The Journal of Immunology

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J Immunol 1999; 162:3063-3069; ;
http://www.jimmunol.org/content/162/5/3063

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

EBV Structural Antigens, gp350 and gp85, as Targets for Ex Vivo Virus-Specific CTL During Acute Infectious Mononucleosis: Potential Use of gp350/gp85 CTL Epitopes for Vaccine Design[1]

Rajiv Khanna,[2] Martina Sherritt, and Scott R. Burrows

For many years, EBV vaccine development efforts have concentrated on the use of structural Ag, gp350, and have been directed toward Ab-mediated blocking virus attachment to the target cell. There is increasing evidence to suggest that the development of neutralizing Abs in vaccinated animals does not always correlate with protection; nevertheless, it has been postulated that gp350-specific T cell-mediated immune responses may have an effector role in protection. This hypothesis has largely remained untested. In the present study, we demonstrate that CTL from acute infectious mononucleosis patients display strong ex vivo reactivity against the EBV structural Ags, gp85 and gp350. Moreover, long-term follow up studies on infectious mononucleosis-recovered individuals showed that these individuals maintain gp350- and gp85-specific memory CTL, albeit at low levels, in the peripheral blood. These results strongly suggest that CTL specific for EBV structural proteins may play an important role in the control of EBV infection during acute infection. More importantly, we also show that prior immunization of HLA A2/K^b transgenic mice with gp350 and gp85 CTL epitopes induced a strong epitope-specific CTL response and afforded protection against gp85- or gp350-expressing vaccinia virus challenge. These results have important implications for future EBV vaccine design and provides evidence, for the first time, that CTL epitopes from EBV structural proteins may be used for establishing strong antiviral immunity against EBV infection. *The Journal of Immunology,* **1999, 162: 3063–3069.**

It is now well established that long-term protection from persistent viral infection requires the development of virus-specific memory T cells that recognize viral Ags in association with either class I or class II MHC molecules. Because immunization with whole viral proteins is unable to elicit an efficient CTL response, interest has been directed toward designing vaccines based on defined epitope sequences. This is particularly the case with oncogenic viruses, because individual viral genes introduced in recombinant vectors have the potential to initiate tumorigenic processes. Two broad approaches are currently being considered to design an effective vaccine for controlling EBV-associated diseases (for review, see Ref. 1). These include directing immune responses to either EBV structural Ags or latent Ags.

In the last few years, most of the vaccine development efforts have concentrated on the use of a subunit preparation of gp350 (recombinant and affinity-purified) and have been directed toward blocking virus attachment to the target cell in the oropharynx (2). The general approach has been to immunize cotton-top marmosets with gp350 and determine their ability to restrict the outgrowth of EBV-positive lymphomas in these animals. Indeed, highly purified gp350, when administered s.c. in conjunction with adjuvants (muramyl dipeptide or immunostimulatory complexes), induced high levels of serum-neutralizing Abs and inhibited tumor formation in cotton-top tamarins (3). A number of recombinant vectors including vaccinia-gp350 and adenovirus 5-gp350 have also been successfully used in these animals to block tumor outgrowth (4). The precise mechanism by which gp350 affords protection from lymphomas in cotton-top tamarins remains unclear. The fact that development of neutralizing Ab titers in vaccinated animals does not always correlate with protection indicates that gp350-specific T cell-mediated immune responses may also have an effector role (5). Furthermore, Yao and colleagues (6) showed that very low levels of neutralizing anti-gp350 Abs are present in the saliva of healthy EBV-immune donors, which suggests that such Abs are unlikely to be the basis of long-term immunity in healthy seropositive individuals.

In the present study, we have addressed the question of whether EBV structural Ags stimulate specific T cell responses, using a novel method (7) designed to identify CTL epitopes within gp350 and gp85. Using this protocol, we firstly identified a number of HLA A2-binding peptides from gp350 and gp85. Strong ex vivo cytotoxic T cell activity against four of these peptides was noted from PBLs from infectious mononucleosis (IM)[3] patients. Moreover, these effectors also showed efficient recognition of target cells infected with recombinant vaccinia-encoding gp350 and gp85. These individuals maintained gp350- and gp85-specific memory CTL, albeit at low levels, following recovery from acute IM. We also show that prior immunization of HLA A2/K^b transgenic mice with gp350 and gp85 CTL epitopes afforded significant

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Received for publication August 3, 1998. Accepted for publication November 12, 1998.

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[1] This work was supported by grants from the National Health and Medical Research Council, Australia. R.K. is supported by an R. Douglas Wright Fellowship from the National Health and Medical Research Council, Australia.

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[3] Abbreviations used in this paper: LMP1, latent membrane protein 1; IM, infectious mononucleosis; LDA, limiting dilution analysis; LCL, lymphoblastoid cell line.

0022-1767/99/$02.00

protection against recombinant vaccinia virus infection, which correlated with the activation of gp350- and gp85-specific CTL in these animals. These results provide evidence that CTL epitopes from the EBV structural proteins could be exploited in the development of an EBV vaccine that confers a protective immunity against EBV infection.

Materials and Methods

IM patients

IM patients, identified on clinical grounds and by heterophile Ab positivity, were bled during the first 5–10 days of illness and, in two cases, on a second occasion 24–36 mo after the resolution of symptoms. These patients were HLA typed for the HLA A2 allele by serotyping in microcytotoxicity and by genotyping. Three patients (SB, LP, and MG) were identified as HLA A2-positive patients, and this was subsequently confirmed by FACS analysis using an HLA A2-specific mAb (American Type Culture Collection, Manassas, VA).

Establishment and maintenance of cell lines

EBV-transformed lymphoblastoid cell lines (LCLs) were established from a panel of IM and healthy EBV-seropositive donors by exogenous virus transformation of peripheral B cells using type 1 (B95.8) or type 2 (Ag876) EBV isolates (8) and were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin plus 10% FCS (growth medium). In addition, the peptide transporter-negative B $\times$ T hybrid cell line 174 $\times$ CEM.T2 (referred to as T2) (9) were used for peptide stabilization assays.

