Cytotoxic T Lymphocytes Recognize an HLA-A2-restricted Epitope within the Hepatitis B Virus Nucleocapsid Antigen

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Summary

The absence of readily manipulable experimental systems to study the cytotoxic T lymphocyte (CTL) response against hepatitis B virus (HBV) antigens has thus far precluded a definitive demonstration of the role played by this response in the pathogenesis of liver cell injury and viral clearance during HBV infection. To circumvent the problem that HBV infection of human cells in vitro for production of stimulator/target systems for CTL analysis is not feasible, a panel of 22 overlapping synthetic peptides covering the entire amino acid sequence of the HBV core (HBcAg) and e (HBeAg) antigens were used to induce and to analyze the HBV nucleocapsid–specific CTL response in nine patients with acute hepatitis B, six patients with chronic active hepatitis B, and eight normal controls. By using this approach, we have identified an HLA-A2-restricted CTL epitope, located within the NH_2-terminal region of the HBV core molecule, which is shared with the e antigen and is readily recognized by peripheral blood mononuclear cells from patients with self-limited acute hepatitis B but less efficiently in chronic HBV infection. Our study provides the first direct evidence of HLA class I–restricted T cell cytotoxicity against HBV in humans. Furthermore, the different response in HBV-infected subjects who successfully clear the virus (acute patients) in comparison with patients who do not succeed (chronic patients) suggests a pathogenetic role for this CTL activity in the clearance of HBV infection.

Hepatitis B virus (HBV)[1] infection is a worldwide health problem with an estimate of about 250 million people carrying the virus who can develop chronic hepatitis, liver cirrhosis, and ultimately hepatocellular carcinoma. As in other infections with noncytopathic viruses, a HLA class I–restricted cytotoxic T cell response to endogenously synthesized HBV proteins is believed to be the major determinant of infected cell clearance (1–4).

It is well known that CTLs recognize short amino acid (AA) sequences derived from the intracellular processing of viral antigens in association with HLA class I molecules on the surface of the infected cells (5–10). Since HBV does not efficiently infect human cells in vitro, the use of short synthetic peptides mimicking the processed antigen fragments can be a rational strategy to stimulate the HBV-specific CTL response (11–15). Based on this concept, a panel of short peptides representing the entire HBV nucleocapsid region were synthesized and used for CTL analysis.

Our results demonstrate that HBV nucleocapsid–specific CTL are present in the peripheral blood of patients with acute HBV infection and that this response in the context of HLA-A2 haplotype is focused on a CTL epitope included within the 11–27 sequence of the HBV core antigen (HBcAg). The observation that patients with acute and chronic HBV infection express different levels of CTL response may reflect immune events of pathogenetic relevance with respect to viral clearance and disease evolution.

Materials and Methods

Patients. Nine patients with acute hepatitis type B (AVH-B), six patients with chronic HBV infection, and eight healthy subjects were studied. The diagnosis of AVH-B was based on the finding of elevated values of serum glutamic pyruvic transaminase (SGPT) (at least 10 times the upper limit of the normal), associated with the detection of IgM antibodies against HBcAg in the serum and the recent onset of jaundice and other typical symptoms of acute hepatitis. All patients with acute HBV infection recovered com-

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[1] *Abbreviations used in this paper:* AA, amino acid; AVH-B, acute hepatitis type B; HBcAg, Hepatitis B virus core antigen; HBV, hepatitis B virus; SGPT, serum glutamic pyruvic transaminase.

pletely from the illness, with normalization of transaminase and clearance of hepatitis B surface antigen from the serum. The diagnosis of chronic hepatitis type B was based on biochemical and histological findings (16); SGPT levels ranged from 60 to 355 (normal <35) IU/liter. All patients were antibody negative to HIV, hepatitis C, and δ viruses.

Antigens. rHBcAg was obtained from bacterial extracts of *Escherichia coli* K12 strain HB101 harboring the recombinant plasmid carrying the HBcAg coding gene as previously described (17).

