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# Activation of a Heterogeneous Hepatitis B (HB) Core and e Antigen-Specific CD4$^+$ T-Cell Population during Seroconversion to Anti-HBe and Anti-HBs in Hepatitis B Virus Infection†

M.-C. JUNG,[1,2]* H. M. DIEPOLDER,[1] U. SPENGLER,[1,2] E. A. WIERENGA,[3] R. ZACHOVAL,[2] R. M. HOFFMANN,[2] D. EICHENLAUB,[4] G. FRÖSNER,[5] H. WILL,[6] AND G. R. PAPE[1,2]

*Institute for Immunology[1] and Max von Pettenkofer-Institut, Institute of Hygiene and Microbiology,[5] University of Munich, 80336 Munich, Department of Internal Medicine II, Klinikum Grosshadern, University of Munich, 81377 Munich,[2] Department of Internal Medicine, Hospital Schwabing, 80803 Munich,[4] and Pette Institut, University of Hamburg, 20251 Hamburg,[6] Germany, and Laboratory of Cell Biology and Histology, University of Amsterdam, The Netherlands[3]*

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**Overcoming hepatitis B virus infection essentially depends on the appropriate immune response of the infected host. Among the hepatitis B virus antigens, the core (HBcAg) and e (HBeAg) proteins appear highly immunogenic and induce important lymphocyte effector functions. In order to investigate the importance of HBcAg/HBeAg-specific T lymphocytes in patients with acute and chronic hepatitis B and to identify immunodominant epitopes within the HBcAg/HBeAg, CD4$^+$ T-cell responses to hepatitis B virus-encoded HBcAg and HBcAg/HBeAg-derived peptides were studied in 49 patients with acute and 39 patients with chronic hepatitis B. The results show a frequent antigen-specific CD4$^+$ T-cell activation during acute hepatitis B infection, a rare HBcAg/HBeAg-specific CD4$^+$ T-cell response among HBeAg$^+$ chronic carriers, and no response in patients with anti-HBe$^+$ chronic hepatitis. An increasing CD4$^+$ T-cell response to HBcAg/HBeAg coincides with loss of HBeAg and hepatitis B virus surface antigen (HBsAg). Functional analysis of peptide-specific CD4$^+$ T-cell clones revealed a heterogeneous population with respect to lymphokine production. Epitope mapping within the HBcAg/HBeAg peptide defined amino acids (aa) 1 to 25 and aa 61 to 85, irrespective of the HLA haplotype, as the predominant CD4$^+$ T-cell recognition sites. Other important sequences could be identified in the amino-terminal part of the protein, aa 21 to 45, aa 41 to 65, and aa 81 to 105. The immunodominant epitopes are expressed in both proteins, HBcAg and HBeAg. Our findings lead to the conclusion that activation of CD4$^+$ T lymphocytes by HBcAg/HBeAg is a prerequisite for viral elimination, and further studies have to focus on the question of how to enhance or induce this type of T-cell response in chronic carriers. The immunodominant viral sequences identified may have relevance to synthetic vaccine design and to the use of peptide T-cell sites as immunotherapeutic agents in chronic infection.**

The clinical consequences of hepatitis B virus (HBV) infection are extremely variable, including clinical syndromes such as fulminant, acute, and chronic hepatitis, hepatocellular carcinoma, and the asymptomatic carrier state. The underlying mechanisms which are responsible for the diversity of clinical syndromes are poorly understood. Summarizing the results obtained so far, it has been suggested that variations in the immune response to HBV infection account for the different outcomes of infection (9, 19, 28). Thus, it is anticipated that the study of immune responses to HBV-encoded proteins will increase our understanding of immune-mediated viral clearance mechanisms and immunopathology during HBV infection.

Immune defense against virus infection and/or immunopathology involves both nonspecific and antigen-specific phases, with recovery from most primary infections thought to be dependent on classical CD8$^+$ cytotoxic T lymphocytes and CD4$^+$ helper T lymphocytes, which respond to viral antigens. The mechanisms by which these effector cells resolve viral infection remain controversial; however, there is circumstantial support for direct cytolysis as well as evidence for secreted antiviral factors produced by CD4$^+$ and CD8$^+$ T lymphocytes.

For an appropriate response, it is necessary that the T-cell receptor recognize a bimolecular ligand composed of a processed viral immunogenic peptide bound to a major histocompatibility complex (MHC) molecule on the surface of an antigen-presenting cell or target cell. The amino acid sequence and structure of the processed peptide mainly determine the contact to both structures, the MHC molecule and the T-cell receptor, which is a prerequisite for lymphocyte activation and function (6). The importance of the amino acid sequence of the viral peptide for T-lymphocyte activation sheds new light on the consequences of viral mutations. Extensive studies in mice and a limited analysis in humans have shown that among the HBV antigens, the nucleocapsid proteins core antigen (HBcAg) and e antigen (HBeAg) play a predominant role as inducer antigens of T helper (Th) cell function and targets for cytotoxic T lymphocytes (1, 8, 10, 13).

The c gene encodes 183 to 185 amino acid residues of a nucleocapsid protein and is preceded by the precore (pre-C) region, starting with an initiation codon and encoding 29 amino acid residues. The nonparticulate form of HBcAg, identified as HBeAg, is lacking 34 residues at the carboxy terminus and contains an additional N-terminal precore sequence of 29 residues (reviewed in reference 14). Although HBcAg and HBeAg show substantial amino acid sequence homology, they are serologically distinct, and the immune responses to these antigens appear to be regulated independently. Despite the

* Corresponding author. Mailing address: Medizinische Klinik II, Klinikum Grosshadern, Marchioninistrasse 15, 83177 Munich 70, Germany. Phone: 498970952222. Fax: 49897004418.

† Dedicated to G. Riethmüller on the occasion of his 60th birthday.

fact that HBcAg is an internal component of the virus, anti-HBc antibodies are produced by virtually 100% of HBV-infected patients. In contrast, antibodies to HBeAg may not develop at all or may appear at various times after the appearance of anti-HBc and are frequently correlated with viral clearance and remission of liver disease during chronic infection. The occurrence of HBV surface antigen (HBsAg) antibodies (antienvelope) indicates viral elimination. Antibody production to viral proteins is T-cell dependent with the exception of antibodies to HBcAg (20). Studies in mice indicate that the Th cell response to HBcAg/HBeAg supports the production of HBc and HBe antibodies as well as antienvelope antibodies that are virus neutralizing (21).

In addition, Th lymphocytes may mediate viral clearance through direct hepatocyte lysis and through lymphokine production. Depending on distinct lymphokine profiles, $CD4^+$ T lymphocytes have been divided into Th subsets (2, 22). The Th1 subset produces gamma interferon (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor (TNF) and promotes cell-mediated effector responses, whereas Th2 lymphocytes produce IL-4, IL-5, IL-6, and IL-10, cytokines which influence B-cell development and can augment humoral responses. The biological relevance of these T-cell subpopulations has been demonstrated in several experimental models of infectious diseases, including AIDS, in which Th1 cells are believed to give rise to immunoprotection while Th2 cells indicate progressive disease (4). Their role in HBV infection has yet to be investigated.

Since HBcAg/HBeAg-specific Th lymphocytes have been discussed to induce B- and T-cell functions which are important for viral elimination, remission, and acute exacerbation of liver disease, we examine in this study the Th lymphocyte response to HBcAg/HBeAg in patients with acute and chronic hepatitis B with respect to differences between acute and chronic infection and to the correlation of the T-cell response with serological changes. To investigate the role of these lymphocytes in patients infected with precore mutants unable to produce HBeAg and often suffering a severe disease, patients with precore mutant infection were also included in this study. With regard to vaccines and T-cell therapy, immunogenic epitopes within the HBcAg/HBeAg and epitopes not cross-reacting with HBcAg were identified by using peptides derived from HBcAg/HBeAg. The functional capacity of HBcAg/HBeAg-specific $CD4^+$ T lymphocytes was assayed by analyzing the lymphokine profile of HBcAg/HBeAg-specific $CD4^+$ T lymphocyte clones derived from a patient with acute hepatitis B during seroconversion to anti-HBe.

## MATERIALS AND METHODS

**Patients.** Peripheral blood mononuclear cells (PBMC) from patients with acute ($n = 49$) and chronic ($n = 39$) HBsAg-positive hepatitis were included in this study. Diagnosis of HBV infection was based on determination of viral antigens and antibodies with commercially available kits (Abott Laboratories). Acutely infected patients presented with the typical picture of acute hepatitis with high serum transaminases and high titers of anti-HBc immunoglobulin M (IgM). Chronic hepatitis was confirmed by the clinical course and by histological and biochemical findings. Because of an infection with a precore mutant among the chronic carriers, five patients were HBeAg negative and HBe antibody positive but HBV DNA positive.

