Asymptomatic Human CD4+ Cytotoxic T-Cell Epitopes Identified from Herpes Simplex Virus Glycoprotein B

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The identification of “asymptomatic” (i.e., protective) epitopes recognized by T cells from herpes simplex virus (HSV)-seropositive healthy individuals is a prerequisite for an effective vaccine. Using the PepScan epitope mapping strategy, a library of 179 potential peptide epitopes (15-mer overlapping by 10 amino acids) was identified from HSV type 1 (HSV-1) glycoprotein B (gB), an antigen that induces protective immunity in both animal models and humans. Eighteen groups (G1 to G18) of 10 adjacent peptides each were first screened for T-cell antigenicity in 38 HSV-1-seropositive but HSV-2-seronegative individuals. Individual peptides within the two immunodominant groups (i.e., G4 and G14) were further screened with T cells from HLA-DR-genotyped and clinically defined symptomatic (n = 10) and asymptomatic (n = 10) HSV-1-seropositive healthy individuals. Peptides gB161-175 and gB166-180 within G4 and gB661-675 within G14 recalled the strongest HLA-DR-dependent CD4+ T-cell proliferation and gamma interferon production. gB166-180, gB661-675, and gB666-680 elicited ex vivo CD4+ cytotoxic T cells (CTLs) that lysed autologous HSV-1- and vaccinia virus (expressing gB)-infected lymphoblastoid cell lines. Interestingly, gB166-180 and gB666-680 peptide epitopes were strongly recognized by CD4+ T cells from 10 of 10 asymptomatic patients but not by CD4+ T cells from 10 of 10 symptomatic patients (P < 0.0001; analysis of variance posttest). Inversely, CD4+ T cells from symptomatic patients preferentially recognized gB661-675 (P < 0.0001). Thus, we identified three previously unrecognized CD4+ CTL peptide epitopes in HSV-1 gB. Among these, gB166-180 and gB666-680 appear to be “asymptomatic” peptide epitopes and therefore should be considered in the design of future herpes vaccines.

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are ubiquitous viruses that infect a majority of people worldwide (3, 22, 68). Shedding of reactivated HSV is estimated to occur at rates of 3 to 28% in adults who harbor latent HSV in their sensory neurons (32, 66-68). However, the vast majority of these individuals do not experience recurrent herpetic disease and are designated “asymptomatic patients” (22, 40, 68). In contrast, in some individuals (symptomatic patients), reactivation of latent virus leads to induction of “pathogenic” HSV-specific CD4+ and CD8+ T cells (22, 68) and recurrent disease, ranging from rare episodes occurring every 5 to 10 years to outbreaks occurring monthly or even more frequently among a small proportion of subjects (22, 26, 60). Interestingly, for genital herpes, symptomatic and asymptomatic patients have similar virus shedding rates (68). It is likely that the same is true for ocular and oro-facial herpes, since shedding rates in tears and saliva of asymptomatic individuals have been reported to be as high as 33.5% (22, 29, 40, 42). Latently infected patients are at risk of developing severe immunopathology, such as blinding herpetic stromal keratitis (HSK) (primarily due to HSV-1), painful genital ulcerations (primarily due to HSV-2), and in rare cases, fatal HSV encephalitis (reviewed in reference 72).

