Development of a Lipopeptide-based Therapeutic Vaccine to Treat Chronic HBV Infection

I. Induction of a Primary Cytotoxic T Lymphocyte Response in Humans

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Abstract

Our goal is to use peptide epitopes that are recognized by cytotoxic T lymphocytes (CTL) as immunogens for the development of prophylactic and therapeutic vaccines with chronic hepatitis B virus (HBV) infection being our first therapeutic target. Because most CTL peptide epitopes are poor immunogens, we specifically modified them by covalently attaching two additional components: a T helper peptide epitope and two lipid molecules. Using the murine influenza virus CTL epitope NP 147–155 as a model system, we found this construct to be highly immunogenic, and a single injection resulted in memory CTL induction that persisted for > 1 yr. Based on the animal studies, a vaccine was designed and tested for both safety and its ability to induce a primary CTL response in normal subjects. The three vaccine components included HBV core antigen peptide 18–27 as the CTL epitope, tetanus toxoid peptide 830–843 as the T helper peptide, and two palmitic acid molecules as the lipids. A dose escalation trial (5, 50, and 500 μg) carried out in 26 normal subjects showed that the vaccine was safe and able to induce a primary HBV-specific CTL response. A dose–response curve was observed and five out of five subjects responded to the 500-μg dose. (*J. Clin. Invest.* 1995. 95:341–349.) Key words: peptide • immunological memory • murine cytotoxic T lymphocytes • T cell help

Introduction

Evidence has shown that major histocompatibility complex (MHC) class I–restricted cytotoxic T lymphocytes (CTL)[1] can play a central role in the prevention, control, and cure of infectious diseases as well as cancer (1–7). Most of the direct evidence that has demonstrated the capacity of CTL to act prophylactically and therapeutically has come from animal studies. For example, in the murine lymphocytic choriomeningitis virus model, virus-specific CTL have been shown to be capable of both preventing the establishment of a chronic viral infection and eliminating the infection once it has become established (6, 7). Although this form of immunity can be stimulated as a result of natural infection or through the use of live vectors, subunit approaches using inactivated viruses, recombinant proteins, and antigenic peptides, with few exceptions, elicit a poor class I–restricted CTL response (8–13).

Recent advances have greatly facilitated the potential for using peptides as CTL immunogens. These include the demonstration that CTL recognize a small antigenic peptide, typically 8–10 amino acids in length, bound to MHC class I molecules on the surface of infected cells or cancer cells (14, 15) and the development of rapid methods for the identification of these "optimal" antigenic peptides (16). Nevertheless, a major obstacle in the development of the peptide-based vaccines has been the finding that when the optimal peptides are injected alone they are extremely inefficient at inducing a CTL response (10–13).

Animal studies have shown that antigenic peptides can be used to induce a CTL response when administered with certain adjuvants (17), and in liposomes (18), or by direct attachment of lipids (10–12). However, we are not aware of studies that have been carried out in humans using antigenic peptides to specifically elicit a primary CTL response. Our goal has been to design a safe and effective peptide-based immunostimulant that is capable of inducing disease-specific CTL that can both prevent and treat infectious diseases and cancer. As a first target, we have chosen to develop a therapeutic vaccine, called Theradigm™-HBV (Cytel, San Diego, CA), to treat chronic hepatitis B virus (HBV) infection, a disease that afflicts ~ 300 million people worldwide (19). This strategy is based on the previous demonstration that patients who successfully cleared HBV developed a strong HLA class I–restricted response and that the response is weak or undetectable in patients with chronic hepatitis (20–22). Moreover, withdrawal of corticosteroids from patients with chronic HBV infection frequently results in an immunological rebound associated with viral DNA reduction (23), and there is a strong association between the effectiveness of interferon α in clearing chronic HBV infection and the induction of an inflammatory response in the liver (24). We therefore reasoned that the CTL response plays a key role in HBV clearance and that a vaccine capable of inducing a CTL response to HBV should be capable of eradicating chronic infection. Presented here are preclinical results that support the basic design of the Theradigm molecular structure as well as clinical results that demonstrate the molecule's safety and its ability to induce HBV-specific CTL.

Methods

Peptide and lipopeptides. Peptides were synthesized according to standard t-BOC or f-MOC solid-phase synthesis methods (16). Lipopeptides

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Received for publication 10 June 1994 and in revised form 26 August 1994.

1. *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus; HTL, helper T lymphocyte; LU, lytic unit; NK, natural killer; TT, tetanus toxoid.

J. Clin. Invest.

0021-9738/95/01/0341/09 $2.00
Volume 95, January 1995, 341–349

were prepared by coupling the preformed symmetrical anhydride of palmitic acid to the amino terminus of the resin-bound KSS-elongated peptide. Purity of peptides was > 95% and of lipopeptides was > 85% as determined by HPLC. Peptides and lipopeptides were dissolved in DMSO at the concentration of 10–20 mg/ml (stock solutions) and diluted in PBS or medium before use. Peptides and lipopeptides used for the preclinical studies (see Table I) were CTL peptide, the H-2 K^d-restricted CTL epitope derived from the influenza virus A/PR/8/34 nucleoprotein (FLU NP 147–155, TYQRTRALV); helper T lymphocyte (HTL) peptide, the IA^d-restricted HTL epitope derived from ovalbumin (OVA 323–336, ISQAVHAAHAEINE); HTL–CTL peptide, obtained by linking the HTL epitope to the amino terminus of the CTL epitope via a three alanine spacer; $(PAM)_2$–CTL peptide, obtained by linking two palmitic acid molecules to the NH_2 terminus of the KSS-elongated CTL peptide; and $(PAM)_2$–HTL–CTL peptide, obtained by linking two palmitic acid molecules to the NH_2 terminus of the KSS-elongated HTL–CTL peptide. Peptides and lipopeptides used in the clinical studies were CTL peptide, the HLA-A2.1–restricted CTL epitope derived from the HBV core antigen (HBcAg 18–27, FLPSDFFPSV); HTL peptide, the DR promiscuous HTL epitope derived from tetanus toxoid (TT 830-843, QYIKANSKFIGITE); and $(PAM)_2$–HTL–CTL peptide (see Fig. 1).

