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Expression of Epstein–Barr virus nuclear antigens in anti-IgM-stimulated B cells following recombinant vaccinia infection and their recognition by human cytotoxic T cells

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SUMMARY

Cytotoxic T lymphocytes (CTL) recognizing Epstein–Barr virus (EBV) nuclear antigens (EBNA) are an important host defence mechanism in restricting the proliferation of EBV-infected B cells. Previously, B-type lymphoblastoid cell lines (LCL) infected with vaccinia recombinants encoding for the EBNA proteins have been used to identify A-type-specific CTL epitopes. However, to localize the CTL epitopes encoded by both A- and B-type transformants, B-type LCL are an inappropriate host for vaccinia. In the present study, an alternative host cell for vaccinia infection is described. Initial studies demonstrated that anti-IgM (μ-chain specific)-stimulated human B cells allowed vaccinia virus to replicate more efficiently than either phytohaemagglutinin-stimulated lymphocytes (PHA blasts) or CTL and expressed EBNA proteins following recombinant vaccinia infection. Furthermore, the presentation and recognition of target epitopes expressed on vaccinia-infected anti-μ-stimulated B cell blasts were comparable to that on similarly infected LCL. Anti-μ-stimulated B cells were used to define the CTL epitopes recognized by a panel of CTL clones from an EBV-immune donor. Using recombinant vaccinia-infected anti-μ-stimulated B cells, the CTL response from this donor was mapped to the EBNA6 protein. Most importantly, *in vitro* stimulation of unfractionated mononuclear cells with vaccinia-infected anti-μ B cells activated a memory CTL response. Based on the vaccinia results, screening of peptides from EBNA6 localized the epitope for the majority of the EBNA6-specific CTL clones to the sequence EENLLDFVRFM, apparently in association with HLA-B44. This work clearly demonstrates that anti-μ-stimulated B cells not only provide an efficient model for localizing the CTL epitope(s) but also raises the possibility of reactivating a memory T-cell response to any gene product expressed by recombinant vaccinia.

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpes virus that establishes a life-long persistent infection and is strongly associated with two B-cell malignancies, endemic Burkitt's lymphoma and oligoclonal lymphomas, to which immunocompromised individuals are especially susceptible.[1] Proliferation of EBV-infected B cells is controlled by CD4 and CD8 cytotoxic T lymphocytes (CTL) specifically activated to recognize a functionally defined lymphocyte-detected membrane antigen.[2,3] Two types of EBV (A and B) are recognized that show DNA sequence divergence within the BAM H1 WYH and E regions of the genome[4–6] and encode a family of EBV nuclear antigens, EBNA 1, 2, 3, 4, 5 (leader protein) and 6, which are expressed in all latently infected EBV-transformed lymphoblastoid cell lines (LCL). An alternative nomenclature also in current use designates these EBV nuclear antigens as EBNA 1, 2, 3a, 3b, 3 EBNA-leader protein. Earlier studies from this laboratory reported the isolation of CD4 and CD8 cytotoxic cell recognize A-type but not B-type transformants (A-type sp T-cell clones) from two A-type infected individuals.[7]

Recently, recombinant vaccinia viruses capable of ex sing individual EBNA proteins have been used as a mea identifying EBV CTL epitopes encoded by A-type but n type EBV.[8] Our initial approach in identifying these epi was to screen B-type EBV transformants infected with va expressing A-type EBNA sequences. While this app proved very successful in defining A-type-restricted epito could not be applied to the identification of epitopes encod both A- and B-type EBV, as the majority of immune d recognize determinants common to both A-type and E transformants.[9] For this reason we sought an EBV-ne target cell that could support a vaccinia infection. The abi LCL to support vaccinia infection suggested that peri

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lymphocytes on stimulation by certain antigen(s) and/or lymphokines, might be susceptible to vaccinia virus infection. In the present report, we describe the different stimuli that render peripheral blood lymphocytes susceptible to vaccinia infection resulting in (i) expression of EBNA proteins, (ii) recognition of recombinant vaccinia-infected cells by CTL, and (iii) activation of a memory CTL response to a single EBV nuclear antigen. In addition, we have compared phytohaemagglutinin (PHA) blasts, CTL and anti-μ-stimulated B cells to LCL with respect to their susceptibility to vaccinia infection and recognition by specific CTL.