To generate PHA blasts, PBMC were stimulated with PHA (1 μg/ml) (Commonwealth Serum Laboratories, Melbourne, Australia), and, after 3 days, growth medium containing MLA-144 supernatant and rIL-2 (10 U/ml) was added (10). PHA blasts were propagated with biweekly replacement of IL-2 (10U/ml) and MLA supernatant (no further PHA added) for up to 6 wk.

Establishment and preparation of CTL effectors

Acute IM PBMC effectors for use in ex vivo cytotoxicity assays were resuspended in growth medium supplemented with rIL-2 and used directly in a cytotoxicity assay (see below). To generate polyclonal CTL, 2×10^6 PBMCs from HLA A2-positive donors were cocultivated for 7 days with irradiated (8000 rad) T2 cells (responder:stimulator ratio of 25:1) presensitized with synthetic peptides (1 μg/ml) (11). On days 7 and 14, these cultures were restimulated with peptide-sensitized T2 cells. After 18 days of culture in growth medium, the cells were used as polyclonal effectors in a standard ^{51}Cr-release assay against peptide-sensitized autologous PHA blasts.

Synthesis of peptides

Peptides, synthesized by the Merrifield solid phase method, were purchased from Chiron Mimotopes (Melbourne, Australia), dissolved in dimethyl sulfoxide, and diluted in serum-free RPMI 1640 medium for use in standard CTL assays. The purity of these peptides was tested by mass spectrometery and showed >90% purity.

MHC stabilization assays

HLA A2-binding peptides within the gp85 and gp350 Ags were identified using a protocol described elsewhere (7). These predicted peptides were then used in a standard MHC stabilization assay using T2 cells. Briefly, T2 cells (2×10^5) were incubated with 200 μl of each of the peptides (200 μg/ml) at 26°C for 14–16 h in serum-free medium (AIM-V; Life Technologies, Gaithersburg, MD), followed by incubation at 37°C for 2–3 h. After the incubations, HLA A2 expression was measured by FACS using a monoclonal HLA A2-specific Ab (MA2.1; American Type Culture Collection).

Vaccinia virus recombinants

Recombinant vaccinia constructs encoding the EBV structural Ags gp350 (Vacc.gp350) and gp85 (Vacc.gp85), nuclear Ag 2 (EBNA2) and a vaccinia virus construct made by insertion of the pSC11 vector alone and negative for thymidine kinase (Vacc.TK$^-$) have been previously described (12). Target cells were infected with recombinant vaccinia virus at a multiplicity of infection of 10:1 for 1 h at 37°C, as described earlier (13, 14). After overnight infection, cells were washed with growth medium and processed for CTL assays or for immunoblotting to assess the expression of recombinant EBV Ags (15).

Cytotoxicity assay

Target cells were either infected with recombinant vaccinia viruses or presensitized with synthetic peptide epitopes and then incubated with ^{51}Cr for 90 min. Following incubation, these cells were washed in growth medium and used as targets in a standard 5-h ^{51}Cr-release assay (8).

Limiting dilution analysis (LDA)

PBMC from post-IM donors were distributed in graded numbers (twofold dilutions) from 6.25×10^3 to 5×10^4 cells per well in round-bottom microtiter plates. Approximately 5×10^4 γ-irradiated (2000 rad) peptide-sensitized (1 μg/ml) autologous PBMC were added to give a total volume of 100 μl. Twenty-four replicates were used at each concentration in each experiment. Cultures were fed on days 4 and 7 with 50 μl of medium supplemented with 20 U of rIL-2 and 30% (v/v) supernatant from MLA-144 cultures. On day 10, each CTL microculture was split into two replicates and used as effectors in a standard 5-h ^{51}Cr-release assay against autologous PHA blasts precoated with structural (gp350 or gp85) or latent (EBNA3 or LMP2A) Ag peptide epitopes or left uncoated. Wells were scored as positive when the percent specific ^{51}Cr release for peptide-sensitized target cells exceeded the mean release from untreated control wells by 3 SD. LDA was performed by the method of maximum likelihood estimation (7). Data from all experiments were compatible with the hypothesis of single-hit kinetics ($p > 0.4$), and precursor estimates are given with 95% confidence limits.

Immunization of HLA A2/K^b transgenic mice with gp350 and gp85 CTL epitopes

HLA A2/K^b transgenic mice used in this study have been described elsewhere (16) (a kind gift from Dr. L. Sherman, Scripps Research Institute). These mice express a chimeric class I molecule composed of the α1 and α2 domains of the human A*0201 allele and the α3 domains of the mouse H-2K^b class I molecules. Peptide immunizations were conducted as described by Vitiello and colleagues (17). Briefly, these animals were twice immunized (at a 14-day interval) s.c. with 50 μg/mouse of CTL epitopes emulsified in IFA together with 5 μg tetanus toxoid as a source of help. Four weeks following peptide immunization, animals were assessed for gp350- and gp85-specific CTL response. For assessing these CTL responses, splenocytes (3×10^6 cells/ml) were cocultured with syngeneic, irradiated (2000 rad) peptide-coated LPS blasts (3×10^5 cells/ml) and 3 μg/ml human β_2-microglobulin. CTL activity was tested on day 6 using a standard ^{51}Cr-release assay.

Vaccinia protection assay

For protection experiments, groups of 8-wk-old female A2/K^b transgenic mice were immunized with CTL epitopes as described above. On day 28, mice were challenged with Vacc.gp85 and Vacc.gp350 i.p. (1×10^7 plaque-forming units in 100 μl PBS). After 4 days of challenge, these animals were sacrificed and vaccinia titers measured in both ovaries by plaque assay on confluent CV1 cells.