Considering the wealth of data addressing the role of T cells in animal models, it is surprising how few reports explore the immunologic basis of symptomatic and asymptomatic HSV infections in humans. The HSV-1-specific CD4+ T-cell responses in the cornea are much more likely to cause pathology than those in the genital tract or anus/buttocks region. Indeed, the involvement of CD4+ T cells that produce Th1 cytokines (interleukin-2 [IL-2] and gamma interferon [IFN-γ]) in HSK has been established with the mouse model of ocular herpes, and corneal herpetic diseases can be abrogated by depletion of CD4+ T cells or neutralization of Th1 cytokines (24, 25, 50, 51, 62, 63). HSV-specific CD4+ T cells are key participants in the development of recurrent herpetic sores in symptomatic individuals (14, 21, 39, 46, 47, 65, 75, 79). Paradoxically, HSV-specific CD4+ T cells may also be important in controlling the severity of genital herpes infection (1, 47, 49). CD4+ T-cell infiltrates appear to correlate with HSV clearance from mucocutaneous genital lesions (38). Individuals with severe immunodeficiency involving a lack of CD4+ T cells can have severe recurrent herpes with a longer duration of symptomatic lesions (17, 43, 57), thus supporting the thought that CD4+ T cells are one of the main mediators of protective immunity against recurrent herpes (34, 47, 75, 76). Early in the development of recurrent lesions, HSV-1-specific IFN-γ-producing CD4+ T cells that display a cytotoxic activity predominate in the mononuclear infiltrate surrounding HSV-1-infected epidermal cells.
which strongly express HLA-DR (47). For many years, the association of HLA-DR haplotypes with susceptibility to both labial and genital herpes has been appreciated (27, 41, 44, 55). Among the HLA-DR alleles, DRB1*04, DRB1*07, DRB1*11, and DRB1*13 are most frequent in the populations studied (26, 35, 41, 55). DRB1*13 is frequently seen in symptomatic HSV infection, while DRB1*04 is most frequent in individuals with asymptomatic HSV infection (41). Obvious and immediate consequences of variations in HLA-DR allele distribution are changes in functional epitopes presented to the host's CD4+ T cells (26, 27, 35, 41, 44, 47, 49, 55).

We hypothesize that the clinical spectrum of herpes, ranging from asymptomatic infection to frequently distressing outbreaks, may be reflected in T-cell recognition of different sets of epitopes of one or several HSV protein antigens (AgS). Thus, recognition by CD4+ T cells of a set of viral epitopes designated “symptomatic” might be associated with severe immunopathological diseases, while recognition of “asymptomatic” epitopes might, in turn, lead to immunoprotection. However, identification of HSV target Ags and the sets of epitopes recognized by “asymptomatic” versus “symptomatic” CD4+ T cells (i.e., “protective” versus “pathogenic” T cells) is far from being completed. Due to the lack of an animal model with a high incidence of recurrent eye disease, it is currently not feasible to demonstrate that “symptomatic” T-cell epitopes are pathogenic. However, if people with a history of severe recurrent disease (i.e., symptomatic people) tend to develop T cells that recognize a subset of epitopes (i.e., symptomatic epitopes) that differ from those recognized by T cells from asymptomatic people (i.e., asymptomatic epitopes), it would be logical to exclude such symptomatic epitopes from vaccines on the grounds that they may enhance rather than diminish recurrent disease.

Results from a number of studies indicate that HSV glycoprotein B (gB), one of the major HSV Ags that produce protective immunity in both animal models and humans (7, 10, 12, 14, 19, 45, 61), is recognized by CD4+ T cells from both symptomatic and asymptomatic HSV-seropositive humans (14, 39, 45, 75–77). In the late 1980s and early 1990s, Zarling and coworkers generated gB-specific CD4+ T-cell clones from HSV-2-seropositive severely symptomatic patients who had recurrent HSV-2 genital infections approximately every 8 weeks and from HSV-1-seropositive symptomatic patients who suffered frequent recurrent oral lesions (75–77). gB-specific CD4+ T cells have also been cloned from HSV-seropositive healthy asymptomatic individuals (76, 77). Five of these CD4+ cytotoxic T-lymphocyte (CTL) clones lysed autologous HSV-1- or both HSV-1- and -2-infected lymphoblastoid cell lines (LCLs), and their cytotoxicity was restricted to HLA-DR molecules (73, 76). In another study, HSV-specific CD4+ CTL clones were recovered from recurrent herpes lesions of five patients (33, 38). In the late 1990s and early 2000s, Cunningham and coworkers reported that gB is a major target for IFN-γ-producing CD4+ CTLs recovered from skin lesions (47, 49). Although the above studies suggest that gB is a target of both “symptomatic” and “asymptomatic” CD4+ T cells, no specific “symptomatic” or “asymptomatic” gB epitopes have ever been defined.

In this study, we hypothesize that different sets of HSV-1 gB epitopes might be recognized by CD4+ T cells from symptom-
allele frequency and specificities of the sequence-specific primers used for typing the DRB1, DRB3, DRB4, and DRB5 genes was amplified by PCR and was able to distinguish the polymorphic probes immobilized on paper strips. HLA-DRB1 allele frequency was calculated by the following formula:

\[
\text{frequency} = \frac{N}{R}
\]

where \(N\) is the number of samples positive for the Ag and \(R\) is the number of samples tested.