**Antigens.** Recombinant HBcAg (rHBcAg; Biogen, Geneva, Switzerland) was obtained from bacterial extracts of *Escherichia coli* K-12 strain HB101 harboring an expression plasmid carrying the HBc coding gene as described elsewhere (23). HBe- and HBc-specific peptides (Fig. 1), as predicted from cloned viral DNA of proven infectivity, were chemically synthesized (Multiple Peptide Systems, San Diego, Calif.) and were more than 90% pure as determined by high-pressure liquid chromatography (data not shown).

**Cells, culture conditions, and establishment of specific clones.** PBMC were isolated on Ficoll-Isopaque gradients (Pharmacia, Uppsala, Sweden) as described before (3). The interphase cells were suspended in RPMI 1640 medium

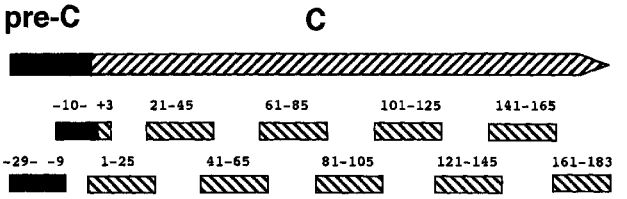


FIG. 1. HBcAg/HBeAg-derived synthetic peptides used in this study. Solid bars indicate protein sequences expressed exclusively on HBeAg. Position 1, ATG of the core.

(GIBCO, Grand Island, N.Y.) supplemented with 2% L-glutamine, 1% penicillin/streptomycin, and 10% human AB serum. For the establishment of peptide-specific T-cell clones, $5 \times 10^6$ PBMC from an acutely infected patient were cultured for 8 days in the presence of a peptide composed of aa 61 to 85 (peptide aa 61–85; 10 μg/ml), expanded for 2 days in the presence of IL-2 (20 U/ml), and subsequently cloned (150 cells) by limiting dilution (0.3 cell per well) in the presence of phytohemagglutinin (PHA, 2 μg/ml). Thus, 37 clones were generated, of which 21 turned out to be peptide specific. Throughout the study, stimulation of cells was done with virus-specific HBcAg at a concentration of 1 mg/ml. Peptides were used at a concentration of 10 μg/ml.

**Proliferation assays.** Unfractionated PBMC ($10^5$ per well) were incubated in 96-well plates for 6 days in the presence of rHBcAg or synthetic peptides in 150 μl of RPMI 1640 medium–10% AB serum. The assay cultures were pulsed for 16 h with 2 μCi of [$^3$H]thymidine per well (specific activity, 80 μCi/mmol; Radiochemical Centre, Amersham, United Kingdom). The cells were collected and washed on filters (Dunn, Asbach, Germany) with a Skatron LKB harvester, and the amount of radiolabel incorporated into DNA was estimated with a beta counter (Beckmann LS 1801). Triplicate cultures were routinely assayed, and the results represent mean counts per minute (cpm) or stimulation indices. Stimulation indices were calculated by dividing the mean cpm in the antigen-stimulated cultures by that in cultures without antigen.

**Phenotypic analysis of peptide-specific clones.** Phenotypic analysis of T-cell clones was done by incubating the T cells for 60 min at 4°C with a labeled antibody (anti-CD45 [Coulter, T200-RD1], anti-CD14 [MT42-fluorescein isothiocyanate (FITC) conjugate; E. Rieber, Institute for Immunology], anti-CD3 [MT301-FITC; Dr. Rieber], anti-CD4 [CD4-Tricolor; Medac], anti-CD8 [CD8-Tricolor; Medac], anti-CD16 [LeuUC-PE; Becton Dickinson], and anti-CD56 [NKH-1-RD1; Coulter]). The cells were fixed in 1% paraformaldehyde, and staining was analyzed with a FACScan (Becton Dickinson).

**HLA class II restriction of peptide-specific clones.** T-cell clones were stimulated in the presence of the specific peptide aa 61–85 and in parallel with the peptide and 10 μl of anti-DR, anti-DP, or anti-DQ antibody. The antibodies were purchased from Becton Dickinson (Hamburg, Germany). Proliferation assays were done as described above.

To further confirm human leukocyte antigen (HLA) class II restriction of the antigen-specific clones, proliferation assays were done with irradiated partially HLA-matched B lymphoblastoid cell lines as antigen-presenting cells.

**Preparation of supernatants from peptide-specific clones.** Peptide aa 61–85-specific T-cell clones ($2 \times 10^5$ cells per 200 μl) were stimulated with a combination of anti-CD3, anti-CD2, and soluble affinity-purified anti-CD28 monoclonal antibodies (MAbs). Stimulation was performed in RPMI 1640–10% inactivated fetal calf serum at 37°C in a humidified atmosphere containing 5% $CO_2$. Cell supernatants were collected after 24 h and stored in aliquots at −80°C.

**Cytokine assays.** Cytokine assays were done as follows. Secretion of IL-4 (24), IL-5 (18), and IFN-γ (30) was measured by sandwich enzyme-linked immunosorbent assay (ELISA) techniques, as described elsewhere. IL-10 was determined in an ELISA kit provided by Cytoscreen (Biosource International, Camarillo, Calif.).

**Characterization of HBV DNA.** Five hundred microliters of serum was incubated with 0.5% sodium dodecyl sulfate (SDS)–10 mM Tris-HCl (pH 7.5)–10 mM EDTA–10 mg of proteinase K per ml at 37°C overnight. The serum DNA was extracted twice with phenol-chloroform and precipitated with ethanol in the presence of 30 μg of tRNA. The pellet was dissolved in 100 μl of 10 mM Tris-HCl–1 mM EDTA. The HBV DNA sequence was amplified with oligonucleotide primers located upstream of the pre-C initiation codon (5′-GTCAAC GACCGACCTTGAGGC-3′) and downstream of the C gene stop codon (5′-CCCACCTTATGAGTCCAAGG-3′). Symmetric and asymmetric amplifications were performed as previously described (21). The amplified DNA was directly sequenced by the dideoxynucleotide chain termination method with 5′-$^{32}$P-labeled primers and a dideoxy sequencing kit (U.S. Biochemicals, Denver, Colo.).

## RESULTS

**Comparison of T-cell reactivity between patients with acute and chronic hepatitis.** Single measurements for cell samples