MATERIALS AND METHODS

Cell culture

LCL were established by exogenous virus transformation of peripheral B cells from selected donors using A-type (B95.8 and IARC-BL74) or B-type (Ag876 and L4) EBV isolates,[7] and were routinely maintained in RPMI-1640 containing 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin plus 10% foetal calf serum (FCS) (growth medium). LCL were designated with the donors initial followed by the transforming virus source (e.g. LC/Ag876 designates B lymphocytes from donor LC transformed with virus from the Ag876 cell line). The simian CV-1 and TK$^-$ 143B cell lines used for growth and titration of vaccinia viruses were maintained in minimal essential medium (MEM) with 10% FCS.

To generate anti-μ B-cell blasts, unfractionated mononuclear cells (UM cells) separated on Ficoll–Paque (Pharmacia, Uppsala, Sweden) from venous blood was used as the source of B lymphocytes. T cells were removed from UM cells using the E-rosetting.[10] The enriched B lymphocytes were cultured in growth medium containing anti-IgM (μ-chain specific) coupled to acrylamide beads (Bio-Rad, Richmond, CA), recombinant human interleukin-4 (rIL-4; 50 U/ml; Genzyme, Boston, MA) and highly purified human recombinant IL-2 (rIL-2; 20 U/ml).[11,12] After 48–72 hr, B-cell blasts were suspended in growth medium supplemented with rIL-2 (20 U/ml). The B cells continued to divide two to three times/week for 3 weeks in the presence of rIL-2. These cells are referred to as anti-μ B-cell blasts.

To generate PHA blasts, UM cells were stimulated with PHA (Commonwealth Serum Laboratories, Melbourne, Australia) and after 3 days growth medium containing MLA 144 supernatant and rIL-2 was added.[13] PHA blasts were propagated with bi-weekly replacement of IL-2 and MLA supernatant (no further PHA added) for up to 6 weeks.

Vaccinia virus recombinants

The detailed description of recombinant vaccinia constructs for different EBNA genes (except EBNA1) has been described previously.[8] All EBV sequences were derived from the B95.8 strain of virus. The *Sspl* fragment [encompasses T7 promoter:-EBNA1 with minimal flanking TK gene sequence (< 100 bases)] from the pTF7-5:EBNA1 plasmid was inserted into pSC11 plasmid opposite to the P7.5 promoter for EBNA1 expression. Although EBNA1 expression is under T7 DNA polymerase, the baseline expression is comparable to the level of EBNA1 in a LCL. The EBNA2 coding sequence was contained within a genomic clone comprising nucleotides 48475–50305.[14] The EBNA3 sequence from the cDNA clone T216,[6] the EBNA5 sequence from the CDNA clone T65[15] and the EBNA6 sequence from the cDNA clones T36 and T27 were the same as described previously.[16] All constructs had the potential to encode the relevant full-length EBV protein. A vaccinia construct negative for thymidine kinase (Vacc.TK$^-$) with no EBV latent genes was used as a control.

Source and generation of CTL clones

Generation of EBV-specific CTL clones. UM cells (10^6/ml) from two donors, DM (HLA A24, A29, B44, Bw47) and LC (HLA A1, B8, B18), were cultivated with irradiated (8000 rads) autologous A-type (B95.8) LCL (responder to stimulator ratio of 200:1) in 2-ml culture wells (Linbro, Flow Labs, McLean, VA) for 3 days in growth medium. CTL clones generated by seeding in 0·35% agarose were established from these donors and maintained as described elsewhere.[7] CTL were screened in a standard 4-hr ^{51}Cr-release assay for specific reactivity against autologous A-type LCL. The CTL generated after stimulation with LCL were also assessed for their susceptibility to vaccinia infection.

