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Definition of a Minimal Optimal Cytotoxic T-Cell Epitope within the Hepatitis B Virus Nucleocapsid Protein†

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Residues 11 to 27 of the hepatitis B virus nucleocapsid antigen contain a cytotoxic T-cell epitope that is recognized by cytotoxic T cells from virtually all HLA-A2-positive patients with acute hepatitis B virus infection. Using panels of truncated and overlapping peptides, we now show that the optimal amino acid sequence recognized by cytotoxic T cells is a 10-mer (residues 18 to 27) containing the predicted peptide-binding motif for HLA-A2 and that this peptide can stimulate cytotoxic T cells able to recognize endogenously synthesized hepatitis B core antigen. Since patients with chronic hepatitis B virus infection fail to mount an efficient cytotoxic T-cell response to it, this epitope might serve as the starting point for the design of synthetic peptide-based immunotherapeutic strategies to terminate persistent viral infection.

Cytotoxic T lymphocytes (CTLs) normally recognize endogenously synthesized viral proteins in the form of short antigenic peptides bound by human histocompatibility leukocyte antigen (HLA) class I molecules that transport the processed viral peptides to the surface of the infected cells (16, 36).

During acute hepatitis B virus (HBV) infection, we have detected an HLA class I-restricted CTL response to HBV core antigen residues 11 to 27 ($HBcAg_{11-27}$) from virtually all HLA-A2-positive patients who successfully cleared the virus (2, 28). In contrast, patients with chronic HBV infection do not mount efficient CTL responses to this epitope, suggesting that $HBcAg_{11-27}$-specific CTLs play a critical role in terminating HBV infection and that defective clearance of HBcAg-expressing hepatocytes may be critical for virus persistence within the infected host.

The aim of the present study was to identify the minimal optimal sequence recognized by $HBcAg_{11-27}$-specific CTLs in order to more thoroughly characterize the CTL response to this epitope, with the hope that it might contribute to the

TABLE 1. CTL recognition of truncated HBcAg synthetic peptides

Peptide	Amino acid at position:																		CTL response (% ^{51}Cr release)[a]
	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
11–27	A	T	V	E	L	L	S	F	L	P	S	D	F	F	P	S	V			87
12–27		T	V	E	L	L	S	F	L	P	S	D	F	F	P	S	V			86
13–27			V	E	L	L	S	F	L	P	S	D	F	F	P	S	V			98
14–27				E	L	L	S	F	L	P	S	D	F	F	P	S	V			70
15–27					L	L	S	F	L	P	S	D	F	F	P	S	V			87
16–27						L	S	F	L	P	S	D	F	F	P	S	V			84
18–27								F	L	P	S	D	F	F	P	S	V			85
20–29										P	S	D	F	F	P	S	V	R	D	0
19–27									L	P	S	D	F	F	P	S	V			45
18–26								F	L	P	S	D	F	F	P	S				0
17–25							S	F	L	P	S	D	F	F	P					0
16–24						L	S	F	L	P	S	D	F	F						0
15–23					L	L	S	F	L	P	S	D	F							0
14–22				E	L	L	S	F	L	P	S	D								0
13–21			V	E	L	L	S	F	L	P	S									0

[a] The amount of ^{51}Cr release in the culture supernatants was quantitated after 4 h of incubation, and the level of lysis was determined by using the following formula: $[(E - M)/(D - M)] \times 100$, where E is the amount of experimental ^{51}Cr release, M is the amount of ^{51}Cr released in the presence of culture medium (which ranged between 15 and 25% of the releasable counts), and D is the total amount of ^{51}Cr released in the presence of 10% Triton X-100. Results similar to those illustrated here (patient 1) were also obtained with PBLs from patients 2 and 3. Identical results were obtained when peptides were incubated with target cells in the absence of serum (not shown).

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design of immunogenic strategies to induce a CTL response that results in clearance of virus-infected cells in HLA-A2-positive patients with chronic HBV infection.

Three HLA-A2-positive patients (patients 1, 2, and 3) with acute HBV infection were studied. Diagnosis of acute hepatitis was based on the finding of elevated values of serum glutamic pyruvic transaminase activity (at least 10 times the upper limit of normal), associated with the detection of hepatitis B surface antigen and immunoglobulin M anti-HBc antibodies in the serum and the recent onset of jaundice and other typical symptoms of acute hepatitis. All patients recovered completely from the illness, with normalization of serum transaminase and clearance of hepatitis B surface antigen from the serum. All patients were antibody negative to delta Ag and to hepatitis C virus. For this study, several HLA-A2-restricted polyclonal CTL lines specific for $HBcAg_{11-27}$ were generated from all three patients by peripheral blood lymphocyte (PBL) stimulation with peptide 11–27 and HBcAg, as previously described (2, 28).