| | aa -29 -9 | -10 +3 | 1- 25 | 21- 45 | 41- 65 | 61- 85 | 81- 105 | 101- 125 | 121- 145 | 141- 165 | 161- 183 | HBcAg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1,4 | 1,4 | 0,8 | 0,8 | 0.7 | 3,9 | 1,6 | 1,2 | 1,4 | 1,1 | 1,1 | 3,6 |
| 2 | 0,8 | 1 | 0,9 | 0,8 | 1 | 0,9 | 0,9 | 1,1 | 0.9 | 3 | 1,2 | 3,6 |
| 3 | 1,2 | 1,2 | 3,2 | 1 | 1 | 4,9 | 1,3 | 1,5 | 4 | 0,9 | 1 | 8,2 |
| 4 | 1,3 | 1,4 | 1 | 1,7 | 1,5 | 5,4 | 5,8 | 7,2 | 3,7 | 3 | 1 | 10,3 |
| 5 | n.t. | n.t. | 4,8 | 10,1 | 4,1 | 1,5 | 7 | 1,9 | 2,6 | 1,1 | 0,9 | 14,7 |
| 6 | 1,9 | 0,7 | 8,1 | 19,7 | 4,6 | 7 | 38 | 28 | 2,5 | 19,7 | 6,8 | 35,3 |
| 7 | 1,2 | 1,5 | 6,9 | 0,8 | 1,8 | 4,5 | 4,6 | 2,4 | 1 | 1,1 | 1,9 | 5,4 |
| 8 | 5,2 | 3 | 5,7 | 7,3 | 5,8 | 2,9 | 4,5 | 2,6 | 1,9 | 1,6 | 0,5 | 7,6 |
| 9 | n.t. | n.t. | 4,7 | 5,5 | 1,5 | 13,3 | 3 | 3 | 1,7 | 0,8 | 1,3 | 5,7 |
| 10 | 1 | 0,8 | 20 | 1,4 | 4,5 | 16,8 | 1,6 | 2 | 1,4 | 2,7 | 0,4 | 22,7 |
| 11 | 0,5 | 1,1 | 8,4 | 2 | 1,2 | 3,2 | 5 | 1,8 | 0,7 | 3,7 | 1,8 | 4,7 |
| 12 | 1,3 | 1,1 | 2,7 | 1 | 1,9 | 0,6 | 3,5 | 1,3 | 0,6 | 0,7 | 0,7 | 5,2 |
| 13 | 1,2 | 1 | 7,8 | 1,4 | 4,3 | 2,1 | 2,9 | 2,7 | 1,5 | 1 | 0,7 | 5,8 |
| 14 | 1,4 | 1,3 | 2 | 13,5 | 10,5 | 4,7 | 2,2 | 1,5 | 2,4 | 0,7 | 0,7 | 4,7 |
| 15 | 0,9 | 1 | 1,1 | 3,3 | 2,1 | 3,9 | 2,2 | 1,6 | 5,1 | 1 | o,8 | 5,5 |
| 16 | 0,8 | 0,9 | 1,8 | 1,1 | 1,1 | 6,8 | 3,6 | 3 | 1,9 | 1,1 | 1,8 | 3,6 |
| 17 | n.t | n.t. | 0,7 | 2,9 | 2,4 | 0,7 | 3,7 | n.t | 0,8 | 3 | 6,9 | 32 |
| 18 | n.t. | n.t. | 1,6 | 1,4 | 3,4 | 4,1 | 1,8 | 2,4 | 3 | 0,9 | 0,7 | 6,8 |
| 19 | 1 | 0,9 | 4,4 | 3,1 | 1,1 | 1,1 | 1,2 | 1,3 | 0,6 | 1 | 0,7 | 3,5 |
| 20 | n.t. | n.t. | 1,9 | 6,2 | 5,9 | 3,6 | 2 | 1,1 | n.t. | 0,8 | 0,7 | 4 |
| 21 | 1,4 | 0,6 | 0,8 | n.t. | n.t. | 10 | 1,1 | 1,4 | 4,8 | 1,5 | 1 | 9 |
| 22 | n.t. | n.t. | 11 | 1,3 | 1,7 | 8 | 1,7 | n.t. | 2,2 | 0,7 | 0,6 | 14 |
| 23* | n.t. | n.t. | 3,6 | 3,3 | 1,6 | 1,2 | 2,2 | 1,3 | 1,7 | 0,4 | 0,7 | 4,1 |
| 24* | 2 | 1,3 | 2,7 | 1,1 | 2,4 | 1,5 | 1,4 | 1,2 | 4,6 | 1,8 | 1,4 | 3,4 |
| 25* | 3,4 | 4,8 | 4,5 | 1,4 | 1,5 | 1,4 | 2 | 1,9 | 1,3 | 2,3 | 1,6 | 11 |
| 26* | n.t. | n.t. | 1,4 | 1,6 | 0,7 | 2 | 5,6 | 1.9 | 1,1 | 2 | 1 | 31 |
| 27* | 4,4 | 7,9 | 7,2 | 13 | 16 | 12 | 12 | 8,9 | 7,9 | 3,5 | 2 | 6 |

FIG. 2. Response of peripheral blood lymphocytes to HBcAg and to HBcAg/HBeAg-derived peptides in 22 patients with acute hepatitis B and five patients with chronic hepatitis B. PBMC ($10^5$) were cultured for 5 days in the presence of the respective antigen, and activation was measured as [$^3$H]thymidine incorporation. Results are expressed as the stimulation index, which is the ratio between mean cpm in the presence of antigen and cpm in the absence of antigen. Stimulation indices higher than 3 were considered significant and are indicated as stippled squares. *, chronically infected patients. n.t., not tested.

from 49 acutely and 39 chronically infected individuals revealed that 22 (45%) from patients with acute hepatitis B and 5 (13%) from patients with chronic hepatitis B recognized HBcAg and HBcAg/HBeAg-derived peptides. However, repeated tests during acute infection revealed a significant response in 90% of patients with acute hepatitis. Repeated testing did not increase the percentage of responders among chronically infected individuals. The fine specificity of HBV nucleocapsid recognition by $CD4^+$ T lymphocytes is similar in acute and chronic hepatitis patients (Fig. 2).

**HBcAg/HBeAg-specific T-cell responses in patients infected with precore mutants.** Among the chronically infected patients who were included in the study, five patients were infected with precore mutants unable to secrete HBeAg, as demonstrated by direct sequencing of HBV DNA. The infecting virus in all five patients had a point mutation in the distal pre-C region, converting the TGG codon at nucleotides (nt) 3177 to 3179 into a TAG stop codon. Since the majority of investigators argue that a T-cell response to HBcAg/HBeAg might represent the pressure of the immune system responsible for the occurrence of precore mutants, we investigated the HBcAg/HBeAg-specific $CD4^+$ T-cell response in these patients. The proliferative response of PBMC from three patients was measured once, whereas two of them could be followed up for at least 4 months. Activation of $CD4^+$ T lymphocytes by HBcAg and HBcAg/HBeAg-derived peptides was not demonstrable in any of the five patients. As a positive control, the peripheral blood lymphocytes responded adequately to stimulation with PHA (Fig. 3).

**T-cell proliferative responses to peptides: identification of immunogenic epitopes within the HBc/HBe protein.** To define immunogenic epitopes within HBcAg/HBeAg, the proliferation of peripheral blood lymphocytes derived from patients with acute hepatitis ($n$ = 49) and chronic hepatitis ($n$ = 38) after stimulation with protein-derived peptides (Fig. 2) was tested. The results shown in Fig. 2 refer to patients who re-

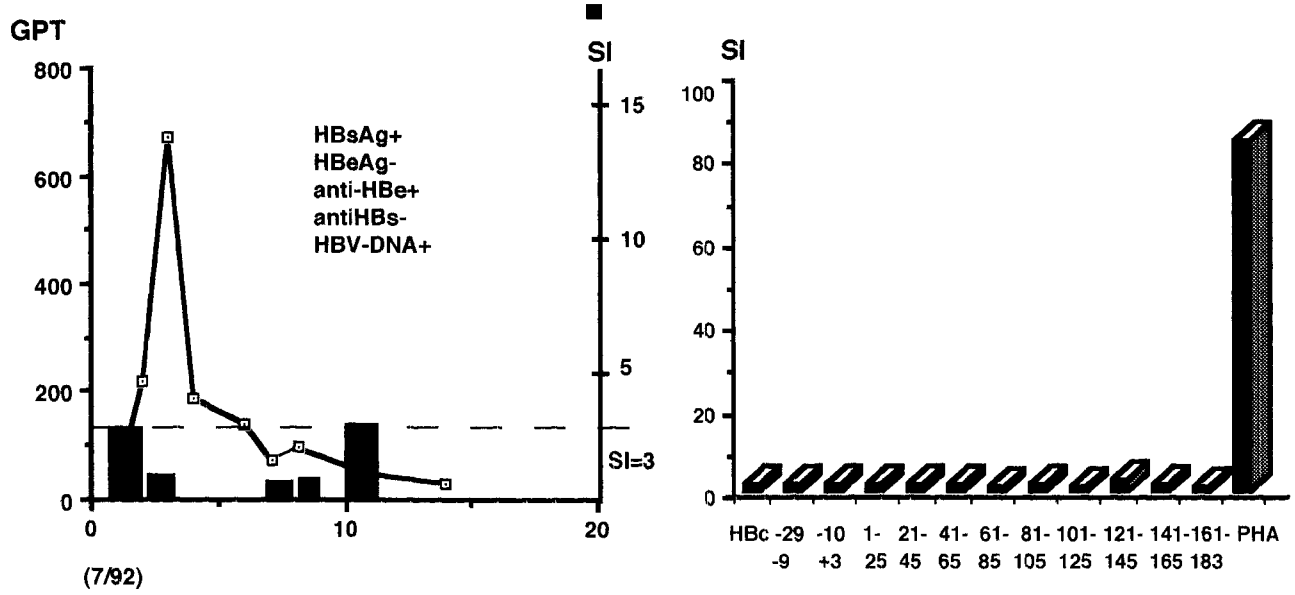


FIG. 3. Data for a patient infected chronically with an HBV precore stop mutant. PBMC were repeatedly tested during active disease for proliferation upon stimulation with HBcAg and HBcAg/HBeAg-derived peptides. Solid bars on the left side indicate stimulation indices (SI) of PBMC after stimulation with HBcAg. The SI was never significantly elevated, that is, >3. The right side demonstrates the proliferation results of PBMC from the same patient to HBcAg and HBcAg/HBeAg-derived peptides 7 months after infection as a representative example. Note that none of the peptides induces a specific response, but the lymphocytes responded adequately to nonspecific stimulation with PHA.

sponded to HBcAg and at least one of the peptides. Examination of the fine specificity of T-cell recognition of HBcAg/HBeAg showed that HBcAg/HBeAg-specific T cells from 27 patients recognized multiple but distinct sites within the HBcAg/HBeAg sequence. Cells from all patients showed proliferative responses to one or more T-cell epitopes (Fig. 2), and each epitope was recognized by cells from at least two patients. Peptide aa 61–85 was most frequently recognized (59% of the patients). Other important sequences could be identified in the amino-terminal part of the protein, aa 1 to 25 (51%), aa 21 to 45 (37%), aa 41 to 65 (33%), and aa 81 to 105 (40%) (Fig. 2). The carboxy-terminal part of the protein contains sequences that are immunogenic for only a few patients; aa 101 to 125 is recognized by 11%, aa 121 to 145 by 22%, aa 141 to 165 by 11%, and aa 161 to 183 by 7.4% of the patients (Fig. 2). Except for aa 161 to 183 and a part of aa 141 to 165, all epitopes described are shared by HBcAg and HBeAg.