A panel of 22 overlapping synthetic peptides (10–20 AA long) corresponding to the entire sequence of the core- and pre-core region–encoded polypeptides (subtype ayw) was kindly provided by Cytel (La Jolla, CA) or purchased from Multiple Peptide System (La Jolla, CA). AA sequences of the peptides used in this study and their location within the HBV core and e molecules are illustrated in Fig. 1 *A*.

Induction of HBV-specific CTL. PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and resuspended at 4×10^6 cells/ml (24 wells/plate) in RPMI 1640 containing 10% human AB serum. Cells were stimulated either with rHBcAg (1 µg/ml) or with four distinct peptide pools (MIX 1, MIX 2, MIX 3, MIX 4) each one consisting of five to six synthetic peptides (Fig. 1 *A*). Individual peptides were used at the concentration of 5 µg/ml. Fresh medium containing 10% FCS and 20 U/ml of rIL-2 was added on days 3 and 5 of culture and the cytotoxicity assay was performed on day 7.

Cytotoxicity Assays. After 7 d of culture, stimulated PBMC were tested for their cytolytic activity against autologous macrophages or EBV-transformed B (EBV-B) cells as targets: (*a*) incubated overnight in medium alone; (*b*) incubated overnight with rHBcAg; (*c*) pulsed for 2 h with the different peptide mixtures; or (*d*) pulsed

for 2 h with individual peptides. Target cells labeled with 100 µCi of ^{51}Cr (Na^{51}CrO₄; Amersham International, Buckinghamshire, England) for 1 h at 37°C were cocultured with activated PBMC at an E/T ratio of 50 or 100:1 in 96-well round-bottomed plates (similar results were obtained at both E/T ratios). The amount of ^{51}Cr released in the culture supernatants was evaluated after 4 h of incubation and the percentage of ^{51}Cr release was calculated as follows: 100× [(cpm E − cpm SR)/(cpm MR − cpm SR)]; where E indicates the ^{51}Cr activity present in the supernatant of experimental culture, and SR and MR represent the spontaneous and maximum amounts of ^{51}Cr released by target cells cultured in medium alone or in the presence of 10% Triton X, respectively.

Restriction Experiments. After 7 d of culture, MIX 4–activated CTL were tested against autologous or allogeneic, partially HLA-matched macrophages, or EBV-B cells as targets either prepulsed with peptide 11–27 or incubated in medium alone. The cytolytic activity was measured 4 h later as above described.