Activation of memory CTL response with recombinant vaccinia. Anti-μ B-cell blasts from donor DM were infected with recombinant vaccinia expressing EBNA-6 for 14–16 hr, irradiated (8000 rads) and used to stimulate autologous UM cells (responder to stimulator ratio of 50:1). After 3 days, activated cells were seeded in 0·35% agarose and clones harvested as described earlier.[7] Clones were subsequently stimulated weekly with irradiated CM/B95.8 and screened for specific reactivity against autologous and allogeneic LCL and anti-μ B-cell blasts infected with vaccinia constructs encoding for different EBNA proteins.

Screening for expression of EBV latent proteins

LCL, anti-μ B-cell blasts, PHA blasts and CTL were exposed to the recombinant vaccinia virus, encoding EBNA5 (Vacc. EBNA5 was used as a representative for the group of vaccinia constructs), at a multiplicity of infection (MOI) of 10:1 for 1 hr at 37°. After incubation, infected cells were cultured in growth medium for 14–16 hr. The protein extracts from infected cells were separated by SDS–PAGE and probed with EBNA5-specific monoclonal antibodies (mAb JF186).[17] In addition, infected cells were also processed for immunofluorescence.[18] Briefly, recombinant vaccinia-infected cell smears were fixed with acetone/methanol (50% v/v) at 4° for 20 min. After fixation, cell smears were washed three times with phosphate-buffered saline (PBS; 0·2 M; pH 7·2) containing 1% FCS. Subsequently, cells were permeabilized with 0·5% NP40 in PBS with 1% FCS for 5 min at room temperature. After washing smears with PBS/1% FCS, slides were incubated with mAb JF186 (specific for EBNA5), anti-vaccinia antibody (kindly provided by Dr M. Lavin, Queensland Institute of Medical Research, Australia) or anti-β galactosidase (Boerhinger-Mannheim, Mannheim, Germany) for 1 hr at room temperature. The smears were extensively washed with PBS/1% FCS and incubated with FITC-labelled anti-mouse Ig (Silenus, Australia) or FITC-labelled anti-sheep Ig (CSL, Melbourne, Australia) for 1 hr at room temperature. Cell smears were washed in PBS/1% FCS and examined under the fluorescence microscope in the presence of n-propyl gallate.

Table 1. Susceptibility of activated lymphocytes to recombinant vaccinia infection

Cell type	PFU/ml of supernatant*
PHA blasts	$1{\cdot}7 \times 10^3$
CTL	$1{\cdot}3 \times 10^3$
Anti-μ B-cell blasts	$2{\cdot}5 \times 10^5$
LCL	$8{\cdot}3 \times 10^5$

* Mean of duplicate determinations average over twofold serial dilutions. Different cell populations were infected with recombinant vaccinia at MOI of 10:1, infected cells cultured for 18 hr and culture supernatant assessed for plaque-forming units.

Assay for virus infection and replication

Anti-μ B-cell blasts, LCL, PHA blasts and CTL (usually 1×10^6 cells) were infected with recombinant vaccinia virus (Vacc. EBNA5) at a MOI of 10:1 for 1 hr at 37°. Cells were washed twice with RPMI-1640/10% FCS and resuspended in medium with an excess of sheep anti-vaccinia virus antibodies to neutralize all extracellular virus. After 30 min at 37°, cells were washed twice, suspended to original volume and counted. These cells were cultured for 18 hr in 24-well plates. Supernatants from these cultures were collected and titres of infectious virus (plaque-forming units; PFU) determined on TK^- 143 monolayers.

Cytotoxicity assay on recombinant vaccinia virus-infected targets

Anti-μ B-cell blasts and B-type LCL were infected with recombinant vaccinia viruses at a MOI of 10:1, as described above. After 14–16 hr, cells were pelleted and incubated with ^{51}Cr for 90 min, washed three times and used as targets in a standard 4-hr ^{51}Cr-release assay. The effector cells were added to the assay at effector to target ratios between 5:1 and 10:1. To identify the CTL epitope recognized by EBV-specific CTL clones from donor DM, a series of peptides from EBNA6 were synthesized (20–25 amino acids)[19] by using the known sequence of the B95.8 strain of EBV. Peptides selected were primarily based on recombinant vaccinia CTL assay and those that corresponded to predicted algorithms.[20,21] Peptides were dissolved in RPMI-1640 and distributed into U-well microdilution plates (200 μg/ml, 20 μl/well) and frozen at −70° until required. ^{51}Cr-labelled anti-μ B-cell blasts were added to each well (2×10^5/ml, 50 μl/ml) and incubated at 37°. After 1 hr, 130 μl of cloned autologous CTL were added to the reaction mixture (final effector to target ratio as indicated), and the assay conducted as previously described.[13]