Results

Identification of HLA A2-binding peptides within gp85 and gp350

To identify potential HLA A2-restricted epitopes within gp85 and gp350, the amino acid sequence was analyzed by a computer program designed to predict HLA-binding peptides, based on an estimation of the half-time disassociation of the HLA-peptide complex (http://bimas.dcrt.nih.gov/molbio/hla bind/index.html) (18). A total of 20 peptides (13 from gp85 and 7 from gp350) with an estimated half-time disassociation score of >100 for gp85 and >50 for gp350 were selected (Table I). These peptides were then tested for HLA A2-binding efficiency using HLA A2-positive T2 cells. Representative data from a series of experiments is presented in Fig. 1. This analysis showed that seven of these peptides significantly increased the expression of HLA A2 on T2 cells, suggesting that these peptides might be potential HLA A2-restricted epitopes.

Table I. *Identification of potential HLA A2-binding peptides within gp85 and gp350*

	Ranking	Residue	Peptide Sequence	Score[b]
gp85	1	177–185	FLMGTYKRV	1775.663
	2	317–325	WLAKSFFEL	1082.903
	3	672–680	GLYEERAHV	912.522
	4	685–693	ILYFIAFAL	674.026
	5	2–10	QLLCVFCLV	488.951
	6	225–233	SLVIVTTFV	382.536
	7	681–689	VLAIILYFI	224.537
	8	684–692	IILYFIAFA	196.407
	9	542–550	LMIIPLINV	181.738
	10	1–9	MQLLCVFCL	181.738
	11	7–15	FCLVLLWEV	133.298
	12	658–666	YLLLTTNGT	126.883
	13	420–428	TLFIGSHVV	105.510
gp350	1	871–879	VLTLLLLLV	271.948
	2	152–160	LIPETVPYI	126.481
	3	863–871	VLQWASLAV	118.238
	4	875–883	LLLLVMADC	71.872
	5	67–75	QLTPHTKAV	69.552
	6	861–869	MLVLQWASL	61.737
	7	873–881	TLLLLLVMA	42.278

[a] To identify the potential HLA A2-binding peptides within gp85 and gp350, a computer-based program was employed as described elsewhere (18). This program can be directly accessed through http.// bimas.dcrt.nih.gov/molbio/hla_bind/index.html.

[b] Estimate half-time disassociation from HLA A2 allele.

Recognition of the gp85 and gp350 peptide epitopes by IM effectors ex vivo

The seven HLA A2-binding peptides, which included four peptides from gp85 and three peptides from gp350, were next tested for CTL recognition by effectors from IM patients. In addition, we also included an HLA A2-restricted CTL epitope from EBV latent membrane protein 1 (LMP1) as a positive control (11). PBMCs from three HLA A2-positive IM patients, SB, LP, and MG, were resuspended in IL-2-supplemented growth medium and used as effectors in a standard ^{51}Cr-release assay against HLA-matched PHA blasts sensitized with the gp85, gp350, or LMP1 peptides. Representative data from two different experiments is shown in Fig. 2, *A–C*. Effectors from all three IM patients showed clear recognition of the reference LMP1 peptide (YLQQNWWTL), consistent with our earlier finding that this peptide is recognized by EBV-specific CTL. More importantly, these IM patients also showed strong ex vivo recognition of target cells sensitized with selected gp85 and gp350 peptides, and the level of CTL recognition of target cells sensitized with these peptides was comparable with that seen with the LMP1 peptide (YLQQNWWTL). Interestingly, each of these individuals showed a distinct pattern of reactivity against these peptides. IM patient SB showed strong reactivity against peptides SLVIVTTFV (gp85) and VLQWASLAV (gp350) (Fig. 2*A*), while the LP and MG effectors recognized target cells preloaded with peptides LMIIPLINV (gp85) and VLQ WASLAV (gp350) (Fig. 2, *B* and *C*). Furthermore, ex vivo effectors from patient LP also recognized target cells infected with Vacc.gp350 and Vacc.gp85 (Fig. 2*D*).

In vitro expansion of gp85 and gp350 peptide epitope reactive CTL

The data presented above clearly demonstrate that gp85 and gp350 include CTL determinants that can bind HLA A2 molecules and are efficiently recognized by ex vivo effectors from IM patients. To determine whether gp85- or gp350-reactive CTL can be detected following recovery from IM, PBMCs from the two donors, SB and LP, were collected at 24–36 mo post-IM, respectively, and were

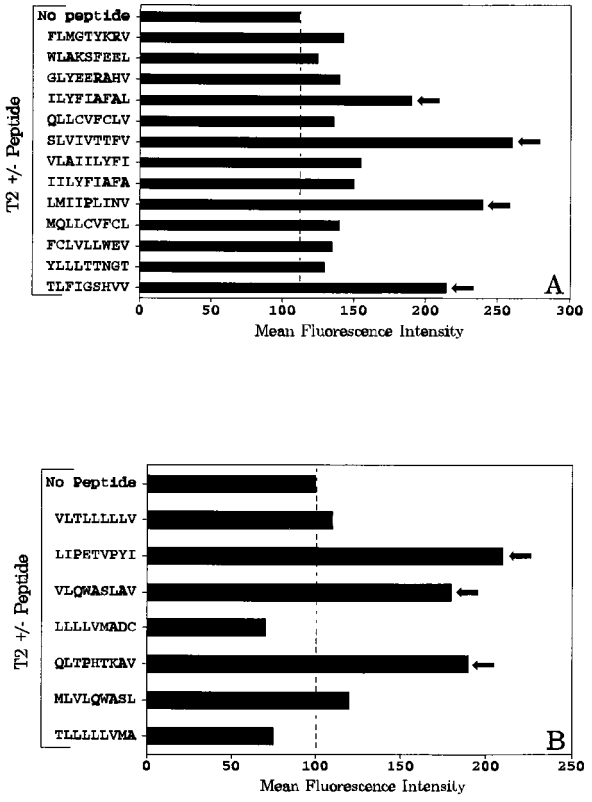

FIGURE 1. MHC stabilization analysis on T2 cells using potential HLA A2-binding peptides within gp85 (*A*) and gp350 (*B*). T2 cells were initially incubated with 200 μl of each of the peptides (200 μg/ml) for 14–16 h at 26°C followed by incubation at 37°C for 2–3 h. HLA A2 expression on these cells was analyzed by FACS using the BB7.2 Ab. The dotted line indicates the background mean fluorescence intensity for HLA A2 on T2 cells without any peptide. The gp85 and gp350 peptides showing significant stabilization of HLA A2 molecules on T2 cells are indicated by arrows.