Results and Discussion

Four pools of synthetic peptides covering the entire HBV core and e molecules (Fig. 1 *A*) were initially used to stimulate PMBC of a patient with AVH-B in an attempt to induce nucleocapsid-specific CTL. After 7 d of stimulation either with the peptide pools or with a recombinant preparation of core antigen, cytolytic activity was tested in a 4-h cytotoxicity assay against autologous macrophage targets pre-pulsed either with the pools of peptides or with exogenous core antigen (Fig. 1 *B*). No detectable cytolytic activity was induced by MIX 1, 2, and 3 containing also previously identified
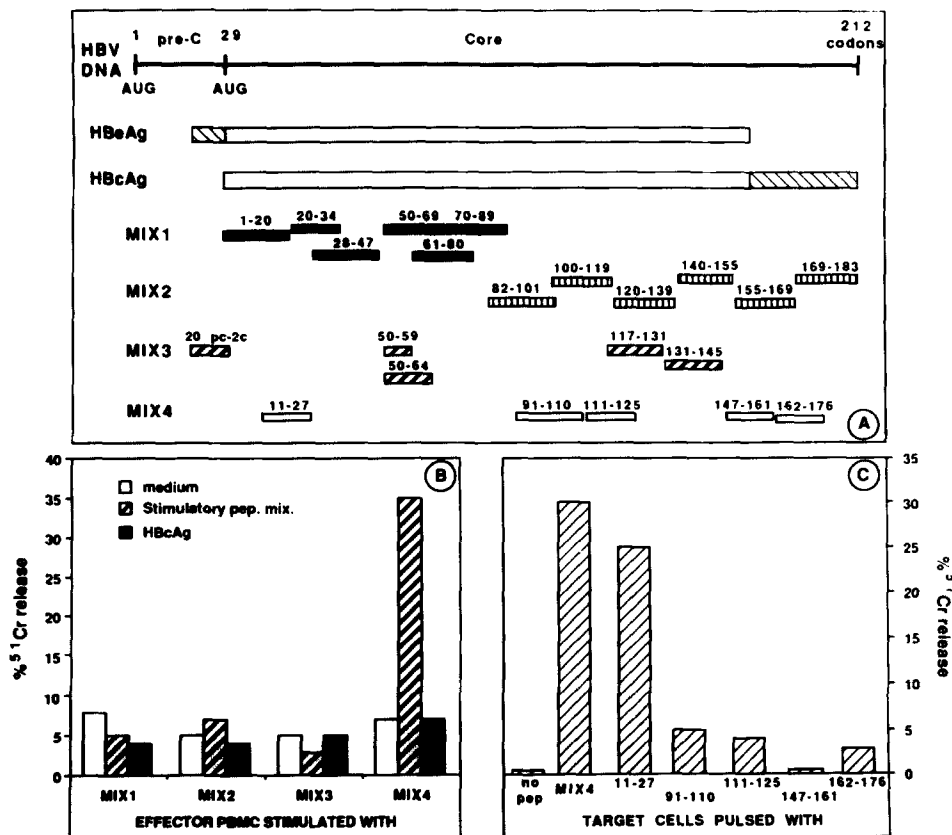
Figure 1. Activation of HBV-specific CTL by PBMC stimulation with synthetic peptides. (*A*) Synthetic peptide sequences (with indication of the distinct peptide mixtures used for CTL analysis), HBcAg and HBeAg molecules, and HBV core open reading frame. (*B*) Cytolytic activity of PBMC stimulated for 7 d with the peptide mixtures against autologous targets prepulsed either with the same stimulatory mixture or with exogenous HBcAg. (*C*) Lysis of autologous target cells prepulsed with the individual peptides included in MIX 4, by MIX 4–stimulated PBMC. MIX 4 or peptide 11-27-pulsed target cells were never lysed by PBMC stimulated with MIX 1, MIX 2, MIX 3, or exogenous HBcAg (not shown). The E/T ratio used was always 100:1.
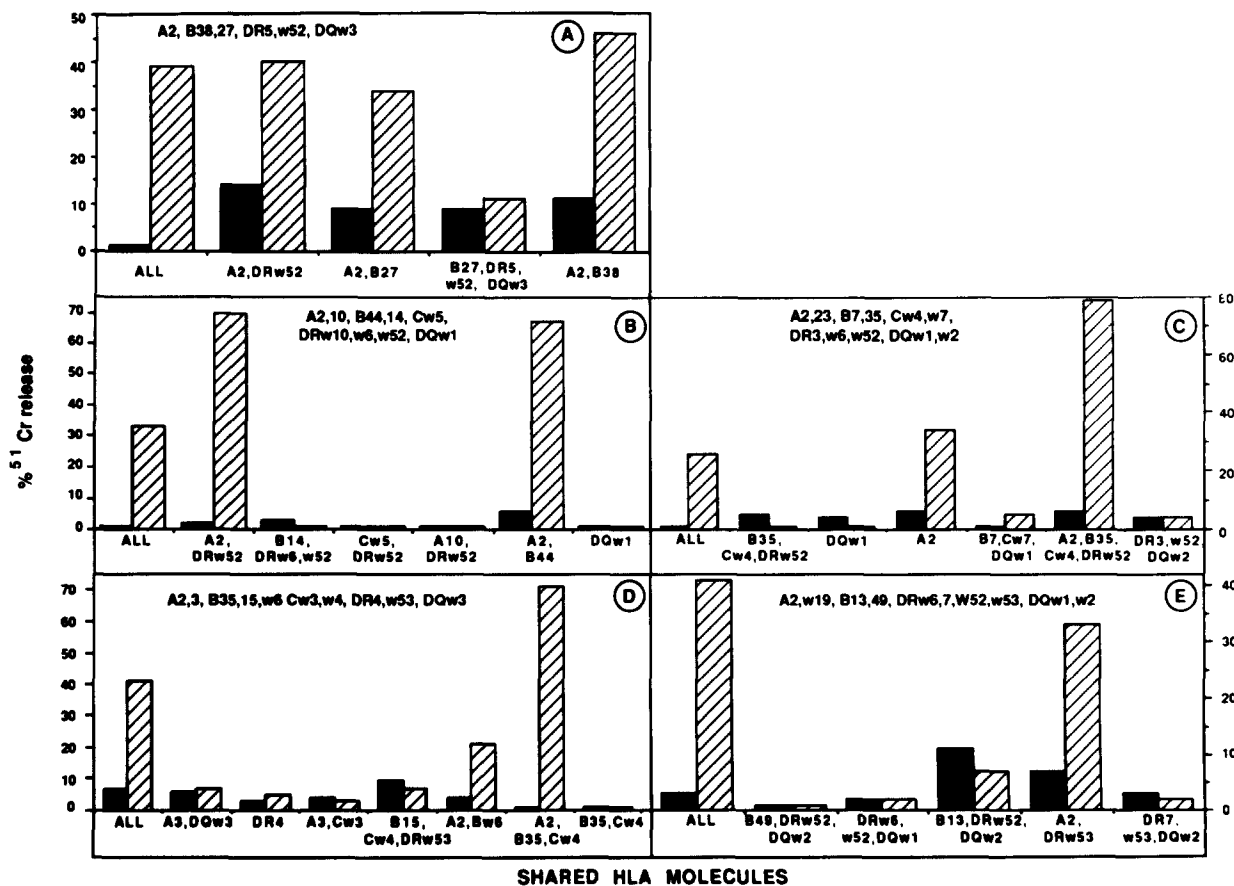
Figure 2. HLA restriction of peptide 11-27 recognition. PBMC from five patients with AVH-B were stimulated for 7 d with MIX 4, and their cytolytic activity was tested against autologous or allogeneic, partially HLA-matched macrophages, or EBV-B cell lines as targets (E/T ratio of 50:1) either pre-pulsed with peptide 11-27 (▨) or incubated in medium alone (■). The HLA haplotype of each patient (*top* of each panel) and the HLA molecules shared by patients and target cells (*bottom of each panel*) are indicated.