RESULTS

Recombinant vaccinia infection of anti-μ-stimulated B cells

The ability of anti-μ B-cell blasts, LCL, PHA blasts and CTL to support and replicate vaccinia virus infection was determined. Table 1 shows the number of PFU produced by vaccinia virus recovered from supernatants of Vacc.EBNA5-infected anti-μ B-cell blasts, LCL, PHA blasts and CTL. Vaccinia virus recovered from supernatants of anti-μ B-cell blasts produced a higher number of PFU/ml compared to PHA blasts and CTL. The PFU/ml produced by the anti-μ B-cell blast supernatant was comparable to supernatants recovered from LCL (Table 1).

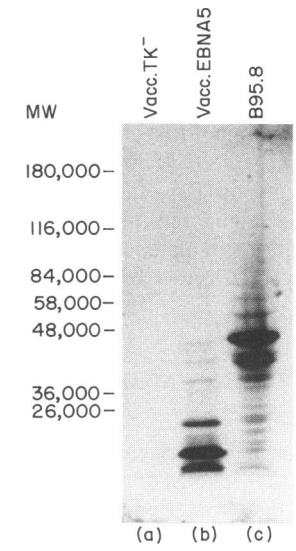

Figure 1. Immunoblot of protein extracts made from anti-μ B-cell blasts infected with recombinant vaccinia virus carrying coding sequence for EBNA5 (b). Anti-μ B-cell blasts infected with Vacc.TK^- (a) served as a negative control. B95.8-transformed LCL (c) served as a positive control. Blots were probed with mAb JF186 for EBNA5 expression.

Immunoblotting was used to detect the expression of EBNA5 in recombinant vaccinia (Vacc.EBNA5)-infected anti-μ B-cell blasts. EBNA5 in vaccinia-infected B cells was identified as a ladder of proteins in the 18,000–48,000 MW region of the gel (Fig. 1). A similar ladder of low molecular weight EBNA5 proteins was also frequently seen in LCL,[17] although LCL which were used as a control cell line (Fig. 1) showed strong expression of high molecular weight EBNA5 species when reacted with the same mAb (JF186).

Monoclonal antibodies specific for EBNA5 (JF186) and β-galactosidase and a polyclonal anti-vaccinia virus-specific immune serum were also used to confirm the expression of proteins and vaccinia infection in B cells by indirect immunofluorescence. Anti-μ B-cell blasts infected with recombinant Vacc.EBNA5 demonstrated positive fluorescence (80% cells) using either mAb JF186, anti-vaccinia antibody or mAb anti-β-galactosidase (Fig. 2a–c). There was no fluorescence in Vacc. TK^--infected anti-μ B-cell blasts stained with mAb JF186 (Fig. 2d). No fluorescence was observed in Vacc.EBNA5-infected PHA blasts or CTL using either anti-EBNA5 or anti-β-galactosidase mAb.

Recognition of EBV latent proteins by CTL clones

Since PHA blasts and CTL were unable to support a significant level of vaccinia infection, all subsequent studies involved a comparison of anti-μ B-cell blasts and LCL. In the experiment presented in Fig. 3, an A-type specific CTL clone from donor LC (clone 7), shown previously to recognize an epitope in EBNA3,[13] was used to compare the CTL recognition of autologous vaccinia-infected anti-μ B-cell blasts and autologous B-type LCL. The results demonstrate that B-cell blasts infected with Vacc.EBNA3 were specifically recognized by clone 7. Ex-

