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Altered Helper T Lymphocyte Function Associated with Chronic Hepatitis B Virus Infection and its Role in Response to Therapeutic Vaccination in Humans

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Theradigm-hepatitis B virus (HBV) is an experimental lipopeptide vaccine designed to stimulate induction of HBV-specific CTL responses in HLA-A2 individuals. Previous studies had demonstrated high immunogenicity in healthy volunteers, but comparatively weak CTL responses in chronically infected HBV patients. Herein, we examined helper T lymphocyte (HTL) responses in chronically infected patients. Despite normal proliferation and IL-2 secretion, IL-12 and IFN-γ secretion in vitro in response to the vaccine was reduced compared with healthy volunteers. A similar pattern of cytokine secretion was observed following mitogen stimulation, suggesting a general altered balance of Th1/Th2 responses. Further analysis indicated that HTL recall responses to whole tetanus toxoid protein were reduced in chronically infected subjects, and reduced responsiveness correlated with the outcome of Theradigm-HBV immunization. Finally, experiments in HBV transgenic mice indicated that the nonnatural Pan DR HTL epitope, PADRE, is capable of inducing high levels of IFN-γ secretion and that its inclusion in a lipopeptide incorporating an immunodominant Ld-restricted CTL epitope resulted in breaking tolerance at the CTL level. Overall, our results demonstrate an alteration in the quality of HTL responses induced in chronically infected HBV patients and suggest that use of a potent HTL epitope may be important to overcome CTL tolerance against specific HBV Ags. The Journal of Immunology, 1999, 162: 3088–3095.

Several studies have emphasized the association between self-limiting acute hepatitis and multispecific CTL responses (1, 2). Spontaneous and IFN-related clearance of chronic hepatitis B virus (HBV) infection are also associated with the resurgence of a vigorous class I-restricted cellular immune response (3). In all such cases, the CTL response is polyclonal and specific for multiple viral proteins, including the HBV envelope, core, and polymerase Ags. In contrast, in patients with chronic hepatitis the CTL activity is usually absent or weak and antigenically restricted.

The crucial role of CTL in the resolution of HBV infection has been further underlined by studies in HBV transgenic mice. Adoptive transfer of HBV-specific CTL into mice transgenic for the HBV genome resulted in suppression of virus replication. This effect was primarily mediated by a nonlymphokine-based mechanism (4–7).

As is the case for class I-restricted responses, class II-restricted T cell responses are usually detected in patients with acute hepatitis but are absent or weak in patients with chronic hepatitis (8). However, in contrast with the clear association of CTL responses with the resolution of HBV infection, the association of an HBV-specific helper T lymphocyte (HTL) response with clearance of HBV infection has been less clearly documented. Because hepatocytes do not express MHC class II molecules, HTL responses are likely to recognize specific viral Ags phagocytosed and presented by professional APC (9). In this context, although HTL responses may directly contribute to the clearance of HBV infection through the secretion of cytokines that suppress viral replication (10), their primary role in disease resolution is probably mediated by supporting the induction and expansion of virus-specific CTL and B cells.

These data suggest that a vaccine capable of inducing HBV-specific CTL responses similar in quality and magnitude to those observed in acute hepatitis could represent a safe and effective treatment for chronic HBV infection. The modular immunotherapeutic, Theradigm-HBV, was designed based on this line of reasoning (11).

Theradigm-HBV is composed of an HLA-A2-restricted, HBV-derived CTL epitope covalently linked to an universal HTL epitope and palmitoylated at the N-terminus. The palmitic acid moiety enhances the immunogenicity of the construct and obviates the need for an oil-based adjuvant (11). The HLA-A2-restricted hepatitis B core Ag (HBcAg) 18–27 CTL epitope was selected for inclusion in Theradigm-HBV. This epitope is recognized by specific CTL derived from HLA-A2.1 patients with acute, resolving HBV infection (1, 2). The HBcAg 18–27 epitope is also highly conserved among different HBV isolates, binds all common HLA-A2 subtypes analyzed to date, and is immunogenic in HLA-A2Kβ transgenic mice and primary human CTL cultures (12–14).