HBcAg helper epitopes (18), whereas PBMC stimulated with MIX 4 expressed significant cytotoxicity against target cells pulsed with the same mixture. Interestingly, PBMC stimulated with the whole core molecule could not recognize MIX 4–pulsed targets and, conversely, PBMC activated by MIX 4 did not lyse macrophages incubated with exogenous HBcAg (data not shown). These findings suggest that the AA sequence within MIX 4 responsible for CTL activation is not generated by the intracellular processing of exogenous core protein.

The specific peptide 11-27 was then identified as being responsible for the induction of the cytotoxic activity by MIX 4 (Fig. 1 C). The single-letter code AA sequence of this peptide is ATVELLSFLPSDFFPSV, which is conserved among the major subtypes of HBV. It is noteworthy that this peptide overlaps with a previously identified sequence containing an immunodominant helper epitope (AA sequence 1–20) recognized by approximately 70% of patients with acute HBV infection (18). These cytotoxic and helper epitopes are contiguous but certainly distinct since peptide 1–20 (included in MIX1) did not induce a cytotoxic response, whereas the

cytotoxic peptide 11-27 was totally unable to stimulate PBMC proliferation in any of the acute patients studied (not shown).

The nature of the effector cytotoxic cells induced by peptide stimulation was then investigated by restriction experiments designed to define the specific restriction element involved in the recognition of peptide 11-27. MIX 4–activated PBMC did not lyse allogeneic, peptide 11-27–pulsed EBV-B cells sharing the HLA class II molecules with the PBMC donor. In contrast, the cytolytic activity was selectively detected against target cells expressing the HLA-A2 molecule (Fig. 2 A). Moreover, CTL activity was inhibited by anti-CD8 but not anti-CD4 antibodies (data not shown). The observation that peptide 11-27 is recognized by CTL in association with HLA-A2 antigens suggests that this peptide likely mimics a specific CTL epitope generated by the intracellular processing of endogenously synthesized HBcAg in infected cells since this processing pathway mainly leads to association of the processed antigen with HLA class I molecules (19–22).