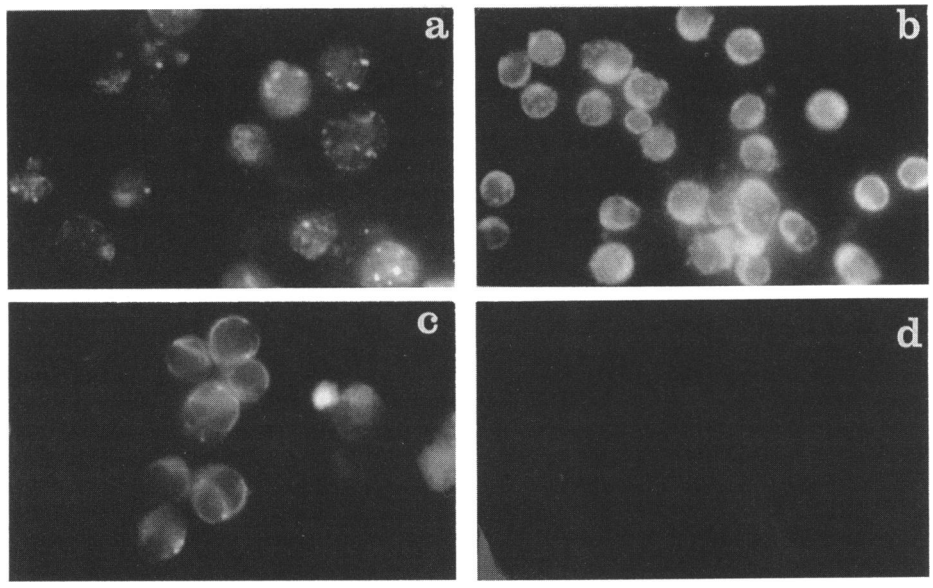

Figure 2. Immunofluorescence patterns of anti-μ B-cell blasts infected with Vacc.EBNA5. B-cell blasts were infected for 12–14 hr (MOI 10:1) with Vacc.EBNA5 (a,b,c) and Vacc. TK$^-$ (d). The cells were fixed in methanol/acetone, permeabilized and stained by indirect immunofluorescence using anti-EBNA5 mAb (JF186) (a,d), anti-vaccinia antibody (b) and anti-β-galactosidase mAb (c).

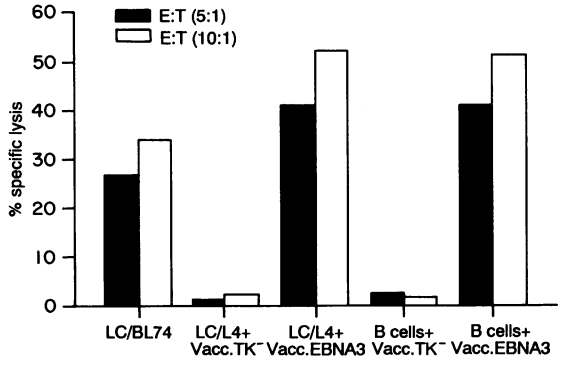

Figure 3. Recognition of autologous LCL LC/BL74 (A-type) and Vacc.EBNA3 or Vacc.TK$^-$-infected autologous LC/L4 (B-type) and anti-μ B-cell blasts by LC CTL Clone 7. Results are expressed as percentage specific lysis observed in a standard 4-hr Cr-release assay; the two columns represent results obtained at an effector:target (E:T) of 5:1 and 10:1.

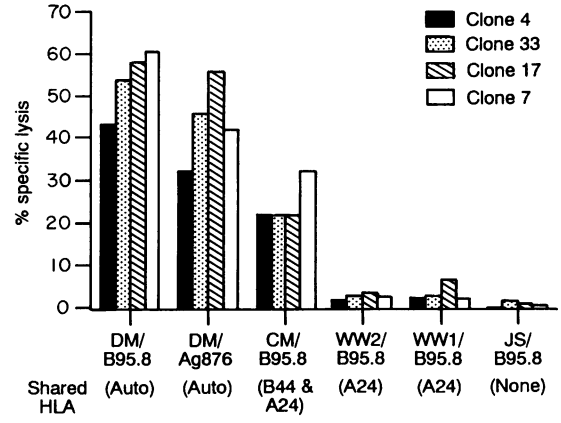

Figure 4. Percentage specific lysis by CTL clones (Clones 4, 17, 33 and 7) from donor DM of autologous LCL DM/B95.8 (A type) and DM/Ag876 (B-type) and allogenic LCL (A-type) from HLA-related and unrelated donors. Results are expressed as in Fig. 3.

pression of EBNA3 in the different target cells (B-cell blasts or B-type LCL) induced comparable levels of lysis, significantly higher than those from the same target cells infected with Vacc.TK$^-$ (Fig. 3).