Peptide binding assays specific for HLA-DR molecules. PBMCs were stimulated with PHA (1 \(\mu\)g/ml) in RPMI 1640 plus 10% fetal calf serum (FCS) for 3 days. Half of the medium was then replaced with fresh medium containing IL-2 (10 \(\mu\)g/ml) and IL-7 (20 \(\mu\)g/ml). On day 6, the PBMCs were infected with VVgB, an empty vaccinia virus control (VVC), or HSV-1, each at an MOI of 10. The next day, infected LCLs were restimulated in a CD107a/b degranulation assay as we recently described (13). In order to demonstrate whether the strong T-cell responses detected against synthetic peptides gB151-175, gB165-180, gB161-175, and gB161-175 can be detected from naturally processed gB, PHA-stimulated blasts were infected with HSV-1, VVgB, and T-cell stimulation was measured by IFN-\(\gamma\) production in peptide-stimulated PBMCs. The serum was isolated and centrifuged for 10 min at 800 \(\times\) g. IL-2 (5 ng/ml; R&D Systems) was added to the culture every other day starting on day 3. On day 7, cells were restimulated with mitomycin C-treated autologous individual LCLs were generated as we recently described (13). Briefly, LCLs were derived by stimulating PBMCs with PHA (1 \(\mu\)g/ml) in RPMI 1640 plus 10% fetal calf serum (FCS) for 3 days. Half of the medium was then replaced with fresh medium containing IL-2 (10 \(\mu\)g/ml) and IL-7 (20 \(\mu\)g/ml). On day 6, the PBMCs were infected overnight with VVgB, an empty vaccinia virus control (VVC), or HSV-1, each at an MOI of 10. The next day, infected PBMCs were washed three times and treated with mitomycin C (50 \(\mu\)g/ml) for 30 min. The PBMCs were then washed three more times before a 4-h incubation with effector CD4+ T cells at the indicated effector/target ratio. Cytotoxic activity was assessed in a CD107a/b degranulation assay as we recently described (13). In order to demonstrate whether the strong T-cell responses detected against synthetic peptides gB151-175, gB165-180, gB161-175, and gB161-175 can be detected from naturally processed gB, PHA-stimulated blasts were infected with HSV-1, VVgB, and T-cell stimulation was measured by IFN-\(\gamma\) production in peptide-stimulated PBMCs. The serum was isolated and centrifuged for 10 min at 800 \(\times\) g. IL-2 (5 ng/ml; R&D Systems) was added to the culture every other day starting on day 3. On day 7, cells were restimulated with mitomycin C-treated autologous individual LCLs were generated as we recently described (13). Briefly, LCLs were derived by stimulating PBMCs with PHA (1 \(\mu\)g/ml) in RPMI 1640 plus 10% fetal calf serum (FCS) for 3 days. Half of the medium was then replaced with fresh medium containing IL-2 (10 \(\mu\)g/ml) and IL-7 (20 \(\mu\)g/ml). On day 6, the PBMCs were infected overnight with VVgB, an empty vaccinia virus control (VVC), or HSV-1, each at an MOI of 10. The next day, infected PBMCs were washed three times and treated with mitomycin C (50 \(\mu\)g/ml) for 30 min. The PBMCs were then washed three more times before a 4-h incubation with effector CD4+ T cells at the indicated effector/target ratio. Cytotoxic activity was assessed in a CD107a/b degranulation assay as we recently described (13). In order to demonstrate whether the strong T-cell responses detected against synthetic peptides gB151-175, gB165-180, gB161-175, and gB161-175 can be detected from naturally processed gB, PHA-stimulated blasts were infected with HSV-1, VVgB,
or VVC and used as target cells. The response to VVC was subtracted from the response to VVgB (not shown).

T-cell cytotoxicity assay. In order to detect cytolytic CD4+ T cells recognizing gB peptides in freshly isolated PBMCs, we used the CD107a/b cytotoxicity assay as we recently described (13). On the day of the assay, nonstimulated or gB peptide-stimulated PBMCs were incubated at 37°C for 5 to 6 h with BD-Golgi Stop, costimulatory Abs anti-CD28 and anti-CD49d (1 μg/ml), and 10 μl of CD107a-fluorescein isothiocyanate (CD107a-FITC) and CD107b-FITC. At the end of the incubation period, the cells were harvested into separate tubes, washed once with fluorescence-activated cell sorting (FACS) buffer, and then stained with Cy-phycoerythrin-conjugated anti-human CD4 for 30 min. The cells were then washed again and analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, CA).