This possibility is further supported by separate experiments, to be reported in detail elsewhere (23), showing the
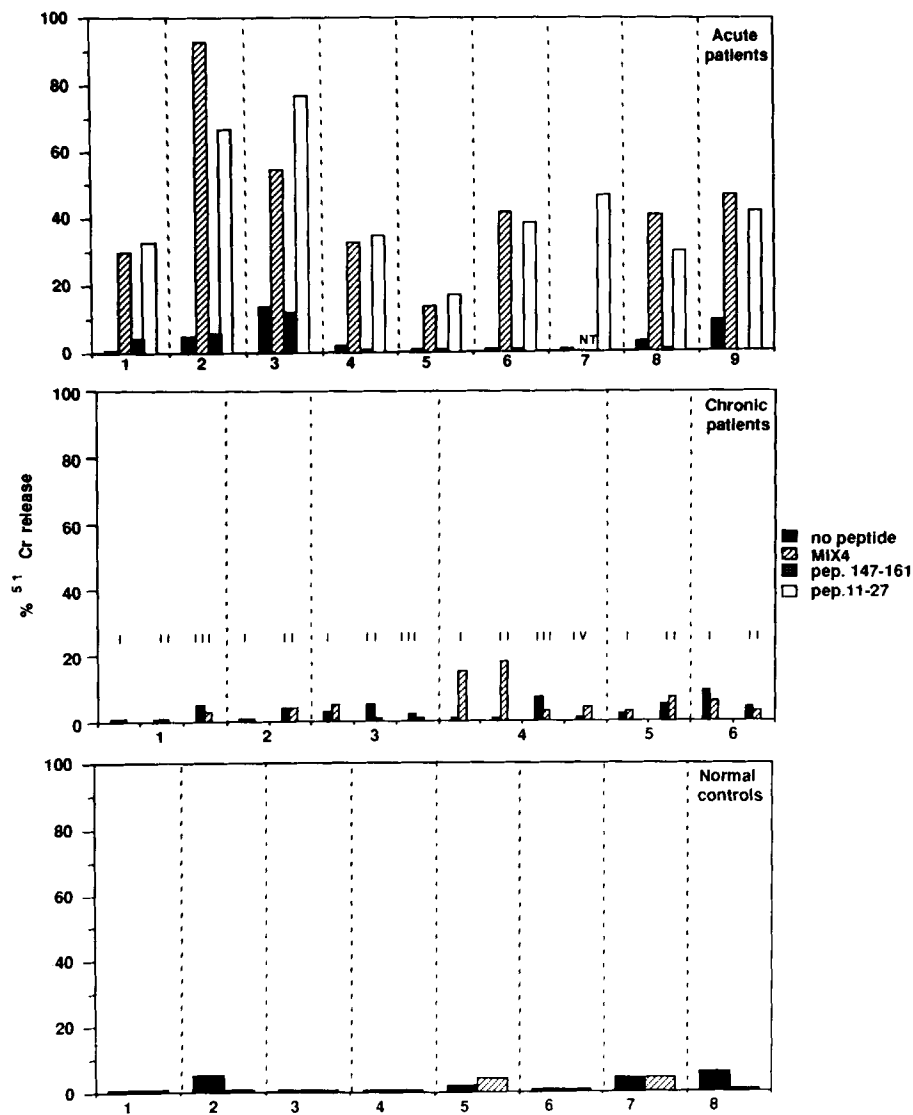
Figure 3. CTL activity of PBMC from HLA-A2-positive acute and chronic HBV-infected patients and normal subjects after stimulation with MIX 4. MIX 4-activated PBMC from nine HLA-A2 patients with AVH-B (*top*) were tested against autologous macrophages (E/T ratio 50:1) incubated in medium alone, or pre-pulsed with MIX 4, or with the immunogenic peptide 11-27, or with an irrelevant control peptide (pep. 147-161). All patients were repeatedly tested during the course of illness and a representative experiment for each subject is illustrated. Patient no. 7 was not tested against autologous macrophages pulsed with MIX 4 and peptide 147-161 (NT, not tested). MIX 4-stimulated PBMC from six HLA-A2 subjects with chronic HBV infection (*middle*) and eight HLA-A2 healthy subjects (*bottom*) were tested for their cytotoxic activity (E/T ratio 50:1) against autologous targets pulsed with the same peptide mixture used for induction. By using the individual peptides, cytotoxicity against MIX 4 in patient 4 (*middle*) was shown to be directed against peptide 11-27 (not shown). Roman numbers (*middle*) designate distinct experiments performed at different time points in individual patients. Patients 1, 2, 5, and 6 (*middle*) were HBeAg and HBV-DNA positive, patient 3 was anti-HBe positive and HBV-DNA negative, and patient 4 was anti-HBe and HBV-DNA positive.

