Five new cytotoxic T cell epitopes identified within Epstein–Barr virus nuclear antigen 3

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Epstein–Barr virus (EBV) CD8+ cytotoxic T lymphocyte (CTL) epitopes are currently being considered for inclusion into subunit vaccines. Here we describe the characterization of five new CTL epitopes within EBV nuclear antigen 3 (EBNA3), confirming EBNA3 as a major target for CTL recognition.

Epstein–Barr virus (EBV) is a herpesvirus that infects most of the human population. Following primary infection a lifelong latent infection of B cells is established. When primary infection is delayed until adolescence, as occurs in 10 to 20% of individuals in Western societies, there is an approximately 50% chance of developing infectious mononucleosis. There are two EBV types, A and B. The A type appears to predominate in the majority of lymphoid infections of healthy seropositive individuals. In such individuals latently infected B cells appear to be controlled by CD8+ cytotoxic T lymphocytes (CTLs) specific for the latent antigens, which include the EBV nuclear antigens (EBNAs) 2 to 6 and the latent membrane proteins (Moss et al., 1992). As whole virus or recombinant virus vaccines based on full-length latent proteins are considered potentially oncogenic (Wang et al., 1985), an EBV vaccine based on CTL epitopes derived from the latent antigens is currently being developed (Moss & Suhrbier, 1993). EBNA3 from EBV type A appears to be a rich source of CTL epitopes; CTL clones specific for EBNA3 can be isolated from at least two-thirds of healthy EBV seropositive donors (Khanna et al., 1992). Although several CTL epitopes within the latent proteins have been identified, only one, FLRGRAYGL, has been defined at the peptide level within EBNA3 (Moss et al., 1992; Zhang et al., 1993).

This report describes the localization of five new CTL epitopes within EBNA3 using a peptide net overlapping by 10 amino acids spanning the EBNA3 protein. The peptides were based on the only available A type EBNA3 sequence, which was derived from virus strain B95.8 (Sample et al., 1990). Although strain variations undoubtedly exist, two EBV CTL epitopes showed no variation in several EBV type A isolates from human donors (Apolloni et al., 1992).

Initially, bulk CTL effectors were generated by stimulating unfractionated peripheral blood lymphocytes from healthy seropositive donors with irradiated (80 Gly; 80 J/kg) autologous EBV type A-transformed lymphoblastoid cell lines (LCLs) on days 0 and 7. Interleukin-2 (IL-2) was not added to these cultures, as its presence favoured the expansion of non-specific T cells (data not shown). On days 10 to 11 the unfractonated bulk CTL cultures were used to screen the EBNA3 peptides individually on chromium-labelled autologous phytohaemagglutinin (PHA)-stimulated T cell blasts; PHA blasts are relatively resistant to non-specific lysis and give low background killing values. Peptide sensitization of PHA blasts was performed for 1 to 2 h at 37 °C. The serum in the medium degrades most of the peptide during this time (Widmann et al., 1991), ameliorating the problem of unlabelled target inhibition caused by free peptide sensitizing effector cells. An example of a representative screen is given in Fig. 1. Toxicity testing of all the peptides was performed prior to screening by adding peptide to PHA blasts in the absence of bulk CTL effectors. Two neighbouring peptides were found to be toxic; the high lysis values obtained with these peptides during screening were therefore disregarded. This HLA B8 donor responded to peptides AGFLRGRAYGL-LLRTEGEH and PSTETAQAWNAGFLRGRAYG (Fig. 1) that contain the known B8-restricted epitope, FLRGRAYGI, and eight of the amino acids from this epitope respectively. The B95.8 strain of EBV, derived from a marmoset cell line, differs from several human cell line-derived type A strains of EBV, which present the epitope FLRGRAYGL (Apolloni et al., 1992). This donor also responded to the peptide ASRRDQAKWR-LQTLAAGWPM, indicating that a new epitope might be localized within this sequence.

EBV-specific CTL clones from the same donor were then generated and expanded in the presence of IL-2 (Misko et al., 1984; Wang et al., 1984; Rosenberg et al., 1985).
Possible new epitope within ASRRDQAKWRLQTLAAGWPM

Peptide AGFLRGRAYGIDLLRTEGEH which contains the previously identified HLA B8 epitope FLRGRAYGL (see text)

Peptides previously identified as toxic (see text)

Fig. 1. Example of bulk CTL cultures from donor CF (A1, A2; B8, B44) tested on 20-mer peptides overlapping by 10 covering the B95.8 EBNA3 sequence (Falk et al., 1991). Bulk CTL effectors were generated from unfractionated peripheral blood mononuclear cells by stimulation in 24-well plates (2 × 10⁶/well) with irradiated 80 Gy (80 J/kg) autologous lymphoblastoid cell line transformed with EBV type A strain BL36 (LCL CF/BL36) (responder: stimulator ratio of 20:1) on days 0 and 7. After 10 days culture in RPMI-1640 supplemented with 10% fetal calf serum they were used as effectors in a 6 h standard chromium release assay with autologous peptide-sensitized PHA blasts as target cells (effector to target ratio of 20:1). Chromium-labelled PHA blasts were incubated for 1 h at 37 °C with 100 μM of each 20-mer peptide prior to the addition of the effectors. Peptides were synthesized using the pin technology. A C-terminal glycine ester link was used in the preparation of the peptides leaving glycine-free acidic C termini and free amine N termini (Valerio et al., 1991).