Cell lines. B10.D2-derived SV 40 transformed fibroblasts; K562, a human erythroleukemia natural killer (NK)-sensitive cell line; 3A4-721.221-A2.1 [.221(A2.1)], an EBV transformed cell line that had been mutagenized and selected to be class I negative and transfected with the HLA-A2.1 gene (25); and .221(A2.1)-core, an HBV-core EBO-transfectant (26) of the .221(A2.1) cell line were used in various aspects of the study. Cell lines were grown in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) containing 10% FCS, 4 mM L-glutamine, 50 μg/ml gentamicin (all from Irvine Scientific, Santa Ana, CA) and 5×10^{-5} M 2-mercaptoethanol (RPMI 10% FCS). Transfected cell lines were grown in selection media, that is 221(A2.1) was grown in the presence of 200 μg/ml of G418 sulfate (Life Technologies Laboratories, Grand Island, NY) and .221(A2.1)-core in the presence of 200 μg/ml of G418 and 200 U/ml of hygromycin sulfate (Sigma Chemical Co., St. Louis, MO).

Immunization of mice and evaluation of murine CTL activity. 8–12-wk-old female BALB/cJ mice (Jackson Laboratory, Bar Harbor, ME) were immunized subcutaneously in the base of the tail with peptides or lipopeptides in 0.1 ml PBS or with 300 HAU of PR8 influenza virus in the form of allantoic fluid.

2–3 wk later, 3×10^7 splenocytes from individual mice were stimulated with 40 ng/ml FLU NP 147–155 peptide in upright 25-cm^2 flasks. Each flask contained 10 ml RPMI–10% FCS. After 6 d, splenocytes from each flask were collected and assayed for cytolytic activity using a ^{51}Cr-release assay.

Theradigm-HBV clinical trial and evaluation of human CTL activity. Healthy HLA-A2.1–positive male volunteers aged 18–45 y who were seronegative for markers of HBV infection were divided into three dosage groups. Each group consisted of six subjects who received Theradigm-HBV and three subjects who received the formulation buffer (i.e., PBS, 10% DMSO, 0.01% trifluoroacetic acid). Subjects were assigned treatment in a randomized fashion that was blinded to the subjects, investigator, and sponsor. Groups were immunized sequentially at escalating doses of 5, 50, and 500 μg/subject. Theradigm-HBV or placebo was administered by injecting 0.5 ml s.c. into the upper forearm. Subjects receiving Theradigm-HBV were boosted with an identical dose to that used for priming 28–35 d after the initial immunization. The booster injection was given in the same anatomical location as the primary inoculation. 60–100 ml of blood was collected from each volunteer 7 d before vaccination, immediately before receiving the day zero primary immunization, and 7, 14, and 28 d after primary and booster immunizations. PBMC isolated from preimmunization samples were used as antigen presenting cells for restimulating the CTL culture. Before blood collection, each subject was clinically monitored for adverse reactions to immunization. PBMC from each blood sample were isolated by Ficoll gradient separation and stored in liquid nitrogen for later use.

To determine the CTL responses in immunized subjects, $3–4 \times 10^6$ PBMC collected at various pre- and postvaccination timepoints were stimulated in vitro with 10 μg/ml HBcAg 18–27 peptide (27). PBMC were cultured in 24-well plates in 1 ml RPMI medium supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μg/ml gentamicin, and 10% pooled human AB serum (RPMI–10% HS medium). Three and 6 d later, wells were fed with 1 ml medium containing rIL-2 (10 U/ml; Sandoz Pharma AB, Basel, Switzerland). After 7 d of culture, the cells were restimulated with peptide. To restimulate T cells, autologous PBMC (3,500 rad irradiated, $3–4 \times 10^6$ cells/well) were allowed to adhere for 2 h at 37°C in RPMI–2% HS medium containing 10 μg/ml HBcAg 18–27 peptide and 3 μg/ml human β2-microglobulin (Scripps Laboratory, La Jolla, CA). After removing nonadherent PBMC by gentle washing, $1–2 \times 10^6$ T cells were added to the peptide-pulsed adherent PBMC in 1 ml RPMI–10% HS medium containing 0.5 μg/ml peptide. 2 d later, cells received 1 ml rIL-2–containing medium (10 U/ml). Cells were fed or split into fresh wells on a 2- or 3-d cycle with rIL-2 medium. 7 d after restimulation, T cells were assayed for cytolytic activity.

^{51}Cr release assay. To assay murine or human T cells for cytolytic activity, T cells were plated at varying concentrations in 96-well U-bottomed plates. Assay medium consisted of RPMI–10% FCS. Target cells ($3–5 \times 10^6$ cells) were routinely labeled with 300 μCi ^{51}Cr sodium chromate (NEN Research Products, Boston, MA) for 60 min at 37°C and $5–10 \times 10^3$ target cells were added to each well. B10.D2 SV 40–transformed fibroblasts were used as targets to assess murine CTL activity. Soluble FLU NP 147–155 peptide (2 μg/ml) was added to target cells. In some experiments, B10.D2 were infected with A/PR/8/34 influenza virus as previously described (28).

To measure CTL activity in human cultures, cells were tested against .221(A2.1) cells (4×10^6/ml) that had been cultured overnight in RPMI–10% FCS medium with or without 10 μg/ml HBcAg 18–27 peptide and 3 μg/ml human β2-microglobulin and then washed and labeled with ^{51}Cr. To decrease the antigen nonspecific cytolytic activity of NK cells during the ^{51}Cr release assay, microtiter wells received the NK-sensitive tumor cell line K562 added in 20-fold excess of labeled targets. To examine CTL recognition of endogenously synthesized antigen, the .221(A2.1) HBV core cell line was used as a target cell. After incubation for 6 h at 37°C, 100 μl of supernatant was removed from each well and quantitated for ^{51}Cr. The percent specific lysis was determined by the following formula: % lysis = 100 × ([experimental release − spontaneous release] ÷ [maximum release − spontaneous release]). Spontaneous release for all target cells used never exceeded 15% of the maximum release. To more readily compare responses, the data are expressed as lytic units/10^6 effector cells (i.e., murine splenocytes or human PBL) with 1 lytic unit (LU) being defined as the number of effector cells required to induce 30% lysis of 5×10^3 B10.D2 or 1×10^4 .221(A2.1) ^{51}Cr-labeled target cells during a 6-h assay. Specific CTL activity is obtained by subtracting the lytic unit obtained in the absence of antigen from the lytic unit obtained in the presence of antigen.