capacity of peptide-induced CTL to recognize endogenous HBcAg encoded by recombinant vaccinia and Epstein Barr-based plasmid vectors in autologous EBV-B cells.

To assess the immunodominance in the HLA-A2 haplotype of the CTL epitope contained in peptide 11-27, eight additional subjects with self-limited AVH-B, six patients with chronic active hepatitis B, and eight healthy subjects without evidence of previous exposure to HBV (all HLA-A2 positive) were repeatedly tested. All the acute patients serially studied (every 7-10 d) during the symptomatic and the recovery periods of disease efficiently recognized autologous target cells pulsed with peptide 11-27 as well as with MIX 4 (Fig. 3, *top*). Restriction experiments performed with MIX 4-stimulated PBMC from four of them confirmed that CTL recognition of peptide 11-27 is HLA-A2 restricted (Fig. 2, *B-E*). Patient to patient variations in the pattern of response to peptide 11-27 were observed during the course of the illness. The cytolytic activity was generally detectable during

the symptomatic phase when SGPT values were high (Fig. 4, *A* and *B*); in some patients the response was long lasting, being still detectable when SGPT levels had already become normal (Fig. 4, *C* and *D*); in other subjects the lytic activity became undetectable 2 or 3 wk after the transaminase peak when SGPT levels were still slightly altered (Fig. 4 *B*). The lack of a constant association between CTL activity and SGPT levels could be explained considering that the peripheral blood compartment only partially reflects immune events taking place in the liver at the site of antigen synthesis and cellular injury (24).