Statistical analysis. Data are expressed as means ± standard errors. Statistical significance was estimated by analysis of variance followed by Dunnett’s test to identify differences between groups. Differences were considered significant when P values were <0.05. All P values were two-tailed unless stated otherwise.

RESULTS

Prevalence of gB epitope-specific IFN-γ-producing CD4+ T cells in healthy HSV-seropositive patients. We first assessed the ability of HSV-specific CD4+ T cells from 38 HSV-1-seropositive, HSV-2-seronegative individuals to respond to a PepScan library of 179 gB peptides divided into 18 groups of 10 peptides each (G1 to G18). In an IFN-γ-producing CD4+ T cell assay, we used the CD107a/b cytotoxicity assay as we recently described (13). On the day of the assay, nonstimulated or gB peptide-stimulated PBMCs were incubated at 37°C for 5 to 6 h with BD-Golgi Stop, costimulatory Abs anti-CD28 and anti-CD49d (1 μg/ml), and 10 μl of CD107a-fluorescein isothiocyanate (CD107a-FITC) and CD107b-FITC. At the end of the incubation period, the cells were harvested into separate tubes, washed once with fluorescence-activated cell sorting (FACS) buffer, and then stained with Cy-phycoerythrin-conjugated anti-human CD4 for 30 min. The cells were then washed again and analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, CA).

The highest frequency of IFN-γ-producing CD4+ T-cell responses was detected against G4 (47.5%), while 42% of individuals responded to G14 and G9. In comparison, only 5 to 38% of individuals showed positive T-cell responses to the remaining 16 groups of peptides (G1, G2, G3, G5, G6, G7, G8, G10, G11, G12, G13, G15, G16, G17, and G18) (5%). The highest magnitude of response, against G4 and G14, was also recorded for 11% of HSV-1-seropositive individuals (Fig. 3). The lowest magnitude of response was detected against G18. All of the donors responded similarly to the PHA and heat-inactivated HSV-1 positive controls. The G6 group of peptides appeared to be recognized by a large percentage of individuals and to stimulate the highest magnitude of T-cell responses. However, G6 induced a significantly smaller proportion of responses in the >100 SFC range than did G4 (5% versus 11%). In addition, a smaller percentage of individuals responded to G6 than to G4 and G14 (38% versus 47.5% and 42%). Besides G6, the G9 group of peptides appeared to be next, after G4 and G14, to be recognized by a large percentage of individuals. However, G9 induced a smaller proportion of responses in the >100 SFC range than G4 and G14 did (0% versus 11%). In addition, although a majority of individuals
responded to G9, their T-cell responses were at a low magnitude (i.e., 37% of individuals responded in the range of 20 to 50% SFCs) compared to the responses to G4 and G14 (31% and 15%). The borderline negative responses obtained with G6 and G9 were confirmed by the inability of individual peptides within G6 and G9 to stimulate significant CD4 T-cell responses, based on both the magnitude and percentage of responders (not shown). Thus, both G6 and G9 do not contain an immunodominant epitope and were therefore excluded from further studies. Altogether, these results indicate that G4 and G14 might contain one or several immunodominant gB epitopes.

