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Complete List of Authors:	Malmassari, Silvina; Institut Pasteur, INSERM U 812, Departement de Virologie Deng, Qiang; Institut Pasteur, INSERM U 812, Departement de Virologie Fontaine, Helene; AP-HP, Unité d☐Hépatologie, Hôpital Cochin Houitte, Diane; Commissariat à l☐Energie Atomique, Département d'Ingénierie et d'Etudes des Protéines Rimlinger, François; INSERM U785; Institut Pasteur, Département de Virologie Thiers, Valérie; INSERM U785; Institut Pasteur, Département de Virologie Maillere, Bernard; Commissariat à l☐Energie Atomique, Département d'Ingénierie et d'Etudes des Protéines Pol, Stanislas; AP-HP, Unité d☐Hépatologie, Hôpital Cochin Michel, Marie-Louise; Institut Pasteur, INSERM U 812, Departement de Virologie
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Silvina Laura MALMASSARI[1,2], Qiang DENG[1,2], Hélène FONTAINE[3], Dianne HOUITTE[4], François RIMLINGER[2,5], Valérie THIERS[2,5], Bernard MAILLERE[4], Stanislas POL[3], Marie-Louise MICHEL[1,2]

[1] INSERM U812, Pathogenèse des Hépatites Virales B et Immunothérapie; [2] Institut Pasteur, Département de Virologie, Paris, France; [3] AP-HP, Unité d'Hépatologie, Hôpital Cochin, Paris, France; [4] Département d'Ingénierie et d'Etudes des Protéines, Commissariat à l'Energie Atomique (CEA), Gif sur Yvette, France; [5] INSERM U785, Villejuif, France

KEYWORDS: T helper, core promoter mutation, IFN-gamma, IL-10, promiscuous epitope

Contact Information

Marie-Louise Michel

INSERM U812, Pathogénèse des Hépatites Virales B et Immunothérapie; Institut Pasteur, Département de Virologie

28, rue du Docteur Roux

75724 Paris Cedex 15

France

Telephone number: +33 1 4568 8849

Fax number: +33 1 4061 3841

E-mail: maloum@pasteur.fr

E-mail addresses of all authors

S. Malmassari: smalmass@pasteur.fr

Q. Deng: qdeng@pasteur.fr

H. Fontaine: helene.fontaine@cch.aphp.fr

D. Houitte: diane.houitte@cea.fr

B. Maillere: Bernard.maillere@cea.fr

F. Rimilinger: rimlinger@vjf.inserm.fr

V. Thiers: thiers@vjf.inserm.fr

S. Pol: stanislas.pol@cch.aphp.fr

M.-L. Michel: maloum@pasteur.fr

Abbreviations

HBV, hepatitis B virus; HBx, hepatitis B X protein; HCC, hepatocellular carcinoma; CTL, cytotoxic T lymphocyte; Th, T helper; IFN, interferon; IL, interleukin; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; Elispot, enzyme-linked immunosorbent spot; SFC, spot-forming cell

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ABSTRACT

The hepatitis B X (HBx) protein is a crucial component in hepatitis B virus (HBV) infection *in vivo* and has been implicated in hepatocellular carcinoma. In this study, we aimed to detect and characterize peripheral HBx-specific T cells in chronically infected patients at the inactive carrier state of the disease. HBx-specific IFN-γ-secreting T cells were found in 36 of 52 patients (69%) and 78% (28/36) of responding patients had T cells targeting epitopes in the carboxy-terminal part of HBx. IL-10 secretion following the stimulation of T cells with HBx-derived peptides was weak or undetectable. IFN-γ-secreting T cells recognized a previously unknown immunodominant CD4+ T-cell epitope, HBx 126-140 (EIRLKVFVLGGCRHK), in 86% (24/28) of patients. This peptide bound several HLA-DR molecules (HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*1301 and HLA-DRB5*0101). Its coding sequence overlaps a domain of the HBV genome encompassing the basic core promoter (BCP) region. Taking into account the selection of viral core promoter mutants during HBV infection, we found that HBV variants with BCP mutations were present in patient sera. We further demonstrated that these viral mutant sequences activated T cells specific for the immunodominant epitope only weakly, if at all. This is the first study linking BCP mutations and HBx-specific T-cell responses. Therefore, wild type and variant peptides may represent potentially tools for monitoring the HBV-specific T-cell responses involved in sequence evolution during disease progression. Finally, the degenerate HLA-DR binding of this promiscuous, immunodominant peptide would make it a valuable component of vaccines for protecting large and ethnically diverse patient populations.

INTRODUCTION

An effective vaccine against hepatitis B virus (HBV) infection has been available for more than two decades, but 400 million people —more than 5% of the world's population — are chronically infected with HBV, and more than 1 million people die each year from HBV-related liver cirrhosis and hepatocellular carcinoma (HCC) (1, 2).

The hepatitis B X (HBx) protein is a key element in HBV infection *in vivo* and has been implicated in HCC development. HBx is well conserved among mammalian hepadnaviruses and is produced very early after infection and throughout chronic infection. The potentially oncogenic functions of HBx include the transcriptional activation of genes encoding proteins regulating cell growth, apoptosis modulation and the inhibition of nucleotide excision repair following DNA damage. HBx exerts its effects by interacting with cellular proteins and activating cell signaling pathways (3, 4).

The pathogenesis of HBV infection involves the selection and expression of several common viral mutants. HBV genes have overlapping open reading frames. A mutation in the HBV genome may therefore have effects on several proteins. The HBx gene overlaps regions of crucial importance for viral replication, such as the direct repeat sequences DR1 and DR2, the preC/C gene promoter and the enhancer II region. The common double mutation in the HBV basic core promoter (BCP) region A1762T/G1764A corresponds to a double mutation in codons 130 and 131 of the HBV X gene. The change in HBx amino-acid sequence (K130M and V131I) resulting from these T-A point mutations is associated with severe liver damage and HCC (5-8). These substitutions may be associated with an additional mutation at position 127 in the HBx protein, which has been detected in patients with HCC or fulminant hepatitis (9, 10). Natural mutations in the HBx gene are thought to lead to progression to chronic disease due to the abolition of anti-proliferative and apoptotic effects, causing uncontrolled growth and multistep hepatocarcinogenesis (11). The selection and expression of natural HBx mutants may have major implications for T-cell recognition of this protein.

As HBV is mainly not directly cytopathic, the immune response to viral antigens is thought to be responsible for both liver disease and viral clearance following HBV infection. Patients with acute viral infection who successfully clear the virus display a multispecific polyclonal cytotoxic T-

lymphocyte (CTL) response specific for a number of epitopes within the core, polymerase and envelope proteins (12-15). HBV-specific T helper (Th) cells are also activated and multispecific Th1-like responses are detected in patients successfully clearing HBV after acute infection (16). This HBV-specific T-cell response is weak or undetectable in patients who develop chronic infection (17). Little is known about the CTL directed against HBx protein in HBV-infected individuals (18, 19) or about HBx-specific CD4+ T cells and their cytokine profile during the course of viral infection (20).

We characterized peripheral HBx-specific T cells in 52 patients with chronic HBV infection at the inactive carrier state of the disease, by measuring interferon-gamma (IFN-γ) and interleukin-10 (IL-10) secretion after the activation of peripheral blood mononuclear cells (PBMC) with 15-mer peptides spanning the HBx sequence. We identified an immunodominant, promiscuous T-cell epitope, HBx 126-140, located in the carboxy-terminal part of the protein and recognized by IFN-γ-secreting CD4+ T cells in most patients. HBV core promoter mutations, which frequently occur during chronic infection, modify the sequence of this HBx-derived immunodominant CD4+ T-cell epitope. These mutant viral sequences were recognized by T cells specific for the HBx wild-type epitope only weakly, if at all.

MATERIALS AND METHODS

Patient population

Fifty-two subjects with chronic HBV infection, in the inactive carrier state of the disease (21) with less than 100,000 HBV copies/ml were enrolled (see Table online). All were hepatitis B surface antigen (HBsAg)-positive, hepatitis B e antigen (HBeAg)-negative, anti-HBe antibodies-positive, had normal transaminase levels and no or low underlying liver disease. This group of patients is heterogeneous as it contains i) patients with persistently low HBV DNA levels, even falling to undetectable levels (< 200 cp/ml) either spontaneously or after effective antiviral treatment and ii) patients with fluctuating levels of HBV DNA being nevertheless < 100,000 cp/ml. This sub-group may include patients carrying HBV viruses with preC or BCP mutations (22). Patients had received no antiviral treatment for at least six months before inclusion. All patients were 18 to 60 years old, had no

immunosuppression or infections associated with human immunodeficiency virus, hepatitis C virus or hepatitis D virus, or liver diseases other than HBV infection, and consumed less than 40 g of alcohol/d. HLA-DR genotyping was carried out with the Olerup SSP™ Genovision kit (Saltsjöbaden, Sweeden). Two blood samples were collected from each patient at a mean of 8 + 4 month interval. This study was approved by the ethics committee of hospital, and all participants gave informed, written consent for participation, in line with French ethical guidelines.

Synthetic peptides

Synthetic peptides were purchased from NeoMPS (Strasbourg, France). The consensus sequence of the HBx protein, obtained by comparing published HBx-encoding sequences in Genbank — MAARLCCQLDPARDVLCLRPVGAESRGRPLSGPLGTLSSPSPSAVPTDHGAHLSLRGLPVCAF SSAGPCALRFTSARRMETTVNAHQILPKVLHKRTLGLSAMSTTDLEAYFKDCLFKDWEELGE EIRLKVFVLGGCRHKLVCAPAPCNFFTSA — was covered by 29 15-mer peptides with 10-residue overlaps. Individual or pooled peptides were used to stimulate PBMCs *in vitro* and for the Elispot assay. Three peptide pools were used: pool A (peptides x1 to x10), pool B (peptides x11 to x20) and pool C (peptides x21 to x29). Two additional peptides corresponding to variant sequences of the wild-type x26 peptide (HBx 126-140, EIRLKVFVLGGCRHK) were used: V2 (EIRLMIFVLGGCRHK) and V3 (ETRLMIFVLGGCRHK). Peptides were prepared at 1 mg/ml in water or 20% DMSO if required and stored at -20 °C until use.

HLA-DR peptide-binding assays COULD BE PROVIDED ON LINE

HLA-DR molecules were purified from homologous EBV cell lines by affinity chromatography, as previously described (23, 24). Binding to various HLA-DR molecules was assessed by competitive ELISA, as previously described (23, 24). We used the individual peptides of pool C and a 20-mer HBc-derived peptide (core 50-69; PHHTALRQAILCWGELMTLA) as competitors (25). Maximal binding was determined by incubating the biotinylated peptide with the MHC class II molecule in the absence of competitor. Binding specificity for each HLA-DR molecule was ensured by the choice of the biotinylated peptides as described previously (23, 24). Concentration of the peptide that prevented

50% of binding of the biotinylated peptide was evaluated (IC_{50}). The reference peptide is the unlabelled form of the biotinylated peptide and corresponds to high binder. Their IC50 are the following: 2 nM for DRB1*0101; 403 nM for DRB1*0301; 38 nM for DRB1*0401; 6 nM for DRB1*0701; 6 nM for DRB1*1101; 170 nM for DRB1*1301; 26 nM for DRB1*1501; 15 nM for DRB3*0101; 10 nM for DRB4*0101 and 6 nM for DRB5*0101. Data are expressed as relative activity: ratio of the IC_{50} of the peptide by the IC_{50} of the reference peptide.