**Association of the HBcAg/HBeAg-specific T-cell response with serological changes during acute HBV infection.** To approach the function of the HBcAg/HBeAg-specific $CD4^+$ T lymphocyte response, we looked for the association of T-cell reactivity with serological changes during acute hepatitis B infection by consecutive testing. Patients who lost HBeAg and/or developed anti-HBe and/or anti-HBs were selected, and their T-cell response to HBcAg/HBeAg-derived peptides was analyzed.

We concentrated on the question of seroconversion to anti-HBe because the occurrence of these antibodies, at least in the majority of patients in our country, correlates with loss of viral replication and remission of liver disease. Figure 4 shows that the loss of HBeAg and/or the emergence of HBe antibodies was in every case associated with an increasing T-cell response to the whole core protein and peptides. Since core- and HBe-specific B cells are distinct populations in vivo, we studied T-cell help for anti-HBc antibody production in parallel by quantitative determination of anti-HBc titers in serum. Figure 4 shows that the new appearance of anti-HBe antibodies was accompanied by a slight increase in anti-HBc antibodies. Long-term observation (Fig. 4 and 5) in patients with acute hepatitis B demonstrates that T-cell activation in response to HBc/HBe antigens is a transient phenomenon which is not present at the very beginning of the disease and is lost after clearance of the virus. We could also monitor the HBcAg/HBeAg-specific T-cell response during seroconversion to anti-HBs in two patients. In these patients (Fig. 5), loss of HBsAg or appearance of anti-HBs was associated with a strong T-cell response to HBcAg/HBeAg-derived peptides.

**Immunogenicity of protein sequences expressed exclusively on HBeAg.** The amino acid sequence of the unprocessed HBe protein is almost identical to that of HBcAg. The only difference is that 29 additional amino acids of the so-called pre-C sequence precede the HBc protein sequence. To determine whether human T lymphocytes are activated by protein sequences expressed exclusively on HBeAg and not expressed on HBcAg, we asked whether the additional N-terminal residues of HBeAg contain T-cell sites which stimulate human T lymphocytes.

We therefore tested the stimulatory effect of peptides carrying aa −29 to −9 and −10 to +3 in 40 patients with acute hepatitis and 31 patients with chronic hepatitis. As summarized in Fig. 2, only two of the chronically and one of the acutely infected patients responded significantly to these peptides. Note that the two responding carriers are a mother and her son. To exclude the possibility that the time of testing was responsible for the rare answer, we tested some patients consecutively, even during the phase of seroconversion to anti-HBe. Figure 4 shows that even in the phase of seroconversion, lymphocytes which specifically recognize aa −29 to +1 were not demonstrable, suggesting that protein sequences shared by HBcAg and HBeAg are the main activators for $CD4^+$ T lymphocytes. We also used the peptide in different concentrations (Fig. 4), which did not influence the results.

**Establishment of peptide-specific T-cell clones.** Since HBcAg/HBeAg-specific T-cell activation could be associated with the loss of HBeAg and HBsAg as well as the production of HBe and HBs antibodies, T-cell clones were produced from a patient with acute hepatitis B infection for functional analysis of this T-cell reaction (patient 16 in Fig. 2). PBMC were stimulated with the specific peptide (aa 61 to 85) for 8 days, consecutively expanded with IL-2 for 2 days, and then cloned by the limiting dilution technique in the presence of PHA. Using this technique, we obtained 37 clones, of which 21 turned out to specifically recognize peptide aa 61–85 (Table 1). All T-cell clones could be restimulated with HBcAg or the peptide.

**Phenotypic analysis of HBc/HBe peptide-specific T-cell clones.** To characterize the T-cell surface antigens of HBc/HBe-specific T-cell clones mediating certain functions, T-cell clones were incubated with CD45, CD3, CD4, CD8, CD14, CD16, and CD56 MAbs, and staining was analyzed in a FACScan. All T-cell clones expressed CD45, CD3, and CD4. They were negative for CD8, CD14, and CD16 and thus do not express markers characteristic of classical cytotoxic T cells, natural killer cells (CD16), or monocytes and B cells (CD14). CD56 was expressed by one clone.

**HLA class II restriction of peptide-specific T-cell clones.** The T-cell clones were established from a patient with the following HLA type: A26, A28, B13, B27, Cw1, DR7, DRw13, DQw1, DQw2. As could be shown in blocking experiments with antibodies against HLA-DR, HLA-DQ, and HLA-DP (Fig. 6), recognition of specific peptide aa 61–85 by five specific $CD4^+$ clones was diminished in the presence of anti-DR. DR restriction could also be demonstrated more directly by using HLA-DR-matched Epstein-Barr virus blasts as antigen-presenting cells in the proliferation tests (Fig. 6).

**Lymphokine profiles of peptide-specific clones.** The cytokine secretion profiles of seven HBc/HBe-specific T-cell clones selected from a patient with acute hepatitis were investigated after stimulation with a mixture of anti-CD2, anti-CD3, and anti-CD28. The reactivity of the T-cell clones was confirmed by strong proliferative responses in all cases (data not shown). It turns out that HBc/HBe-specific clones, recognizing the same epitope, produce different lymphokine profiles (Table 2). Some of them produced substantial amounts of IL-4 or IL-5 but no IFN-γ, and others secrete IFN-γ alone or in combination with IL-4 or IL-5. IL-10 was determined in the supernatants of three clones. Two of them produced more than 250 pg/ml, whereas IL-10 was not detectable in the supernatant of the other clone.

**Determination of the viral sequence.** The serum-derived HBV DNA from five patients with anti-$HBe^+$ chronic hepatitis and from the patient whose $CD4^+$ T lymphocytes were cloned was sequenced. As expected, all patients with anti-$HBe^+$ and HBeAg-negative hepatitis were infected with so-called precore mutants. Because of a stop codon mutation (TGG to TAG) at nt 3177 to 3179, these mutants are unable to produce HBeAg.

The patient from whom we generated antigen-specific clones was infected with a virus which showed no major mutations such as stop codons, frameshifts, or start codon mutations. However, the sequence which is recognized by the clones (aa 61 to 85) demonstrated one amino acid exchange at position 74 (V to N).