\( gB_{161-175}, gB_{166-180}, gB_{661-675}, \) and \( gB_{666-680} \) are immunodominant peptide epitopes. To identify the immunodominant CD4 T-cell epitopes within G4 and G14, we analyzed the ability of the 10 individual peptides forming each group to induce HSV-specific CD4 T-cell responses in four HSV-seropositive individuals with different HLA-DR haplotypes. Both activation (expression of the CD69 early activation marker) and proliferation (CFSE incorporation) were followed by FACS of gated CD4 T cells, as described in Materials and Methods. Among the 10 peptides forming G4, peptides \( gB_{161-175} \) and \( gB_{166-180} \) induced the strongest CD4 T-cell activation (Fig. 4A) and proliferation (Fig. 4B), with \( gB_{166-180} \) being the immunodominant peptide epitope within this group. In G14, peptides \( gB_{661-675} \) and \( gB_{666-680} \) induced the strongest CD4 T-cell activation (Fig. 4C) and proliferation (Fig. 4D). Overall, T-cell responses induced by individual peptides within G4 were higher than those induced by peptides in G14. As a positive control, CD4 T-cell responses to PHA were similar in all tested donors. Altogether, the results show that peptides \( gB_{161-175} \) and \( gB_{166-180} \) among G4 peptides and peptides \( gB_{661-675} \) and \( gB_{666-680} \) among G14 peptides are immunodominant peptide epitopes. In addition, the results indicate that regardless of HLA-DR haplotype, \( gB_{161-175}, gB_{166-180}, gB_{661-675}, \) and \( gB_{666-680} \) induced significant magnitudes of CD4 T-cell activation (expression of the CD69 early activation marker) and proliferation (CFSE incorporation), pointing to the promiscuity of these epitopes.

In order to depict the magnitude and frequency of CD4 T-cell responses induced by each HSV-1 gB peptide, we employed multiple immunological assays. Thus, to rank as an immunodominant peptide epitope, the peptide must induce strong responses (i.e., T-cell activation, proliferation, CD69 upregulation, and IFN-\( \gamma \) production) in at least four different donors. Looking at these immunological criteria, \( gB_{686-700} \) did
induce T-cell stimulation but failed to induce cell division (Fig. 4B). Similarly, although gB 176–190 induced a moderate proliferation of T cells, it also induced one of the lowest and least significant T-cell stimulations (Fig. 4C). Therefore, both gB176–190 and gB686–700 peptides were ranked as negative peptides, and gB166–180 and gB666–680 were ranked as immunodominant peptide epitopes.

In vitro binding to soluble HLA-DR molecules confirms gB161–175, gB166–180, gB661–675, and gB666–680 as strong peptide epitopes. The four immunodominant gB responder peptides (i.e., gB161–175, gB166–180, gB661–675, and gB666–680), as well as four nonresponder control peptides (i.e., gB871–895, gB886–900, and gB890–904), were synthesized and tested in vitro for binding to seven soluble HLA-DR molecules (Table 1). This panel of available HLA-DR molecules consists of the most common HLA class II haplotypes, regardless of ethnicity (78). The relative binding capacity (IC₅₀ [nanomolar]) for each peptide was calculated as the concentration of competitor peptide required to inhibit 50% of the binding of an allele-specific biotinylated peptide (indicator peptide), as described in Materials and Methods. Based on an upper threshold of 250 nM, which characterizes high-affinity peptide binders (11, 78), gB161–175, gB166–180, gB661–675, and gB666–680 bound to three or more different HLA-DR molecules, while the remaining peptide epitopes did not bind to any of the HLA-DR molecules tested. This suggests that the gB161–175, gB166–180, gB661–675, and gB666–680 peptide epitopes contain at least one universal T-cell epitope or several overlapping epitopes presented by multiple HLA-DR molecules.

T-cell responses to peptides gB161–175, gB166–180, and gB661–675 are HLA-DR dependent. In order to ascertain that T-cell responses to the four immunodominant gB peptides are...
HLA-DR dependent, HLA blocking experiments were performed where antigen-presenting cell presentation of individual peptides to CD4^+ T cells was assessed in the presence and absence of MAb specific to the monomorphic region of HLA-DR or HLA-DQ molecules. Figure 5 shows that CD4^+ T-cell proliferation induced by gB161–175, gB166–180, and gB661–675 was significantly (P < 0.005) abrogated in the presence of an anti-HLA-DR blocking MAb (4A) but not in the presence of an anti-HLA-DQ MAb (4B). CD4^+ T-cell proliferation induced by gB666–680 was not affected by either anti-HLA-DR MAb or anti-HLA-DQ MAb. These results were confirmed for HSV-seropositive individuals with different HLA-DRB1 haplotypes. Altogether, these results show that T-cell responses to peptides gB161–175, gB166–180, and gB661–675 are HLA-DR dependent.