Use of anti-μ B-cell blasts to define the protein location of CTL epitopes

One of the important potential uses of B-cell blasts is to define the protein location of CTL epitopes that are present on A-type and B-type transformants. To test their use in this regard, a selection of four EBV-specific CTL clones from donor DM (Clones 4, 7, 17 and 33) were chosen and their specificity illustrated in Fig. 4. As can be seen, all clones recognized both the A- and B-type transformant apparently restricted through HLA B44. To determine the antigen location of the epitope recognized by these clones, anti-μ B-cell blasts from donor DM were infected with a panel of recombinant vaccinia constructs and their lysis by the autologous CTL clones defined. Each of the four EBV-specific CTL clones from donor DM recognized autologous anti-μ B-cell blasts infected with Vacc.EBNA6 but failed to recognize B-cell blasts infected with any of the other constructs (Fig. 5). These experiments illustrate the use of anti-μ B-cell blasts in conjunction with recombinant vaccinia virus to localize CTL epitopes present on both A- and B-type transformants.

Use of vaccinia-infected anti-μ B-cell blasts to stimulate a memory CTL response

A further important use of the B-cell blasts revolves about their ability to activate a specific memory response after infection

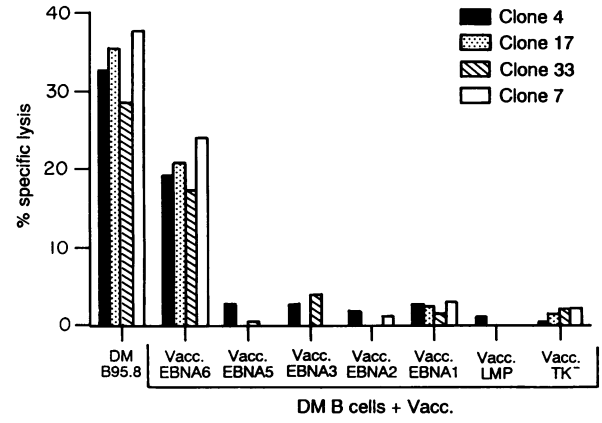

Figure 5. Specific lysis by EBV-specific CTL clones from donor DM of autologous LCL (DM/B95.8) and Vacc.EBNA6, 5, 3, 2, 1, LMP and TK$^-$ infected DM B-cell blasts. Vacc.TK$^-$ was used as a control recombinant vaccinia. Results are expressed as in Fig. 3.

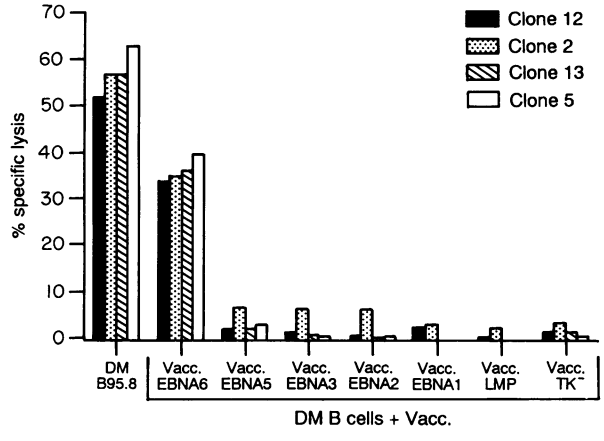

Figure 6. Functional analysis of CTL clones from donor DM (Clones 12, 2, 13 and 5) generated from an *in vitro* stimulation with autologous B-cell blasts infected with Vacc.EBNA6 and tested on autologous A-type LCL (DM/B95.8) and DM B-cell blasts infected with vaccinia recombinants encoding from EBNA6, 5, 3, 2, 1, LMP or TK$^-$ (control recombinant). Results are expressed as in Fig. 3.