***In vitro* expansion of the PBMC**

PBMC were suspended at 3 x 10^6 cells per ml in complete medium (RPMI 1640 medium, Life Technologies, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% human AB serum (Institut Jacques Boy, Reims, France) plus 20 ng/ml IL-7 (Roche, Meylan, France) and 100 pg/ml IL-12 (R&D Systems Inc., MN, USA), in 24-well plates. Cells were stimulated by incubation with peptide pools A, B and C (1 µg/ml of each peptide) or with individual peptides (10 µg/ml). Half the medium was replaced every three to four days with complete medium supplemented with recombinant IL-2 (50 IU/ml) (Roche, Meylan, France). After 10 to 14 days of culture, HBx-specific IFN-γ- and IL-10-producing cells were quantified by Elispot assays and intracellular cytokine staining.

Elispot assay COULD BE PROVIDED ON LINE

Sterile nitrocellulose HA 96-well plates (Millipore, Bedford, MA) were coated with 15 µg/ml anti-IFN-γ mAb (clone 1-DIK; Mabtech, Stockholm, Sweden) in 0.1 M bicarbonate buffer (pH 9.6) or with 10 µg/ml anti-IL-10 mAb (clone B-N10, Diaclone, Besançon, France) in PBS (pH 7.0). The wells were blocked and washed (26), then filled, in triplicate, with *in vitro*-stimulated cells (1 to 2 x 10^5/well) in complete medium and the appropriate peptides (1 µg/ml), with medium alone used as a negative control and phorbol myristate acetate (25 ng/ml)/ionomycin (2 mg/ml) or staphylococcal enterotoxin B (500 ng/ml) (Sigma, St. Louis, MO, USA), as positive control. After 20 h of incubation at 37°C, plates were washed and incubated with 1 µg/ml biotinylated anti-IFN-γ mAb (clone 7B6-1;

Mabtech) or with 20 µg/ml biotinylated anti-IL-10 mAb (clone B-T10; Diaclone) for 2 h at room temperature. Plates were then washed and antibody binding was detected as previously described (26). A Zeiss Elispot automatic counter was used to score the number of spots.

The specificity and cut-off of Elispot assays were determined with PBMC from healthy individuals (n=9) and with PBMC from hemochromatosis patients (n=2). These PBMC were subjected to *in vitro* expansion with HBx-derived peptides and tested in Elispot assays in experimental conditions identical to those used for PBMC from chronic HBV carriers. The cut-off of Elispot assays were 62 IFN-γ- and 40 IL-10-spot-forming cells (SFC) per million PBMC, calculated as mean + 2 sd SFC per million PBMC from HBV-negative subjects. The response was considered positive if the median number of SFC in triplicate wells was at least twice than in control wells without peptide and was superior to the cut-off values.

Inhibition of Elispot assays

Class II HLA-restriction was determined, after *in vitro* expansion, by incubating PBMC for 90 min at 37°C with 10 µg/ml of anti-class II HLA antibodies: anti-HLA-DR (L243) from ATCC, anti-HLA-DQ (SPVL3) and anti-HLA-DP (B7/21) kindly provided by Dr. Y. van de Wal (Department of Immuno-hematology and Blood Bank, Leiden, The Netherlands). Anti-class I HLA-A2 antibody (BB7-2) was used as a negative control. Pre-incubated PBMC were then tested in Elispot assays, as described above.

Intracellular staining COULD BE PROVIDED ON LINE

Populations of PBMC expanded *in vitro* were incubated overnight either with 500 ng/ml staphylococcal enterotoxin B (Sigma) as a positive control, with medium alone as a negative control or with individual HBx-derived peptides (1 µg/ml) and Brefeldin A (2 µg/ml) (Sigma). After washing, cells were stained with appropriate combinations of monoclonal antibodies — anti-CD3-APC (clone HIT3a; BD Pharmingen), anti-CD4-PE (clone RPA-T4; BD Pharmingen), anti-CD4-APC (clone RPA-T4; Serotec) and/or anti-CD8-PerCP (clone SK1; BD Biosciences) — for 30 minutes at 4°C and

washed again. Cells were fixed, permeabilized and stained with anti-human IFN-γ-FITC antibody (clone 4S.B3; BD Biosciences) and with anti-human IL-10-PE antibody (clone JES3-9D7; Serotec) for 30 minutes at 4°C and washed again. At least 50,000 lymphocyte-gated events were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with Cellquest (BD Biosciences). Background staining was assessed with an isotype-matched control monoclonal antibody and subtracted from all values.

RESULTS

Presence of HBx-specific IFN-γ-secreting T cells in chronic HBV carriers

We analyzed the HBx-specific IFN-γ-secreting T-cell response by Elispot assays and the use of three peptide pools (A, B, C) covering the entire HBx sequence to stimulate PBMC from 52 chronic inactive carriers of HBV. HBx-specific IFN-γ-secreting T cells were found in 36 of the 52 patients studied (69%): 28 (78%) had T cells recognizing epitopes in the carboxy-terminal part of HBx (pool C), 17 (42%) had T cells specific for the central region of the protein (pool B) and 9 (25%) had T cells specific for epitopes in the amino-terminal part of the protein (pool A) (Figure 1A). The diversity of HBx-specific IFN-γ-secreting T-cell responses to pools A, B and C is shown in Figure 1B. No more than 10% of patients displayed specific responses to all three peptide pools.

Mapping of the IFN-γ-secreting T-cell response to individual pool C peptides

As most HBx-specific IFN-γ-secreting T cells recognized the carboxy-terminal part of the protein, we mapped the single epitopes targeted by T cells in this region. PBMC from the 28 patients with pool C-specific T cells were stimulated with the entire peptide pool C and with individual pool C peptides, and then tested in Elispot assays (Figure 2). T-cell activation following PBMC culture with the entire peptide pool resulted from specific stimulation with a single peptide, as 24 of the 28 patients (86%) had T cells specific for the x26 epitope (compare left and middle panels of Figure 2). Moreover, x26-specific T cells were the only T cells reactive against the domain of the protein covered by peptide

pool C in at least 13 of the 28 patients (46%), and an absence of T cells recognizing the x26 epitope was noted in only four of these patients (patients P62, P34, P53 and P38). We analyzed PBMC from 11 uninfected individuals to check the specificity of these responses. None had T cells responding to pool C or x26 peptides in Elispot assays after *in vitro* expansion (data not shown).

T cells specific for individual pool C peptides other than x26 were detected in 8 patients (Figure 2, right panel). In the absence of x26-specific T-cell reactivity, T cells recognized the x22 peptide (patients P62, P53 and P38). In 5 of the 24 patients with x26-specific T cells, weak reactivity to x21 to x25 and x27 was also observed (patients P31, P46, P59, P28 and P23, right panel, Figure 2). With the exception of patient P23, whose T cells were more strongly activated with x25 than with x26, specific T-cell reactivity was 5 to 10 times higher for x26 than for other pool C single peptides (compare middle and right panels).

In conclusion, IFN-γ-secreting T cells recognizing the carboxy-terminal domain of HBx targeted a single immunodominant epitope. As this epitope was recognized by T cells from a large number of patients expressing different HLA molecules, x26 peptide may be considered a promiscuous epitope.

HBx-derived peptides activated IFN-γ- and IL-10-secreting T cells in chronic HBV carriers

Even if HBx-specific IFN-γ-secreting T cells were found in PBMC from most of the studied patients, we asked whether IL-10 secretion can be detected in PBMC from IFN-γ-Elispot-negative patients. We quantified and compared IFN-γ and IL-10 secretion after the *in vitro* expansion of PBMC from 31 chronic HBV carriers with and without pool C-specific IFN-γ-secreting T-cell responses. In 13 of the 31 patients (42%), neither IFN-γ- nor IL-10-producing specific T cells were detected. No IL-10 secretion was observed in 11 patients (35%) with IFN-γ-secreting T-cell responses of various magnitudes (Figure 3A). Finally, only 7 of the 31 patients (23%) with generally strong IFN-γ-secreting T-cell responses had detectable numbers of specific IL-10-secreting T cells (Figure 3B). Except in patient P26, the frequency of IFN-γ-secreting T cells was always higher than that of IL-10-secreting T cells (Figure 3B). The IFN-γ-secreting T-cell response was around ten times higher than the IL-10-producing T-cell response in Elispot (range: 4.8 - 27.4 fold, median; 11.5).

IL-10 secretion was observed in only two and one of the 24 studied patients after the activation of PBMC with peptide pools A and B, respectively. IFN-γ secretion by activated T cells was detected simultaneously in these three patients with IL-10-producing T cells. Peptide pool A activated 505 IFN-γ- and 170 IL-10-secreting T cells per million PBMC from patient P7 and 104 IFN-γ- and 313 IL-10-SFC per million PBMC from patient P27. Finally, 652 IFN-γ- and 79 IL-10-SFC pool B-specific T cells per million PBMC were detected for patient P41.

Overall, IL-10 secretion after T-cell activation with HBx-derived peptides was weak or undetectable. Studies focusing on the carboxy-terminal region showed that peptide pool C or x26 activated IFN-γ-production more efficiently than IL-10 production.

Phenotype of x26-specific T cells

We investigated whether the x26 epitope activated CD4+ or CD8+ T cells, by intracellular IFN-γ staining of PBMC stimulated *in vitro* with x26. The phenotype of CD3+ IFN-γ-producing x26-specific T cells from a representative patient (P30) is shown in Figure 4A. The promiscuous, immunodominant x26 peptide specifically stimulated CD4+ but not CD8+ T cells. This result was confirmed in 10 patients with x26-positive response.

The x26-specific T-cell response of patient P26 was further characterized by intracellular staining of both IFN-γ and IL-10, to identify more precisely the T cells producing either or both cytokines. We found that 7.66% CD4+ T cells produced IFN-γ and 0.73 % produced IL-10 (Figure 4B, left and central panels). However, most CD4+ T cells activated by x26 (6.95% of total CD4+ T cells) produced IFN-γ but not IL-10 (Figure 4B, right panel). Less than 10% of the x26-specific CD4+ T-cell population (0.73% of total CD4+ T cells) produced IL-10 together with IFN-γ. No activated T cells producing IL-10 only were observed.

This is consistent with the small number of IL-10-producing T cells identified by Elispot and highlights the more precise quantification of IFN-γ and IL-10 by intracellular staining than by Elispot assays when these cytokines are produced simultaneously (compare Figures 3B and 4B).