Briefly, PBLs were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and cultured at 4×10^6 cells per ml in RPMI 1640 containing 10% AB serum plus 5 μg of the $HBcAg_{11-27}$ peptide per ml and 1 μg of recombinant HBcAg (Biogen, Geneva, Switzerland) (27) per ml in a 24-well plate (Corning). Recombinant interleukin 2 (20 U/ml) (Hoffmann-La Roche, Basel, Switzerland) was added after 4 days, and the CTL activity was tested after 7 days of culture against HLA-A2-positive Epstein-Barr virus (EBV)-transformed human B-cell lines preincubated with peptides. The truncated peptides encompassing the entire sequence of the HBV nucleocapsid region between positions 11 and 29 (ayw subtype) were synthesized on a peptide synthesizer (430A; Applied Biosystems, Foster City, Calif.) as previously described (35). The peptides were then purified by reverse-phase high-performance liquid chromatography (HPLC). The purities of the peptides were substantiated by amino acid sequence and/or component analysis. They were routinely >95% pure after HPLC. EBV B cells were labeled with 100 μCi of sodium [^{51}Cr]chromate for 1 h at 37°C, washed three times, diluted in RPMI containing 10% fetal calf serum, and incubated for 1 h with the appropriate peptide. Target cells were then cocultured with CTL lines at an effector/target ratio of 20:1 in 96-well round-bottom plates.

On the basis of the recent finding that naturally processed peptides isolated from major histocompatibility complex class I molecules are usually 8 or 9 amino acids long (22, 24, 29, 37) and previous observations defining 9-residue peptides as the minimal length recognized by CTLs (26), the fine specificity of the $HBcAg_{11-27}$ response was assessed with a panel of overlapping 9-mers spanning core residues 11 to 27. Cytotoxic activity was detected exclusively against $HBcAg_{19-27}$, LPSDFFPSV, but not against the other 9-mers (Table 1). Since the long 11–27 peptide was recognized approximately 100-fold better than the 9-mer 19–27 and since short natural peptides, because of their higher binding affinities to HLA class I molecules (9, 10), are usually more efficiently recognized by CTLs than longer synthetic analogs (12, 29, 34), the data suggested that residues 19 to 27 represented part but not all of the minimal optimal epitope present within $HBcAg_{11-27}$.

Sequence analysis of in vivo processed peptides eluted from purified HLA-A2 molecules and comparison with the known HLA-A2-restricted T-cell epitopes have recently led to the definition of an HLA-A2 peptide-binding motif with leucine or isoleucine at position 2 and valine or a residue