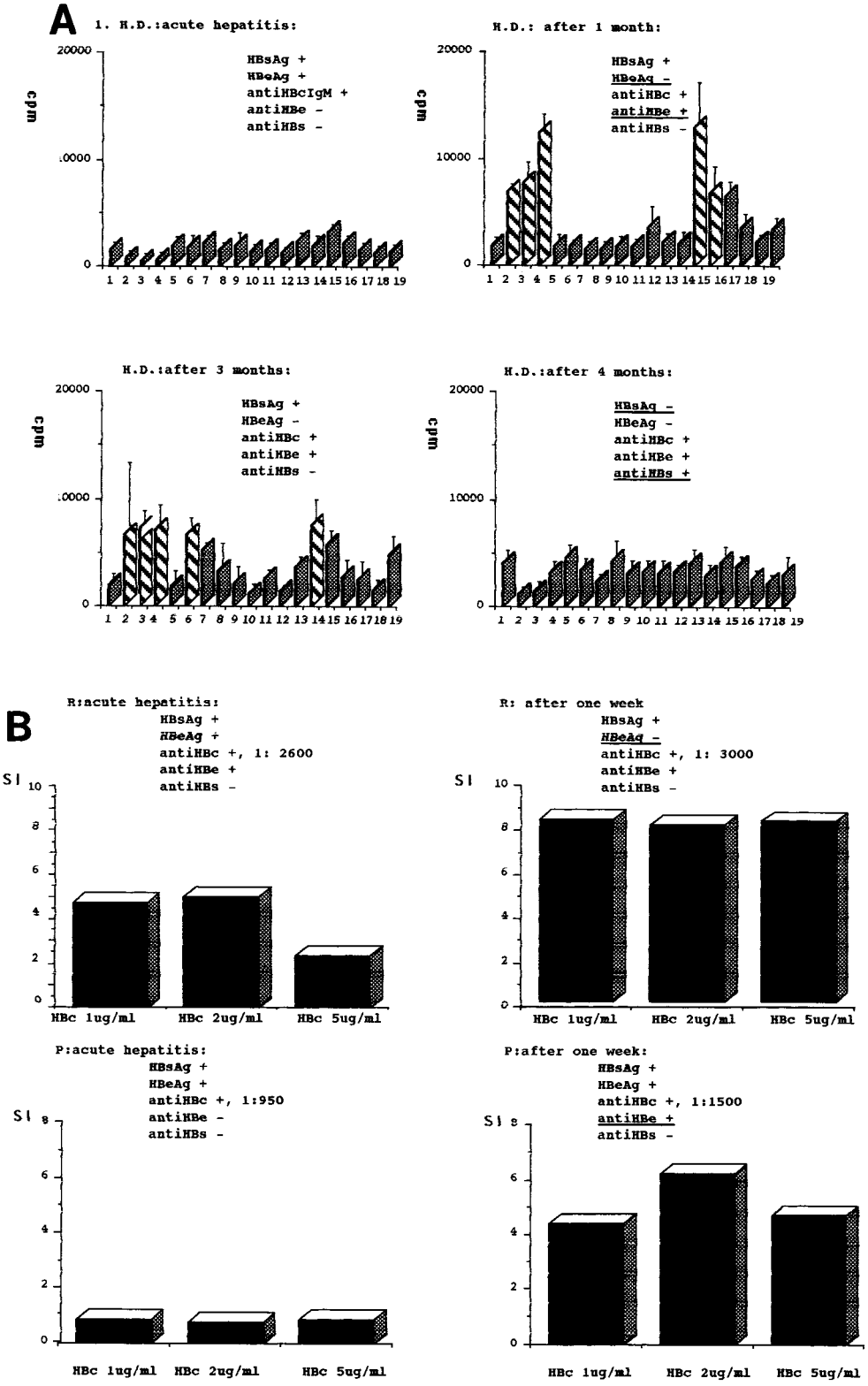


FIG. 4. HBcAg/HBeAg-specific T-cell activation in the course of acute HBV infection in three different patients with acute hepatitis B. (A) Peripheral blood lymphocytes from a patient with acute hepatitis B were cultured for 5 days in the presence of HBcAg or HBcAg/HBeAg-derived peptides. The cultures were pulsed for 16 h with 2 μCi of [$^3$H]thymidine. Increased T-cell proliferation, as indicated by the hatched bars, was seen during seroconversion to anti-HBe. Results are presented as cpm + standard deviation (SD). 1, medium; 2, 3, and 4, HBcAg at 1, 2, and 5 μg/ml, respectively; 5, 6, and 7, aa −29 to −9 at 1, 5, and 10 μg/ml, respectively; 8, 9, and 10, aa −10 to +3 at 1, 5, and 10 μg/ml, respectively; 11, aa 1 to 25 (10 μg/ml); 12, aa 21 to 45 (10 μg/ml); 13, aa 41 to 65 (10 μg/ml); 14, aa 61 to 85 (10 μg/ml); 15, aa 81 to 105 (10 μg/ml); 16, aa 101 to 125 (10 μg/ml); 17, aa 121 to 145 (10 μg/ml); 18, aa 141 to 165 (10 μg/ml); 19, aa 161 to 183 (10 μg/ml). (B) Determination of anti-HBc titer was done in parallel. Although the antigen-specific T-cell response is significantly enhanced when these patients eliminate HBeAg or seroconvert to anti-HBe, the anti-HBc titer increases only slightly. SI, stimulation index.

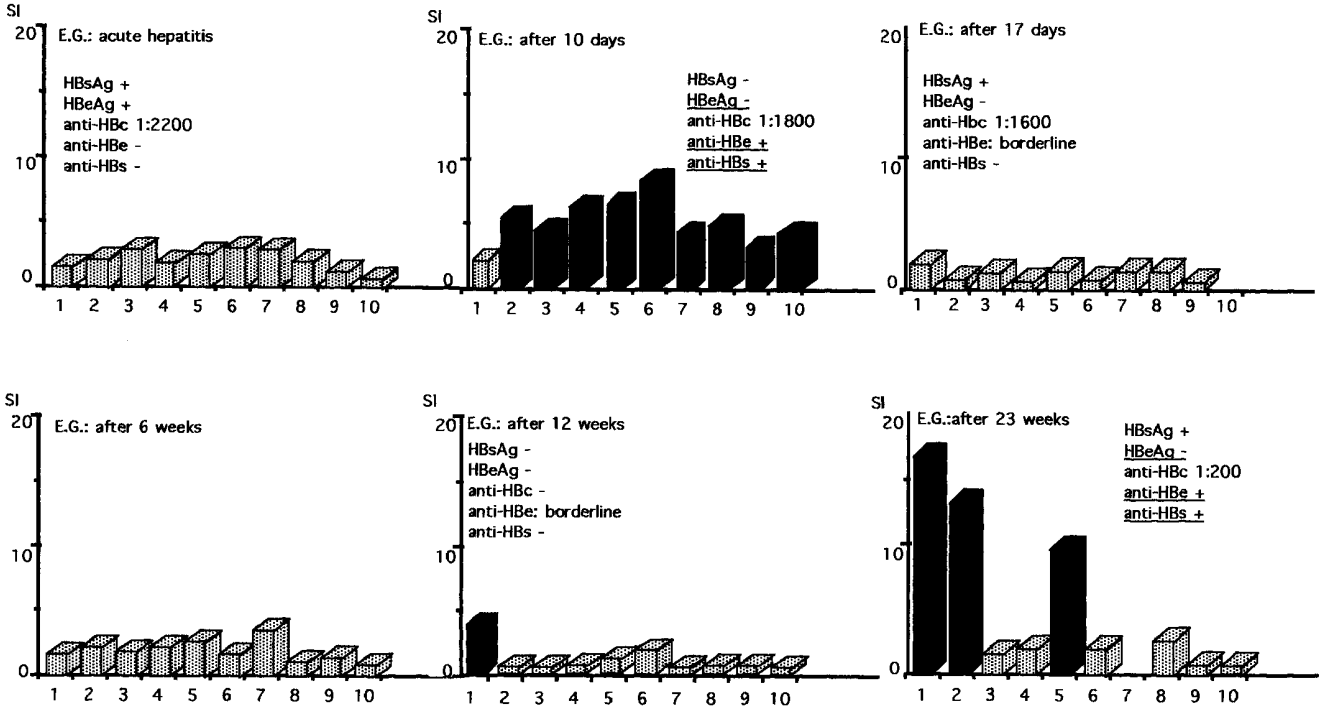


FIG. 5. HBcAg/HBeAg-specific proliferative response of peripheral blood lymphocytes from two patients in the course of acute hepatitis B. An increased HBcAg/HBeAg T-cell activation is temporarily associated with loss of HBeAg and HBsAg. It is noteworthy to mention that in the first panel, the anti-HBc titer decreases while the HBcAg/HBeAg-specific T-cell response is enhanced. 1, HBcAg (1 μg/ml); 2, aa 1 to 25; 3, aa 21 to 45; 4, aa 41 to 65; 5, aa 61 to 85; 6, aa 81 to 105; 7, aa 101 to 125; 8, aa 121 to 145; 9, aa 141 to 165; 10, aa 161 to 185. Peptides 2 through 10 were used at 10 μg/ml.