with recombinant vaccinia virus. UM cells from donor DM were co-cultured with autologous anti-μ B-cell blasts infected with Vacc.EBNA6 (responder to stimulator ratio of 50:1). CTL clones were isolated, restimulated with DM/B95.8 and expanded with rIL-2. 12/16 CTL clones displayed an EBV-specific pattern of lysis by criteria similar to that previously reported.[7]

To define the EBNA specificity of EBV-specific CTL clones, autologous anti-μ B-cell blasts were infected with a panel of vaccinia constructs and their lysis by different CTL clones assessed. 11/12 EBV-specific CTL clones recognized DM anti-μ B-cell blasts infected with Vacc.EBNA6 (data from a selection of four EBV-specific CTL clones is shown in Fig. 6). The EBNA specificity for one EBV-specific clone could not be localized by using the available panel of vaccinia constructs. It should be stressed that the anti-μ B-cell blasts were used in the primary stimulation (with irradiation but without fixation) and that the CTL clones were subsequently stimulated with DM/B95.8. These experiments confirm that anti-μ B-cell blasts infected with recombinant vaccinia virus can effectively stimulate a memory T-cell response.

Recognition of synthetic peptides as CTL epitopes

Based on the results obtained from vaccinia experiments, EBV peptides from EBNA6 were screened for their ability to act as targets for EBV-specific CTL lysis after adsorption onto autologous B-cell blasts. All four CTL clones from donor DM recognizing Vacc.EBNA6 lysed autologous B cells in the presence of peptide EENLLDFVRFM (ORF BERF4 residues 290–309) (Table 2). To define the peptide epitope(s) recognized by CTL clones generated from Vacc.EBNA6 stimulation, clones recognizing EBNA6 were checked for reactivity to peptide EENLLDFVRFM. 6/9 DM CTL clones recognized peptide EENLLDFVRFM, when presented on autologous anti-μ B-cell blasts. However, peptide epitope(s) for the remaining CTL clones could not be defined by the available panel of peptides (data from a selection of four Vacc.EBNA6-specific CTL clones is shown in Table 2).

DISCUSSION

The present experiments clearly demonstrate that anti-μ stimulated B cells can be efficiently infected with recombinant vaccinia virus resulting in the expression and presentation of EBNA proteins. Normally, T and B lymphocytes, both sessile and recirculating are metabolically quiescent (G0), and in a resting phase of the cell cycle. In this state cells have little cytoplasmic volume with minimal biosynthetic capability[22] and are poor hosts for viral infection.[23,24] Upon activation, lymphocytes can support the synthesis of viral proteins and nucleic acids.[23] Based on these studies, we attempted to define an EBV-negative lymphocyte population that could support vaccinia infection and express the recombinant protein. Since our attempts to infect activated T cells (CTL or PHA blasts) with vaccinia were not successful, our attention was turned to anti-μ B-cell blasts which express levels of B-cell activation antigens[25] similar to those present on LCL. It has been suggested that anti-μ mediates receptor cross-linking and internal signalling followed by induction of new lymphokine receptors which allow subsequent differentiation events supported by lymphokines (IL-4 and/or IL-2) and leads to increased virus infection.[26] We have shown that activation of B cells with anti-μ allows active vaccinia infection with a significant production of infectious virus particles comparable with LCL. Moreover, the majority of infected anti-μ B-cell blasts expressed EBNA proteins when monitored by immunofluorescence and immunoblotting.