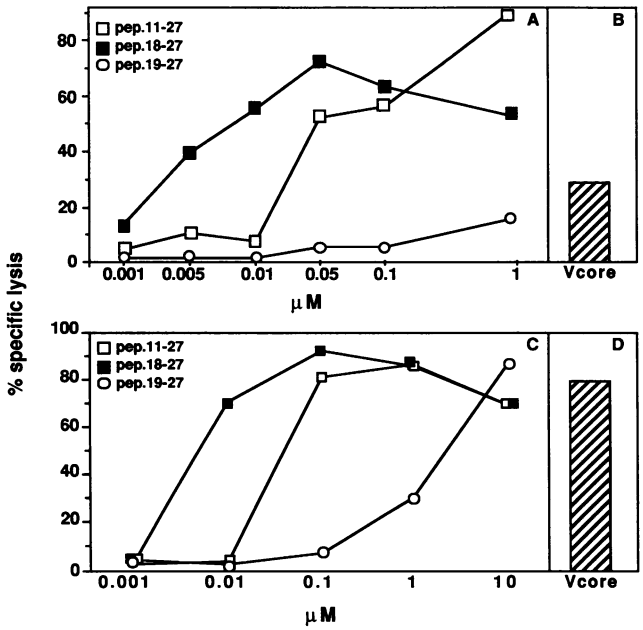

FIG. 1. CTL recognition of HBcAg peptides 18–27, 19–27, and 11–27 and endogenously synthesized core antigen. Two different eukaryotic expression systems were employed to study CTL recognition of endogenously synthesized core antigen. The HBV envelope and core open reading frames were inserted into the EBV-based plasmid vector EBO-pLPP (6) that is maintained as an episome when transfected into EBV-transformed human B-cell lines. EBO-core and EBO-env code for the HBV core and envelope polypeptides (subtype ayw), respectively. The construction and characterization of these vectors have been reported in detail elsewhere (17). Additionally, recombinant vaccinia viruses that express the HBV core (V_{core}) polypeptide (subtype ayw) were constructed and used as described in reference 32. CTL lines produced by stimulating PBLs from patient 1 for 7 days with peptide $HBcAg_{11-27}$ as described in the text (A and B) or by stimulation with peptide $HBcAg_{11-27}$ for 1 week followed by restimulation with irradiated (7,000 rads) EBO-core-transfected EBV B cells (plus autologous irradiated [3,000 rads] PBLs [5×10^5/ml] and recombinant HBcAg) for another week (C and D) were tested against allogeneic HLA-A2-positive target cells (effector/target ratio, 10:1) that were either preincubated with the indicated peptides or infected with recombinant vaccinia virus expressing the HBV core gene (V_{core}) or with wild-type vaccinia virus (Vwt) as a negative control. For target cell infection, vaccinia viruses were used at a multiplicity of infection of 10 for 1 h, with 5 h of expression. In panels B and D, core-specific lysis was calculated by subtracting the specific lysis of targets infected with control V_{wt} from the specific lysis of V_{core}-infected targets. Lysis of target cells in the absence of peptides was 0% (A and C); lysis of target cells infected with V_{wt} was 24% (B) and 8% (D). Identical results were obtained for all three patients studied when peptide $HBcAg_{18-27}$ instead of peptide $HBcAg_{11-27}$ was used for PBL stimulation.

with aliphatic hydrocarbon side chains at the C terminus (13, 20). This motif matches very well the three-dimensional structure of the HLA-A2 cleft that shows the presence of binding pockets which can accommodate complementary amino acid side chains such as those provided by leucine and valine at positions 2 and 9, respectively, of the A2 motif (4, 5, 15, 30) and which are therefore believed to act as anchor residues.

Since leucine is in position 1 instead of 2 (as it is in the predicted A2 motif) (Table 1) in $HBcAg_{19-27}$, we asked

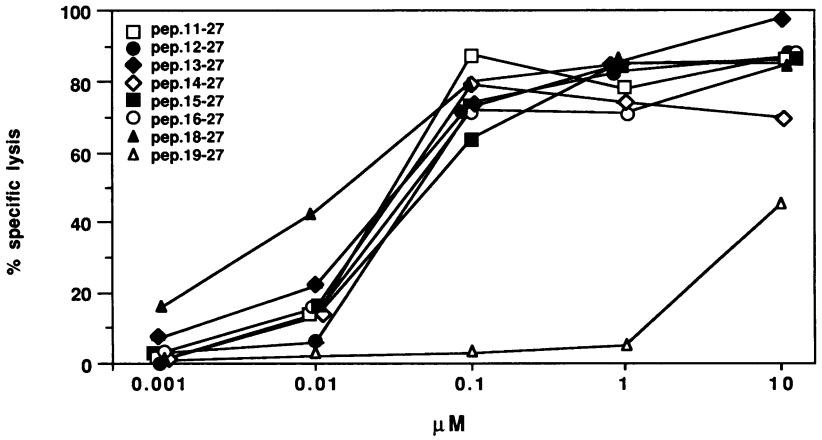

FIG. 2. Recognition of peptide $HBcAg_{11-27}$ and truncated peptides of different lengths by HBV nucleocapsid-specific CTLs. Target cells were incubated with the indicated concentrations of peptides and cocultured with effector CTLs from patient 3 (produced by PBL stimulation with peptide $HBcAg_{11-27}$ for 7 days as described in the text followed by restimulation with phytohemagglutinin [1 μg/ml] in the presence of allogeneic irradiated [5,000 rads] PBLs [5×10^5/ml] at an effector/target ratio of 5:1). Lysis in the absence of peptides was 2%.