The tetanus toxoid (TT)-derived peptide 830–843 was selected as the universal HTL epitope. This epitope has been shown to be highly degenerate in its HLA-DR binding and is capable of functioning as an HTL epitope in individuals expressing many different DR types (15). Furthermore, an HTL epitope unrelated to HBV
was intentionally selected to avoid potential problems related to Ag-specific tolerance in the class II HTL compartment resulting from chronic HBV infection.

Previous studies in normal, uninfected volunteers have shown that Theradigm-HBV is highly immunogenic in humans (11, 16). The magnitude of the CTL responses induced by Theradigm-HBV immunization was in fact found to be comparable to CTL responses associated with clearance of acute viral infection (16). A strong correlation between the induction of CTL and HTL responses was also apparent, demonstrating that HTL responses are crucial for the development or maintenance of CTL responses (11, 16).

Subsequent clinical studies demonstrated that while individuals chronically infected with HBV were overall hyporesponsive to immunization, nonetheless Theradigm-HBV was capable of inducing HBV-specific CTL responses in a dose-dependent fashion (J. Heathcote et al., manuscript in preparation). These data indicate that although some level of Ag-specific T cell tolerance at the CTL level is associated with chronic HBV infection, HBV-specific CTL precursors are present in HBV patients, arguing against a complete deletion of precursors as the mechanism of induction of such tolerance. In addition, while HBV-specific precursors are not activated by the large amount of viral Ags expressed during the course of chronic infection, nevertheless they can be activated, at least to a certain extent, by deliberate immunization with a therapeutic construct.

In the present study, we have further characterized the immune responses following Theradigm-HBV immunization in both normal subjects and HBV patients. While proliferative responses in chronically infected HBV patients to the HTL epitope included in the construct appeared normal, the cytokine profile observed suggested the induction of a Th0/Th2 HTL responses rather than a Th1 response. Decreases in recall proliferative responses to whole HBV were also observed, and decreased proliferative responses to TT in turn correlated with the outcome of Theradigm-HBV immunization. Altered lymphokine profiles in chronically infected HBV patients were detectable even in response to mitogenic stimulation of PBMC, further illustrating a generalized and previously unappreciated alteration of HTL responses associated with chronic HBV infection.

Materials and Methods

Peptides and lipopeptides

Peptides were synthesized according to standard F-moc solid phase synthesis methods (17). Lipidated HTL-CTL peptide constructs were designed with the HTL epitope (TT830-843, OVA323-336, or PADRE) at the amino terminus. A 3-amino acid spacer separated the HTL epitope from the ACE-ACE-ACE-ACE-ACE peptide. The peptides and the protecting groups on the amino acids were cleaved using trifluoroacetic acid, ethanedithiol, water (9.5:2.5:2.5, v/v/v) or trifluoroacetic acid, thioanisole, phenol, ethanedithiol, water (10 ml:0.5 ml:0.75 g:0.25 ml:0.5 ml) depending upon the protecting group present on the amino acids.

Unlipidated peptides were purified using conventional HPLC methods. Lipopeptides were purified by repetitive washes with 50% acetic acid–water v/v, followed by two washes with H2O and lyophilization.

The purity and identity of all compounds were checked by analytical HPLC and mass spectrometry. Purity was ≥75% for lipidated peptides and ≥90% for unlipidated peptides.