HLA class II restriction of x26 peptide

We used three experimental approaches to characterize the HLA class II-restriction of x26. After *in vitro* expansion, PBMC were incubated with anti-HLA-DR, anti-HLA-DQ or anti-HLA-DP antibodies and tested in Elispot assays. Prior incubation with anti-HLA-DR antibodies inhibited IFN-γ-secretion upon stimulation with x26 by at least 80%. No such effect was observed after the prior incubation of PBMC with anti-HLA-DP or anti-HLA-DQ antibodies, or with irrelevant control anti-HLA-A2 antibodies (Figure 5). The x26 epitope is therefore presented by HLA-DR molecules.

We assessed the capacity of x26 and of other pool C peptides to bind to various purified HLA-DR molecules. An HBcAg-derived peptide, c59-60, described as HBV promiscuous epitope (25) was tested in parallel (Table 1). High-affinity binding was observed for x25 peptide with at least 4 of the 10 HLA-DR molecules tested (DRB1*0301, DRB1*1101, DRB1*1301 and DRB1*1501). The x26 peptide could be presented by the HLA-DRB1*0101, HLA-DR01*0401, HLA-DRB1*1301 and HLA-DRB5*0101 molecules. DRB1*1301 molecules could bind x22, x23 and x27 peptides as well, while x22 also bound to DRB4*0101. In comparison, the HBcAg-derived promiscuous epitope exhibited a good affinity for DRB1*0301, DRB1*1301 and DRB1*1501.

Finally, we genotyped the HLA-DR molecules of 30 inactive HBV carriers with or without x26-specific T cells. For the HLA-DRB1 gene, the prevalence of alleles DRB1*0301 (9/30), DRB1*0401(9/30), DRB1*1101 (9/30) and DRB1*1301 (7/30) was high among the studied patients. In 16 patients, the presence or absence of HLA-DR alleles binding x26 (HLA-DRB1*0101, HLA-DR01*0401, HLA-DRB1*1301 and HLA-DRB5*0101) was found to be related to specific IFN-γ-secreting T-cell reactivity (Table 2).

Recognition of viral mutants by x26-specific T cells

Some HBx mutations in basic core promoter mutant viruses concern the x26 epitope. An analysis of sequences from 40 cloned HBV genomes published in GenBank (http://www.ncbi.nlm.nih.gov) shows that the frequent codon 130 and 131 (K130M and V131I) mutations were present in 12 of the 40 sequences. Codon 127 (I127T) mutation occurred in 4 of the 40 HBx sequences. We therefore evaluated the recognition of viral sequence variants by x26-specific T cells. For PBMC stimulation *in*

vitro, we used x26 peptide, covering the wild-type epitope, separately or mixed with the peptides V2 and V3, corresponding to viral mutant sequences. No activation of x26-specific T cells by the variant peptides V2 and V3 was found in 10 of 13 patients with x26-specific T-cell responses (Figure 6). When the three peptides were used for in vitro expansion (Figure 6 right panel), markedly fewer cells were recalled in only three patients in Elispot assay with the variant peptides. None of 12 patients with x26-negative T-cell responses in IFN-γ Elispot assays had T-cell responses to variant peptides (not shown).

We investigated whether mutant peptides activated IL-10- rather than IFN-γ-secreting T cells, by assessing cytokine secretion after the stimulation of PBMC with V2 and V3. No IL-10-secreting T cells were found in 15 studied patients with (n=6) or without (n=9) x26-specific IFN-γ-secreting T cells. In conclusion, viral mutant sequences activated T cells specific for the x26 epitope much less efficiently and no cross-recognition of variant sequences by x26 specific-T cells was found.

Sequencing of HBV viral DNA from patients

We next investigate if HBV genome from patients may have viral mutations affecting the x26 epitope. Sequencing of the x26 encoding HBV DNA region could be performed only on three samples (P48, P59, P46) with HBV viral load exceeding $2x10^4$ copies /ml and detectable HBV DNA after nested PCR (see online figure). For patients P48 and P59, A-T and G-A mutations at nucleotides 1762 and 1764 were found in HBV genome, changing amino acid in the HBx protein at positions K130M and V131I. Clinical data from patient P48 shows that this patient was infected at birth with an HBeAg-negative mutant (see online Table). X26 T-cell response was found negative in this patient (see Table 2). In contrast, in patient P59 carrying a virus with BCP mutation, x26-specific T cells were detected in PBMC taken at the time of DNA sequencing (Figure 2). Finally, the amplified virus from patient P46 with x26-specific T-cell response (Figure 2) showed a wild type BCP sequence.

This indicates that HBV variants with BCP mutations can be found in some of our patients at the inactive carrier state of the disease.

DISCUSSION

HBV-specific CD4+ T cells play an important role in HBV infection, secreting the Th1 cytokines that downregulate HBV replication (27) and by contributing to the induction and maintenance of efficient CD8+ T-cell and B-cell responses (28). CD4+ T-cell epitopes have been identified in the core protein (25), HBe antigen (29), envelope (30) and polymerase proteins (31). Immune responses to HBx protein are poorly documented, with only one study dealing with CD4+ T-cell responses (20). In an attempt to define more accurately the breadth and epitope specificity of T-cell responses to HBx protein, we carried out a systematic analysis of T cells from 52 chronic HBV carriers. Following *in vitro* stimulation with pools of peptides covering the HBx sequence, IFN-γ-secreting T cells specific for HBx were detected in 69% of patients, and in 67% (24/36) of them CD4+ T cells could be defined. This contrasts with the low prevalence of the Th cell responses against structural HBV proteins usually detected during chronic HBV infection. In this study, using the core 50-69 peptide (25, 32) for stimulation of PBMC and our cutured Elispot assay, IFN-γ-secreting T-cell response was found in 3 out of 8 patients, but with a ten fold lower number of specific T cells (data not shown). Previous studies have found that HLA-class II-restricted nucleocapsid antigen-specific Th cell responses are only detectable transiently during hepatitis exacerbation (14, 33). The CD4+ T-cell response to envelope proteins is markedly reduced during chronic HBV infection (1, 34). The high prevalence of IFN-γ-secreting HBx-specific T cells reported here may be due to the protein itself or to the clinical status of the patients, i.e. HBV carriers in the inactive stage of the disease with less than 100,000 HBV copies/ml. The presence of HBx-specific CD4+ T cells during chronic infection may reflect T-cell activation due to the release of HBx protein by apoptotic hepatocytes during viral replication. The persistence of HBx-specific T cells could be related to the small amounts of HBx produced by infected hepatocytes, preventing the deletion or anergy of specific T cells occurring with other highly expressed viral antigens, such as HBsAg or HBcAg. The impact of viral load on antiviral T-cell responses has been characterized in mouse models of chronic infection and in humans (35). T-cell responses to HBV antigens are detected more easily in patients with less than 10^7 copies/ml or after

successful antiviral treatment (36, 37). Consistent with these data, we found x26-specific T cells after stimulation of PBMC from only 1 of 13 patients with chronic active hepatitis and $>10^5$ copies/ml (data not shown).

In contrast to the high prevalence of IFN-γ-secreting HBx-specific T cells, IL-10-secreting T cells were detected in very few of the studied patients, always in the presence of IFN-γ production. This is consistent with previous reports of IL-12-induced IFN-γ/IL-10-secreting T cells generated in response to chronic infection (38, 39). Regulatory T cells specific for HBx, producing IL-10, are therefore unlikely to exist in our group of patients (40). This contrasts with HCV persistent infection, which is associated with enhanced IL-10 production in response to non-structural HCV antigens such as NS3, IL-10-producing T cells in the liver and weak CD4+ Th 1 reactivity in the periphery (16, 41).

We observed a high prevalence of peripheral CD4+ T-cell responses principally targeting the C-terminal part of HBx. This finding is in agreement with a previous report characterizing T-cell clones recognizing peptides within this domain of HBx (20). Remarkably, most of our patients had IFN-γ-secreting CD4+ T cells recognizing a previously unidentified single peptide, HBx 126-140 referred here to x26. In addition, x26 peptide was immunogenic in the context of multiple HLA-class II molecules and may therefore be considered a promiscuous epitope. Nevertheless, some patients with x26-specific T cells lack the HLA-DR molecules that bound the peptide with high affinity *in vitro*. Other class II molecules not tested here are therefore probably able to bind x26. The partial correlation between the HLA-DR-restriction of the peptides and the pattern of DR alleles from different donors is consistent with published findings (31, 42).

Chronic HBV infection evolves from an initial HBeAg-positive phase, through HBe seroconversion to an HBeAg-negative phase in which replication levels are lower. This process is characterized by a progressive switch from viral quasi-species dominated by “wild-type” variants lacking precore or core promoter mutations, to viral quasi-species in which precore or core promoter variants predominate (43, 44). But it was recently shown that precore and core promoter mutations exist in a substantial proportion of patients before HBeAg clearance (22). What role do x26-specific IFN-γ-secreting T cells play against both wild-type and mutated x26-derived sequences? The effector properties of x26-

specific T cells may constitute an immune system pressure against which these mutant viral variants are selected.

The lack of recognition of variant HBx peptides, corresponding to BCP mutant sequences, by x26-specfic T cells could not be explained by a decreased binding capacity of these sequences to HLA-DR molecules, as shown by comparative binding studies with the x26, V2 and V3 peptides and purified class II molecules (data not shown). Although core promoter mutations may appear early in HBV infection (8), the possibility of initial infection with the mutant virus cannot be excluded. This is the case for patient P48 who was infected at birth with an HBeAg-negative virus, and lacked for x26-specific T-cell response. On the other hand, a wild-type sequence virus was found in patient P46 concomitantly with detectable x26-specific T cells. However, for other patients such as P59 who were initially infected with a "wild-type" variant lacking core promoter mutations, x26-specific memory T cells may exist and can be detected in our assay despite the presence of a core promoter variant viral quasi-species at the time of blood collection. Our study shows that HBV strains with mutations affecting the immunodominant HBx epitope are likely to induce weaker T-cell responses, favoring the accumulation of such mutant strains.

Mutations resulting in HBx protein truncation have also been associated with low levels of HBV replication and decreases in hepatitis activity in anti-HBe antibody-positive HBV carriers (45). Thus, at least some of the studied chronic carriers may produce truncated HBx. The presence of BCP mutants or truncated HBx mutants might account for the lack of T-cell reactivity specific for the wild-type x26 peptide in some of our patients. Therefore, x26 and variant peptides could be used for the immunomonitoring of HBV sequence changes during disease progression. This hypothesis should be tested in longitudinal studies in groups of patients differing in clinical status.

In addition, the immunogenicity, the promiscuous HLA-DR binding and the efficient activation of specific IFN-γ-secreting T cells of this newly described HBx epitope suggest that it is a potential candidate for use in therapeutic vaccines for patients with chronic HBV infection.