T cell proliferation assay. PBMC (1.5×10^5/well) were stimulated in 96-well flat-bottomed plates with or without 10 μg/ml TT 830–843 peptide in RPMI–10% HS medium. Routinely, cells were plated in replicate sets of four to six wells per condition. After 7 d, 135 μl of medium was removed from each well and replaced with fresh medium containing rIL-2 (20 U/ml final). 48 h later, 1 μCi methyl[^{3}H]-thymidine (ICN Radiochemicals, Irvine, CA) was added to each well. Cells were harvested on glass fiber mats the next day and quantitated for ^{3}H incorporation.

CD4/CD8 phenotyping. To determine the cell surface phenotype of CTL, $\sim 5 \times 10^5$ cells were treated with 100 μl of a pool of monoclonal antibodies containing phycoerythrin-conjugated anti-CD4 and FITC-conjugated anti-CD8 (Becton Dickinson Immunocytometry Systems, San Jose, CA; cat. 347327 and 347313, respectively, 1:20 final dilution). As control, another aliquot of cells was treated in a similar manner with irrelevant IgGγ1 antibody conjugated with FITC or phycoerythrin (Becton Dickinson 349041 and 49043, respectively). Cells were incubated for 60 min on ice and then washed three times with cold PBS containing 0.5% BSA and 0.02% sodium azide. Cells were then analyzed

on a FACScan® flow cytometer (Becton Dickinson) to determine the percentage of CD4- and CD8-bearing cells.

Statistical methods. To determine the relationship between dose of Theradigm-HBV injected and CTL response induced, a linear-regression model was fit between the $\log_{10}$ of the dose injected and the $\log_{10}$ of the response obtained. The minimum CTL response was set at 1 LU and dose zero was set at 1. This permitted logging dose and response with minimal distortion of the untransformed variables.

To determine the relationship between the HTL and CTL response, Fisher's exact two-tailed test was used. A positive HTL response was defined as a proliferative response of > 5,000 cpm with a stimulation index > 3; a positive CTL response in one individual sample was defined as > 10 LU (mean + 3 SD of the day zero responses from all subjects).

Results

Our goal is to use synthetic peptides representing MHC class I–restricted CTL epitopes as immunogens to produce disease-specific prophylactic and therapeutic vaccines. A major problem in using synthetic CTL epitopes to stimulate an immune response in vivo is the finding that such peptides tend to be extremely poor immunogens when used alone. We therefore first set out to specifically modify the CTL epitope to enhance its immunogenicity.

Modification of peptides to enhance immunogenicity. Although the available published data are controversial, there is evidence suggesting that two essential elements may be required to allow a CTL epitope to function as an immunogen. One element is a source of T cell help (29–32). To provide this, a second peptide was included, one that stimulated MHC class II–restricted HTL. The rationale was that if the CTL epitope and the HTL epitope could be delivered to the same antigen presenting cell, it would result in the production of IL-2 and other cytokines locally at the appropriate time when CTL precursor cells are being activated by recognition of the CTL epitope and require cytokines for further activation and proliferation. We hypothesized that the second required element was lipid in the form of fatty acids. Lipid molecules have been shown to augment cell-mediated immunity by mechanisms that are only partially understood (10).

Initial experiments to test the ability of various peptide modifications to enhance CTL epitope immunogenicity were carried out using the H-2 K^d-restricted influenza nucleoprotein peptide (NP 147–155) as the CTL epitope. Chicken ovalbumin (OVA) 323–336 peptide, which is restricted by IA^d, was selected as the HTL epitope and palmitic acid was chosen as the lipid. Immunogenicity experiments were carried out in BALB/c ($H\text{-}2^d$) mice comparing various peptide and lipopeptide combinations administered in saline. CTL activity was detected using target cells incubated with the FLU NP 147–155 peptide or target cells infected with the PR8 strain of influenza virus and thus producing antigen endogenously. The results of a comparative experiment are shown in Table I. By grouping the relative immunogenicity of the various peptides and lipopeptides tested as weak (mean L.U. of 0–1), moderate (mean L.U. of 2–10), or high (mean L.U. of > 10), a distinct pattern can be defined. Thus the CTL peptide alone (group 1) is only weakly immunogenic when administered at either the 10- or 100-nmol dose and tested against the NP 147–155 peptide or influenza PR8-infected targets. The immunogenicity of a peptide mixture containing the CTL epitope plus the HTL epitope (group 2) produced a slightly different pattern. Although this combination of peptides was only weakly immunogenic at the 10-nmol dose, it was moderately immunogenic at the 100-nmol dose, suggesting that the HTL epitope may increase the immunogenicity of the CTL epitope. The covalently linked peptide containing both the HTL and the CTL epitopes (group 3) was observed to be only weakly immunogenic when administered in saline.

Palmitoylation of the CTL epitope (group 4) produced a molecule with weak but definite immunogenicity at the 10-nmol dose and weak to moderate activity at the 100-nmol dose. This finding is consistent with those reported by others (11), who found that lipid modification of CTL epitopes enhanced their immunogenicity. Immunogenicity was enhanced further by administering a mixture of palmitoylated-CTL peptide and HTL peptide (group 5). These results suggested that both the lipid and the HTL epitope were playing a role in enhancing the immunogenicity of the CTL epitope. The most potent immunogen by far was produced by covalently linking all three of the elements together (group 6). This linked lipopeptide was found to be highly immunogenic at both the 10- and 100-nmol doses. The level of immunity induced by the linked construct was equivalent to that generated by priming mice with live influenza virus (group 7). Extensive dose titration studies using this linked lipopeptide showed that it was immunogenic when as little as 0.1 nmol (0.3 μg) was administered as a single dose (data not shown). Thus modification of the CTL epitope by conjugation with a lipidated HTL epitope produced a highly immunogenic antigen that was ≥ 10-fold more potent than the other combinations tested.