Human study population

Twenty-six healthy HLA-A2 individuals who tested seronegative for markers of HBV infection as determined by standard clinical laboratory assays were enrolled in a Phase I clinical trial as described previously (16). The phase II clinical trial population consisted of 90 chronically HBV-infected HLA-A2 patients that were positive for hepatitis B surface Ag (HBsAg), hepatitis B e Ag (HBeAg), and HBV DNA (J. Heathcote et al., manuscript in preparation). Both cohorts were immunized with the experimental therapeutic vaccine, Theradigm-HBV, which consists of a lipopeptide containing the TT830-843 HTL epitope covalently linked to the HBeAg 18–27 CTL epitope and palmitoylated at the N-terminus. Normal subjects received doses of either 0.05, 0.5, 0.5, 5, or 15 mg Theradigm-HBV. Subjects were immunized at 6-wk intervals and received up to four injections of Theradigm-HBV. Blood was drawn the second and fifth week following each injection. During the course of Theradigm-HBV immunization, HBV patients did not receive any other antiviral or immunomodulatory therapy.

Mice

B6D2F1 were obtained from the Jackson Laboratories (Bar Harbor, ME); 8- to 12-wk-old mice were used in all experiments. The HBV transgenic mice used in this study have been described previously (4–7). The 1.3.32 lineage of HBV transgenic mice transcribe the 3.5- and 2.1-kb HBV RNAs in their hepatocytes and express both HBeAg and HBsAg as well as replicative HBV DNA intermediates and complete virus particles. Mice from the lineage 1.3.32 were extensively backcrossed onto the C57BL/6 parental background and mated with BALB/c By1 mice to generate H-2b restricted, HBeAg-specific CTLs.

T cell proliferation assays

Human T cell proliferation assays were performed as described previously for the characterization of responses normal to volunteers immunized with Theradigm-HBV (11, 16). Briefly, human PBMC were cultured at 1.5 × 10^5 cells/well in a 96-well flat-bottom plate in replicates of four to six wells for each condition. TT830-843 was added at 10 µg/ml, whole TT (Connaught, Swiftwater, PA) was added at 0.5 µg/ml or PHA (Murex Biotech Limited, Dartford, England) was added at 4 µg/ml final concentration. After 7 days, 1 µCi of [3H]Tdr (ICN, Irvine, CA) was added to each well of the PHA cultures and incubated for an additional 18 h. Human cultures stimulated with TT or TT830-843 were fed after 7 days with 100 µl of medium containing IL-2 at a final concentration of 10 U/ml. After an additional 2 days in culture, 1 µCi of [3H]Tdr was added to each well and harvested after 18 h.

Proliferative responses in mice were determined as described previously (18). Briefly, 1 × 10^6 murine splenocytes/well were plated in 96-well microtiter tissue culture plates in the presence of a log-dose peptide titration (100–0.01 µg/ml) of either PADRE or OVA323-336 peptides. After three days, 1 µCi of [3H]Tdr was added to each well and incubated for an additional 18 h. Cells from both human and mouse assays were harvested onto glass filters (cell harvester 1295–001, LKB Wallac, Gaithersburg, MD), and [3H]Tdr incorporation was measured (β plate counter 1205, LKB Wallac).

Cytokine secretion assays

Frozen human PBMC samples were thawed in RPMI 1640, 2% human serum (Gemini Bioproducts, Calabasas, CA) containing 30 µg/ml DNase (Calbiochem, La Jolla, CA) and washed two times. Cells were resuspended in RPMI 1640 with 10% human serum and cultured at 1.5 × 10^5 cells/well in a 24-well plate. Cultures were stimulated with either 10 µg/ml TT830-843 or 4 µg/ml PHA. The cultures were incubated for 3 days, after which the supernatants were collected and stored at −80°C for subsequent analysis. Human cytokines were quantitated using human cytokine kits (Endogen, Woburn, MA) according to manufacturers’ protocols. The IL-12 detection kit recognizes both the p70 and p40 forms of IL-12.

For cytokine assays using murine cells, splenocytes from individual mice were treated to remove the RBC with RBC lysing buffer (Sigma, St. Louis, MO) and washed twice in RPMI, 10% FCS. Splenocytes were cultured at 4 × 10^6 splenocytes/well in a 24-well plate. Cultures were either stimulated with either 10 µg/ml of PADRE or OVA323-336. Culture supernatants were collected after 3 days. Cytokine secretion was measured using Endogen ELISA reagents (Endogen) according to manufacturers’ protocols.