1984) to approximately 10³ cells. One-hundred to 200 cells of each clone were tested using the rapid visual CTL assay (Burrows et al., 1992) for specificity to the positive peptide (ASRRDQAKWRLQTLAAGWPM in this example). Clones with specificity for the peptide were then expanded and tested on overlapping nonamers that covered the 20-mer sequence identified above, using a standard chromium release assay and autologous PHA blast targets (Fig. 2a). EBV type A/B specificity (Fig. 2b; Table 1) and HLA restriction (Fig. 3) were similarly determined using a panel of target cells. The new epitopes identified by this protocol are described in Table 1.

This combination of procedures represents a rapid and reliable method of CTL epitope identification that has allowed us almost to double the number of defined EBV CTL epitopes in a short period. Precursor frequency analysis for a number of epitopes (data not shown) indicates that this method is unreliable for detection of CTL specificities below a precursor frequency of about 1 in 10000.

The specificity for EBV type A strains of CTL clones recognizing four of the five new epitopes (Fig. 2b; Table 1) was consistent with differences in the A and B type EBNA3 protein sequences in the epitope regions. Only clones specific for SVRDRLARL were able to recognize transformants of both EBV strains, which have sequence identity in this epitope region. If all the possible nonamers from the two proteins were compared about 35% would have sequence identity.

All of the HLA B8 donors which were examined, CF, LC and YW, recognized the two HLA B8-restricted epitopes, with YW also recognizing the A2-restricted epitope SVRDRLARL (Table 1; Fig. 1). In all cases the response to FLRGRAYGL appeared to dominate (data not shown).

Defining HLA restriction for three of the five epitopes was not possible on the basis of HLA serotyping alone, indicating that the epitopes or the CTL clones were restricted by subtypes. Using a panel of Burkitt's lymphoma (BL) lines covering B35.1 to B35.4 (Andersson et al., 1991) (but not B35.5 or B35.6), the CTLs from donor NB was shown to recognize the HLA B35.3 BL sensitized with YPLHEQHGM (Fig. 3c). There was no specific response to YPLHEQHGM detected from the B35 donors CS and SB (data not shown). Testing a panel of LCLs from Chinese donors showed that the SVRDRLARL-specific CTL from donor YW was restricted by an A2 subtype (Fig. 3d). These restrictions could be caused by major histocompatibility complex restriction, in which a given peptide associates with only one subtype, and/or T cell receptor restriction, in which the CTL cannot recognize its cognate peptide on a different subtype (Brooks et al., 1993). We were unable to determine the restriction of the LX CTL (Fig. 3b). The A24 restriction for the
RRDQAKWRLQ
RDQAKWRLQ
DQAKWRLQT
QAKWRLQTL
AKWRLQTDA
KWRLQTDA
WRQLTAAGW
RLQTLAAG

CF/BL36
CF/B95.8
CF/Ag876
CF/L4

Fig. 2. (a) Minimalization of ASRRDQAKWRLQTLAAGWPM. A CTL clone from donor CF [generated as described previously by Misko et al. (1984)] specific for the above peptide by visual assay was tested against overlapping nonamers covering the central 18 amino acids of the 20-mer (the first and last two amino acids in the 20-mer peptide were excluded from nonamer peptides since no activity was apparent in the preceding and following 20-mers that overlapped by 10 amino acids). Peptides were synthesized using pin technology and had free acidic C termini and free amine N termini (Valerio et al., 1991). Autologous chromium-labelled PHA blasts were sensitized with 1 gM of each nonamer for 1 h at 37 °C and used as target cells in a standard 5 h chromium release assay (E:T ratio of 2:1). (b) The A/B type specificity of the clone is illustrated by lysis of a panel of LCLs transformed with different virus strains. (BL36 and B95.8 are A type strains, Ag876 and L4 are B type strains; Apolloni et al., 1992.) Standard 5 h chromium release assays were performed (E:T ratio of 2:1).