Duration of the CTL response. For a prophylactic or therapeutic vaccine to be effective, it is important that a long-lasting "memory" CTL response be induced. To test the ability of the $(PAM)_2$–HTL–CTL construct to induce long-lasting immunity, groups of mice (five per group) were injected with 10 nmol of the construct described above. At various times after immunization, groups of animals were killed and their splenocytes restimulated in vitro with the CTL epitope (FLU NP 147–155) and then assayed for CTL activity. Results showed that FLU NP 147–155-specific CTL activity was first detected in the spleen on day 6 (mean±SD from four animals, 2±2 LU), continued to increase on day 7 (10±7 LU), and peaked at day 8 (21±7 LU). Table II shows the levels of CTL activity detected in the splenocytes taken from mice 2, 4, 8, 16, 26, and 55 wk after immunization. From the results, it is clear that immunization with the lipopeptide induced memory CTL that persisted for > 1 yr.

Testing of Theradigm-HBV in humans. Based on the results obtained in the animal model, a three-piece covalently linked construct called Theradigm-HBV was designed for use in humans (Fig. 1). The lipid molecules, palmitic acid, were the same as those used in the animal model studies. For the HTL epitope TT 830–843 was selected. Although this peptide is MHC DR restricted, it had been shown to be highly "degenerate" in its DR specificity and able to function as a HTL epitope in most individuals (33). The HBV core antigen (HBcAg) peptide 18–27, which had been shown to be HLA A2.1–restricted and recognized by CTL obtained from patients with acute HBV infection (20, 21, 26, 27, 34) and CTL induced in vitro from the blood of patients with chronic HBV infection (Chisari, F. V., unpublished observation), was selected as the CTL epitope. Preclinical testing of Theradigm-HBV in HLA-A2.1 transgenic animals demonstrated its ability to induce HBcAg 18–27-specific CTL that were able to lyse target cells transfected with the HBV core gene, that is, synthesizing the core protein endogenously (data not shown). Based on these

Table I. Modification of the Influenza NP 147–155 CTL Epitope to Enhance Immunogenicity

Group	Peptide, lipopeptide, or virus administered	Specific CTL activity			
		NP 147–155 peptide	Influenza infected	NP 147–155 peptide	Influenza infected
		10 nmol	*LU/10⁶ splenocytes*	*100 nmol*	
1	CTL	0‡	1	0	0
		0	0	0	0
		0	0	0	0
		0 (0.0)§	0 (0.2)	0 (0.0)	0 (0.0)
2	CTL + HTL	0	0	4	4
		0	0	12	11
		0	1	0	1
		0 (0.0)	0 (0.2)	4 (5.0)	5 (5.3)
3	HTL–CTL	0	0	1	1
		0	0	0	0
		0	0	1	1
		0 (0.0)	0 (0.0)	1 (0.8)	5 (1.8)
4	$(PAM)_2$–CTL	0	0	6	2
		1	1	2	3
		3	0	1	0
		3 (1.8)	3 (1.0)	1 (2.5)	1 (1.5)
5	$(PAM)_2$–CTL + HTL	6	8	2	1
		2	0	34	25
		3	8	2	4
		4 (3.8)	4 (5.0)	9 (11.8)	0 (7.3)
6	$(PAM)_2$–HTL–CTL	>22	8	8	11
		>22	>22	14	11
		>17	>17	>17	>17
		29‖ (>22.5)	32 (>18.8)	26 (>16.3)	28 (>16.8)
		PR8			
7	PR8 influenza virus	30	20		
		25	25		
		22	27		
		35 (28.0)	35 (26.7)		

BALB/c mice were injected subcutaneously with 10 or 100 nmol/mouse of the indicated peptides or peptide combinations (group 1–6) with the PR8 influenza virus (group 7). Four animals were injected with each different preparation. 3 wk after immunization, splenocytes were removed and stimulated in vitro with the NP 147–155 peptide. CTL activity was assayed 1 wk later using ^{51}Cr-labeled B10.D2 fibroblasts as targets. Target cells were tested in the absence or presence of antigen (NP 147–155 peptide) or infected with the PR8 virus. * The data are expressed as lytic units/10^6 effector cells with 1 LU being defined as the number of effector cells required to induce 30% lysis of 5×10^3 B10.D2 ^{51}Cr-labeled target cells during a 6-h assay. Specific CTL activity is obtained by subtracting the LU obtained in the absence of antigen from the LU obtained in the presence of antigen. CTL activity in the absence of antigen was never above 1 LU i.e., at an effector to target ratio of 100:1 the ^{51}Cr released was never above 30%. ‡ Each number represents the specific CTL activity from an indivdual mouse. § Mean response of the four animals tested. ‖ Representative % ^{51}Cr release data: the specific CTL activity of 29 LU was derived from the following % ^{51}Cr release results: at E:T ratios of 3:1, 9:1, 26:1, 79:1 the % ^{51}Cr released was, respectively, 2, 4, 8, and 17 in the absence of antigen and 22, 48, 65, and 76 in the presence of antigen.

preclinical studies, a phase I clinical study was conducted in normal volunteers.

Safety and tolerability. Adverse events related to Theradigm-HBV were mild, reversible, and generally related to skin reactions at the site of inoculation. These local reactions consisted of erythema or swelling, required no therapy, typically lasted 24–48 h, and were not associated with regional adenopathy or systemic symptoms. These observations suggest that Theradigm-HBV given in the dosage schedule tested is both safe and well tolerated in normal individuals.

Induction of CTL activity. Peripheral blood lymphocytes obtained before and at various times after the primary and booster immunizations were stimulated in vitro and tested for HBV-specific CTL activity. The placebo group contained a total of nine subjects, the 5- and 50-μg groups each contained six subjects, and the 500-μg group contained five subjects (one subject dropped from the study for unrelated reasons). The results are presented in two ways (Figs. 2 and 3). Fig. 2 shows the cumulative CTL activity for each individual (i.e., the sum of the activity detected in the day 7, day 14, and day 28 blood samples after both the primary and booster injections), as well as the mean±SD for each dose group.