Assays for murine CTL activity

Eleven to 14 days after immunization, 3 × 10^6 splenocytes, harvested from individual mice, were stimulated with 10.0 µg/ml HBV surface 28–39 peptide in upright 25 cm^2 flask containing 10 ml RPMI 1640 (Life Technologies, Grand Island, NY)-10% FCS. After 6 days, splenocytes from each flask were collected and assayed for cytolytic activity using a standard
4-h $^{51}$Cr-release assay using peptide-loaded P815 mastocytoma cells originally obtained from DBA/2 mice (19). Target cells ($3 \times 10^6$) were routinely labeled with 300 $\mu$Ci $^{51}$Cr-sodium chromate (NEN Research Products, Boston, MA) for 60 min at 37°C, in the absence or presence of 2 $\mu$g/ml of the HBV surface 28–39 peptide. The percent release data was transformed into 30% lytic units (LU) per 10$^6$ cells (LU30) (11) to more readily compare CTL responses observed in different samples. One LU corresponds to the number of effector cells required to induce 30% lysis of 10$^4$ $^{51}$Cr-labeled target cells during a 4-h assay. Specific CTL activity is obtained by subtracting the LU30 obtained in the absence of the Ag from the LU30 obtained in the presence of Ag.

**Results**

**Comparison of HTL proliferative responses in normal subjects and chronically infected HBV patients**

To characterize immune responses to Theradigm-HBV, PBMC from chronically infected individuals were examined for their capacity to proliferate in response to the universal HTL epitope, T8830-843, which is a component of Theradigm-HBV. Proliferative responses were evaluated both in terms of frequency of responding individuals (average stimulation index (SI) $\geq 10$) as well as magnitudes of the positive responses. The results of this analysis are shown in Fig. 1.

The responder frequencies in chronically infected HBV patients were directly related to the immunizing dose and found to be similar to those detected in normal subjects (Fig. 1A). In the 0.05-mg dose group, $\sim$10% of the individuals responded to the HTL epitope. As dosing was increased, responder frequency increased to 60% in the 10-mg dose group. In contrast, responder frequency was reduced in the 15-mg dose group; this observation may be a consequence of the small sample size or might reflect the induction of tolerance at the highest dose tested. As in the case of responder frequencies, the magnitude of the T8830-843-specific proliferative responses in the HBV patients was found to be similar to the magnitude of responses observed in normal subjects (Fig. 1B).

**Relationship between HTL and CTL responses**

The results of previous clinical studies in normal subjects suggested that HTL responses are important in the development of
CTL responses to Theradigm-HBV (11, 16). To determine whether this was also the case in patients chronically infected with HBV, the correlation between HTL and CTL responses was analyzed. As observed in normal subjects, HBV patients that developed HTL responses to TT830-843 were more likely to also develop HbcAg 18–27-specific CTL responses (Table I). This data indicates that in individuals chronically infected with HBV, the development of an HTL response following Theradigm-HBV immunization is strongly associated with the development of a CTL response.

As mentioned above, >90% of the normal subjects who responded to the HTL epitope also mounted a CTL response (16). In contrast, only slightly >50% of the HBV patients that responded to the HTL epitope also mounted a CTL response. This finding suggests that the quality of the HTL response might differ in an aspect critical for the elaboration of an CTL response. Alternatively, a defect in the CTL precursors themselves might render them unreceptive to antigenic stimulation and/or HTL support, leading to suboptimal activation or expansion.