Five of the six chronic patients (each one tested two to four times, following the same experimental protocol used for the acute patients), did not show cytolytic activity against autologous targets pulsed with peptide 11-27 (contained in MIX 4), while one patient displayed detectable, though very low levels of cytotoxicity against the relevant peptide sequence (Fig. 3, *middle*, patient 4). The lytic activity against peptide
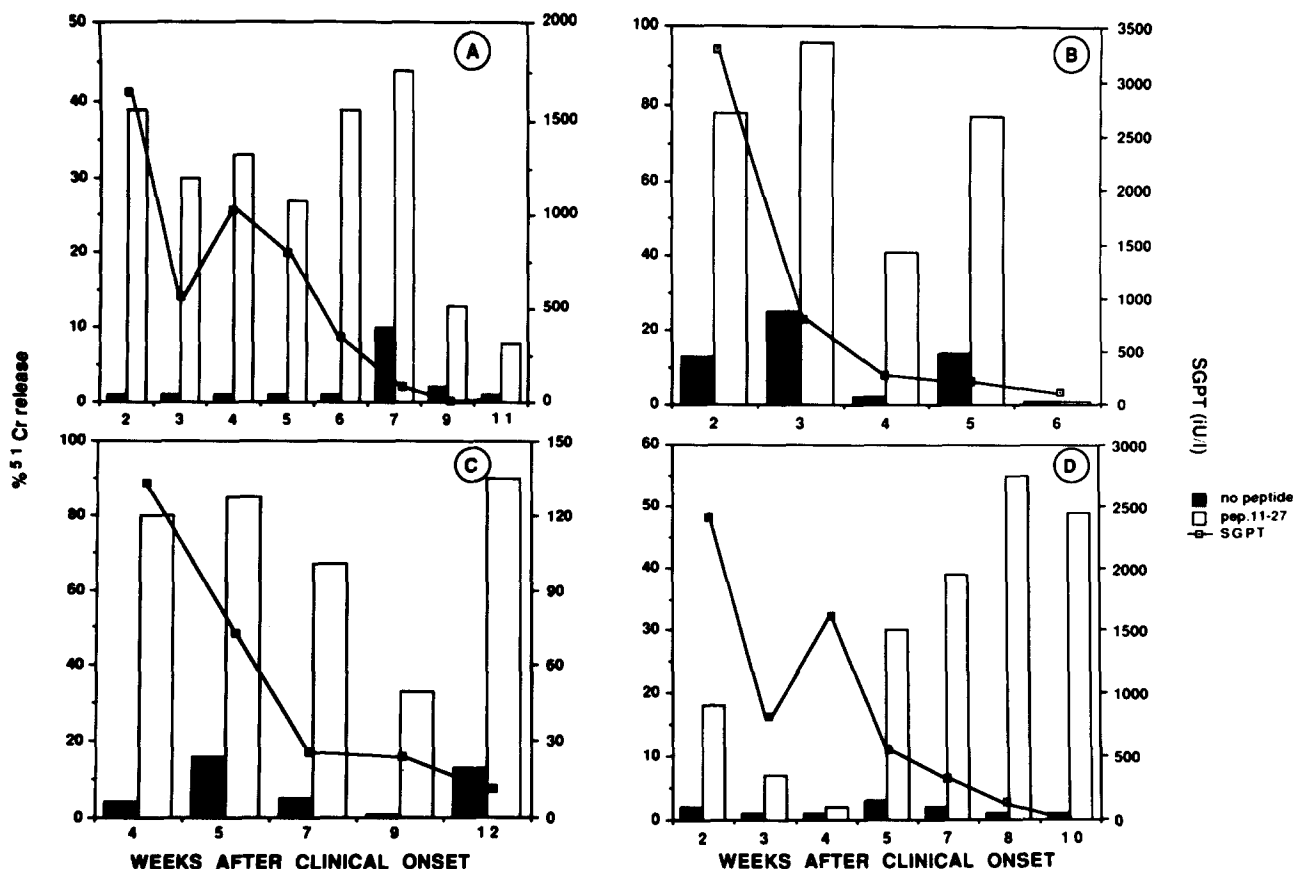
Figure 4. Serial determinations of the cytolytic activity expressed by PBMC from four representative patients with AVH-B during the course of acute infection. SGPT levels assayed at the same time points are also illustrated. PBMC were stimulated with MIX 4 and then tested against peptide 11-27-pulsed autologous target cells.

11-27 was not present in normal HLA-A2-positive control subjects (Fig. 3, *bottom*). This shows that the cytolytic activity displayed by HBV patients is not due to in vitro priming and that peptide stimulation selectively expands a specific T cell population pre-primed in vivo by HBV infection.

In conclusion, our results indicate a clear association between the CTL response to core peptide 11-27 and acute HBV infection in patients who succeed in clearing the virus. Whether the absent or weak CTL response to the core epitope identified in the present study among the population of chronic patients actually reflects an immune defect respon-

sible for the inability to clear the virus or a preferential intrahepatic sequestration in chronic disease remains to be further investigated using intrahepatic T cells. The experimental approach defined in this study will hopefully provide the appropriate tools to answer these central issues in HBV pathogenesis. Finally, the finding that the NH_2-terminal portion of the core molecule contains two contiguous and probably overlapping immunodominant epitopes for helper and cytotoxic T cells might be valuable for future preventive and therapeutic strategies against HBV infection.

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