal line from donor JS described in Fig. 2 also consistently gave the same pattern of reactivity. Four of the vaccinia-deleted EBNA 2A recombinants produced the same sensitization to CTL lysis as the Vacc–full-length EBNA 2A recombinant; in contrast the Vacc–Δ and Vacc–HB recombinant viruses produced no sensitization. It was significant that these latter two recombinant viruses were distinct from all others in having overlapping deletions affecting a region near the N terminus of EBNA 2A protein (see Fig. 3*B*).

A final series of experiments was conducted with CTL clones derived from a second donor, LC, whose EBV-specific CTL response was similarly dominated by a type A virus-directed component. Here again the autologous type B virus-transformed LCL was tested for CTL recognition after its infection with vaccinia recombinants expressing the various type A EBNA proteins. Results from one such CTL

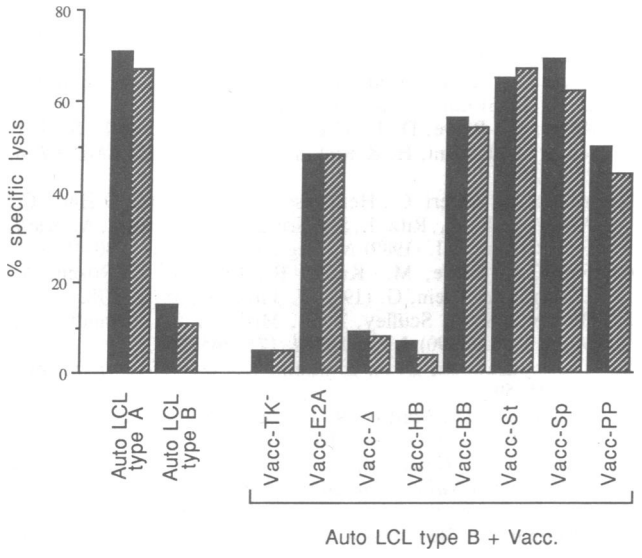

FIG. 4. Functional analysis of CTL clone 6 from donor JS, generated by an *in vitro* stimulation as described in Fig. 2 and tested on autologous type A virus (B95.8)-transformed LCL cells, and on the corresponding type B virus (QIMR-JSM)-transformed cells either uninfected or infected with recombinant vaccinia viruses carrying EBNA 2A gene deletions, with the full-length EBNA 2A-vaccinia recombinant, or with the control recombinant (Vacc–TK⁻). Results are expressed as in Fig. 2 for effector/target ratios of 5:1 (■) and 2:1 (▨).

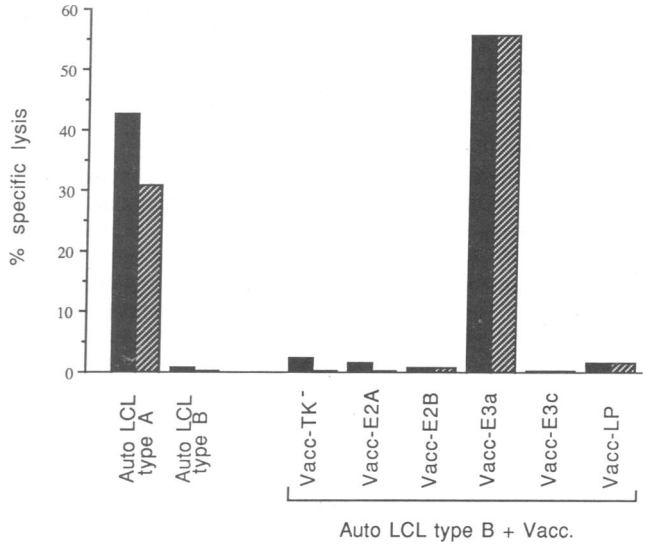

FIG. 5. Functional analysis of CTL clone 28 from donor LC generated from an *in vitro* stimulation with autologous type A virus (BL74)-transformed LCL cells (8) and tested on autologous type A virus (B95.8)-transformed LCL cells and on the corresponding type B virus (AG876)-transformed cells either uninfected or infected with recombinant vaccinia viruses as in Fig. 2. Results are expressed as in Fig. 2 for effector/target ratios of 6:1 (■) and 3:1 (□). In the same experiment, EBV-specific CTLs from the HLA mismatched donor LY (HLA-A1, A24, B27, B35) gave 48% specific lysis of the autologous LY LCL but <10% specific lysis of each LC LCL target (with or without recombinant vaccinia virus infection) (data not shown).

clone, shown in Fig. 5, represent those obtained with all eight individual clones tested from donor LC. In this case, clear sensitization was seen after infection with the EBNA 3a-encoding vaccinia recombinant, producing levels of lysis even higher than seen for the autologous type A virus-transformed LCL in the same experiment. Once again allogeneic effector/target combinations included as controls confirmed specificity of the above recognition (see Fig. 5 legend).