gB166–180, gB661–675, and gB666–680-specific CD4^+ T cells display lytic activity. In order to detect whether CD4^+ T cells specific to the gB161–175, gB166–180, gB661–675, and gB666–680 peptides display cytotoxic activity, we used the CD107a/b degranulation assay on freshly isolated T cells. Figure 6 shows that peptides gB166–180, gB661–675, and gB666–680 were able to induce significant degranulation/mobilization of CD107 on the surfaces of specific CD4^+ T cells. gB166–180 induced the highest cytotoxic activity, suggesting that this peptide contains a dominant CTL epitope. gB161–175 was unable to elicit CD4^+ cytotoxic T cells, suggesting a helper function of this epitope.

gB166–180, gB661–675, and gB666–680 peptide epitopes are naturally processed and presented from native gB. Because CD4^+ CTLs were generated by in vitro stimulation with synthetic peptides, it was of interest to ascertain whether they recognized processed HSV gB-derived native epitopes in addition to the artificial synthetic peptide epitopes. Therefore, we analyzed the ability of CD4^+ T-cell lines generated by gB161–175, gB166–180, and gB666–680 peptides to lyse HLA-DR-positive autologous HSV-1- and VVgB-infected LCLs. Figure 7 shows that CD4^+ T-cell lines specific to gB166–180, gB661–675, and gB666–680 peptides undergo significant CD107a/b degranulation when incubated with HLA-DR-positive autologous target cells infected with VVgB or HSV-1 (strain McKrae) (P < 0.005). As a control, no cytolytic activity was detected when gB166–180, gB661–675, and gB666–680-specific CD4^+ T-cell lines were incubated with target cells infected with empty vaccinia virus (VVC) not expressing gB (not shown). gB161–175 was unable to elicit CTL activity even after several rounds of T-cell line expansion. Altogether, these results indicate that gB166–180, gB661–675, and gB666–680 are targets of CD4^+ CTLs that are able to recognize naturally processed gB epitopes.

T cells from asymptomatic and symptomatic patients recognize different sets of gB peptide epitopes. Finally, we assessed whether CD4^+ T cells from symptomatic and asymptomatic patients recognized different sets of gB epitopes. gB166–180 and gB666–680 T-cell epitope peptides were strongly recognized by CD4^+ T cells from 10 of 10 asymptomatic patients but not by CD4^+ T cells from 10 of 10 symptomatic patients (P < 0.0001; analysis of variance posttest) (Fig. 8). Conversely, CD4^+ T cells from symptomatic patients preferentially recognized gB661–675 (P < 0.0001) (Fig. 8). These findings greatly strengthen our hypothesis that T cells from symptomatic and asymptomatic patients recognize different sets of herpesvirus epitopes. Interestingly, the “asymptomatic” gB166–180 and gB666–680 peptide epitopes appeared to be highly conserved within and between HSV-1 and HSV-2 strains (not shown) and showed high binding affinities for a range of HLA-DR haplotypes (Table 1). Altogether, these results suggest that the “asymptomatic” gB166–180 and gB666–680 peptide...
epitopes are strong candidates to be included in the design of future herpes vaccines, while the “symptomatic” gB661–675 peptide epitope should be excluded.

**DISCUSSION**

This study identified four human CD4+ T-cell peptide epitopes from HSV-1 gB. Among these peptide epitopes, gB166–180 and gB666–680 appeared to be most highly recognized by CD4+ T cells from asymptomatic patients and were therefore designated “asymptomatic epitopes,” while gB661–675 appeared to be most highly recognized by CD4+ T cells from symptomatic patients and was therefore designated a “symptomatic epitope”.