From this type of analysis, two observations can be made. Considering the mean responses, there was a clear dose–response curve: the 5-μg-dose group showed no response compared with the placebo, whereas the 50- and 500-μg-dose groups both showed an increase over the placebo. Statistically, there was a strong positive linear relationship observed between the $\log_{10}$ of the dose injected and the $\log_{10}$ of the response obtained with $P < 0.001$ (45% of the variance was accounted for by the

Table II. A Single Injection of the $(PAM)_2$–HTL–CTL Construct Produces Long-lasting CTL Immunity

Time after immunization	Antigen-specific CTL activity*
wk	*LU/10^6 splenocytes*
2	19±12
4	22±12
8	52±25
16	41±24
26	41±23
55	25±27

BALB/c mice were injected subcutaneously with 10 nmol/mouse of the $(PAM)_2$–HTC–CTL peptide. At the indicated time after immunization, five animals were killed and CTL activity determined as explained in Table I and Methods. *Values are means±SD.

model and inspection of the residuals after fitting the model indicated that the model was adequately specified and the residuals were normal). In addition to illustrating the overall response for each group, we also determined the number of individuals within each group who responded to Theradigm-HBV. For subjects to be considered as responders, three criteria were used. First, there must have been consecutive responses detected unless the only response observed was in the last blood sample taken. Second, when a response was detected after the primary injection, one also had to be observed after the booster injection. Third, that the cumulative CTL response, that is, cumulative lytic units, was > 2.54 SDs above the mean of the placebo group, that is, cumulative ≥ 19 LU. Using these criteria, none of the individuals in the placebo or the 5-μg-dose group were responders, whereas two of six subjects in the 50-μg group and all five of the subjects in the 500-μg group were determined to have responded to Theradigm-HBV.

The CTL activity detected in each of the individual blood samples collected from the subjects receiving active drug is shown in Fig. 3. In the 5-μg-dose group there was little to no CTL activity detected. In the group of subjects receiving the 50-μg dose, there were two responders. The response observed in subject 203 was first detected on day 28 (28 LU) after the primary injection. It was not detected on day 7 after boost but was detected again on both days 14 and 28 after boost. In contrast, the other subject who responded in the 50-μg-dose group was subject 204, who had 20 LU of CTL activity detected on day 28 after the second injection.

In the 500-μg-dose group, all five of the subjects responded to the immunogen. Subject 301 produced the lowest overall response with a peak response of 13 LU detected in the day 14 postboost sample. Subject 302 produced a low response by day 14 after the primary injection, which increased after the booster immunization to a peak of 43 LU by day 14 postboost with a slightly lower level of activity detected in the day 28 postboost sample.

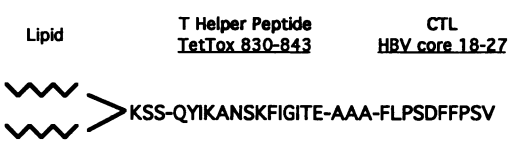

Figure 1. Structure of Theradigm-HBV.

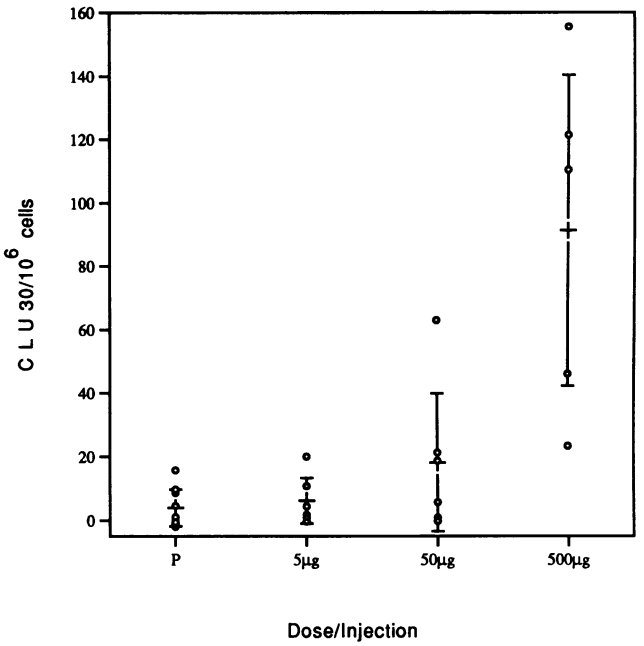

Figure 2. Immune response to Theradigm-HBV in normal volunteers. Human volunteers were injected with the indicated preparations. CTL activity was determined as described in Methods in PBMC samples obtained 7, 14, and 28 d after the primary and the booster injection. CLU represents the sum of the lytic activity from all of the time points for each individual subject.

Blood samples obtained from subject 304 demonstrated a somewhat different pattern. Here 36 LU were detected in the day 14 blood sample after the primary injection, which declined to 18 LU in the day 28 sample. Although no activity was detected in the day 7 postboost sample, 41 LU were detected on day 14 postboost, declining to 11 LU by day 28. Similar to subject 301, subject 305 produced no detectable CTL activity until day 14 postboost. However, in contrast to subject 301, subject 305 produced a very high level of CTL activity with 84 LU detected on day 14 and 69 LU on day 28 postboost. Finally, subject 309 produced an intermediate level of CTL activity with the initial response detected in the day 28 blood sample obtained after the primary injection and a peak response of 22 LU detected on day 7 postboost.