whether the addition of a flanking residue at the N-terminal end of the 9-mer could improve peptide immunogenicity. In the resulting 10-mer, leucine in position 2 should fit into the deep pocket in the middle side of the groove (pocket B), whereas the new N-terminal residue would be available for interaction with the first pocket located at one end of the groove (pocket A), thereby providing more binding energy (23, 25). The 10-mer $HBcAg_{18-27}$ was thus synthesized, and HLA-A2-positive target cells were incubated with different concentrations of either the 9-mer $HBcAg_{19-27}$, the 10-mer $HBcAg_{18-27}$, or the original peptide $HBcAg_{11-27}$ and then tested for recognition by the CTL lines established by stimulation with peptide $HBcAg_{11-27}$. Figure 1 shows that the 10-mer $HBcAg_{18-27}$ is 100- to 1,000-fold better than the 9-mer $HBcAg_{19-27}$ in target cell sensitization and approximately 10-fold better than the original $HBcAg_{11-27}$ peptide.

In additional experiments, target cell sensitization with different concentrations of the 10-mer $HBcAg_{19-28}$ resulted in levels of lysis identical to those observed with $HBcAg_{19-27}$ (data not shown). Furthermore, deletion of the predicted carboxyl- or amino-terminal anchor residue (leucine 19 or valine 27 in peptide 20–29 or 18–26, respectively) completely abrogated the recognition of the HBV core epitope by the CTL lines (Table 1), confirming their critical importance for peptide binding to the HLA-A2 molecules.

Further analysis with a panel of peptides representing progressive N-terminal extensions of the $HBcAg_{18-27}$ peptide indicates that the CTL response is decreased when the peptides are elongated at the N terminus and confirms that peptide $HBcAg_{18-27}$ is the minimal sequence optimally recognized by HLA-A2-restricted HBcAg-specific CTLs (Fig. 2). Similar results have been obtained with attempts at defining the specificity of HLA-A2.1-restricted CTL clones derived from HLA-A2.1 transgenic mice (37a, 38).

These results suggest that the two predicted anchor residues (leucine and valine) must be correctly positioned within the peptide for effective binding to HLA-A2 and that the length restriction of 8 or 9 amino acids is not an absolute constraint for optimal HLA binding and subsequent CTL recognition. As suggested by the analysis of the interaction of viral peptides with the murine major histocompatibility complex class I *H-2K*^{b} and the human HLA-Aw68 (14, 18,

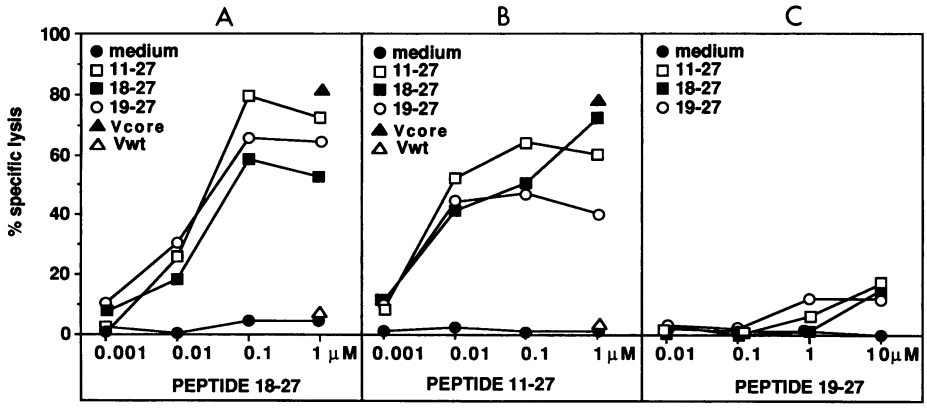

FIG. 3. Relative efficiencies of HBcAg peptides 18–27 (A) 11–27 (B), and 19–27 (C) in CTL induction. PBLs from patient 2 were stimulated with the indicated concentrations of HBcAg peptides 18–27, 11–27, and 19–27. After 7 days of culture, the different lines were tested against target cells pulsed with 10 μM different peptides (10 μM represents the minimal concentration required by peptide $HBcAg_{19-27}$ to sensitize target cells) or infected with wild-type vaccinia (V_{wt}) or V_{core} virus.

25), accommodation of a 10-mer within the HLA groove, which appears to be closed at both ends (3, 14, 25), may be achieved by protrusion of the middle portion of the peptide out of the cleft.