CD4+ T-cell influxes appear to correlate with HSV clearance from mucocutaneous lesions (38). The target HSV protein Ags and derived epitopes remain to be identified. A number of studies indicate that gB is a major CD4+ T-cell target Ag in HSV-seropositive humans (14, 39, 45, 46, 75–77). Our studies suggest that gB epitopes are targets of both “symptomatic” and “asymptomatic” CD4+ T cells, thus supporting previous studies by Zarling et al., Koelle et al., and Cunningham et al. (33, 37, 38, 47–49, 73–77) showing that gB is targeted by T cells from both symptomatic and asymptomatic individuals. Recently, it was reported that CD4+ T-cell responses to HSV-2 gG differ in symptomatic and asymptomatic HSV-2-infected individuals (9, 16, 58). Similarly, HSV-1-infected symptomatic but not asymptomatic individuals respond to ICP8 and VP16 (59). Of the three identified CD4+ cytotoxic gB peptide epitopes described in the current report, gB166–180 and gB666–680 were recognized mostly by T cells from asymptomatic patients, while gB661–675 was recognized mostly by T cells from symptomatic patients. These findings strengthen our hypothesis that T cells from symptomatic and asymptomatic patients recognize different sets of herpesvirus peptide epitopes. To our knowledge, this is the first report to show a difference in the sets of epitopes recognized in a specific HSV protein by CD4+ cytotoxic T cells from HSV-1-infected asymptomatic compared to symptomatic individuals. Although the first goal of the present report was to investigate whether different and discrete sets of HSV-1 gB epitopes are recognized by CD4+ T cells from symptomatic versus asymptomatic individuals, an HLA association of CD4+ T-cell responses to symptomatic versus asymptomatic individuals is possible. Indeed, the set of viral epitopes recognized by CD4+ T cells from symptomatic individuals (i.e., “symptomatic epitopes”) might be associated with particular HLA haplotypes, while the different set of viral epitopes recognized by T cells from asymptomatic individuals (i.e., “asymptomatic epitopes”) might be associated with different HLA haplotypes. Investigating a possible association between HLA haplotypes and herpes disease would be useful to address in future studies, using a larger cohort of symptomatic and asymptomatic individuals.

It must also be remembered that humans are not immunologically naive and often have a large number of memory CD4+ T-cell populations that can cross-react with, and may disproportionately contribute to the responses to, other infectious pathogens. These cross-reactive T cells can become activated and modulate the immune response and outcome of subsequent heterologous infections, a phenomenon termed heterologous immunity (30, 69, 70). Therefore, we cannot exclude that some of the “symptomatic” and “asymptomatic” HSV-specific CD4+ T cells identified in this study are cross-reactive with self or other pathogen-derived epitopes. Such scenarios have been reported recently for murine heterologous infection and may be true for humans, as shown for CD4+ T-cell responses to cytomegalovirus and other pathogens (15, 54, 69, 70). Shedding of reactivated HSV is estimated to occur at rates of 3 to 33.5% in adults who harbor latent HSV-1 in their sensory neurons (32, 66–68). However, the vast majority of these individuals do not experience recurrent herpetic disease and are designated “asymptomatic patients” (22, 40, 68). In contrast, in some individuals (symptomatic patients) reactivation of latent virus leads to either an induction of ineffective
or “symptomatic” HSV-specific T-cell immunity or a greater immunopathological response (20, 22, 68). Recurrent disease ranges from rare episodes occurring once every 5 to 10 years to outbreaks occurring monthly or even more frequently among a small proportion of subjects (22). Interestingly, for genital herpes, symptomatic and asymptomatic patients have similar virus shedding rates (68). It is likely to be the same for ocular herpes, since shedding rates in tears of asymptomatic individuals have been reported to be as high as 33.5% (22, 29, 40, 42).

Identification of the T-cell-mediated immune mechanism(s) by which asymptomatic patients control herpes disease and symptomatic patients do not, or at least the viral epitopes involved, is critical for the rational advance of herpes vaccine development. Among the multitude and complex mechanisms that might be in play are the following. (i) There may be differences in precursor frequencies, proliferative capacities, and functional properties of “symptomatic” versus “asymptomatic” epitope-specific T cells. Indeed, the T-cell repertoires of individuals with the same major histocompatibility complex restriction elements can vary significantly because of “heterologous immunity” and “private specificity” (69, 70). (ii) The differential level of infiltration/homing into sites of infection, i.e., corneas, genital areas, and/or sensory ganglia of T cells, specific to “symptomatic” versus “asymptomatic” epitopes may affect viral production and disease (23, 28, 38). (iii) “Asymptomatic epitopes” might trigger proliferation of “protective” T cells within the sites of infection, while “symptomatic” epitopes might trigger “pathogenic” T cells. (iv) The “symptomatic” epitopes may direct T-cell responses away from those that are best suited to clear the viral infection, with minimal pathogenic reaction. (v) An immunopathogenic T-cell response might occur through stimulating low-affinity oligoclonal responses that inhibit broad-based high-affinity T-cell responses to other well-presented epitopes, thus deviating protective responses to damaging responses. (vi) There may be differences in effector T cells lingering after recent shedding and/or disease versus memory T cells maintained in the absence of antigenic exposure. (vii) Finally, T-cell cross-reactivity with epitopes from other viruses, within or outside the herpesvirus family, can also play a role in protective heterologous immunity versus damaging heterologous immunopathology (31, 71). Regardless of the mechanism(s), if symptomatic individuals tend to generate T cells that recognize a discrete set of “symptomatic” epitopes that differs from the set of “asymptomatic” epitopes, it would be logical to exclude such “symptomatic” epitopes from future herpes vaccines on the grounds that they may enhance rather than diminish recurrent herpes diseases.