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REFERENCES

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995;13:29-60.

2.Ganem D, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. N Engl J Med 2004;350:1118-1129.

3.Bouchard MJ, Schneider RJ. The enigmatic X gene of hepatitis B virus. J Virol 2004;78:12725-12734.

4.Murakami S. Hepatitis B virus X protein: a multifunctional viral regulator. J Gastroenterol 2001;36:651-660.

5.Baptista M, Kramvis A, Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology 1999;29:946-953.

6.Poussin K, Dienes H, Sirma H, Urban S, Beaugrand M, Franco D, Schirmacher P, et al. Expression of mutated hepatitis B virus X genes in human hepatocellular carcinomas. Int J Cancer 1999;80:497-505.

7.Kuang SY, Jackson PE, Wang JB, Lu PX, Munoz A, Qian GS, Kensler TW, et al. Specific mutations of hepatitis B virus in plasma predict liver cancer development. Proc Natl Acad Sci U S A 2004;101:3575-3580.

8.Leon B, Taylor L, Vargas M, Luftig RB, Albertazzi F, Herrero L, Visona K. HBx M130K and V131I (T-A) mutations in HBV genotype F during a follow-up study in chronic carriers. Virol J 2005;2:60.

9.Iavarone M, Trabut JB, Delpuech O, Carnot F, Colombo M, Kremsdorf D, Brechot C, et al. Characterisation of hepatitis B virus X protein mutants in tumour and non-tumour liver cells using laser capture microdissection. J Hepatol 2003;39:253-261.

10.Stuyver L, De Gendt S, Cadranel JF, Van Geyt C, Van Reybroeck G, Dorent R, Gandjbachkh I, et al. Three cases of severe subfulminant hepatitis in heart-transplanted patients after nosocomial transmission of a mutant hepatitis B virus. Hepatology 1999;29:1876-1883.

11.Sirma H, Giannini C, Poussin K, Paterlini P, Kremsdorf D, Brechot C. Hepatitis B virus X mutants, present in hepatocellular carcinoma tissue abrogate both the antiproliferative and transactivation effects of HBx. Oncogene 1999;18:4848-4859.

12.Bertoni R, Sidney J, Fowler P, Chesnut RW, Chisari FV, Sette A. Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. J Clin Invest 1997;100:503-513.

13. Chisari FV. Cytotoxic T cells and viral hepatitis. J Clin Invest 1997;99:1472-1477.

14.Maini MK, Boni C, Ogg GS, King AS, Reignat S, Lee CK, Larrubia JR, et al. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 1999;117:1386-1396.

15.Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, Moss B, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995;181:1047-1058.

16.Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005;5:215-229.

17.Bertoletti A, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? Curr Opin Immunol 2000;12:403-408.

18.Chung MK, Yoon H, Min SS, Lee HG, Kim YJ, Lee TG, Lim JS, et al. Induction of cytotoxic T lymphocytes with peptides in vitro: identification of candidate T-cell epitopes in hepatitis B virus X antigen. J Immunother 1999;22:279-287.

19.Hwang YK, Kim NK, Park JM, Lee K, Han WK, Kim HI, Cheong HS. HLA-A2 1 restricted peptides from the HBx antigen induce specific CTL responses in vitro and in vivo. Vaccine 2002;20:3770-3777.

20.Jung MC, Stemler M, Weimer T, Spengler U, Dohrmann J, Hoffmann R, Eichenlaub D, et al. Immune response of peripheral blood mononuclear cells to HBx-antigen of hepatitis B virus. Hepatology 1991;13:637-643.

21.Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000--summary of a workshop. Gastroenterology 2001;120:1828-1853.

22.Yuan HJ, Ka-Ho Wong D, Doutreloigne J, Sablon E, Lai CL, Yuen MF. Precore and core promoter mutations at the time of HBeAg seroclearance in Chinese patients with chronic hepatitis B. J Infect 2006.

23.Texier C, Pouvelle-Moratille S, Busson M, Charron D, Menez A, Maillere B. Complementarity and redundancy of the binding specificity of HLA-DRB1, -DRB3, -DRB4 and -DRB5 molecules. Eur J Immunol 2001;31:1837-1846.

24.Texier C, Pouvelle S, Busson M, Herve M, Charron D, Menez A, Maillere B. HLA-DR restricted peptide candidates for bee venom immunotherapy. J Immunol 2000;164:3177-3184.

25.Ferrari C, Bertoletti A, Penna A, Cavalli A, Valli A, Missale G, Pilli M, et al. Identification of immunodominant T cell epitopes of the hepatitis B virus nucleocapsid antigen. J Clin Invest 1991;88:214-222.

26.Mancini-Bourgine M, Fontaine H, Scott-Algara D, Pol S, Brechot C, Michel ML. Induction or expansion of T-cell responses by a hepatitis B DNA vaccine administered to chronic HBV carriers. Hepatology 2004;40:874-882.

27.Franco A, Guidotti L, Hobbs MV, Pasquetto V, Chisari FV. Pathogenic effector function of CD4-positive T helper 1 cells in hepatitis B virus transgenic mice. J. Immunol. 1997;159:2001-2008.

28.Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med 1998;188:2199-2204.

29.Jung MC, Diepolder HM, Spengler U, Wierenga EA, Zachoval R, Hoffmann RM, Eichenlaub D, et al. 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. J Virol 1995;69:3358-3368.

30.Celis E, Ou D, Otvos L, Jr. Recognition of hepatitis B surface antigen by human T lymphocytes. Proliferative and cytotoxic responses to a major antigenic determinant defined by synthetic peptides. J Immunol 1988;140:1808-1815.

31.Mizukoshi E, Sidney J, Livingston B, Ghany M, Hoofnagle JH, Sette A, Rehermann B. Cellular immune responses to the hepatitis B virus polymerase. J Immunol 2004;173:5863-5871.

32.Torre F, Cramp M, Owsianka A, Dornan E, Marsden H, Carman W, Williams R, et al. Direct evidence that naturally occurring mutations within hepatitis B core epitope alter CD4+ T-cell reactivity. J Med Virol 2004;72:370-376.

33.Tsai SL, Chen PJ, Lai MY, Yang PM, Sung JL, Huang JH, Hwang LH, et al. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen seroconversion. J Clin Invest 1992;89:87-96.

34.Bocher WO, Herzog-Hauff S, Schlaak J, Meyer zum Buschenfeld KH, Lohr HF. Kinetics of hepatitis B surface antigen-specific immune responses in acute and chronic hepatitis B or after HBs vaccination: stimulation of the in vitro antibody response by interferon gamma. Hepatology 1999;29:238-244.

35.Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol 2003;77:4911-4927.

36.Webster GJ, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, Williams R, et al. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 2004;78:5707-5719.

37.Boni C, Penna A, Ogg GS, Bertoletti A, Pilli M, Cavallo C, Cavalli A, et al. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. Hepatology 2001;33:963-971.

38.Pohl-Koppe A, Balashov KE, Steere AC, Logigian EL, Hafler DA. Identification of a T cell subset capable of both IFN-gamma and IL-10 secretion in patients with chronic Borrelia burgdorferi infection. J Immunol 1998;160:1804-1810.

39.Vingerhoets J, Michielsen P, Vanham G, Bosmans E, Paulij W, Ramon A, Pelckmans P, et al. HBV-specific lymphoproliferative and cytokine responses in patients with chronic hepatitis B. J Hepatol 1998;28:8-16.

40.Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737-742.

41.Accapezzato D, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, Abrignani S, et al. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. J Clin Invest 2004;113:963-972.

42.Bauer T, Weinberger K, Jilg W. Variants of two major T cell epitopes within the hepatitis B surface antigen are not recognized by specific T helper cells of vaccinated individuals. Hepatology 2002;35:455-465.

43.Yuen MF, Sablon E, Yuan HJ, Hui CK, Wong DK, Doutreloigne J, Wong BC, et al. Relationship between the development of precore and core promoter mutations and hepatitis B e antigen seroconversion in patients with chronic hepatitis B virus. J Infect Dis 2002;186:1335-1338.

44.Yim HJ, Lok AS. Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. Hepatology 2006;43:S173-181.

45.Fukuda R, Nguyen XT, Ishimura N, Ishihara S, Chowdhury A, Kohge N, Akagi S, et al. X gene and precore region mutations in the hepatitis B virus genome in persons positive for antibody to hepatitis B e antigen: comparison between asymptomatic "healthy" carriers and patients with severe chronic active hepatitis. J Infect Dis 1995;172:1191-1197.

Legends to Figures

Figure 1. Presence and diversity of HBx-specific IFN-γ-secreting T cells in 52 patients with chronic HBV infection. PBMCs were stimulated *in vitro* with HBx-derived 15-mer peptides covering the whole HBx sequence, divided into pools A, B and C. IFN-γ-secreting T cells were determined by Elispot, using the same peptide pools. The proportion of patients testing positive is indicated on the top of each column. A) Percentage of patients with undetectable HBx-specific T cells (white column) and with HBx-specific T cells activated with each peptide pool (gray columns); B) diversity of recognition of regions within the HBx protein by HBx-specific T cells (gray striped columns).

Figure 2. IFN-γ-secreting T cells upon stimulation with peptide pool C and mapping of the T-cell response to single peptides. Number of IFN-γ-secreting T cells determined by Elispot and expressed as the number of specific spot-forming cells (SFC)/10^6 PBMCs after *in vitro* stimulation with peptide pool C (left panel), individual peptide x26 (middle panel), and single peptides or groups of peptides from pool C with the exception of x26 peptide (right panel). In the right panel, the number of SFC is indicated on each bar. The scale of the right panel differs from that of the left and central panels. ND: not done.

Figure 3. IFN-γ- and IL-10-secreting T cells determined by Elispot assays after *in vitro* stimulation of PBMC with the entire peptide pool C or x26 alone. Number of IFN-γ- or IL-10 spot-forming cells (SFC)/10^6 PBMC (black and gray columns, respectively) of 11 patients with only IFN-γ-secreting T cells (A panel) and 7 patients with both IFN-γ- and IL-10-secreting T cells (B panel). Number of SFC is indicated on the top of each column. The cutoff points for Elispot assays are 62 IFN-γ- and 40 IL-10-SFC/10^6 PBMC.

Figure 4. Phenotype of x26-specific T cells after *in vitro* expansion from PBMC with x26. A) Percentages of IFN-γ-secreting CD3+ CD4+ (left panel) or CD3+ CD8+ (right panel) specific T cells are shown, B) within the CD4+ T-cell population, the percentages of x26-specific CD4+ T cells secreting IFN-γ (left panel), IL-10 (central panel) and IL-10 and/or IFN-γ (right panel) are shown.

Figure 5. Anti-MHC class II antibody-mediated inhibition of IFN-γ secretion by x26-specific T cells. PBMC expanded *in vitro* with peptide x26 were first incubated with anti-class II HLA antibodies — anti-HLA-DR, anti-

HLA-DQ or anti-HLA-DP — or with an irrelevant antibody (anti-HLA-A2). PBMC were then tested in Elispot assays as described in Materials and Methods. Results obtained with three representative patients are shown.