Vaccinia recombinants have recently been used to localize and identify a number of viral determinants recognized by human CTL, including influenza nucleoprotein,[27] RSV nucleoprotein,[28] HSV glycoprotein[29] HIV glycoprotein[30] and A-type-specific EBV nucleoprotein.[8] All of these studies employed LCL as a host for recombinant vaccinia to define the CTL epitopes. Indeed, earlier studies have shown that B-type LCL infected with recombinant vaccinia expressing the A-type EBNA sequence are an effective tool for identifying epitopes present on A-type transformants.[8] However, previous studies from this

Table 2. Recognition of peptide EENLLDFVRFM by DM CTL clones stimulated with DM/B95.8 (Exp. 1) and Vacc.EBNA6-infected DM B-cell blasts (Exp. 2)

Targets	Peptide EENLLDFVRFM	Percentage specific lysis by			
		Clone 4	Clone 7	Clone 17	Clone 33
Exp. 1					
DM B-cell blasts	–	1	0	0	0
	+	58	77	21	55
		Clone 2	Clone 5	Clone 12	Clone 13
Exp. 2					
DM B-cell blasts	–	1	3	0	1
	+	54	0	61	23

^{51}Cr-labelled targets were sensitized with the peptide EENLLDFVRFM (200 μg/ml, 20 μl/well) for 1 hr at 37°. After incubation, the effector cells were added at an effector to target ratio of 10:1.

laboratory have shown that the EBV-specific T-cell response from the majority of sero-positive donors recognize determinants expressed on both A-type- and B-type-transformed LCL.[9] To define the protein location of these determinants, recombinant vaccinia-infected B-type LCL are clearly an inappropriate host since the uninfected cells present the relevant epitope. The anti-μ B-cell blasts provide an alternative EBV-negative host cell that is susceptible to vaccinia infection (Table 1), express the inserted gene (Figs 1 and 2) and present the relevant CTL epitope (Fig. 3). These results suggest that recombinant vaccinia-infected B-cell blasts are an effective method of defining the protein location of EBV CTL epitopes present on both A- and B-type transformants. The advantage of anti-μ B-cell blasts was clearly demonstrated in this study while localizing the T-cell epitopes for donor DM. The majority of CTL clones from this donor recognized by A- and B-type transformants and the relevant epitope was mapped to EBNA6 using recombinant vaccinia-infected B-cell blasts (Fig. 5).

Of more general interest is the observation that vaccinia-infected anti-μ B-cell blasts stimulated a memory T-cell response. The majority of Vacc.EBNA6-stimulated EBV-specific clones (11/12) recognized Vacc.EBNA6-infected B-cell blasts. This result raises the possibility of reactivating a memory T-cell response to any gene product expressed by vaccinia virus. Recombinant vaccinia-infected human fibroblasts have been used to activate a cytomegalovirus-specific memory T-cell response with some success.[31] However, the ease and speed with which anti-μ B-cell blasts can be established makes this cell population preferable to skin fibroblasts.

It has been shown that human and murine influenza-specific CTL predominantly recognize processed linear peptides from intracellular viral proteins associated with MHC class I molecules.[32] The establishment by this laboratory of the first EBV-coded amino acid sequence derived from EBNA3, capable of acting as a target for EBV-specific CTL clones, provided a model for determining the peptide epitopes for CTL recognition.[13] In that report, peptide sequences from *all* of the latent antigens were screened for CTL activity using A-type-specific T cells. Experiments using vaccinia constructs can clearly enhance this technique by specifying the EBNA region from which the epitope is derived. Hence, a much more limited number of peptides can then be selected and screened. In the present report it has been demonstrated using recombinant vaccinia constructs that the active epitope for donor DM CTL clones is encoded by EBNA6. By screening a selected panel of EBNA6 peptides, the determinant was localized to the peptide sequence EENLLDFVRFM. It is interesting to note that only 6/9 EBNA6-specific CTL clones (stimulated with Vacc.EBNA6) from donor DM lysed autologous anti-μ B-cell blasts sensitized with peptide EENLLDFVRFM while the CTL epitope for the three other clones could not be specified with the available panel of peptides. This observation indicated that donor DM CTL can recognize more than one peptide epitope on EBNA6 (other than EENLLDFVRFM).

By extending the experimental approach described here, we intend to use vaccinia recombinants encoding different EBV proteins to localize other possible CTL determinants and to use synthetic peptides within the specified gene to define more CTL epitope(s) important for recognition. This should permit investigation on CTL responses in a larger number of subjects, something that has previously been difficult because of the requirement for autologous host cell lines not infected with EBV.

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