It has been demonstrated that stimulation with peptides may yield CTLs that recognize the peptide but not the native antigen. This has been attributed to a specific induction of CTLs with low affinity for the corresponding endogenous antigen (8) or to selection of CTLs recognizing amino acid sequences distinct from that of the natural peptide fragment generated by intracellular antigen processing (31). Indeed, stimulation of PBLs from patients with acute hepatitis B (primed in vivo by the natural HBV infection) with either the longer peptide, $HBcAg_{11-27}$, or the 10-mer $HBcAg_{18-27}$ sometimes expands a CTL population able to recognize synthetic peptides more efficiently than endogenous antigen (Fig. 1A and B). To prevent this problem, CTLs initially stimulated with a peptide were then restimulated with HLA-A2-positive EBV B-cell transfectants that stably express the HBV core protein (17) in order to selectively expand CTL lines able to recognize endogenously synthesized nucleocapsid antigen with high efficiency. CTL lines generated by this approach were tested for their abilities to lyse target cells infected either with a recombinant vaccinia virus (32) that expresses the HBV core protein (V_{core}) or with a wild-type control vaccinia virus or target cells incubated with different concentrations of the 9-mer, 10-mer, or the original peptide, $HBcAg_{11-27}$ (Fig. 1C and D). As previously reported (2, 17), highly efficient recognition of endogenous core antigen was observed upon restimulation with EBV B-cell transfectants (Fig. 1D). The observation that the 10-mer $HBcAg_{18-27}$ is the minimal optimal antigenic peptide even for cytotoxic T cells selected by restimulation with endogenously synthesized HBcAg (Fig. 1C) suggests that it could represent the naturally processed peptide. Elution experiments with HLA-A2-positive HBV-infected hepatocytes designed to address directly this issue are in progress.

Having demonstrated that $HBcAg_{18-27}$ is more efficiently recognized as a target antigen than $HBcAg_{11-27}$ and $HBcAg_{19-27}$, we examined the relative potencies of the three peptides with respect to induction of the CTL response. In this study, PBLs from two HLA-A2-positive acute hepatitis B patients were stimulated with increasing concentrations of the different peptides. After 7 days of stimulation, the cytolytic activity was tested against target cells incubated with an optimal concentration (10 μM) of each of the three peptides or infected with recombinant vaccinia virus.

PBLs stimulated with concentrations as low as 10 nM of the 10-mer $HBcAg_{18-27}$ or the original $HBcAg_{11-27}$ peptide expressed significant levels of cytolytic activity (Fig. 3). In contrast, 1,000-fold higher concentrations (10 μM) of the 9-mer $HBcAg_{19-27}$ elicited very weak levels of cytolytic activity, reminiscent of the inefficiency of this peptide as a target antigen as well (Fig. 1 and 2). Endogenously synthesized HBcAg was recognized with identical efficiencies by CTLs induced with $HBcAg_{18-27}$ and those induced with $HBcAg_{11-27}$ (Fig. 3).

Finally, deletion of valine at the C terminus of $HBcAg_{18-27}$ completely abrogated the ability of the peptide to induce cytolytic activity (not shown) as well as its capacity to be recognized as a target, as mentioned above.

In conclusion, these results demonstrate that some synthetic peptides containing an HLA-A2 allele-specific binding motif derived from antigenic proteins of human pathogens can be used to identify immunogenic epitopes recognized by HLA class I-restricted CTLs. Since CTLs specific for the minimal sequence $HBcAg_{18-27}$ recognize endogenously synthesized antigen, the data suggest that peptide 18–27 can stimulate memory T cells which are likely relevant to the immunopathogenesis of HBV infection. In view of the selective association of this nucleocapsid-specific CTL response with the acute stage of self-limited HBV infection and its wide recognition in the context of the HLA-A2 haplotype (28), $HBcAg_{18-27}$ might represent the starting point for the design of a vaccine capable of inducing in vivo an efficient anti-HBV-specific CTL response in HLA-A2-positive individuals, including chronically infected patients who have failed to mount an efficient CTL response during natural infection. The evidence that CTLs induced in vivo by immunization with peptides (either as such [1, 7, 21] or in the form of lipopeptides [11]) can actually protect against viral (33) and bacterial (19) infections further supports the potential feasibility of this application. In pursuit of this objective, we have recently shown that $HBcAg_{18-27}$ can prime in vivo an HLA class I-restricted CTL response in HLA-A2.1 transgenic mice (37a).

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