TABLE 1. HBcAg/HBeAg-specific proliferation of CD4+ clones derived from a patient with acute hepatitis B[a]

| Clone no. | Proliferation (mean cpm ± SD) | |
|---|---|---|
| | Medium (control) | Peptide aa 61–85 |
| 1 | 28,226 ± 2,742 | 152,432 ± 9,079 |
| 2 | 7,156 ± 1,528 | 162,586 ± 12,407 |
| 3 | 17,818 ± 5,851 | 228,180 ± 14,966 |
| 4 | 63,328 ± 10,832 | 984,907 ± 30,377 |
| 5 | 11,093 ± 4,530 | 194,724 ± 16,767 |
| 6 | 152,594 ± 427 | 239,452 ± 41,039 |
| 7 | 24,686 ± 6,508 | 93,133 ± 3,444 |
| 8 | 85,932 ± 10,497 | 272,401 ± 14,572 |
| 9 | 49,821 ± 7,526 | 452,021 ± 35,225 |
| 10 | 18,331 ± 2,841 | 120,838 ± 20,957 |
| 11 | 21,131 ± 2,262 | 155,859 ± 22,251 |
| 12 | 6,963 ± 2,457 | 213,700 ± 11,482 |
| 13 | 27,150 ± 6,272 | 359,735 ± 26,780 |
| 14 | 19,205 ± 3,174 | 181,723 ± 19,519 |
| 15 | 3,720 ± 1,115 | 150,094 ± 13,060 |
| 16 | 41,102 ± 29,694 | 450,340 ± 26,971 |
| 17 | 58,802 ± 20,288 | 429,146 ± 24,426 |
| 18 | 9,116 ± 1,147 | 77,561 ± 9,040 |
| 19 | 27,501 ± 2,036 | 189,120 ± 27,252 |
| 20 | 27,871 ± 4,955 | 159,072 ± 34,977 |
| 21 | 6,927 ± 648 | 26,449 ± 2,215 |

[a] PBMC were stimulated with specific peptide aa 61–85 for 10 days, consecutively expanded with IL-2 for 2 days, and then cloned by the limiting dilution technique in the presence of PHA. Using this technique, we obtained 37 clones, of which 21 turned out to specifically recognize peptide aa 61–85.

## DISCUSSION

Since CD4+ T lymphocytes are centrally involved in the majority of immunological host defense mechanisms against viral infection, it was the aim of this study to precisely characterize the virus-specific CD4+ T-lymphocyte response in patients with HBV infection. There were several reasons to investigate the immune response of CD4+ T lymphocytes to HBcAg/HBeAg.

(i) HBcAg/HBeAg-specific Th lymphocytes have been shown to provide help for anti-HBe and anti-HBs antibody production (21) and are believed to support the generation of HBcAg/HBeAg-specific cytotoxic T lymphocytes. Since HBcAg and HBeAg share the majority of their amino acids (12) and T lymphocytes do not recognize native proteins but processed viral immunogenic peptides (26), it is likely that both proteins, HBcAg and HBeAg, induce T-cell help and are cross-reactive at the T-cell level (7).

(ii) Because of their immunogenicity, HBcAg/HBeAg have been proposed as an alternative HBV vaccine and therapeutic tool to enhance the virus-specific immune response during chronic infection. For that purpose, immunogenic epitopes are of major interest and have to be identified.

(iii) The T-cell-directed immune response to HBcAg/HBeAg may contribute to multiple genetic alterations in the precore/core gene, which result in the abrogation of HBeAg synthesis.

We therefore analyzed the CD4+ T lymphocyte response to HBcAg/HBeAg and peptides in 49 patients with acute and 39 patients with chronic hepatitis B. Assessment of the T-cell response at single time points detected HBcAg/HBeAg-specific T-cell reactivity in almost 50% of acutely infected patients and in 13% of the chronically infected patients. Serial testing in both patient groups revealed specific responses in 90% of

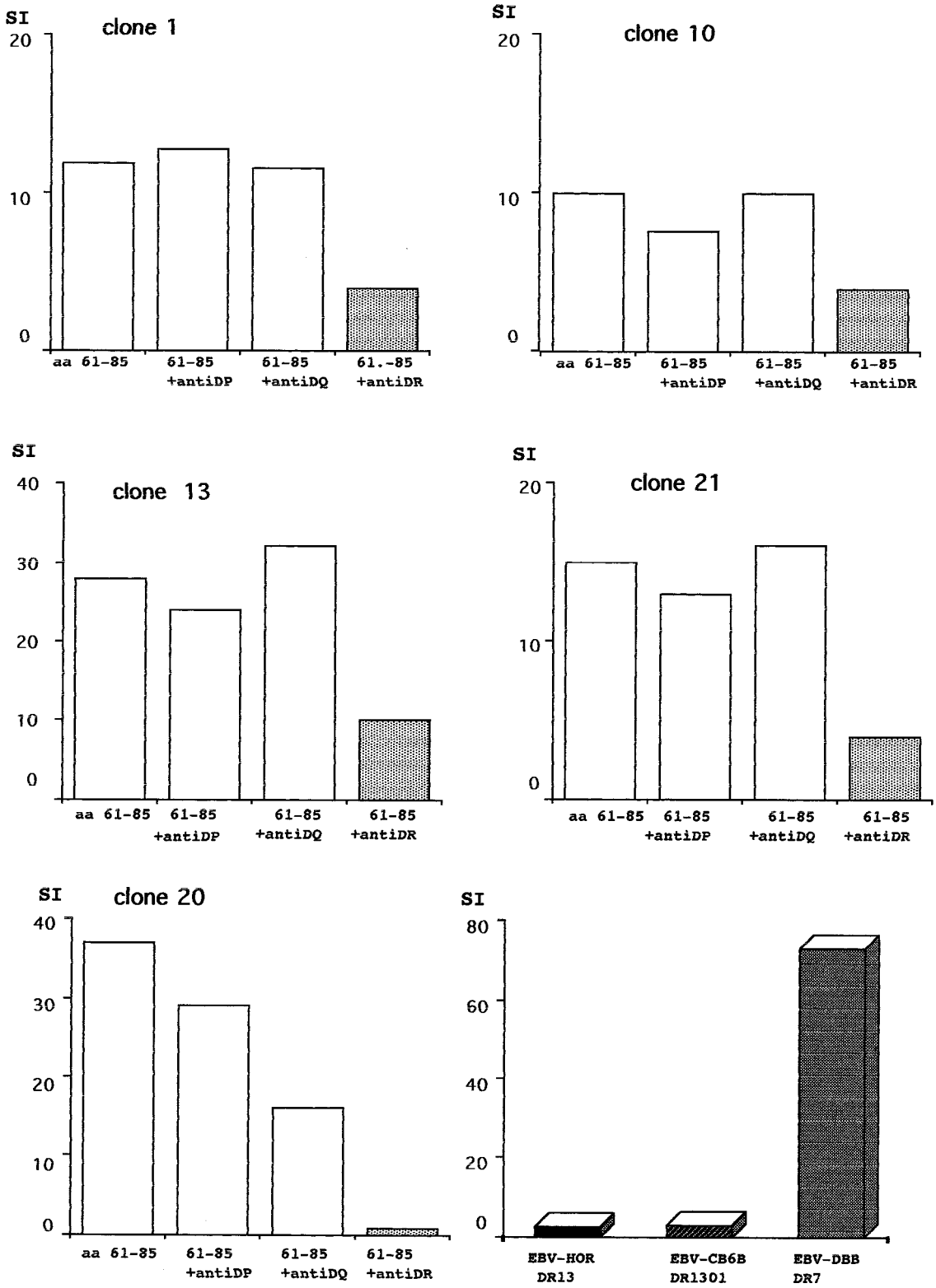


FIG. 6. HLA class II restriction of peptide-specific $CD4^+$ T-cell clones. Proliferation assays with five different T-cell clones specific for HBcAg/HBeAg peptide aa 61–85 were done in the presence of either peptide alone or peptide with the respective HLA class II antibody (anti-DR, anti-DP, or anti-DO; 10 μl). The antigen-specific response was impressively blocked by the addition of anti-DR antibodies. The last panel on the right side shows, as a representative example, DR restriction more directly. Clone 20 was stimulated with specific peptide aa 61–85 in the presence of HLA-matched Epstein-Barr virus (EBV) blasts expressing the patient's HLA-DR, either DR7 or DR13. Clone 20 responded to the specific antigen only in the presence of HLA-DR7.