Figure 6. Recognition of viral mutants by x26-specific T cells. PBMC from 13 patients with known x26-specific IFN-γ-producing T cells were expanded in vitro separately with x26 peptide (left panel) or with a mixture of x26, V2 and V3 peptides (right panel). IFN-γ-secretion after the activation with each of the three peptides in Elispot assays is shown.

Figure online. Amino acid sequence of x26 region.

Legends to Tables

Table (available online). Clinical, virological and immunological characteristics of patients included in this study.

Table 1. Binding of HBx-derived peptides to immunopurified class II HLA molecules. Data are expressed as relative activity: ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide. The relative activities of pool C peptides and an HBcAg-derived peptide (c50-69) are shown. Boldface indicates relative binding affinity below 100 and corresponds to good binders.

Table 2. Comparative analysis of patients' HLA-DR genotypes, x26-binding HLA-DR molecules and x26-specific IFN-γ-secreting T-cell responses. Brackets for DRB1 genotype indicate that heterozygosity could not be confirmed with the current assay. (#) HLA-DR molecules binding x26, as shown in Table 1 for DRB1*0101, DRB1*0401, DRB1*1301 and DRB5*0101. (##) determined by Elispot assays and expressed as ranges of IFN-γ-SFC per million PBMC: + 100-500; ++ 500-1000; +++ 1000-2000 and ++++ >2000.

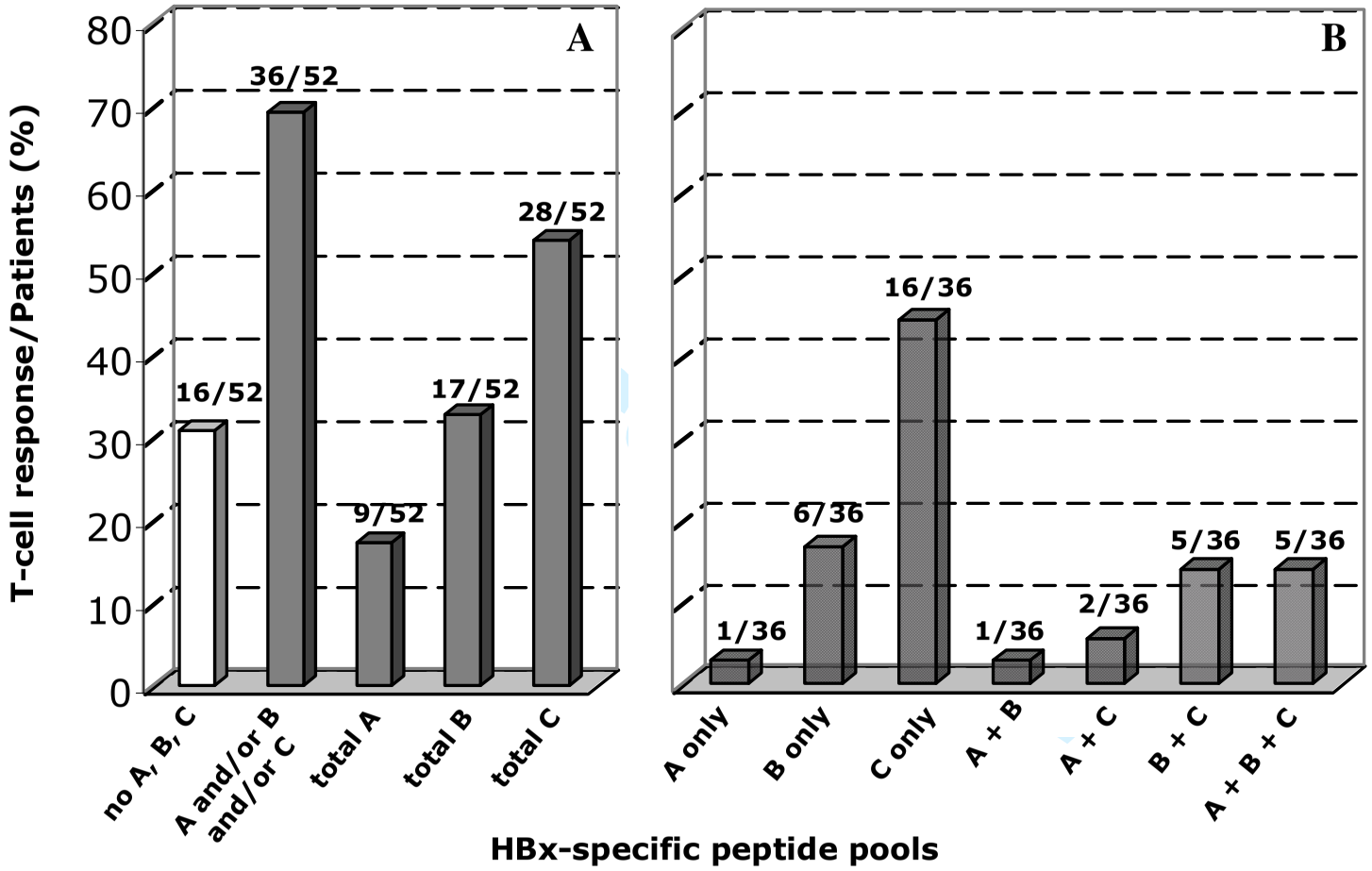

Figure 1. Malmassari *et al.*

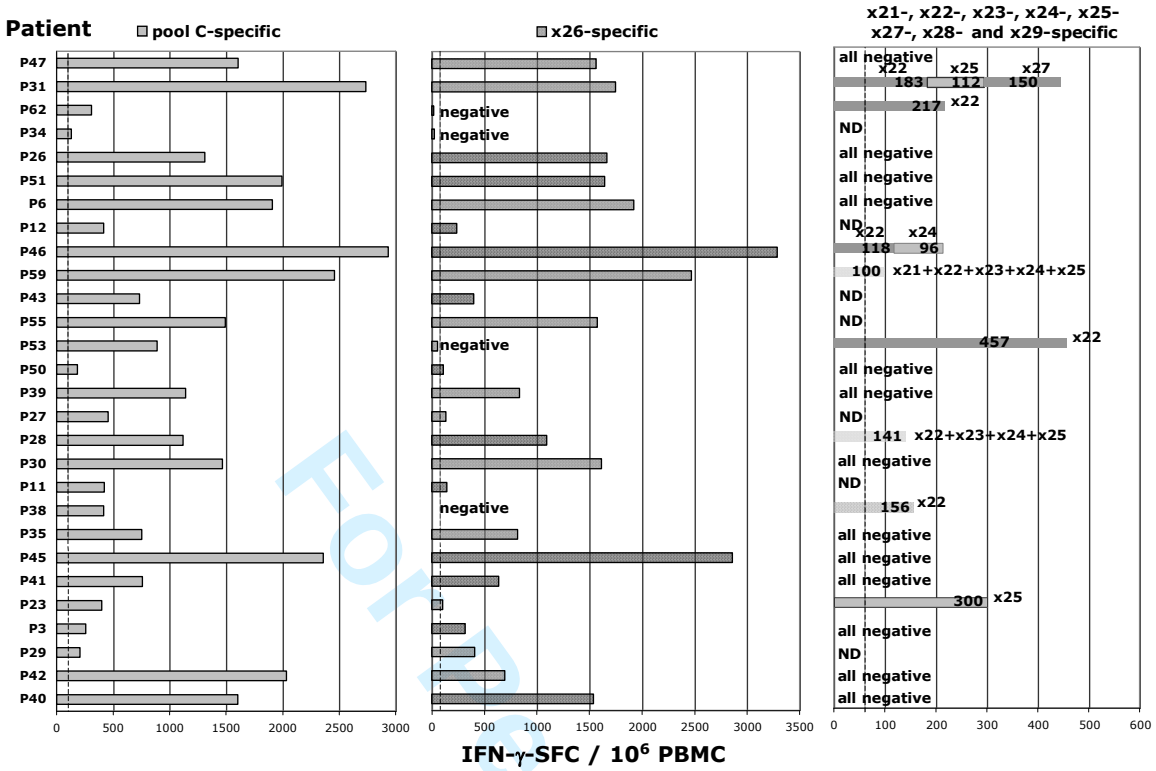

Figure 2. Malmassari et al.