Recognition of endogenously synthesized antigen. For a subunit-based vaccine to be useful in the prevention and/or treatment of disease, it must be able to induce CTL capable not only of recognizing peptide pulsed target cells but also able to recognize the relevant virus-infected cell or tumor cell. Since HBV does not reproducibly infect cells in vitro, HLA A2.1–positive EBV-transformed B cells that had been transfected with the HBV core gene were used as target cells expressing endogenously generated antigen. Shown in Fig. 4 are the results obtained when the cells present in the blood samples obtained on day 14 postboost from subjects 302 (*A*) and 304 (*B*) were tested for their ability to lyse HBV core antigen transfected targets vs. nontransfected targets in the presence or absence of the HBcAg 18–27 peptide. Cells from both individuals lysed target cells producing naturally processed HBV core antigen. The extent of lysis was similar (191 and 60 LU) to that obtained with nontransfected target cells in the presence of HBcAg 18–27 peptide (186 and 139 LU). We also determined the percentage of CD4 vs. CD8 T cells present in these cell suspensions.

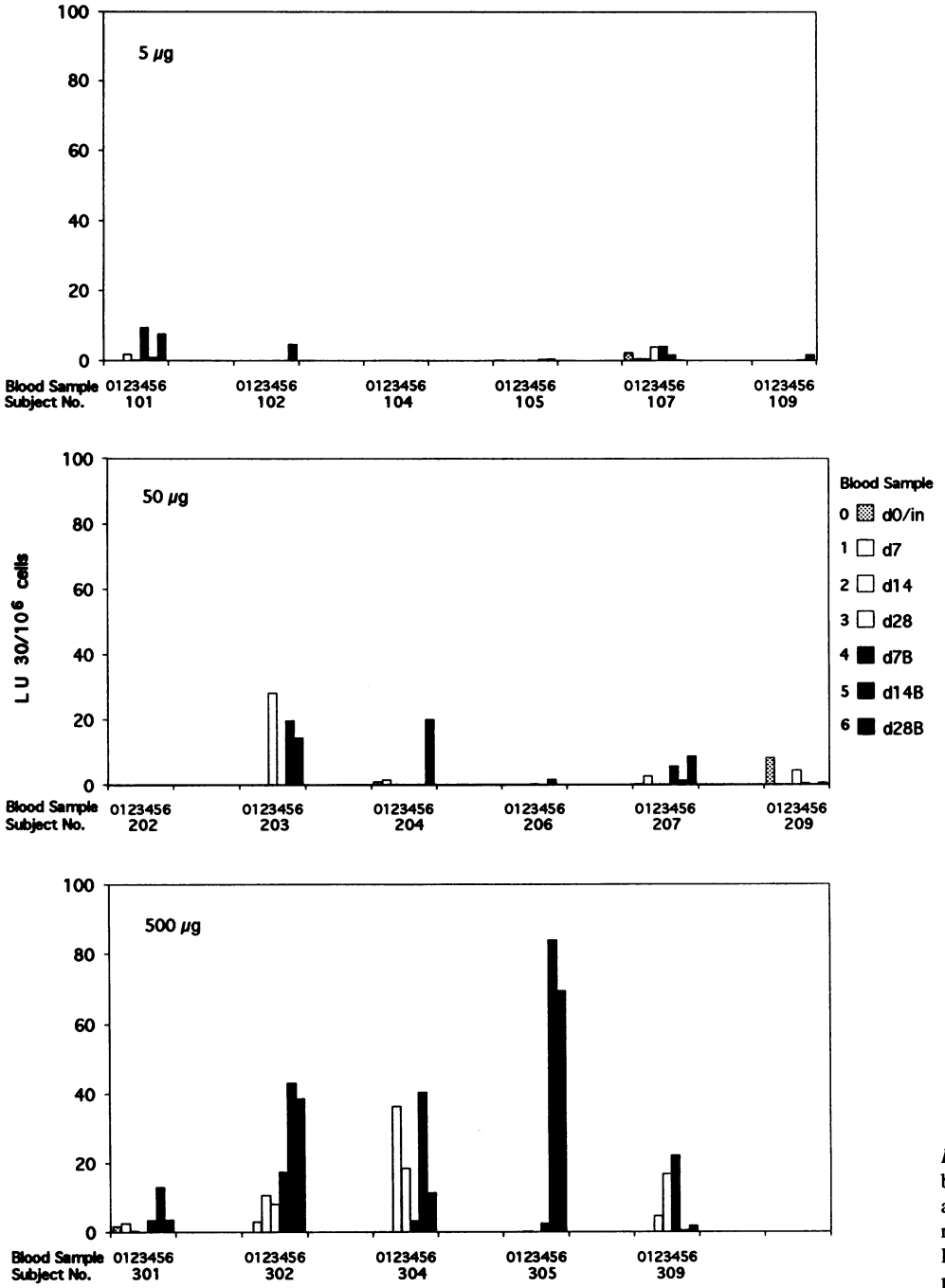

Figure 3. CTL activity in individual blood samples taken from the 5-, 50-, and 500-μg-dose groups. Each bar represents the CTL activity present in the PBMC obtained at the indicated time point.

As expected for class I–restricted antigen-specific CTL response, the great majority of the cells were CD8 positive. For example, effector cells obtained from subject 302 were 2.2% CD4+ and 81.2% CD8+ and effector cells from subject 304 were 3.3% CD4+ and 72.8% CD8+.

Requirement for T helper activity for CTL induction. One of the key modifications made to enhance the immunogenicity of the CTL epitope was the addition of an HTL epitope. To evaluate the contribution of the HTL response in the induction of the CTL response, we assessed the correlation between the peak level of CTL activity and the peak T cell proliferative response to TT 830–843 for the 50- and 500-μg-dose groups after the primary and the booster injections (Table III). For the primary injection, the association between the HTL and CTL response was significant with $P = 0.003$ using Fisher's exact two-tailed test. In this case, the four top CTL responses also had the four highest proliferative responses to the helper epitope, whereas the seven low CTL responses were also low for T cell proliferation. However, after the booster injection, no significant association was found between the CTL response and the HTL proliferative response. For instance, in the 50-μg-dose group, subject 204 produced a substantial CTL response but had little detectable HTL response. In contrast, subject 209 produced a moderate proliferative response to the HTL epitope but had only a very low level of CTL activity. In the 500-μg-dose group the correlation was better. After boosting, only subject 301 produced a CTL response, albeit a low response, in the absence of a positive HTL proliferative response.