TABLE 2. Lymphokine profiles of peptide-specific clones[a]

| Clone no. | IFN-γ (ng/ml) | IL-2 (U/ml) | IL-4 (ng/ml) | IL-5 (ng/ml) | IL-10 (pg/ml) |
|---|---|---|---|---|---|
| 1 | 0.5 | 0.9 | 0.08 | — | |
| 5 | 3.9 | | 0.3 | 11 | |
| 7 | 1.8 | <0.1 | 1.9 | 1.4 | |
| 9 | 1.3 | 9.7 | 0.3 | — | >250 |
| 10 | — | 0.9 | 5.4 | 3.4 | >250 |
| 20 | — | | 0.1 | 9 | — |
| 21 | 1.2 | | 1.1 | — | |

[a] $CD4^+$ clone cells ($2 \times 10^5$), which specifically recognize peptide aa 61–85, were stimulated with a mixture of CD3, CD2, and CD28 antibodies. After 24 h, cytokine secretion in the supernatants was determined. Detection levels of the assays: IL-5, >3,000 pg/ml; IL-4, >200 pg/ml; IFN-γ, >100 pg/ml; IL-10, >5 pg/ml.

acutely infected patients. A strong T-cell response to HBcAg/HBeAg in acutely infected patients and a weak and rare T-cell response in chronically infected individuals have been described before by an Italian group (8) and by our group (13). It has been discussed that one reason for the absence of antigen-specific T lymphocytes in the periphery of chronically infected patients might be the result of a redistribution of those cells in the liver. In the meantime, Tsai et al. demonstrated HBcAg/HBeAg-specific T-cell proliferations in peripheral blood derived from chronically infected patients (29). However, these T-cell responses to HBcAg/HBeAg corresponded to acute exacerbations of chronic type B hepatitis and HBeAg seroconversion, whereas none of our patients with chronic hepatitis B infection who were either $HBeAg^+$ or anti-$HBe^+$ because of an infection with a precore mutant had an exacerbation period or seroconverted to anti-HBe during the observation period. Several explanations for the absence of detectable peripheral HBcAg/HBeAg recognition in patients with chronic hepatitis B are possible. A small number of specific peripheral T lymphocytes, anergy of T lymphocytes, and inefficient antigen presentation or lymphokine production by the antigen-presenting cell are among the possibilities. Although only a few chronically infected patients responded to HBcAg-derived peptides, the data obtained suggest that the weak T-cell response in chronic HBV infection is not necessarily explained by the inability to generate T lymphocytes specific for immunogenic HBcAg/HBeAg determinants: The fine specificity of HBV nucleocapsid recognition by $CD4^+$ T cells seems not to differ impressively in acute and chronic patients (patient 27 in Fig. 2). Whatever explains the rare and inefficient T-cell response to HBcAg/HBeAg during chronic infection, it may contribute to the chronic carrier state, because this T-cell response is required for an effective antiviral immune response. One mechanism by which IFN-α seems to mediate its effect during treatment is the enhancement of the HBcAg/HBeAg-specific $CD4^+$ T lymphocyte response, which corresponds to the "flare up" of transaminases and induces HBe seroconversion (5). By observing and investigating chronically infected patients during IFN treatment, we demonstrated that those patients who responded to IFN-α treatment showed significant HBcAg/HBeAg-specific T-cell proliferation, whereas those who did not respond to antiviral treatment did not show any T-cell response to HBcAg/HBeAg (Fig. 7).

Interestingly, another group (8) recently failed to detect nucleocapsid-specific $CD8^+$ T lymphocytes in chronic HBV carriers, the generation of which is dependent on the presence of $CD4^+$ T lymphocytes. Since virus-specific $CD8^+$ T lymphocytes are needed to lyse virus-infected cells, this observation further supports the pivotal function of antigen-specific $CD4^+$ T lymphocytes as well. The importance of the HBcAg/HBeAg-specific $CD4^+$ T lymphocytes in HBV infection in acute B hepatitis has been shown by our data. The present analysis revealed significant temporal correlations between the activation of $CD4^+$ T lymphocytes by HBcAg/HBeAg and the seroconversion phase to anti-HBe and anti-HBs in acutely infected patients, implying that the response of HBcAg/HBeAg-specific $CD4^+$ T lymphocytes is necessary for viral elimination. We agree with the results reported by Ferrari et al. (8), who demonstrated an increased T-cell response to the nucleocapsid proteins during clearance of the HBV envelope antigens from the serum but could not find a correlation between HBcAg/HBeAg-specific T-cell responses and seroconversion to anti-HBe. The results of our study extend these data by showing that activation of HBcAg/HBeAg-specific T lymphocytes supports anti-HBe production. Anti-HBc titers have been determined in parallel to investigate the contribution of HBcAg/HBeAg-specific T lymphocytes to anti-HBc production. The data show that an increased antigen-specific activation of $CD4^+$ T lymphocytes may correspond with a slight increase in the anti-HBc titer.

Thus, HBcAg/HBeAg-primed $CD4^+$ T lymphocytes are believed to competently help to activate B cells specific for HBeAg, HBcAg, and HBsAg. The ability of HBcAg/HBeAg-specific T lymphocytes to prime antibody production to HBsAg determinants has been demonstrated in mice (21). This phenomenon might be explained by the hypothesis that anti-HBs-producing B cells, after specific uptake of viral particles, simultaneously present immunogenic peptides derived from several viral proteins, including HBcAg. These B lymphocytes could thus stimulate and receive help from HBcAg/HBeAg-specific $CD4^+$ T lymphocytes. The occurrence of HBcAg-specific T-cell activation during clearance of HBV envelope antigens supports this hypothesis and may also explain why vaccination with core particles has been reported to confer protection against challenge by HBV (11). Although they have not directly examined HBc/HBeAg-specific T cells, Maruyama et al. (16) concluded from the correlation between antienvelope antibody production, HBeAg, and HBV DNA levels that HBcAg/HBeAg-specific Th cells in humans mediate antienvelope production. From these observations, it is tempting to speculate that T-cell activation by HBcAg/HBeAg is necessary for viral elimination and potentially useful for vaccination.

To further analyze the details of this antigen-specific reaction, the antigenic sites involved in T-cell response have been mapped by using synthetic peptides. Analysis of the $CD4^+$ T-cell responses in acutely and chronically infected patients revealed immunogenic epitopes within the overlapping sequence of HBeAg and HBcAg. Two synthetic peptides corresponding to residues 1 to 25 and 61 to 85 were found to activate HBcAg/HBeAg-primed T cells in more than 50% of the patients irrespective of their HLA haplotypes, suggesting that these peptides bind to different HLA class II molecules. Using different peptides, Ferrari et al. (7) identified aa 50 to 69, 117 to 131, and 1 to 20 as highly immunogenic. The finding that aa 1 to 20 represents an important region within HBcAg/HBeAg was confirmed by our results with peptide aa 1–25, which is obviously able to bind to several different HLA molecules. Interestingly, this protein region partly overlaps an HLA-A2-restricted T-cell epitope for cytotoxic T lymphocytes (aa 18 to 27) (1). Because the majority of peptides used in both studies were different and therefore not comparable, we could neither confirm nor exclude the immunogenicity of certain regions. Using the immunogenic peptides aa 50–69 and aa 117–131, which have been demonstrated to be immunodominant (7), we