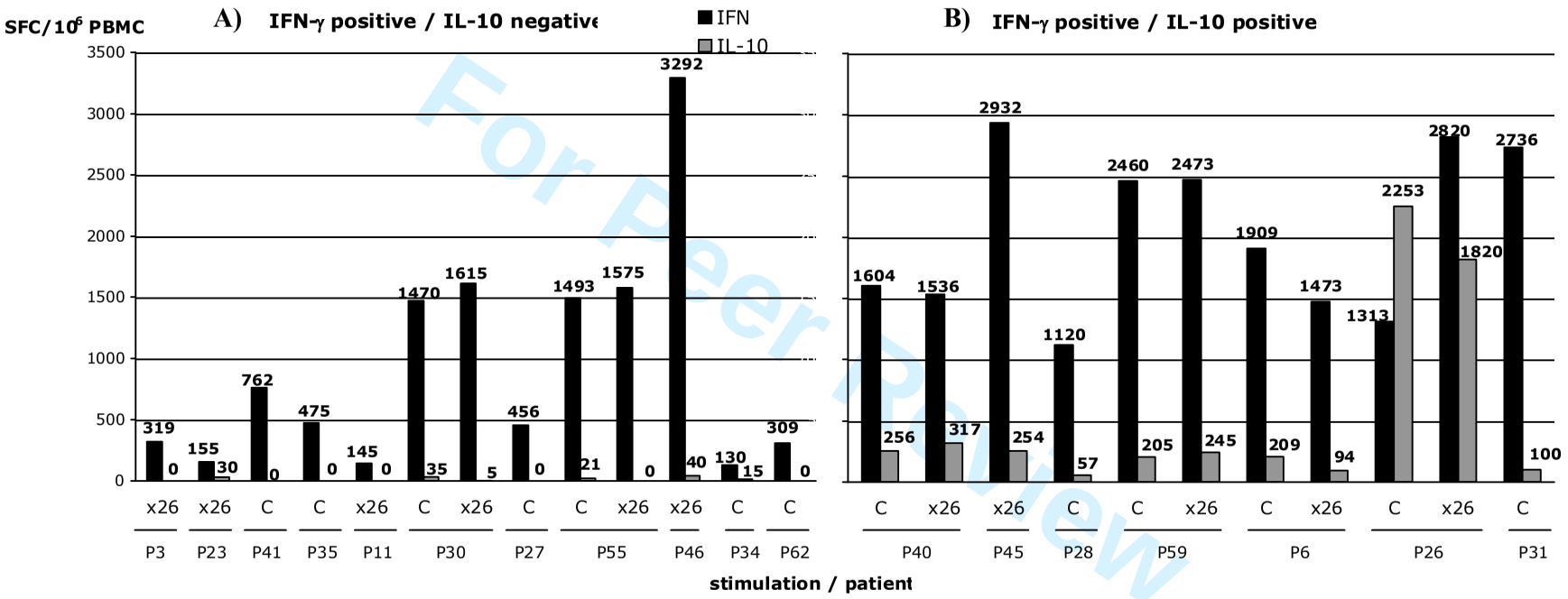

Figure 3. Malmassari *et al.*

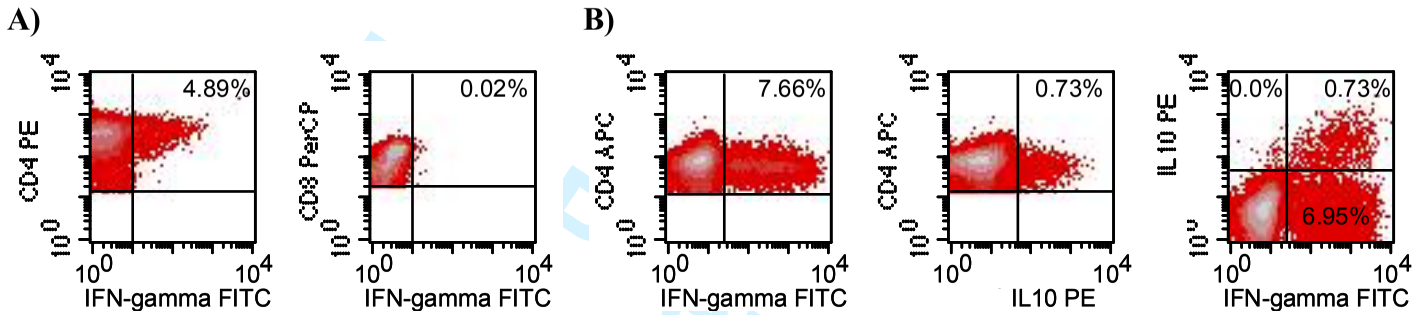

Figure 4. Malmassari *et al.*

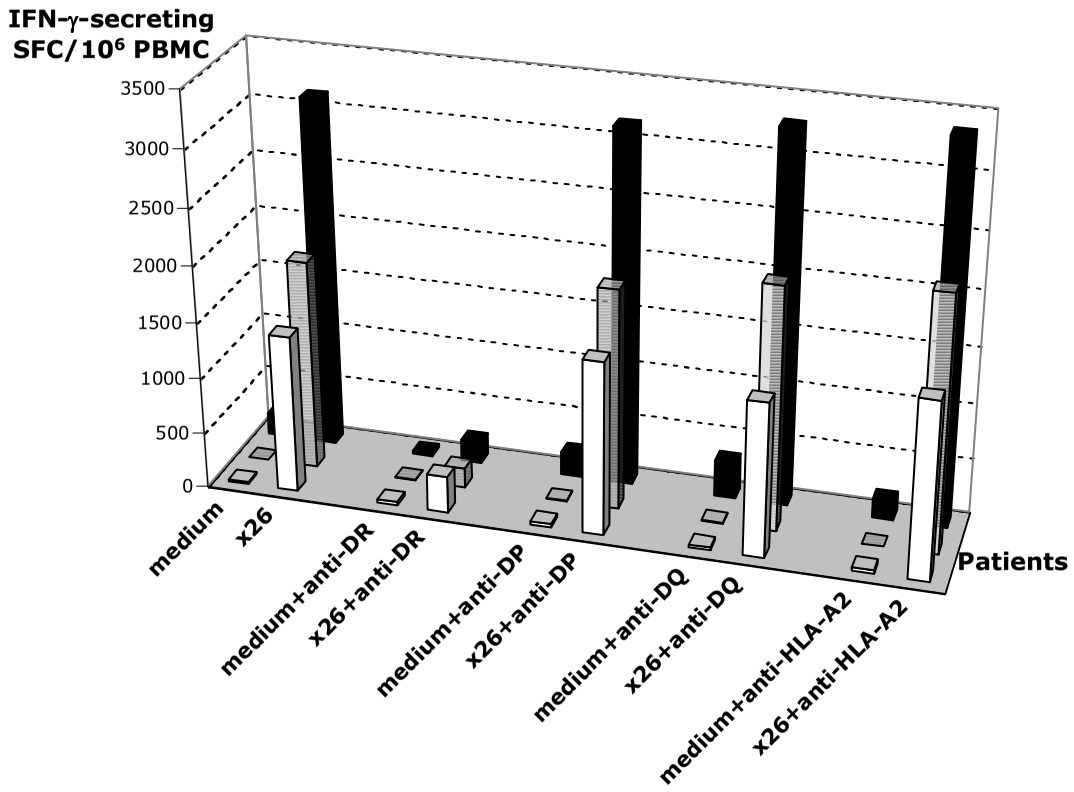

Figure 5. Malmassari *et al.*

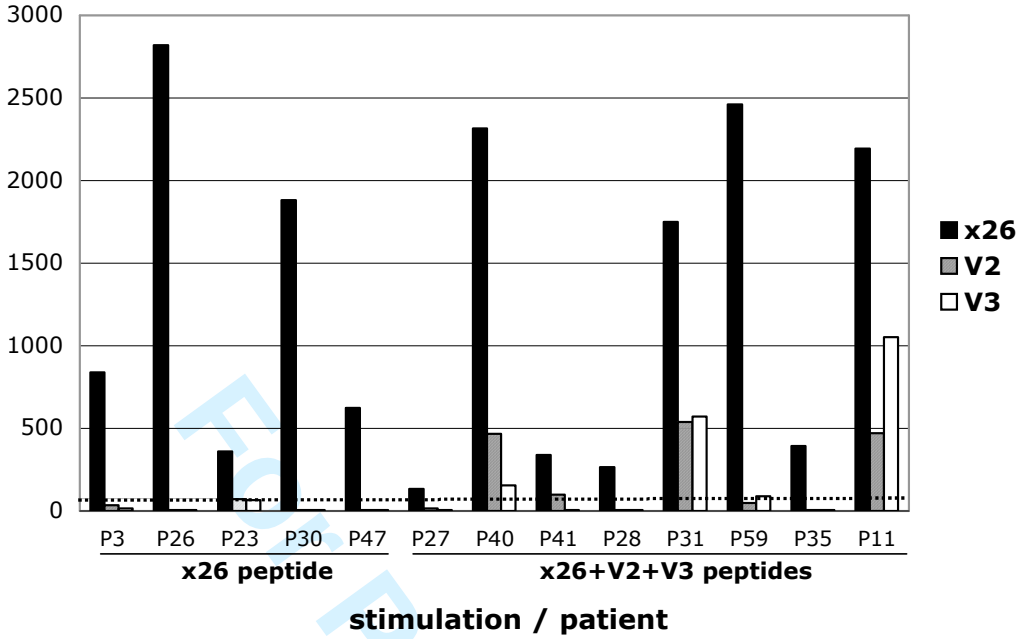

Figure 6. Malmassari *et al.*

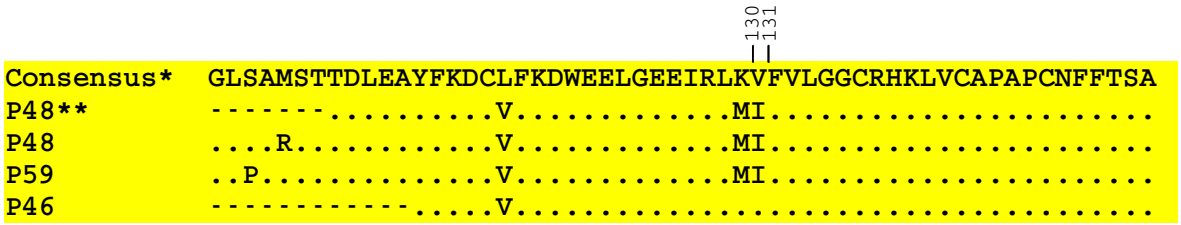

Figure available online

Amino acid sequence of x26 region. Sequencing of HBV viral DNA was performed for three patients: P48, P59 and P46. Part of the preC gene was amplified using two couples of primers in a nested PCR (1680-1962 outer primers and 1705-1940 inner primers). PCRs were performed using a Thermal Cycler 9700 Gold (Perkin Elmer, CA) under the following conditions: 5 cycles, each comprising 15 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C; then 35 cycles, each comprising 15 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C. PCR results were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide. *HBx consensus sequence obtained by comparing published HBx-encoding sequences in Genbank (see Materials and Methods); **two different samples from P48 taken at one year interval. Amino acid positions of mutant sequences on HBx protein are indicated.

Peptides	HLA class II molecules									
	DRB1 *0101	DRB1 *0301	DRB1 *0401	DRB1 *0701	DRB1 *1101	DRB1 *1301	DRB1 *1501	DRB3 *0101	DRB4 *0101	DRB5 *0101
x21	>5,848	>25	>261	>1,547	>1,754	>59	>377	155	135	>1,805
x22	4,455	>25	247	>1,547	>1,754	**41**	245	783	**77**	>1,805
x23	4,160	>25	>261	>1,547	>1,754	**41**	>377	>656	>1,032	>1,805
x24	2,412	>25	>261	>1,547	>1,754	>59	>377	>656	>1,032	1,122
x25	814	**1**	158	135	**4**	**6**	**40**	347	519	>1,805
x26	**1**	>25	**17**	>1,547	900	**55**	112	>656	224	**31**
x27	1,662	>25	>261	1,111	1,400	**10**	>377	>656	>1,032	>1,805
x28	766	>25	133	>1,547	>1,754	>59	195	>656	154	313
x29	2,932	>25	200	540	>1,754	>59	144	>656	549	1,700
c50-69	215	**7**	131	360	408	**15**	**20**	>656	285	171

Table 1.

Binding of HBx-derived peptides to immunopurified class II HLA molecules. Data are expressed as relative activity: ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide. The relative activities of pool C peptides and an HBc-derived peptide (c50-69) are shown. Boldface indicates relative binding affinity below 100 and corresponds to good binders.