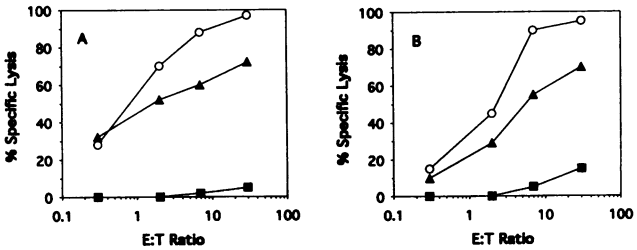

Figure 4. Capacity of CTL to kill HBcAg transfected target cells. PBMC obtained from individuals 302 (*A*) and 304 (*B*) at day 14 after boost (see Fig. 3) were restimulated in vitro twice as described in Methods. CTL activity was tested on ^{51}Cr-labeled .221(A2.1) target cells in the absence (■) or presence (○) of the HBcAg 18-27 CTL epitope and on ^{51}Cr-labeled .221(A2.1)-core target cells (▲).

Discussion

The results presented demonstrate for the first time that a CTL epitope can be used to induce a primary CTL response in humans. The efficiency of the three-piece modular design of the vaccine construct in inducing CTL activity was demonstrated initially in preclinical animal studies (Table I). Here, it was found that the lipid and the HTL peptide linked to the CTL peptide were necessary for optimal CTL induction since the absence of either resulted in a molecule with substantially lower activity. Significantly, a single injection of this modular lipopeptide induced memory CTL that persisted in mice for > 1 yr (Table II). Our results indicate that T cell help is important in enhancing the immunogenicity of the CTL epitope. In the animal studies (Table I), this effect was observed by either injecting a mixture of the two peptides or by covalently linking them. The fact that the linked lipidated construct had superior activity suggests that it may be more efficient to have the two epitopes delivered to the same antigen presenting cell (32). The importance of HTL activity in the generation of a CTL response remains controversial. Although the CTL response to some viruses (35) and modified peptide antigens (12) appear CD4+ HTL independent (35), several groups looking at CTL responses after peptide immunization have reported that T cell help is required for optimal CTL induction (36–39). In addition, as discussed below, the results we obtained in the human studies also suggest an important role for HTL in optimal CTL activation. Despite this controversy, our results in mice are consistent with the role of HTL for induction of optimal CTL activity.

The present results also indicate that lipid modification of antigenic peptides enhances their immunogenicity (Table I). A linked HTL–CTL peptide that was poorly immunogenic when administered in saline was found to be highly immunogenic when modified by attachment of two palmitic acid moieties to the NH_2 terminus of the HTL epitope via a lysine–serine–serine linker. The lipid modification of peptides to specifically enhance CTL epitope immunogenicity has been observed by others (10–12, 40). Some of the earliest work in this area was reported by Rammensee and co-workers (10, 11), who found that the covalent attachment of tripalmitoyl-*S*-glycerylcysteine-serine-serine (P_3CSS) to an influenza CTL epitope was able to induce an influenza-specific CTL response in mice, whereas administration of the CTL epitope alone did not induce CTL immunity.

How modification of a peptide immunogen with lipid functions in elicitation of an immune response is poorly understood. We postulate that the lipid serves three different roles. One, it leads to the persistence of peptides at the site of injection or in the draining lymph node for sufficient time to induce a CTL response. Using a radiolabeled form of Theradigm-HBV, we have shown that the peptide has a half-life at the site of injection of 5–7 d (data not shown). Second, evidence reported by Bessler and co-workers (41) demonstrates that lipid modification of peptides may help them translocate across the plasma membrane into the cytoplasm. In the case of the Theradigm construct, entry into the cytoplasm by a portion of the injected material would presumably lead to proteolytic liberation of the CTL epitope from the lipid HTL and allow (15) for its delivery to the endoplasmic reticulum where it could associate with newly synthesized MHC class I molecules, which are eventually exported to the cell surface. We hypothesize that the CTL epitope of the lipopeptide construct must be cleaved in vivo in order for it to associate with MHC class I molecules since we have found that in vitro the modular lipopeptide construct is 1,000-fold less efficient in sensitizing targets for lysis by CTL than

Table III. Stimulation of CTL Activity by Theradigm-HBV Is Associated with Induction of HTL Activity

		Maximum CTL activity		Maximum HTL activity	
Injection	Subject	LU/10^6 PBL	LR*	cpm × 10^{-3}	SI‡
Primary	304	36.5	7.0	74.2	8.6
	203	28.3	72.8	21.5	5.9
	309	16.9	13.1	43.8	26.8
	302	10.7	7.3	28.6	4.5
	209	4.5	16.0	0.7	1.5
	207	2.7	§	2.5	2.7
	301	2.6	1.9	0	1.0
	204	1.6	1.6	2.0	1.9
	206	0.3	1.8	0.1	1.2
	305	0.2	1.3	0.6	1.2
	202	0		0.1	1.0
Booster	305	84.0	50.4	6.2	4.9
	302	43.0	49.1	191.0	31.7
	304	40.0	17.9	158.0	41.0
	309	22.0	7.3	35.0	13.9
	204	20.0	14.3	1.6	1.7
	203	19.8	§	8.2	2.9
	301	13.2	6.7	0.2	1.1
	207	8.8	§	10.5	10.0
	206	1.7	1.9	0.7	1.4
	209	0.7	8.0	11.4	5.3
	202	0		2.6	1.5

PBMC samples obtained from individuals injected with 50 μg (200 series) and 500 μg (300 series) of Theradigm-HBV were tested for HTL activity in a T cell proliferation assay in the presence of TT 830–843 as described in Methods. CTL activity was determined as described in Fig. 2 and 3. The highest activity present among samples obtained after either 7, 14, or 28 d postinjection is reported. * LR, the lysis ratio indicates the ratio of CTL activity (^{51}Cr release) observed by target cells in the presence of antigen divided by the CTL activity observed in the absence of antigen. ‡ SI, the stimulation index represents the ratio between T cell proliferative activity obtained in the presence of antigen divided by the activity obtained in the absence of antigen. § CTL activity in the absence of antigen was zero.

the HBcAg 18–27 peptide itself (data not shown). Third, the lipid moiety may also have intrinsic adjuvant activity. For instance, after injection into animals, it induced a small granulomatous reaction characterized after a few days by a mononuclear infiltrate that might be important in the elicitation of the CTL response.