Table 1. Epitopes within EBNA3 recognized by CTL from healthy EBV seropositive donors

<table>
<thead>
<tr>
<th>CTL epitopes of EBNA3*</th>
<th>HLA restriction</th>
<th>A/B specificity†</th>
<th>Donors and HLA typing‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>135QAKWRLQTL166</td>
<td>B8</td>
<td>A</td>
<td>CF: A1 A2 B8 B44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LC: A1 B8 B18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YW: A2 A26 B8 B38</td>
</tr>
<tr>
<td>214RYSIFFDY358§</td>
<td>A24</td>
<td>A</td>
<td>AS: A2 A24 B51 B62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CM: A11 A24 B7 B44</td>
</tr>
<tr>
<td>459HLAOGMAY558</td>
<td>Unknown</td>
<td>A</td>
<td>LX: A24 B15 B38</td>
</tr>
<tr>
<td>584SVRDLARL664</td>
<td>A2</td>
<td>A/B</td>
<td>YW: A2 A26 B8 B38</td>
</tr>
<tr>
<td>322FLRGRAYGL337¶</td>
<td>B8</td>
<td>A</td>
<td>LC, CF, YW</td>
</tr>
</tbody>
</table>

* Superscript numbers on the epitopes refer to EBNA3 residue numbers of the first and last amino acid of the epitope.
† A/B specificity determined using A type strains BL36 and B95.8 and B type strains Ag876 and L4 (Apolloni et al., 1992) (for example see Fig. 2b).
‡ All donors were of Caucasian origin except LX and YW who were of Chinese origin. HLA typing of donors was performed by serology.
§ Not confirmed with clones; analysis of restriction, A/B specificity and minimalization performed with bulk cultures; minimalization showed that two nonamers were equally active indicating that the epitope was an octamer.
¶ Clones displaying subtype specificity. B35,(3) is bracketed because only B35.1, 2, 3 and 4, but not B35.5 and 6, typed lines were available for testing (see Fig. 3).
¶ Previously described epitope recognized by LC (Moss et al., 1992; Apolloni et al., 1992).

RYSIFFDY epitope was based on the observation that two A24 donors responded to this epitope (Table 1).

The HLA B8- and A2-restricted epitopes conform to the reported HLA-binding motifs, QAKWRLQTL (Suhrbier et al., 1993) and SYRDRLARL (Falk et al., 1991), respectively. Curiously the latter also conforms to the B8-binding motif. The B35.3-restricted epitope, YPLHEOHGM, partially conforms to the reported B35-binding motif, which has a P in position 2 and a C-terminal Y residue (Hill et al., 1992). The predictive capability of reported algorithms (Gammon et al., 1991) is no better than random; only two of the new epitopes contain sequences predicted to be T cell epitopes by these algorithms.

A universally immunogenic EBV CTL vaccine is likely to require multiple epitopes from several latent proteins since not all individuals respond to EBNA3 (Khanna et al., 1992; Murray et al., 1992). The vaccine would also have to include sufficient epitopes to cover the HLA subtype heterogeneity of the target population. The race differences observed for HLA allele frequencies and subtype heterogeneity (Lopez de Castro, 1989) is likely to mean that a novel set of CTL epitopes will have to be identified for each population. Although initially daunting, there is a limit to the HLA diversity. Currently 41 different HLA A alleles have been identified, the majority in Caucasians (Bodmer et al., 1991). This theoretical upper limit (if all alleles present an EBV epitope) can be reduced by selecting epitopes restricted by common and invariant A and B HLA alleles and/or
epitopes presented by multiple subtypes (Brooks et al., 1993).

The CTL epitopes do not appear clustered in a particular region of EBNA3, as described for the nef antigen of human immunodeficiency virus type 1 and the circumsporozoite protein of the malaria agent (Culmann et al., 1991; Sinigaglia et al., 1988) (although two epitopes overlap by one amino acid, Y468). All of the EBNA3 CTL epitopes fall outside the repeat regions that represent 22% of the EBNA3 sequence (Sample et al., 1990). Although this is not statistically significant \((P = (1 - 0.22)^6 = 0.2)\), CTL epitopes defined within the other latent EBV antigens (Moss et al., 1992; Zhang et al., 1993) also lie outside repeat regions. Repeat regions from EBNA1 and -5 appear to have specific DNA and protein-binding functions respectively (Dhar and Schildkraut, 1991; Szekely et al., 1993). Repeats within EBNA3 also appear to vary considerably in copy number between different EBV strains; this may reflect adaptive changes by the virus to new host populations (Gratama et al., 1990). The presence of a CTL epitope within a repeat region could dramatically influence the amount of the CTL epitope being presented by different EBV strains. Changes in the amount of epitope being presented are likely to influence the level of CTL activity directed toward the virus. One might speculate that EBV has evolved to avoid CTL epitopes within repeats, so that adaptive changes can occur without compromising the critical balance between replication and destruction by CTLs, which EBV has achieved in all human populations.

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