stimulated with T2 cells presensitized with each of the gp85 and gp350 peptides, which showed strong HLA A2 binding. On day 18, these CTL effectors were tested against peptide-sensitized autologous PHA blasts. Representative data from polyclonal CTL from donor SB are presented in Fig. 3. CTL effectors from donor SB not only showed strong reactivity against peptides SLVIVT TFV and VLQWASLAV but also recognized two other peptides from gp85 (LMIIPLINV and TLFIGSHVV). Donor LP also showed a similar pattern of CTL lysis. Thus peptide TLFIGSHVV was a target for EBV-specific CTL recognition in the memory response of these A2-positive individuals, but this response was not detectable with ex vivo effectors during acute infection. Another important point that needs to be highlighted here is that our attempts to activate gp85- or gp350-specific CTL with autologus LCLs as stimulators were unsuccessful. This result is not surprising because it is well established that in latently infected B cells, gp350 or gp85 Ags are poorly expressed. The LCL-stimulated polyclonal T cell lines from these donors are strongly reactive against latent Ags (data not shown). This observation is consistent with our earlier studies, which showed that CTL responses in healthy virus carriers is often dominated by reactivity to latent Ags (13).

Another explanation for an inability to detect gp350- or gp85-specific CTL reactivity following stimulation with the autologous LCLs is that these responses may constitute a minor component of the total virus-specific CTL response in healthy virus carriers. Indeed, LDA for CTL precursors specific for the gp350 (VLQ

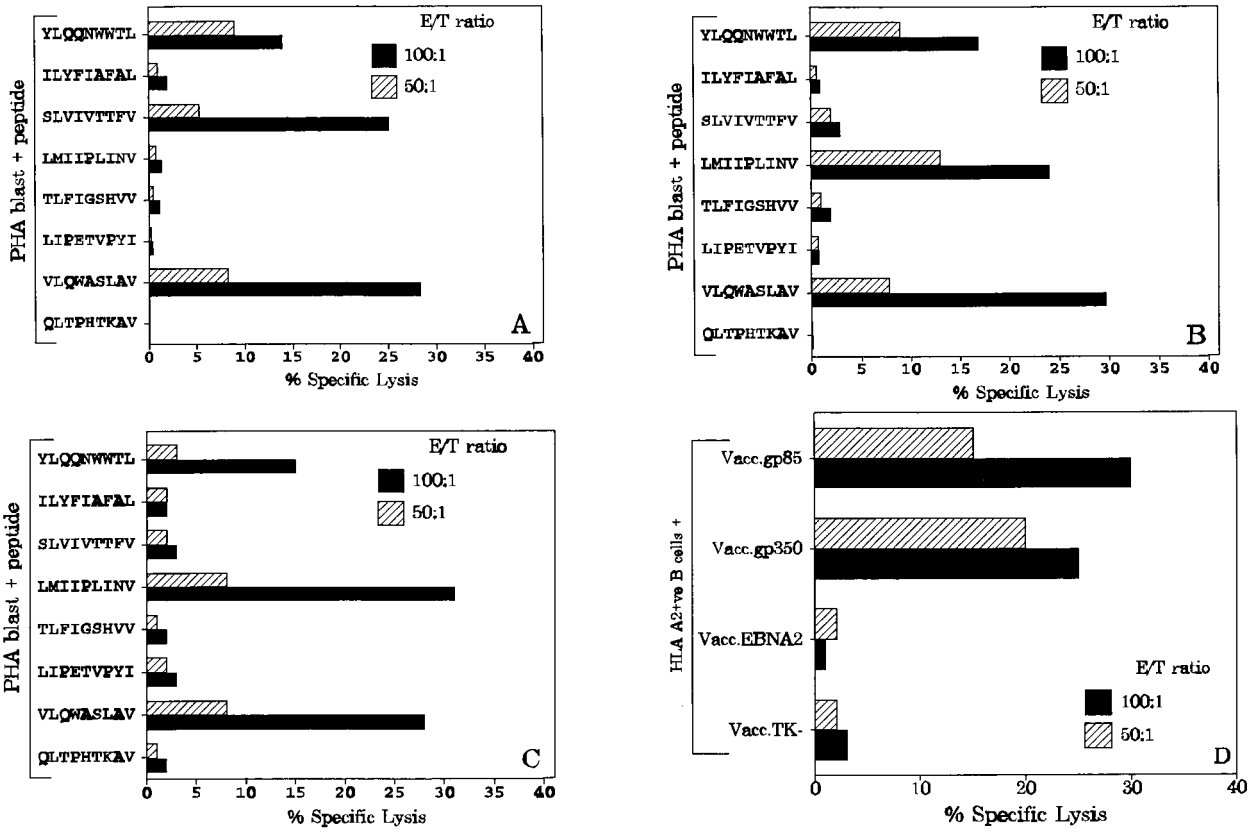

FIGURE 2. gp85 and gp350-specific ex vivo cytotoxic T cell activity in PBLs from IM donors. *A–C*, Ex vivo CTL lysis of peptide-sensitized (1 μg/ml) PHA blasts at two different effector:target (E/T) ratios is shown. Data from IM patient SB, LP, and MG are presented in *A*, *B*, and *C*, respectively. *D*, Ex vivo CTL lysis by PBLs from patient LP of target cells infected with recombinant vaccinia encoding either gp85 (Vacc.gp85) or gp350 (Vacc.350) is shown. Vacc.TK and Vacc.EBNA2 were used as control in the assay.

WASLAV) or gp85 (LMIIPLINV and TLFIGSHVV) peptide epitopes in a post-IM donor showed precursor frequencies of 1/209,211, 1/433,489, and 1/244,823, respectively, while precursor frequencies for the CTL that recognize epitopes within the latent Ags, EBNA3 (YPLHEQHGM) and LMP2A (CLGGLL TMV), were 1/5,244 and 1/293,706, respectively (Fig. 4).