Cytokine profile of specific HTL responses in normal and chronically infected Theradigm-HBV recipients

To investigate these issues further, we characterized the cytokine profile elaborated in response to stimulation with the TT830-843 peptide. Preliminary experiments determined that lymphokine levels in the culture supernatants of both normal subjects and chronically infected HBV patients peaked after 3 days (data not shown). Based on these results, PBMC from normal subjects and chronically infected Theradigm-HBV recipients were stimulated for 3 days with TT830-843 peptide, culture supernatants were then collected, and cytokine secretion was measured by ELISA. Only patients who demonstrated a positive proliferative response were selected for this study because preliminary experiments indicated the patients who failed to mount a proliferative response also failed to secrete any of the cytokines under study (data not shown). Normal HTL responders were also tested as a basis of comparison.

Very similar levels of IL-2 secretion were observed in both populations (Fig. 2). In contrast, secretion of the Th1-associated cytokines IL-12 and IFN-γ were considerably reduced in comparison to normal subjects (Fig. 2). Secretion of the Th2-associated cytokine IL-5 appeared to be elevated, although not significantly (Fig. 2). Similar trends were observed when supernatants were collected at either earlier or later time points, suggesting that the differences were not simply due to an alteration in the kinetics of lymphokine production (data not shown).

These results demonstrate that HTL responses of chronically infected individuals are associated with an apparent decrease in the production of certain Th1-associated cytokines. These results are of note because the TT830-843 HTL epitope is not derived from HBV. As such, the T cell response induced by this epitope would not be expected to be affected by HBV-related, Ag-specific T cell tolerance.

TABLE I. Correlation between helper and CTL responses

<table>
<thead>
<tr>
<th>HTL Response (mean SI ≥10)</th>
<th>CTL Response (mean &gt;2.8 LU)</th>
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<tbody>
<tr>
<td>+</td>
<td>53</td>
</tr>
<tr>
<td>-</td>
<td>13</td>
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</table>

* p = 0.0001 by exact Fisher test.

FIGURE 2. Cytokine profile of PBMCs from HBV patients stimulated with TT830-843. PBMC from normal and chronically infected Theradigm-HBV recipients in the proliferation responders (mean SI > 10), were stimulated in vitro for 3 days with the TT830-843 peptide. Levels of selected cytokine in culture supernatants were determined using standard ELISA assays.

Decreased preimmunization proliferative responses to the recall Ag TT are associated with hyporesponsiveness to Theradigm-HBV immunization

Next, we examined whether these differences were limited to responses to the Theradigm-HBV immunogen or if a more general alteration in the HTL function might be detectable. To this end, we examined the proliferative responses of PBMC derived from normal subjects and HBV patients following stimulation with TT protein. We found that in comparison to PBMC from normal subjects, PBMC from chronically infected HBV patients proliferate less frequently to TT stimulation (Table II). In addition, the magnitude of the proliferative responses also appeared to be somewhat reduced. Taken together, these results suggested a general alteration of HTL immune function in chronically infected HBV patients.

The correlation between these observations and hyporesponsiveness to Theradigm-HBV immunization was also examined. When prevaccination proliferative responses to whole TT were retrospectively analyzed, a significant correlation between prevaccination proliferative responses and the outcome of Theradigm-HBV immunization was revealed (Table III). Taken together, these data suggest that a pre-existing nonspecific alteration of HTL function maybe related to the hyporesponsiveness of chronically infected HBV patients to Theradigm-HBV immunization.

Imbalance of cytokine responses in chronically infected HBV patients following mitogenic stimulation

Similar experiments were performed using PHA as a mitogenic stimulus. PBMC derived from HBV patients exhibited the same altered cytokine profile following 3 days of PHA stimulation as

TABLE II. Comparison of recall TT responses in normal subjects and chronically infected HBV patients

<table>
<thead>
<tr>
<th></th>
<th>Normal Subjects</th>
<th>GBV Patients</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT responder frequency</td>
<td>29/39 (74%)</td>
<td>37/83 (45%)</td>
<td>0.0017</td>
</tr>
<tr>
<td>Magnitude of TT response</td>
<td>90.7 ± 1.7^</td>
<td>65.4 ± 18.4</td>
<td>0.32</td>
</tr>
</tbody>
</table>