Patients	HLA-DR genotype				Presence of HLA-DR with	x26-specific IFN-γ-secreting
	DRB1	DRB3	DRB4	DRB5	x26-binding capacity #	T cells ##
P32	*0701, *0901		√		No	negative
P8	*0301	√			No	negative
P64	*0801, *0901		√		No	negative
P44	*0301	√			No	negative
P34	*0301, *0701	√	√		No	negative
P3	*0301, *1301	√			Yes	++
P41	*0101, *0401			√	Yes	++
P45	*1101, *1301	√			Yes	++++
P11	*0301, *1301	√			Yes	+
P30	*0401, *1101	√	√		Yes	+++
P27	*0401, *1301	√	√		Yes	+
P50	*0101, *0301	√			Yes	+
P59	*0401, *1101	√	√		Yes	++++
P46	*1101, *1301	√			Yes	++++
P12	*0301, *1301	√			Yes	+
P31	*0101, *1501			√	Yes	+++
P23	*0301 (*1367)	√			No	+
P35	*0301, *1101	√			No	++
P6	*0701, *1101	√	√		No	+++
P51	*1101, *1201	√			No	+++
P26	*1101, *1201	√			No	+++
P47	*1101, *1201	√			No	+++
P13	*0401, *1301	√	√		Yes	negative
P1	*0401, *0701		√		Yes	negative
P2	*0801, *1501			√	Yes	negative
P48	*0401, *1401	√	√		Yes	negative
P4	*0401 (*1367)		√		Yes	negative
P38	*1201, *1501	√		√	Yes	negative
P53	*0101, *0401		√		Yes	negative
P62	*0101, *1501			√	Yes	negative

Table 2. Comparative analysis of patients' HLA-DR genotypes, x26-binding HLA-DR molecules and x26-specific IFN-γ-secreting T-cell responses. Brackets for DRB1 genotype indicate that heterozygosity could not be confirmed with

the current assay. (#) HLA-DR molecules binding x26, as shown in Table 1 for DRB1*0101, DRB1*0401, DRB1*1301 and DRB5*0101. (##) determined by Elispot assays and expressed as ranges of IFN-γ-SFC per million PBMC: + 100-500; ++ 500-1000; +++ 1000-2000 and ++++ >2000.

Patient ID	Age	Sex	Viral load at inclusion (copies/ml)	Previous treatment	Metavir score	Possible risk of infection	HBx-specific IFN-γ-secreting T cells*
P2	60	M	5,100	No	ND	Sexual transmission	Positive (x26-)
P3	50	M	<200	No	A0/A1-F0	Blood transfusion	Positive (x26+)
P4	58	F	870	No	ND	Unknown	Negative
P6	57	M	<200	No	ND	Stay in endemic region	Positive (x26+)
P7	48	F	4,700	No	A0-F1	Born in endemic region HBeAg-negative mutant**	Positive (x26-)
P8	29	F	900	No	A0-F0	Born in endemic region	Negative
P9	44	M	69,909	No	A1-F0	Born in endemic region	Negative
P10	31	F	<2,000	No	A0-F0	Unknown	Negative
P11	52	F	10,200	No	A0-F0	Born in endemic region Perinatal transmission	Positive (x26+)
P12	59	M	2,600	No	A0/A1-F1/F2	Born in endemic region HBeAg-negative mutant**	Positive (x26+)
P13	46	M	<200	IFN-α (1991)	A0-F4	Unknown	Negative
P19	26	M	<200	No	A1-F1	Born in endemic region	Negative
P20	43	M	<200	No	ND	Unknown	Negative
P22	60	M	<200	IFN-α (2000)	A1-F4	Born in endemic region	Negative
P23	40	F	13,000	No	ND	Born in endemic region	Positive (x26+)

P24	41	M	34,000	No	A2-F1	Born in endemic region HBeAg-negative mutant**	Negative
P25	21	F	<2,000	No	A0-F0	Born in endemic region HBeAg-negative mutant**	Negative
P26	24	M	<2,000	Lamivudine (1999), adefovir (2003)	A1-F1	Perinatal transmission	Positive (x26+)
P27	56	M	4,200	No	A0-F0	Unknown	Positive (x26+)
P28	31	F	<2,000	No	A0-F0/F1	Born in endemic region	Positive (x26+)
P29	29	M	1,700	No	A0/A1-F1/F2	Born in endemic region	Positive (x26+)
P30	55	M	6,100	IFN-α (1992)	ND	Sexual transmission	Positive (x26+)
P31	60	M	11,000	No	ND	stay in endemic region	Positive (x26+)
P32	35	M	<2,000	Lamivudine (2002)	ND	Born in endemic region	Negative
P33	42	M	1,400	No	ND	Born in endemic region	Positive (x26-)
P34	57	F	2,700	IFN-α (1992)	A0-F2	Horizontal transmission	Positive (x26-)
P35	59	F	<200	No	ND	Blood transfusion	Positive (x26+)
P36	45	F	14,000	No	A0/A1-F0	Unknown	Positive (x26-)
P37	35	F	8,800	No	ND	Born in endemic region	Negative
P38	31	M	<2,000	No	A0/A1-F0	Born in endemic region	Positive (x26-)
P39	38	M	<2,000	No	ND	Born in endemic region	Positive (x26+)
P40	33	F	<2,000	No	A0-F0	Born in endemic region	Positive (x26+)

P41	57	M	<2,000	No	A0-F1/F2	Risk of sexual transmission	Positive (x26+)
P42	37	M	42,000	No	A0/A1-F2	Born in endemic region	Positive (x26+)
P43	21	F	16,700	No	A0-F0	Blood transfusion	Positive (x26+)
P44	47	M	3,000	No	ND	Born in endemic region	Negative
P45	60	M	<2,000	IFN-α (1993)	F3/F4	Born in endemic region	Positive (x26+)
P46	21	M	40,000	No	A0-F0	Born in endemic region	Positive (x26+)
P47	25	M	13,200	No	ND	Born in endemic region	Positive (x26+)
P48	44	M	36,000	No	ND	Perinatal transmission. Mother with a HBeAg-negative mutant	Positive (x26-)
P49	21	F	8,300	No	A0-F0	Perinatal transmission	Positive (x26-)
P50	27	M	5,200	No	A0-F1/F2	Born in endemic region	Positive (x26+)
P51	49	M	<2,000	Peg-FN-α□□□□□□□□□□□□, □□□□□□□□ (2003-04)	A1-F2	Born in endemic region HBeAg-negative mutant**	Positive (x26+)
P52	29	F	2,900	No	A0-F0/F1	Perinatal and/or horizontal transmission	Positive (x26-)
P53	60	M	<2,000	No	A0-F3	Unknown	Positive (x26-)
P54	43	M	4,000	Peg- IFN-α □□□□□□, □□□□□□□□□□	A0/A1-F1/F2	Born in endemic region	Negative

				(2002-03)			
P55	31	M	<2,000	IFN-α (1998)	A1-F1	Perinatal and/or horizontal transmission	Positive (x26+)
P56	35	F	11,400	IFN-α (2000)	ND	Perinatal transmission	Negative
P57	35	M	11,000	□□□□□□□□□□, □□□□□□□□ (1998-2003), IFN-α (2003-04)	A1-F2/F3	Unknown	Positive (x26-)
P59	34	M	20,000	No	ND	Born in endemic region	Positive (x26+)
P62	35	M	<2,000	No	A0-F0/F1	Born in endemic region	Positive (x26-)
P64	29	M	16,600	IFN-α (1998)	A0-F0	Perinatal and/or horizontal transmission	Negative

Table (available online)

Clinical, virological and immunological characteristics of patients included in this study. *Detected by Elispot assays after *in vitro* simulation of PBMC with overlapping 15-mers peptides covering HBx protein. In brackets: presence or absence of the immunodominant T-cell response directed against x26 epitope. ** HBeAg-negative mutant suspected from clinical and serological followed-up of the patient.

Impact of hepatitis B virus basic core promoter mutations on T-cell response to an immunodominant HBx-derived epitope

Silvina Laura MALMASSARI[1,2], Qiang DENG[1,2], Hélène FONTAINE[3], Dianne HOUITTE[4], François RIMLINGER[2,5], Valérie THIERS[2,5], Bernard MAILLERE[4], Stanislas POL[3], Marie-Louise MICHEL[1,2]

[1] INSERM U812, Pathogenèse des Hépatites Virales B et Immunothérapie; [2] Institut Pasteur, Département de Virologie, Paris, France; [3] AP-HP, Unité d'Hépatologie, Hôpital Cochin, Paris, France; [4] Département d'Ingénierie et d'Etudes des Protéines, Commissariat à l'Energie Atomique (CEA), Gif sur Yvette, France; [5] INSERM U785, Villejuif, France

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Contact Information

Marie-Louise Michel

INSERM U812, Pathogénèse des Hépatites Virales B et Immunothérapie; Institut Pasteur, Département de Virologie

28, rue du Docteur Roux

75724 Paris Cedex 15

France

Telephone number: +33 1 4568 8849

Fax number: +33 1 4061 3841

E-mail: maloum@pasteur.fr

E-mail addresses of all authors

S. Malmassari: smalmass@pasteur.fr

Q. Deng: qdeng@pasteur.fr

H. Fontaine: helene.fontaine@cch.aphp.fr

D. Houitte: diane.houitte@cea.fr

B. Maillere: Bernard.maillere@cea.fr

F. Rimilinger: rimlinger@vjf.inserm.fr

V. Thiers: thiers@vjf.inserm.fr

S. Pol: stanislas.pol@cch.aphp.fr

M.-L. Michel: maloum@pasteur.fr

Abbreviations

HBV, hepatitis B virus; HBx, hepatitis B X protein; HCC, hepatocellular carcinoma; CTL, cytotoxic T lymphocyte; Th, T helper; IFN, interferon; IL, interleukin; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; Elispot, enzyme-linked immunosorbent spot; SFC, spot-forming cell

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ABSTRACT

The hepatitis B X (HBx) protein is a crucial component in hepatitis B virus (HBV) infection *in vivo* and has been implicated in hepatocellular carcinoma. In this study, we aimed to detect and characterize peripheral HBx-specific T cells in chronically infected patients at the inactive carrier state of the disease. HBx-specific IFN-γ-secreting T cells were found in 36 of 52 patients (69%) and 78% (28/36) of responding patients had T cells targeting epitopes in the carboxy-terminal part of HBx. IL-10 secretion following the stimulation of T cells with HBx-derived peptides was weak or undetectable. IFN-γ-secreting T cells recognized a previously unknown immunodominant CD4+ T-cell epitope, HBx 126-140 (EIRLKVFVLGGCRHK), in 86% (24/28) of patients. This peptide bound several HLA-DR molecules (HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*1301 and HLA-DRB5*0101). Its coding sequence overlaps a domain of the HBV genome encompassing the basic core promoter (BCP) region. Taking into account the selection of viral core promoter mutants during HBV infection, we found that HBV variants with BCP mutations were present in patient sera. We further demonstrated that these viral mutant sequences activated T cells specific for the immunodominant epitope only weakly, if at all. This is the first study linking BCP mutations and HBx-specific T-cell responses. Therefore, wild type and variant peptides may represent potentially tools for monitoring the HBV-specific T-cell responses involved in sequence evolution during disease progression. Finally, the degenerate HLA-DR binding of this promiscuous, immunodominant peptide would make it a valuable component of vaccines for protecting large and ethnically diverse patient populations.

INTRODUCTION

An effective vaccine against hepatitis B virus (HBV) infection has been available for more than two decades, but 400 million people —more than 5% of the world's population — are chronically infected with HBV, and more than 1 million people die each year from HBV-related liver cirrhosis and hepatocellular carcinoma (HCC) (1, 2).