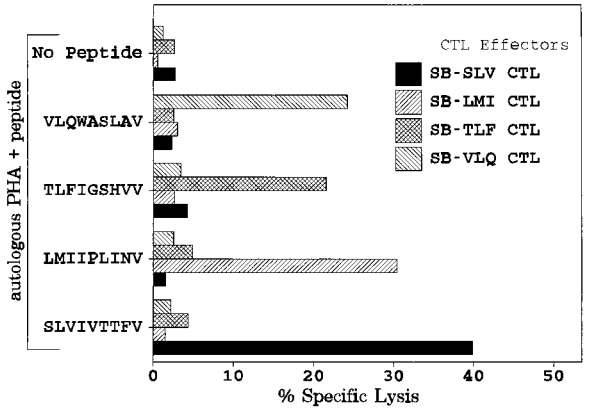

FIGURE 3. Recognition of gp85 and gp350 peptides by polyclonal CTL from an HLA A2-positive IM-recovered (36 mo post-IM) individual. PBMC were cocultivated for 7 days with irradiated T2 cells sensitized with synthetic peptides (indicated on the *y*-axis). On day 18, these cells were used as polyclonal effectors in a standard ^{51}Cr-release assay against peptide-sensitized (1 μg/ml) autologous PHA blasts. An E:T ratio of 20:1 was used in the assay. Individual peptide stimulated CTL effectors used in this experiments are indicated. Data from one representative experiment of three is shown. Results are expressed as percent specific lysis.

Immunization of HLA A2/K^b mice with gp85 and gp350 peptide epitopes induces specific CTL response

Having established that gp85 and gp350 includes CTL epitopes, we extended our studies to explore the possibility of using these peptide epitopes to induce specific CTL response in vivo. HLA A2/K^b transgenic mice were used as an experimental model to address this issue. These mice express a chimeric class I molecule composed of the α1 and α2 domains of the human A*0201 allele and the α3 domain of the mouse H-2K^b class I molecules. These animals were immunized s.c. with gp350 or gp85 CTL epitopes emulsified in IFA together with tetanus toxoid as a source of help. The SLVIVTTFV and TLFIGSHVV peptides from gp85 and the VLQWASLAV peptide from gp350 were used for immunization. Two weeks following immunization, specific CTL response was assessed in each mouse using splenocytes or pooled inguinal lymph node cells as effectors. Data presented in Fig. 5, *A–C* demonstrate that peptide epitopes from gp85 (SLVIVTTFV and TLFIGSHVV) and gp350 (VLQWASLAV) induced strong CTL response in splenocytes. Interestingly, CTL activated from splenocytes with peptide TLFIGSHVV consistently showed strong lysis of targets from all the animals tested, while splenocytes from SLVIVTTFV- and VLQWASLAV-immunized mice showed variable in vitro CTL lysis. In two different sets of experiments, only 50–60% of the animals immunized with SLVIVTTFV and VLQ WASLAV showed strong CTL activity in splenocytes. On the other

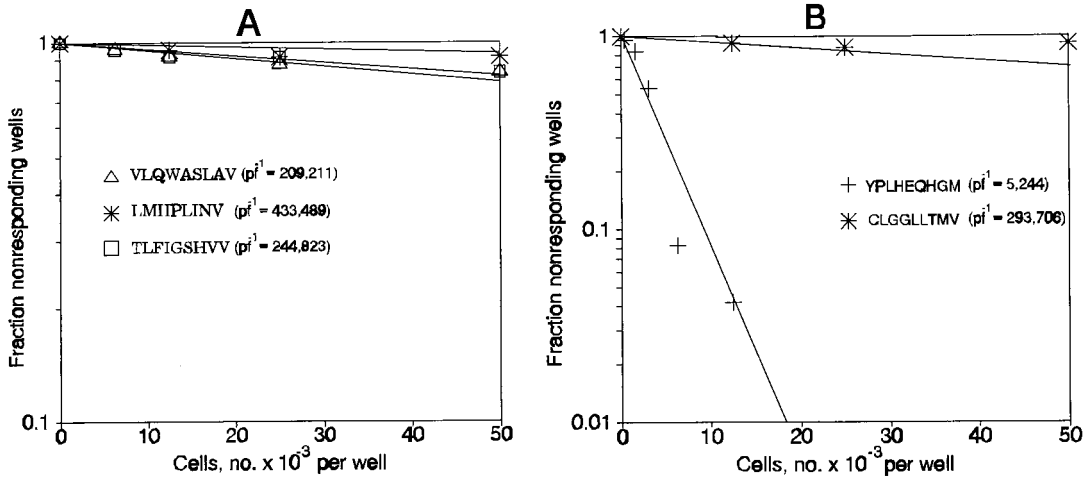

FIGURE 4. CTLp frequencies for the structural (*A*) and latent Ag (*B*) epitopes in a donor following recovery from acute IM. gp350 epitope (VLQ WASLAV) and gp85 epitopes (LMIIPLINV and TLFIGSHVV) (*A*), EBNA3 epitope (YPLHEQHGM), and LMP2A epitope (CLGGLLTMV) were used in this analysis. Peptide epitopes VLQWASLAV, LMIIPLINV, TLFIGSHVV, and CLGGLLTMV are HLA A2-restricted, while YPLHEQHGM epitope is HLA B35-restricted. Using LDA, the frequencies of CTLp for these peptide epitopes were estimated in PBLs from donor LP. PBMC from this donor were stimulated with peptide-sensitized PBMC as described in *Materials and Methods*. Reciprocal values of responder frequencies (f^{-1}) are indicated.

hand, a strong specific CTL activity was consistently observed in pooled lymphocytes from inguinal lymph nodes (Fig. 5*D*).