* Determined by exact Fisher test.
^ Frequency of individuals with mean SI ≥ 10.
^ SI ± SD.
observed following TT830-843 stimulation (Fig. 3). While IL-2 secretion from PBMC of chronically infected individuals was similar that observed from PBMC of normal subjects, IL-12 and IFN-γ secretion were markedly reduced. In addition, a significant increase in secretion of IL-5 was also detected. To determine whether the altered pattern cytokine secretion was generally related to liver disease, similar experiments were performed using PBMC isolated from HCV patients. The altered cytokine pattern characterized in HBV patients was not evident in HCV patients. In contrast to HBV patients, HCV patients secreted normal amounts of IL-5 and IL-12 (data not shown). These data confirm that chronic HBV infection is associated with an altered balance in the secretion of Th1/Th2 lymphokines. Furthermore, these data demonstrate that this phenomenon is not limited to Theradigm-HBV-specific responses but extends to the bulk of the various T cells responsive to PHA stimulation.

**HTL activity of lipopeptides in HBV transgenic mice.**

The data presented above suggest that the use of a more potent HTL epitope might lead to increased effectiveness of lipopeptides, especially in regard to their capacity to overcome T cell tolerance at the CTL level. To examine the relative immunogenicity of constructs containing the different HTL epitopes, we used the HBV transgenic mouse strain, 1.3.32. These mice are transgenic for the entire HBV genome, express high levels of viral Ags, and have been shown to be an useful animal model for studying the role the immune response plays in controlling HBV infection (4–7). These mice were immunized with lipopeptides that contained the dominant L4-restricted HBV surface 28–39 CTL epitope (20) and one of two different HTL epitopes. One construct incorporated the naturally occurring OVA323-336 HTL epitope (18). The other construct incorporated the nonnatural PADRE HTL epitope, which has been optimized for HTL function. PADRE is a synthetic HTL epitope engineered by introducing anchor residues for different DR motifs into a polyalanine backbone. The resulting peptide binds a variety of DR molecules as well as certain mouse class II alleles, including I-Ab, I-Eα, and I-Eβ (18).

As shown in Fig. 4, the PADRE-containing lipopeptide elicited more vigorous responses in terms of proliferation (Fig. 4A) and IL-2 secretion (Fig. 4B) than did the lipopeptide containing the OVA-derived HTL epitope. Little difference was noted in comparing the IL-2 production and proliferative responses obtained with 1.3.32 mice and control mice.

In 1.3.32 mice immunized with the lipopeptide containing the OVA-derived HTL epitope, the level of IFN-γ secretion was somewhat reduced in comparison to control mice immunized with the same construct (Fig. 4C). In contrast, immunization with the PADRE-containing lipopeptide induced higher levels of IFN-γ release in both strains of mice. PADRE was in fact found to induce higher levels of IFN-γ secretion in cultures derived from 1.3.32 transgenic mice than in cultures from control mice (Fig. 4C). In conclusion, these results suggest that the PADRE lipopeptide induced a more vigorous and characteristically Th1-like (as judged from IFN-γ release) response than the lipopeptide incorporating the conventional murine OVA323-336 HTL epitope.