DISCUSSION

EBV-induced growth transformation of resting B cells to LCLs is associated with the expression of a limited number of virus genes encoding the virus latent proteins (3, 4) and also with a dramatic change in cellular gene expression such that the infected cells assume a lymphoblastoid phenotype and constitutively express high levels of cell-surface activation antigens (18) and adhesion molecules (19). Accordingly, ever since HLA-restricted CTL responses operationally specific for LCL cells were first demonstrated in EBV-immune donors, questions have been raised about the relative role of viral antigens versus virus-induced cellular antigens in sensitizing infected cells to such CTL recognition (20, 21). In this context recent work has shown that the EBV-induced up-regulation of cellular adhesion molecules, in particular ICAM-1 and LFA-3, on B cells is critical for efficient conjugate formation between these target cells and CTLs (19). Hence cellular changes play an important role in sensitizing infected B cells to CTL lysis by facilitating cell–cell adhesions important for the cytolytic mechanism but independent of cognate antigen recognition through the T-cell receptor (22). This paper is concerned with the cognate antigens mediating specific CTL recognition in the EBV system and shows that these antigens are of viral origin.

The observation (8) that EBV-specific CTL responses from certain virus-immune donors preferentially recognized autol-

ogous target cells transformed with type A but not with type B EBV isolates immediately suggested that in these cases CTL target epitopes were being derived from a polymorphic viral antigen. Of the eight EBV latent proteins constitutively expressed in LCLs, five are now known to show allelic polymorphism between type A and type B strains—namely, EBNAs 2, 3a, 3b, 3c, and -LP (5–7). For this reason, we constructed recombinant vaccinia viruses capable of expressing individual EBNA proteins (Fig. 1) by cloning relevant genomic sequences or cDNAs into vaccinia vectors under the control of vaccinia *P*7.5 early-late promoter; this strategy is now well established as a means of expressing viral target antigens for CTL assays (13). The results clearly show that in one case (donor JS) the type A EBV-specific CTL response is directed against epitopes of EBNA 2 protein (Fig. 2), whereas in another case (donor LC) the response is directed against epitopes from BERF-1-encoded fragment of EBNA 3a (Fig. 5).

Our experiments clearly show T-cell recognition of EBV nuclear antigens and add to the considerable body of evidence from other viral systems that virus-coded nuclear proteins are frequently the source of CTL-detected target epitopes. Work in the influenza virus system first showed that $CD8^+$ CTLs recognize short peptide fragments, presumably derived from the intracellular processing of virus-coded proteins and presented at the cell surface in conjunction with major histocompatibility complex class I molecules (23). Expression of truncated forms of viral proteins in appropriate target cells is therefore a useful first step in mapping the location of CTL epitopes. Here we have constructed a series of recombinant vaccinia viruses capable of expressing different fragments of the EBNA 2A protein (Fig. 3) and have used these to analyze the EBNA 2-directed CTL response mounted by donor JS. The results indicate that in this case the target epitope is derived from a 100-amino acid fragment of the EBNA 2A protein (amino acids 19–118 of the primary sequence)—i.e., that fragment not encoded by Vacc–HB and Vacc–Δ recombinant viruses (Fig. 4). Omitting the polyproline sequence, which constitutes more than one-third of this fragment in EBNA 2A and is much shorter in EBNA 2B, the fragment differs in 15 amino acids between EBNA 2A and EBNA 2B (5). Short peptide sequences that include such amino acid changes are clearly candidates for the target epitope being recognized in this situation. Work with synthetic peptides has not yet defined this epitope, but experiments that use a series of peptides corresponding to regions of the type A EBNA 3a protein have identified an epitope (amino acids 334–353) that lies within the BERF1-encoded fragment of the molecule and that is recognized by CTLs from donor LC (17).

Additional studies strongly suggest that the CTL responses from donors LC and JS described here are restricted through different HLA class I antigens. For EBNA 3a-specific clones from donor LC (HLA-A1, -B8, -B18) restriction is through HLA-B8 (17). For EBNA 2-specific clones from donor JS (HA-A1, A2, B8, B51) the restricting element has not been unequivocally identified; however, it appears to be an HLA class I antigen other than HLA-B8 because lysis of the autologous type A LCL is blocked by mAbs to an HLA class I framework determinant (8), but the effectors do not recognize type A LCLs from HLA-B8 matched donors (unpublished observations). Such findings serve to emphasize that the identity of CTL target epitopes for individual EBV-induced responses will depend entirely upon the identity of the presenting HLA antigen. We must stress that the present paper has been concerned with EBV type-specific CTL responses, whereas the majority of EBV-immune donors, at least those carrying the type A virus strains prevalent in Western countries, mount responses that show cross-reactive recognition of autologous LCLs transformed with type A or type B isolates (8, 24). By extending the experimental approach described here, identification of the immunodominant EBV latent proteins for such cross-reactive responses should be possible.

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