Prior immunization of HLA A2/K^b mice with gp85 or gp350 CTL epitopes affords protection against recombinant vaccinia virus challenge

Four weeks after peptide immunization with gp85 or gp350 CTL epitopes, HLA A2/K^b mice were challenged with 10^7 plaque-forming units of recombinant vaccinia virus encoding either gp85 or gp350. After 4 days of challenge, these animals were sacrificed and vaccinia titers measured in both ovaries by plaque assay on confluent CV1 cells. Data from one such experiment is presented in Fig. 6. Animals immunized with gp85 and gp350 epitopes showed very low to undetectable virus in their ovaries, while in naive mice very high titers of vaccinia virus were detected. This protection correlated with strong induction of epitope-specific

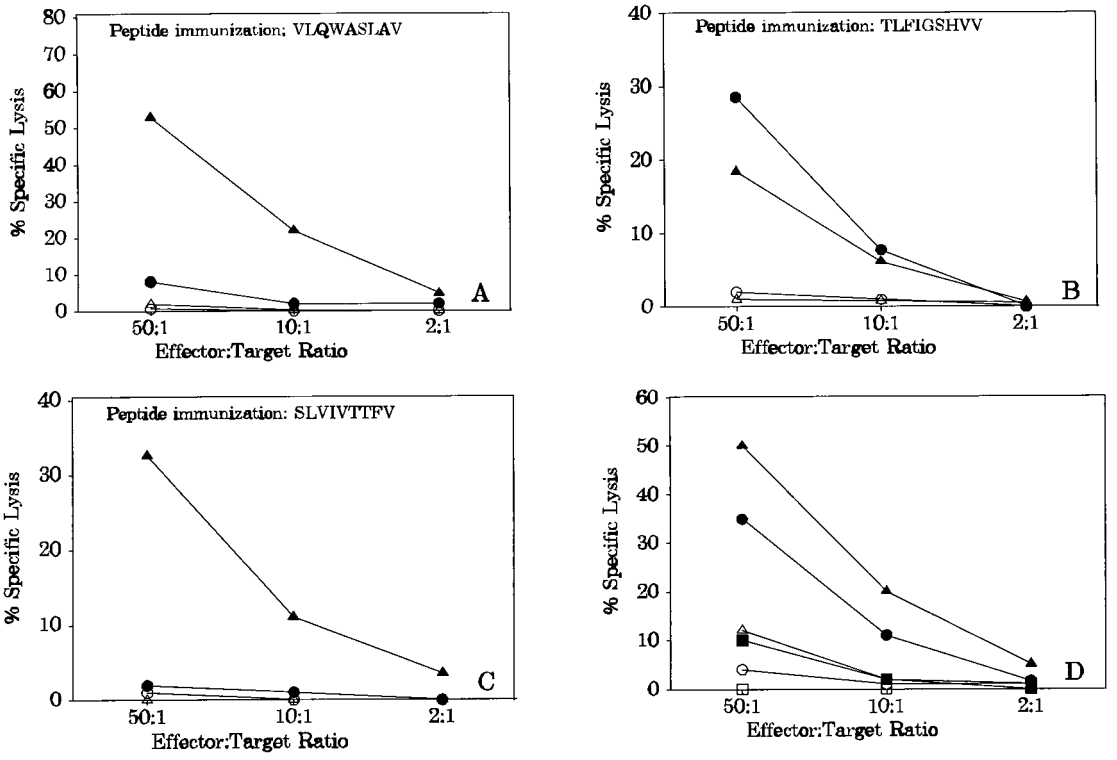

FIGURE 5. Immunization of HLA A2/K^b mice with gp85 and gp350 CTL epitopes induces strong CTL response. Animals were twice immunized (at a 14-day interval) s.c. with individual CTL epitope with tetanus toxoid as a source of help. Four weeks following peptide immunization, animals were assessed for gp350- and gp85-specific CTL response. *A–C*, CTL activity in splenocytes from mice immunized with VLQWASLAV (gp350), TLFIGSHVV (gp85), and SLVIVTTFV (gp85), respectively, is shown. CTL lysis of target cells sensitized with peptide epitopes are shown as filled symbols, while lysis of unsensitized target cells is shown as empty symbols. *D*, CTL activity in pooled inguinal lymph nodes from mice immunized with peptide VLQWASLAV (□ ■), SLVIVTTFV (△ ▲), and TLFIGSHVV (○ ●) is shown. CTL activity was tested on day 6 using a standard ^{51}Cr-release assay.

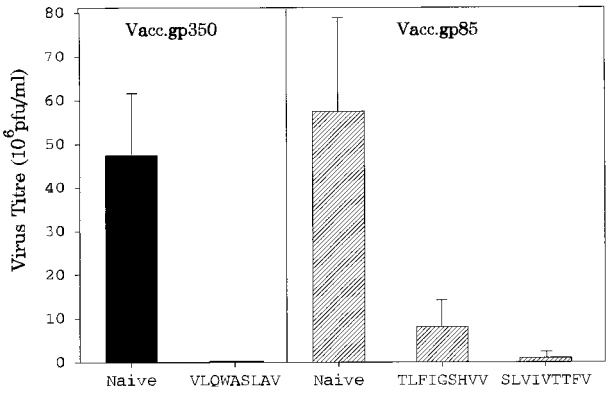

FIGURE 6. Prior immunization of HLA A2/K^b mice with gp85 or gp350 CTL epitopes affords protection against recombinant vaccinia virus challenge. Groups of female A2/K^b transgenic mice, either unimmunized or immunized with CTL epitopes, were challenged with Vacc.gp85 and Vacc.gp350 i.p. After 4 days of challenge, these animals were sacrificed, and vaccinia titer were measured in both ovaries by plaque assay on confluent CV1 cells. The *x*-axis shows peptides used for immunization, while the *y*-axis shows mean ± SE vaccinia virus titer in naive and peptide-immunized mice. The recombinant vaccinia virus used for challenge in these animals is shown in each panel.

CTL responses detected in the splenocytes and lymph node cells collected 4 wk after primary peptide vaccination in HLA A2/K^b transgenic mice.