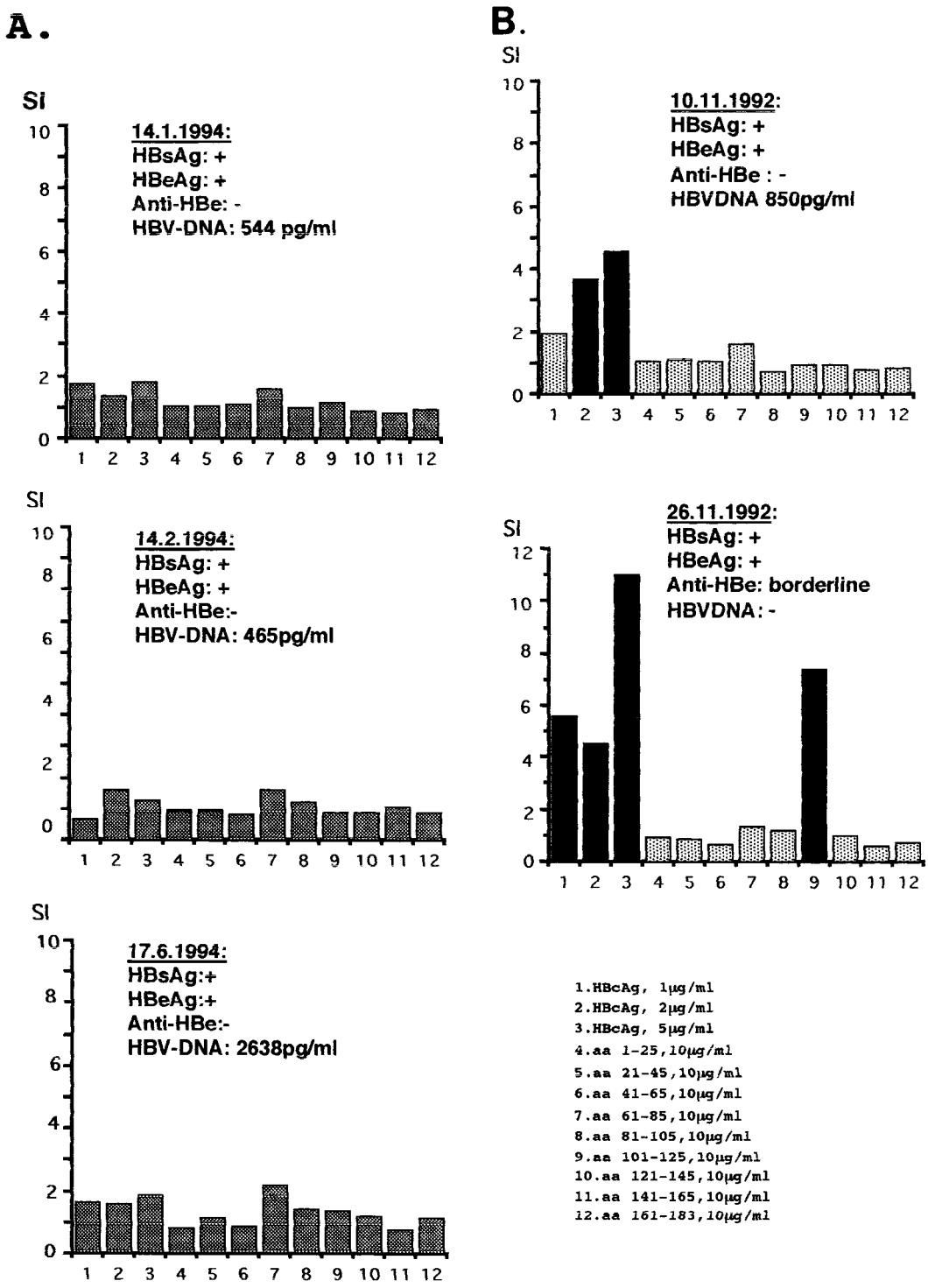


FIG. 7. HBcAg/HBeAg-specific T-cell response of two chronically infected patients. For patient A, treatment with IFN-α from December 1993 to June 1994 was ineffective, whereas patient B, treated from July 1992 to October 1992, responded to antiviral treatment by the loss of HBV DNA and development of anti-HBe antibodies. Responsiveness to treatment was associated with a significant T-cell response to the nucleocapsid of HBV. Dates are shown as day.month.year.

found recognition of aa residues 50 to 69 in 75% and recognition of aa 117 to 131 in 40% of our acutely infected patients (data not shown). This confirms the strong immunogenicity of aa residues 50 to 69 and supports their use in a vaccine, whereas peptide aa 117–131 is, at least in our patients, less frequently stimulatory than previously described. However, comparison of the two studies (reference 7 and the present investigation) clearly shows that by using different peptides, we could identify additional important immunogenic regions within the protein. T-cell responses to the core peptides have

always been associated with a response to the whole core protein.

As mentioned above, the fine specificity of HBV nucleocapsid recognition by $CD4^+$ T cells seems similar in acutely and chronically infected patients. Analyzing the T-cell activation by peptides over weeks or months revealed that $CD4^+$ T lymphocytes from a single patient recognize different peptides at different time points. This is of particular interest, since it has been suggested that the amino acid sequence of the bound peptide mainly affects T-cell function (6, 15). Thus, it is possible that different peptides at different time points during infection could induce selective T-cell effector functions. For the use of viral peptides as therapeutic tools in the future, detailed knowledge of the structure and function of appropriate viral peptides binding to well-defined HLA class II molecules and T-cell receptors is required. Since it is known that even single amino acid changes within a peptide may alter binding to the HLA molecule and/or the T-cell receptor, the epitopes identified in single patients provide a solid basis for functional analysis of mutated viral peptides.

The epitope mapping within HBcAg/HBeAg sought to determine whether $CD4^+$ T lymphocytes can distinguish between HBcAg and HBeAg. Cells from the majority of our patients recognized peptides which are expressed on both antigens, HBcAg and HBeAg. Peptides covering the first 29 aa of the precore gene, which are contained within HBeAg and not within HBcAg, only occasionally induced T-cell proliferation. The same is true for aa 161 to 183, which form the carboxy terminus of HBcAg and are not present in HBeAg. This confirms that HBcAg and HBeAg are mainly cross-reactive at the T-cell level, as already described for the murine system. Thus, the frequent emergence of HBV precore mutants is not explained by the action of T lymphocytes which recognize epitopes expressed exclusively on HBeAg. However, this does not exclude the possibility that T-cell responses to other regions within HBcAg contribute to elimination of HBeAg synthesis. We believe that the major reason for the emergence of HBV precore mutants is that the absence of HBeAg production as an immune target confers a biological advantage to the virus as it encounters the immune response in infected hosts. This view is supported by data presented in this study that patients with chronic anti-$HBe^+$ hepatitis infected with precore mutants did not respond to HBcAg/HBeAg and derived peptides.

If HBcAg/HBeAg-specific Th cells play a predominant immunoregulatory role in the immune response to multiple HBV antigens, it follows that their functional capacities have to be investigated. In an initial approach, we established core peptide-specific clones from an acutely infected patient during seroconversion to anti-HBe and determined their lymphokine profile. As expected, these T lymphocytes were HLA class II restricted, $CD4^+$ positive T-cell clones. Analysis of their lymphokine profiles revealed that the peptide-specific clones produced both Th1- and Th2-like lymphokines. These results fit with the assumption that both Th subsets secreting the appropriate lymphokine profiles are necessary for viral elimination. The Th1 subset produces IFN-$\gamma$, IL-2, and tumor necrosis factor and promotes cell-mediated effector responses (cytotoxicity, proliferation, and upregulation of MHC molecules through interferon production), whereas Th2 lymphocytes produce IL-4, IL-5, IL-6, and IL-10, lymphokines which influence B-cell development and augment humoral responses. The important role of $CD4^+$ Th cell subsets has been suggested for other viral diseases, such as human immunodeficiency virus infection. It has been demonstrated that lymphokines of the Th1 subset support antiviral activity, whereas a preponderance of Th2 $CD4^-$ T lymphocytes promotes viral disease (4). For HBV infection, the importance of $CD4^+$ Th subsets remains unknown. Maruyama et al. proposed in their recent publication the engagement of HBeAg-specific Th subsets in different patient groups (17). The serological profile and absence of liver disease in asymptomatic chronic carriers would be consistent with an exclusive Th2-like response, whereas immune-mediated cytotoxic responses which occur in acute and chronic active hepatitis suggest a Th1-like or a combined Th1- and Th2-like response. They further reported observations in the murine system which indicate that certain HBeAg-specific T-cell site MHC combinations preferentially elicit either a Th1-like or a Th2-like response. With regard to different epitopes recognized by $CD4^+$ T lymphocytes from the same patient, this observation leads to speculations about the different lymphokine profiles induced by different viral proteins. Our preliminary analysis of lymphokines secreted by $CD4^+$ T lymphocytes specific for aa 61 to 85 of HBcAg/HBeAg does not give a clear picture with regard to a Th1 or Th2 lymphokine profile. This, however, does not exclude the possibility that other peptides or smaller peptides of the same region in context with the restricting HLA class II molecule induce the production of selective T-cell lymphokines. These results are encouraging and ask for further studies focusing on antigen-specific Th cell functions, which are mainly influenced by the presence of Th1- or Th2-promoting cytokines at the time of antigen priming but probably also by the structure of the activating viral peptide, the presenting HLA class II molecule, and the T-cell receptor (27).

In summary, analysis of HBcAg/HBeAg-specific $CD4^+$ T lymphocytes during HBV infection indicates a pivotal role of these lymphocytes during hepatitis B infection, i.e., increasing T-cell responses to HBcAg and HBeAg during seroconversion to anti-HBe and anti-HBs in acute viral disease. This finding implies that HBcAg/HBeAg-specific T cells are essential for viral elimination. Comparison of T-cell activity between acutely and chronically infected patients showed that the majority of chronically infected patients fail to mount an efficient T-cell response to HBcAg/HBeAg, which may contribute to the persistent carrier state. $CD4^+$ T-cell epitope mapping within HBcAg/HBeAg reveals the existence of immunodominant epitopes at aa 1 to 25 and aa 61 to 85. HBcAg/HBeAg-specific $CD4^+$ T lymphocytes produce different lymphokines, which may be important for an efficient antiviral immune reaction. It is obvious that these T cells may be a target for therapeutic immunomodulation strategies in the future. However, this will not be a simple approach. For designing such novel strategies as well as for a basic understanding of virus-eliminating mechanisms, much more detailed analyses of the functions and of the functional alteration of these T cells by relevant peptides are required. The epitopes described may be appropriate candidates for innovative approaches to enhance relevant virus-specific T-cell responses in chronic carriers. Alternatively or in addition to the use of immunogenic peptides, lymphokines other than IFN-$\alpha$ should be investigated for the capacity to activate virus-specific effector cells.

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