**Lipopeptides incorporating the PADRE HTL epitope break tolerance at the CTL level**

Next, the CTL responses to HBV surface 28-39 peptide (HBVenv28) induced in either 1.3.32 mice or control mice by injection with the OVA323-336 or PADRE-containing lipopeptides were evaluated. The results are shown in Fig. 5. In the case of the OVA323-336-containing lipopeptide, 90% (9 of 10) of the normal mice responded with an average CTL response of 10.3 LU. As expected, 1.3.32 mice responded poorly, likely because of tolerance at the CTL level. Specifically, only 30% (3 of 10) of the 1.3.32 mice responded with an average CTL response of 4.3 LU. More vigorous responses were induced using the lipopeptide construct incorporating the PADRE HTL epitope. All of the control mice (10 of 10) immunized with this construct responded with average CTL responses of 34.8 LU. More strikingly, vigorous responses were also seen in 1.3.32 mice; 93% (13 of 14) of the 1.3.32 mice exhibited CTL responses averaging 28 LU. Furthermore, the CTL induced in 1.3.32 mice using the PADRE-containing construct were capable of recognizing P815 cells that endogenously express the HBsAg (data not shown). These results demonstrate that PADRE constructs can be used to break tolerance at the CTL level.

**Discussion**

We have compared the HTL responses in normal subjects vs chronically infected HBV patients, following treatment with the experimental therapeutic vaccine, Theradigm-HBV. We found that in both cohorts a vigorous and sustained HTL response was crucial for the development of a good CTL response. In the same series of experiments, a generalized alteration of HTL function was revealed in chronically infected individuals, as demonstrated by altered cytokine profiles and decreased responses to TT. The decreased responses to TT correlated with hyporesponsiveness to Theradigm-HBV vaccination. Further studies in HBV transgenic mice demonstrated that lipopeptides incorporating an immunodominant mouse L4-restricted CTL epitope and the potent HTL epitope, PADRE, could overcome CTL tolerance.

**Table III. Baseline TT responses predict the outcome of Theradigm-HBV immunization**

<table>
<thead>
<tr>
<th>HTL Response: TT (time 0: SI ≥ 10)</th>
<th>CTL Response (mean &gt;2.8 LU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>43% 9/22</td>
</tr>
<tr>
<td>−</td>
<td>15% 5/33*</td>
</tr>
</tbody>
</table>

* p = 0.0215 by exact Fisher test.
The present data has at least two important implications: 1) with regard to the role of HTL in support of CTL generation as well as for the pathogenesis of chronic HBV infection, and 2) in regards to the development of therapeutic vaccines for chronic viral infection and cancer.

The data presented here illustrate a generalized and previously unappreciated alteration in HTL function associated with chronic HBV infection. Secretion of the Th1-associated lymphokines IFN-γ and IL-12 appears to be impaired in favor of increased production of the Th0/Th2-associated lymphokine IL-5. This qualitative alteration of HTL responses does not appear to be Ag specific as it is detected after mitogenic stimulation of PBMC. Several immunological parameters were normal including proliferation, IL-2 secretion, and CD4/CD8 ratios and the recall CTL response to HBV infection. Secretion of the Th1-associated lymphokines IFN-γ and IL-12 appears to be impaired in favor of increased production of the Th0/Th2-associated lymphokine IL-5. This qualitative alteration of HTL responses does not appear to be Ag specific as it is detected after mitogenic stimulation of PBMC. Several immunological parameters were normal including proliferation, IL-2 secretion, and CD4/CD8 ratios and the recall CTL response to HBV infection. Secretion of the Th1-associated lymphokines IFN-γ and IL-12 appears to be impaired in favor of increased production of the Th0/Th2-associated lymphokine IL-5. This qualitative alteration of HTL responses does not appear to be Ag specific as it is detected after mitogenic stimulation of PBMC. Several immunological parameters were normal including proliferation, IL-2 secretion, and CD4/CD8 ratios and the recall CTL response to HBV infection. Secretion of the Th1-associated lymphokines IFN-γ and IL-12 appears to be impaired in favor of increased production of the Th0/Th2-associated lymphokine IL-5. This qualitative alteration of HTL responses does not appear to be Ag specific as it is detected after mitogenic stimulation of PBMC. Several immunological parameters were normal including proliferation, IL-2 secretion, and CD4/CD8 ratios and the recall CTL response to HBV infection.