Discussion

There is increasing interest in formulating an effective vaccine against EBV, designed to not only limit the outgrowth of latently infected B cells in healthy individuals but to also block the development of many EBV-associated malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease. In western societies, the principle aim of such a vaccine would be to protect from IM. In this context, virus load (a large dose of orally transmitted virus and/or overexpansion of the virus-transformed B cell pool beyond a critical threshold) may be a critical determinant of disease risk (1). Therefore, a vaccine capable of either blocking primary EBV infection or significantly reducing the EBV load during primary infection may be adequate to avert clinical symptoms. A similar vaccine will also be able to reduce the immediate risk of lymphoproliferative disease in transplant patients receiving immunosuppressive therapy. On the other hand, EBV-associated malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease arise in patients years after their primary infection, and protection from these longer-term consequences would require a vaccine that ideally confers sterile immunity and prevents the establishment of the carrier state.

EBV structural Ags, primarily gp350, have long been considered as the potential candidates for an EBV vaccine. The suggestion that gp350 is a likely vaccine candidate was based initially upon the observation that this glycoprotein is the principal target of the virus-neutralizing Ab response (19). A number of recombinant formulations of gp350, either presented as a subunit Ag or expressed from recombinant viral vectors, designed to induce high titer neutralizing Abs, have shown significant protection against EBV-induced B cell lymphomas in cotton-top tamarins (2). However, development of neutralizing Abs in vaccinated animals does not always shows limited correlation with protection from EBV infection, although recent results have suggested a role for gp350-specific CTL in this protection (5). If the latter suggestion is correct, it is important to identify the potential CTL determinants within EBV structural proteins because it is now well established that immunization with whole viral proteins is unable to elicit an efficient CTL response. Moreover, a vaccine based on CTL epitopes provides an opportunity to include determinants not only from gp350 but also from other structural Ags, such as gp85. To address this issue we have used a novel protocol to successfully identify CTL epitopes within gp350 and gp85. In the first set of experiments, we identified HLA A2-binding peptides within gp350 and gp85. Subsequent experiments were focussed on IM patients with the HLA A2 allele. Using ex vivo primary effectors, we observed strong reactivity to three different gp350 and gp85 peptides, and the levels of CTL lysis was comparable to that seen with LMP1 peptide. Interestingly, individual IM patients showed distinct patterns of reactivity to each of these peptides. Strong reactivity against peptides SLVIVTTFV (gp85) and VLQWASLAV (gp350) was observed with ex vivo effectors from patient SB, while the LP and MG effectors recognized target cells preloaded with LMIIPLINV (gp85) and VLQWASLAV (gp350) peptides. More importantly, ex vivo effectors from patient LP also recognized target cells infected with Vacc.gp350 and Vacc.gp85. Interestingly, the level of ex vivo CTL lysis directed to epitopes from structural Ags was consistently higher than those seen in the same assays against HLA A2-restricted CTL epitopes from a latent Ag. These results are consistent with recent observations by Steven and colleagues (20) that ex vivo CTL reactivity to lytic Ags in IM patients is significantly higher compared with latent Ags.

In the next set of experiments, we explored the possibility of detecting structural Ag-specific CTL responses in individuals following resolution of IM symptoms. This follow up analysis was conducted 24–36 mo after acute IM. Our initial attempts to isolate gp350- or gp85-specific CTL from post-IM donors by stimulating with the autologous LCL were unsuccessful. Subsequently, we used peptide-loaded T2 cells as stimulators to generate gp350- and gp85-specific CTL. We have recently shown that this method can be successfully used to raise low-frequency EBV-specific CTL precursors (11). Stimulation of PBMC from donors SB and LP raised strong CTL responses to the gp85 and gp350 CTL epitopes. Both donors SB and LP not only showed reactivity against peptides SLVIVTTFV, LMIIPLINV, and VLQWASLAV but also recognized another peptide from gp85, TLFIGSHVV. It is interesting to note here that both donors showed no ex vivo CTL reactivity to TLFIGSHVV during acute IM. One of the important conclusions drawn form these analyses is that, following recovery from acute IM, there is a significant reduction in CTL precursors to the structural Ags, and the response becomes dominated by CTL reactive to the latent Ags. Indeed, LDA for CTL precursors specific for the gp350 or gp85 peptide epitopes in donors SB and LP post-IM showed frequencies of >1/50,000, while much higher precursor frequencies for CTL epitopes within EBNA proteins were seen.

The detection of a strong ex vivo CTL response in IM patients to the structural Ags has important implications for any future vaccine design. As mentioned above, to date, the major emphasis of vaccine design based on EBV structural Ags has been directed toward generating a strong neutralizing Ab response. However, these neutralizing Ab responses fail to correlate with protection against EBV-induced polyclonal lymphomas in cotton-top marmosets. Nevertheless, it is possible that this protection is mediated by structural Ag-specific CTL responses. To address this issue, we employed an experimental animal model system to determine whether gp350 or gp85 CTL epitope-immunized transgenic mice, expressing the human HLA A2 Ag, are capable of 1) generating structural Ag-specific CTL responses and 2) reducing infection

with a recombinant vaccinia virus infection expressing the gp350 or gp85 Ag. These mice not only showed induction of a strong CTL response following immunization, but also acquired strong resistance to virus infection. It is important to mention here that although this experiment does not allow any firm conclusions on the efficacy of a gp350 and/or gp85 CTL epitope-based vaccine in humans, it does clearly show that CTL epitopes from the EBV structural Ags can be used as immunogens to induce an efficient CTL response in vivo. Moreover, this approach also overcomes limitations of whole gp350 or gp85 proteins that might be inefficient at eliciting CTL responses in humans. Obviously, one of the possible obstacles of any epitope-based approach to vaccination in humans is HLA polymorphism because epitope choice is allele-specific. However, this obstacle might be overcome using appropriate mixtures of synthetic peptide epitope or by constructing vectors to express polypeptides in which the relevant epitope sequences are linearly joined together. Indeed, earlier studies from our laboratory have shown that if such an EBV polyepitope sequence is expressed within cells from a recombinant vaccinia vector, all of the constituent epitopes are efficiently presented for CTL recognition (21), indicating the potential of this approach as a vaccine strategy. More recently, work in a murine model has also shown that each of several CTL epitopes combined in a polyepitope construct was capable of eliciting a CTL response in vivo and could protect the animals from subsequent challenge (22). In the long term, it may be possible to combine CTL epitopes from the EBV structural Ags with latent Ag epitopes generating a chimeric protein that fuses the important immunogenic determinants from the two different types of Ags to design an effective vaccine.