Rather than using only predictive computational algorithms to map CD4+ epitopes on HSV-1 gB, we instead employed a systematic strategy, including PepScan library scanning of the whole 904-aa gB sequence followed by multiple functional immunological assays, such as in vitro binding of gB peptides to a panel of HLA-DR molecules, ex vivo IFN-γ ELISPOT assay, CD107 expression, and CD4+ T-cell activation and proliferation analysis. Using these multiple screens, we identified four HSV-1 gB peptides, gB161–175, gB166–180, gB661–675, and gB666–680, that induced IFN-γ-producing CD4+ T cells. Among these peptides, gB166–180, gB661–675, and gB666–680 recalled CD4+ T cells that displayed cytotoxic activity. However, the results reported here do not imply that these are the only human CD4+ T-cell epitopes present on gB. Indeed, the 10-aa regions where these 15-mer peptides overlap might by themselves contain “junctional epitopes.” Shortening or lengthening a peptide by one or a few amino acids might sometimes result in missing junctional epitopes that might be present in the overlapping regions. Thus, to look for these “junctional epitopes,” we expect to synthesize and test a large number of peptide sequences, and the results from these experiments will be the subject of a future report.

A majority of the world’s human population is infected with HSV-1 and/or HSV-2 and would most likely benefit from therapeutic vaccination designed to boost HSV-specific cellular immunity (36). Although a primary goal of this study was to identify human “asymptomatic” CD4+ CTL peptide epitopes on HSV-1 gB, the ultimate goal is to build the knowledge of HSV T-cell epitopes required for the development of a totally synthetic, self-adjuvancing lipopeptide human vaccine. We now plan to construct lipopeptide candidate vaccines incorporating the CD4+ T-cell epitopes (but excluding the “symptomatic” epitopes) identified in this study together with human CD8+ T-cell epitopes recently described for gD (13) and other structural and regulatory herpesvirus proteins (unpublished data). Such a combination would produce CD4+ CD8+ chimeric lipopeptide candidate vaccines similar to those we recently described for mice against ocular and genital herpesvirus infection (79; X. Zhang, A. A. Chentoufi, M. Wu, Z. Zhu, D. Carpenter, A. B. Nesburn, S. L. Wechsler, and L. BenMohamed, submitted for publication). More importantly, each lipopeptide vaccine construct will contain promiscuous CD4+ epitopes identified in this study, thus covering a majority of the population rather than just those having specific HLA-DR alleles.

Although the high degree of HLA polymorphism is often pointed out as a major hindrance to the use of high-affinity epitope-based vaccines, this constraint can be dealt with through the inclusion of multiple supertype-restricted epitopes, recognized in the context of diverse related HLA alleles, and by designing vaccines with higher epitope densities (5, 8, 56, 80). Thus, an effective vaccine could include multiple “asymptomatic” T-cell epitopes present in diverse herpesvirus protein Ags, chosen to represent at least the HLA-DR supertypes, known to provide recognition in up to 95% of the global population, regardless of race and ethnicity (2, 4, 6, 56). Hence, bearing in mind the particular properties that would be required in a possible human vaccine, we have conceived studies to identify HLA class II “promiscuous” T-cell epitopes in HSV protein Ags targeted by CD4+ T cells from “asymptomatic” individuals. These epitopes, along with similarly identified HLA class I supertype-restricted “asymptomatic” CD8+ CTL epitopes, would provide the rationale needed to develop multiepitope CD4+CD8+ vaccines that are broadly recognized in the majority of outbred racial and ethnic populations.

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