Based on the preclinical animal studies, plus the previously described CTL responsiveness to HBcAg 18–27 in HLA-A2–positive patients with acute viral hepatitis, a modular therapeutic vaccine, Theradigm-HBV, was designed to treat chronic hepatitis B virus infection in humans (Fig. 1). The phase I safety evaluation demonstrated that Theradigm-HBV was both safe and well tolerated in normal subjects. The ability of the Theradigm-HBV construct to induce a primary CTL response was also demonstrated (Figs. 2 and 3). This was clearly a primary response since study participants had no evidence of having had HBV infection. They were HBV surface antigen negative and lacked antibodies to both surface and core antigens. In addition, no CTL response to HBcAg 18–27 was detected before immunization with Theradigm-HBV. A clear dose-related response was seen after immunization. The fact that the magnitude of the response to the 500-μg dose was far superior to that achieved with 50 μg suggests that greater responses may be achievable with doses above 500 μg. However, the fact that the response attained at 500 μg was similar in magnitude to that detected in patients with acute HBV infection that go on to clear virus (20–22) suggests that the level of immunity achieved in the phase I study may be clinically relevant.

The results of the phase I study (Fig. 3) demonstrated that a booster effect was seen in response to Theradigm-HBV. Little information is available regarding the effects of booster immunization on T cell immunity. Studies by Schild et al. (11) showed that immunization of mice with "nonimmunogenic" influenza CTL epitopes 3 d before viral challenge led to the detection of enhanced splenic CTL activity compared with animals immunized solely by the virus infection. These results suggest that CTL precursor cells were induced to a state of "latent immunity" leading to augmentation of CTL activity after immunization with virus, an observation that could be interpreted as a booster effect. Using the adjuvant QS-21 and denatured ovalbumin, Newman et al. (42) detected an OVA-specific CTL response after the second injection that doubled in magnitude after a third dose.

The sequential pattern of CTL responses and the heterogeneity detected in individual blood samples observed in the present study are similar to those reported by Miller and co-workers (18) using PBL obtained from Rhesus monkeys that had been immunized and boosted with a synthetic simian immunodeficiency virus CTL peptide in a fusogenic proteoliposome. In the four animals immunized, two patterns of response were observed. Two monkeys showed a clear booster effect. The response peaked at 1 wk after the primary immunization, declined, and then increased again after the booster immunization given at 4 wk. In the other two animals, little or no booster effect was observed.

Similar to the results obtained with Rhesus monkeys, the response observed in the phase I clinical study was heterogeneous (Fig. 3). One response pattern was typified by subjects 203, 302, and 304, who produced detectable activity 2–3 wk after the primary immunization, followed by a decline and then an increase in CTL activity after the booster injection. In the case of subject 302, the booster immunization appeared to greatly increase the magnitude of the response as well. A second pattern observed was characterized by little or no response after the primary immunization but a clear response 2–3 wk after the booster injection. This pattern was observed in subjects 204, 301, and 305.

The cyclic nature of the CTL activity detected in the PBL of subjects immunized with Theradigm-HBV was also similar to the activity observed in the PBL obtained from Rhesus monkeys described above and presumably reflects the trafficking pattern of primed CTL between lymphoid organs and peripheral blood. The appearance followed by the decline in CTL activity detected in the PBL after administration of Theradigm-HBV is in contrast to the persistence of CTL activity detected in the spleens of mice injected with the a similar influenza-specific construct (Table II). In this latter case, memory CTL activity was detectable for $\geq$ 1 yr. Presumably, Theradigm-HBV has a similar capacity to induce long-lived memory in humans, and the failure to demonstrate it is due to our inability to sample the appropriate lymphoid tissue.

The results demonstrate the ability of the Theradigm-HBV molecule to induce CTL not only capable of lysing peptide loaded target cells but also capable of lysing target cells generating antigen endogenously (Fig. 4). It was known that HBcAg 18–27-specific CTL derived from patients with acute HBV infection were able to lyse target cells transfected with the HBV core gene (21, 26) and that HLA-A2.1 transgenic mice injected with the Theradigm-HBV molecule produced HBcAg 18–27-specific CTL capable of lysing the same HBV core gene transfected targets (Vitiello, A., Furze, J., Farness, P., unpublished result). Thus the results presented here are consistent with those leading up to our studies in humans and suggest that the CTL generated by Theradigm-HBV can be expected to recognize HBV infected hepatocytes.

Finally, the HTL epitope present in the Theradigm construct appears to play an important role in the generation of a primary CTL response. This was suggested by the high correlation between the proliferative response and the CTL response after the primary immunization. The correlation between HTL and CTL activity was also evident after the booster immunization, although several exceptions were observed.

In summary, a peptide-based vaccine has been developed that is capable of inducing disease-specific MHC class I–restricted memory CTL. The modular components of the construct consist of a lipidated HTL peptide and a CTL peptide. The first construct of this nature to be tested in humans has been designed to treat chronic HBV infection. This molecule, called Theradigm-HBV, when injected into normal subjects has been found to be safe and well tolerated and effective in inducing HBV-specific CTL. Based on this experience, we believe Theradigm-HBV is an appropriate candidate for future evaluation in individuals chronically infected with HBV.

Acknowledgments

We extend special thanks to the Cytel Theradigm-HBV Project Team members for their extraordinary efforts in carrying out the preclinical and clinical studies. In addition, we thank Robert DeMars for providing the 3A4.221(A2.1) cell line, John Fikes and Brian Livingston for transfection of target cells, Luman Wing for pharmacokinetic studies, Gerard Smits for statistical Analysis, Glenna Marshall for assistance in preparing the manuscript, Jay Kranzler for encouragement and support of the project, and Carlo Ferrari and Patty Fowler for help and advice. This work was funded in part by National Institutes of Health SBIR Grant 1R43 AI32847-01.

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