References

1. Khanna, R., S. R. Burrows, and D. J. Moss. 1995. Immune regulation in Epstein-Barr virus-associated diseases. *Microbiol. Rev. 59:387.*
2. Morgan, A. J. 1992. Epstein-Barr virus vaccines. *Vaccine 10:563.*
3. Morgan, A. J., S. Finerty, K. Lovgren, F. T. Scullion, and B. Morein. 1988. Prevention of Epstein-Barr (EB) virus-induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein gp340 incorporated into immune-stimulating complexes. *J. Gen. Virol. 69:2093.*
4. Morgan, A. J., M. Mackett, S. Finerty, J. R. Arrand, F. T. Scullion, and M. A. Epstein. 1988. Recombinant vaccinia virus expressing Epstein-Barr virus glycoprotein gp340 protects cottontop tamarins against EB virus-induced malignant lymphomas. *J. Med. Virol. 25:189.*
5. Wilson, A. D., M. Shooshstari, S. Finerty, P. Watkins, and A. J. Morgan. 1996. Virus-specific cytotoxic T cell responses are associated with immunity of the cottontop tamarin to Epstein-Barr virus (EBV). *Clin. Exp. Immunol. 103:199.*
6. Yao, Q. Y., M. Rowe, A. J. Morgan, C. K. Sam, U. Prasad, H. Dang, Y. Zeng, and A. B. Rickinson. 1991. Salivary and serum IgA antibodies to the Epstein-Barr virus glycoprotein gp340: incidence and potential for virus neutralization. *Int. J. Cancer 48:45.*
7. Khanna, R., S. R. Burrows, J. Nicholls, and L. M. Poulsen. 1998. Identification of cytotoxic T cell epitopes within Epstein-Barr virus (EBV) oncogene latent membrane protein 1 (LMP1): evidence for HLA A2 supertype-restricted immune recognition of EBV-infected cells by LMP1- specific cytotoxic T lymphocytes. *Eur. J. Immunol. 28:451.*
8. Moss, D. J., I. S. Misko, S. R. Burrows, K. Burman, R. McCarthy, and T. B. Sculley. 1988. Cytotoxic T-cell clones discriminate between A- and B-type Epstein-Barr virus transformants. *Nature 331:719.*
9. Salter, R. D., and P. Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant T×B cell hybrid. *EMBO J. 5:943.*
10. Burrows, S. R., I. S. Misko, T. B. Sculley, C. Schmidt, and D. J. Moss. 1990. An Epstein-Barr virus-specific cytotoxic T-cell epitope present on A- and b-type transformants. *J. Virol. 64:3974.*
11. Khanna, R. 1998. Tumour surveillance: missing peptides and MHC molecules. *Immunol. Cell Biol. 76:20.*
12. White, C. A., S. M. Cross, M. G. Kurilla, B. M. Kerr, C. Schmidt, I. S. Misko, R. Khanna, and D. J. Moss. 1996. Recruitment during infectious mononucleosis of $CD3^+CD4^+CD8^+$ virus-specific cytotoxic T cells which recognise Epstein-Barr virus lytic antigen BHRF1. *Virology 219:489.*
13. Khanna, R., S. R. Burrows, M. G. Kurilla, C. A. Jacob, I. S. Misko, T. B. Sculley, E. Kieff, and D. J. Moss. 1992. Localization of Epstein-Barr virus cytotoxic T-cell epitopes using recombinant vaccinia: implications for vaccine development. *J. Exp. Med. 176:169.*
14. Khanna, R., C. A. Jacob, S. R. Burrows, and D. J. Moss. 1993. Presentation of endogenous viral peptide epitopes by anti-CD40 stimulated human B cells following recombinant vaccinia infection. *J. Immunol. Methods 164:41.*
15. Khanna, R., C. A. Jacob, S. R. Burrows, M. G. Kurilla, E. Kieff, I. S. Misko, and D. J. Moss. 1991. 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. *Immunology 74:504.*
16. Vitiello, A., D. Marchesini, J. Furze, L. A. Sherman, and R. W. Chesnut. 1991. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human-mouse class I major histocompatibility complex. *J. Exp. Med. 173:1007.*
17. Vitiello, A., A. Sette, L. Yuan, P. Farness, S. Southwood, J. Sidney, R. W. Chesnut, H. M. Grey, and B. Livingston. 1997. Comparison of cytotoxic T lymphocyte responses induced by peptide or DNA immunization: implications on immunogenicity and immunodominance. *Eur. J. Immunol. 27:671.*
18. Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *J. Immunol. 152:163.*
19. Thorley-Lawson, D. A. 1980. The suppression of Epstein-Barr virus infection in vitro occurs after infection but before transformation of the cell. *J. Immunol. 124:745.*
20. Steven, N. M., N. E. Annels, A. Kumar, A. M. Leese, M. G. Kurilla, and A. B. Rickinson. 1997. Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T cell response. *J. Exp. Med. 185:1605.*
21. Thomson, S. A., R. Khanna, J. Gardner, S. R. Burrows, B. Coupar, D. J. Moss, and A. Suhrbier. 1995. Minimal epitopes expressed in a recombinant polyepitope protein are processed and presented to $CD8^+$ cytotoxic T cells: implications for vaccine design. *Proc. Natl. Acad. Sci. USA 92:5845.*
22. Thomson, S., S. Elliott, M. Sherritt, K. W. Sproat, B. E. Coupar, A. A. Scalzo, C. A. Forbes, A. Ladhams, X. Y. Mo, R. Tripp, P. C. Doherty, D. J. Moss, and A. Suhrbier. 1996. Recombinant polyepitope vaccines for the delivery of multiple $CD8^+$ cytotoxic T cell epitopes. *J. Immunol 157: 822.*