FIGURE 4. HTL activity of lipopeptides in HBV transgenic mice. A, Proliferative responses of control B6D2F1 mice (hatched bars) and 1.3.32 HBV transgenic mice (shaded bars) following immunization with lipopeptides containing the mouse Ld-restricted HBV surface 28–39 CTL epitope and either the OVA232-336 or PADRE HTL epitopes. Ag-specific T cell proliferation for mouse cells is presented as the net incorporation of [3H]TdR (Δcpm); this is defined as the incorporation of [3H]TdR in the presence of Ag minus the incorporation of [3H]TdR in the absence of Ag. Average responses from four individual B6D2F1 or 1.3.32 transgenic mice are presented. B and C, The IL-2 and IFN-γ secretion observed following stimulation of splenocytes with either OVA323-336 or PADRE for 3 days in vitro, respectively, is shown. Cytokine secretion was determined using standard ELISA assays for quantitative measurement of biologically active mouse IL-2 and IFN-γ. Assays were conducted using pooled culture supernatants.

FIGURE 5. Influence of the helper epitope on the immunogenicity of lipopeptides in HBV transgenic mice. Control B6D2F1 mice or 1.3.32 HBV transgenic mice were injected with 10 μg of lipidated construct dissolved in DMSO/PBS. Eleven days after immunization, animals were sacrificed and splenocytes were stimulated with 10 μg/ml CTL peptide. Following 6 days in culture, CTL activity was measured using P815 cells loaded with the HBV surface 28–39 peptide. The magnitude of the CTL responses is expressed as the geometric mean of positive cultures.
the dominant influenza matrix peptide 58–66 (data not shown), consistent with the lack of clinical signs of general immune suppression in patients affected by chronic HBV infection.

Our data are consistent with studies that analyzed HBV-specific HTL function (21). Bertolletti and colleagues reported that in chronic HBV infection the majority of the liver infiltrating T cells are of Th0 type and are capable of secreting both IFN-γ and IL-5. In contrast, in acute self-limiting hepatitis T cells appear to be predominantly of the Th1 type (22).

High levels of circulating viral Ags might favor the induction of Ag-specific tolerance and/or shifts in the balance of Th1/Th2 responses. Differential engagement of Th1 vs Th2 responses have been addressed in studies by Milich (23), which suggest that Th1 and Th2 subsets of T cells might differ in their sensitivity to tolerance induction (24–26). Dendritic cells can be conditioned by IL-10 to prime preferentially for Th2 responses (27–30). Therefore, a bias toward the Th2 phenotype could be initially established in HBV-specific responses as a result of high levels of circulating Ags but eventually lead to a nonspecific Th2 bias resulting from altered APC function (31). This hypothesis would be consistent with the important role IL-12 plays in the clearance of HBV infection (32). Alterations of the HTL response described herein might not be limited to chronic HBV infection and could be associated with other chronic viral diseases and cancer (33–35), for which alterations of the immune responsiveness have also been described.

The experiments performed in HBV transgenic mice demonstrate that immunization with lipopeptides containing the mouse L4-restricted HBV surface 28–39 CTL epitope, and a potent HTL epitope can break tolerance at the level of HBV-specific CTL responses. This data should be interpreted with the knowledge that the transgenic mice may only incompletely mimic the disease pathogenesis in humans. HBV transgenic mice do not display the altered lymphokine pattern in response to mitogen that we observed in infected humans, and the CTL specificities elicited in transgenic mice are low affinity and consequently have limited histological impact (data not shown). Despite these caveats, the data nonetheless suggest that use of potent T cell help that elicits a more markedly Th1-type lymphokine profile may play an important role in overcoming nonresponsiveness at the CTL level.

Finally, the results presented in this report have implications in terms of the further development of immunotherapy for chronic HBV infection. Based on our results, the combination of Thera-
digm-HBV with other nonspecific immunostimulants such as HBV infection. Based on our results, the combination of Theradigm-HBV to the clinic. In addition, we thank Diana Pack and Mara Capella for assistance in preparation of the manuscript and Ramakrishnan Vadi for technical assistance.

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