The hepatitis B X (HBx) protein is a key element in HBV infection *in vivo* and has been implicated in HCC development. HBx is well conserved among mammalian hepadnaviruses and is produced very early after infection and throughout chronic infection. The potentially oncogenic functions of HBx include the transcriptional activation of genes encoding proteins regulating cell growth, apoptosis modulation and the inhibition of nucleotide excision repair following DNA damage. HBx exerts its effects by interacting with cellular proteins and activating cell signaling pathways (3, 4).

The pathogenesis of HBV infection involves the selection and expression of several common viral mutants. HBV genes have overlapping open reading frames. A mutation in the HBV genome may therefore have effects on several proteins. The HBx gene overlaps regions of crucial importance for viral replication, such as the direct repeat sequences DR1 and DR2, the preC/C gene promoter and the enhancer II region. The common double mutation in the HBV basic core promoter (BCP) region A1762T/G1764A corresponds to a double mutation in codons 130 and 131 of the HBV X gene. The change in HBx amino-acid sequence (K130M and V131I) resulting from these T-A point mutations is associated with severe liver damage and HCC (5-8). These substitutions may be associated with an additional mutation at position 127 in the HBx protein, which has been detected in patients with HCC or fulminant hepatitis (9, 10). Natural mutations in the HBx gene are thought to lead to progression to chronic disease due to the abolition of anti-proliferative and apoptotic effects, causing uncontrolled growth and multistep hepatocarcinogenesis (11). The selection and expression of natural HBx mutants may have major implications for T-cell recognition of this protein.

As HBV is mainly not directly cytopathic, the immune response to viral antigens is thought to be responsible for both liver disease and viral clearance following HBV infection. Patients with acute viral infection who successfully clear the virus display a multispecific polyclonal cytotoxic T-

lymphocyte (CTL) response specific for a number of epitopes within the core, polymerase and envelope proteins (12-15). HBV-specific T helper (Th) cells are also activated and multispecific Th1-like responses are detected in patients successfully clearing HBV after acute infection (16). This HBV-specific T-cell response is weak or undetectable in patients who develop chronic infection (17). Little is known about the CTL directed against HBx protein in HBV-infected individuals (18, 19) or about HBx-specific CD4+ T cells and their cytokine profile during the course of viral infection (20).

We characterized peripheral HBx-specific T cells in 52 patients with chronic HBV infection at the inactive carrier state of the disease, by measuring interferon-gamma (IFN-γ) and interleukin-10 (IL-10) secretion after the activation of peripheral blood mononuclear cells (PBMC) with 15-mer peptides spanning the HBx sequence. We identified an immunodominant, promiscuous T-cell epitope, HBx 126-140, located in the carboxy-terminal part of the protein and recognized by IFN-γ-secreting CD4+ T cells in most patients. HBV core promoter mutations, which frequently occur during chronic infection, modify the sequence of this HBx-derived immunodominant CD4+ T-cell epitope. These mutant viral sequences were recognized by T cells specific for the HBx wild-type epitope only weakly, if at all.

MATERIALS AND METHODS

Patient population

Fifty-two subjects with chronic HBV infection, in the inactive carrier state of the disease (21) with less than 100,000 HBV copies/ml were enrolled (see Table online). All were hepatitis B surface antigen (HBsAg)-positive, hepatitis B e antigen (HBeAg)-negative, anti-HBe antibodies-positive, had normal transaminase levels and no or low underlying liver disease. This group of patients is heterogeneous as it contains i) patients with persistently low HBV DNA levels, even falling to undetectable levels (< 200 cp/ml) either spontaneously or after effective antiviral treatment and ii) patients with fluctuating levels of HBV DNA being nevertheless < 100,000 cp/ml. This sub-group may include patients carrying HBV viruses with preC or BCP mutations (22). Patients had received no antiviral treatment for at least six months before inclusion. All patients were 18 to 60 years old, had no

immunosuppression or infections associated with human immunodeficiency virus, hepatitis C virus or hepatitis D virus, or liver diseases other than HBV infection, and consumed less than 40 g of alcohol/d. HLA-DR genotyping was carried out with the Olerup SSP™ Genovision kit (Saltsjöbaden, Sweeden). Two blood samples were collected from each patient at a mean of 8 ± 4 month interval. This study was approved by the ethics committee of hospital, and all participants gave informed, written consent for participation, in line with French ethical guidelines.

Synthetic peptides

Synthetic peptides were purchased from NeoMPS (Strasbourg, France). The consensus sequence of the HBx protein, obtained by comparing published HBx-encoding sequences in Genbank — MAARLCCQLDPARDVLCLRPVGAESRGRPLSGPLGTLSSPSPSAVPTDHGAHLSLRGLPVCAF SSAGPCALRFTSARRMETTVNAHQILPKVLHKRTLGLSAMSTTDLEAYFKDCLFKDWEELGE EIRLKVFVLGGCRHKLVCAPAPCNFFTSA — was covered by 29 15-mer peptides with 10-residue overlaps. Individual or pooled peptides were used to stimulate PBMCs *in vitro* and for the Elispot assay. Three peptide pools were used: pool A (peptides x1 to x10), pool B (peptides x11 to x20) and pool C (peptides x21 to x29). Two additional peptides corresponding to variant sequences of the wild-type x26 peptide (HBx 126-140, EIRLKVFVLGGCRHK) were used: V2 (EIRLMIFVLGGCRHK) and V3 (ETRLMIFVLGGCRHK). Peptides were prepared at 1 mg/ml in water or 20% DMSO if required and stored at -20 °C until use.

HLA-DR peptide-binding assays COULD BE PROVIDED ON LINE

HLA-DR molecules were purified from homologous EBV cell lines by affinity chromatography, as previously described (23, 24). Binding to various HLA-DR molecules was assessed by competitive ELISA, as previously described (23, 24). We used the individual peptides of pool C and a 20-mer HBc-derived peptide (core 50-69; PHHTALRQAILCWGELMTLA) as competitors (25). Maximal binding was determined by incubating the biotinylated peptide with the MHC class II molecule in the absence of competitor. Binding specificity for each HLA-DR molecule was ensured by the choice of the biotinylated peptides as described previously (23, 24). Concentration of the peptide that prevented

50% of binding of the biotinylated peptide was evaluated (IC_{50}). The reference peptide is the unlabelled form of the biotinylated peptide and corresponds to high binder. Their IC50 are the following: 2 nM for DRB1*0101; 403 nM for DRB1*0301; 38 nM for DRB1*0401; 6 nM for DRB1*0701; 6 nM for DRB1*1101; 170 nM for DRB1*1301; 26 nM for DRB1*1501; 15 nM for DRB3*0101; 10 nM for DRB4*0101 and 6 nM for DRB5*0101. Data are expressed as relative activity: ratio of the IC_{50} of the peptide by the IC_{50} of the reference peptide.

***In vitro* expansion of the PBMC**

PBMC were suspended at 3 x 10^6 cells per ml in complete medium (RPMI 1640 medium, Life Technologies, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% human AB serum (Institut Jacques Boy, Reims, France) plus 20 ng/ml IL-7 (Roche, Meylan, France) and 100 pg/ml IL-12 (R&D Systems Inc., MN, USA), in 24-well plates. Cells were stimulated by incubation with peptide pools A, B and C (1 µg/ml of each peptide) or with individual peptides (10 µg/ml). Half the medium was replaced every three to four days with complete medium supplemented with recombinant IL-2 (50 IU/ml) (Roche, Meylan, France). After 10 to 14 days of culture, HBx-specific IFN-γ- and IL-10-producing cells were quantified by Elispot assays and intracellular cytokine staining.

Elispot assay <u>COULD BE PROVIDED ON LINE</u>

Sterile nitrocellulose HA 96-well plates (Millipore, Bedford, MA) were coated with 15 µg/ml anti-IFN-γ mAb (clone 1-DIK; Mabtech, Stockholm, Sweden) in 0.1 M bicarbonate buffer (pH 9.6) or with 10 µg/ml anti-IL-10 mAb (clone B-N10, Diaclone, Besançon, France) in PBS (pH 7.0). The wells were blocked and washed (26), then filled, in triplicate, with *in vitro*-stimulated cells (1 to 2 x 10^5/well) in complete medium and the appropriate peptides (1 µg/ml), with medium alone used as a negative control and phorbol myristate acetate (25 ng/ml)/ionomycin (2 mg/ml) or staphylococcal enterotoxin B (500 ng/ml) (Sigma, St. Louis, MO, USA), as positive control. After 20 h of incubation at 37°C, plates were washed and incubated with 1 µg/ml biotinylated anti-IFN-γ mAb (clone 7B6-1;

Mabtech) or with 20 µg/ml biotinylated anti-IL-10 mAb (clone B-T10; Diaclone) for 2 h at room temperature. Plates were then washed and antibody binding was detected as previously described (26). A Zeiss Elispot automatic counter was used to score the number of spots.

The specificity and cut-off of Elispot assays were determined with PBMC from healthy individuals (n=9) and with PBMC from hemochromatosis patients (n=2). These PBMC were subjected to *in vitro* expansion with HBx-derived peptides and tested in Elispot assays in experimental conditions identical to those used for PBMC from chronic HBV carriers. The cut-off of Elispot assays were 62 IFN-γ- and 40 IL-10-spot-forming cells (SFC) per million PBMC, calculated as mean + 2 sd SFC per million PBMC from HBV-negative subjects. The response was considered positive if the median number of SFC in triplicate wells was at least twice than in control wells without peptide and was superior to the cut-off values.

Inhibition of Elispot assays

Class II HLA-restriction was determined, after *in vitro* expansion, by incubating PBMC for 90 min at 37°C with 10 µg/ml of anti-class II HLA antibodies: anti-HLA-DR (L243) from ATCC, anti-HLA-DQ (SPVL3) and anti-HLA-DP (B7/21) kindly provided by Dr. Y. van de Wal (Department of Immuno-hematology and Blood Bank, Leiden, The Netherlands). Anti-class I HLA-A2 antibody (BB7-2) was used as a negative control. Pre-incubated PBMC were then tested in Elispot assays, as described above.

Intracellular staining <u>COULD BE PROVIDED ON LINE</u>

Populations of PBMC expanded *in vitro* were incubated overnight either with 500 ng/ml staphylococcal enterotoxin B (Sigma) as a positive control, with medium alone as a negative control or with individual HBx-derived peptides (1 µg/ml) and Brefeldin A (2 µg/ml) (Sigma). After washing, cells were stained with appropriate combinations of monoclonal antibodies — anti-CD3-APC (clone HIT3a; BD Pharmingen), anti-CD4-PE (clone RPA-T4; BD Pharmingen), anti-CD4-APC (clone RPA-T4; Serotec) and/or anti-CD8-PerCP (clone SK1; BD Biosciences) — for 30 minutes at 4°C and

washed again. Cells were fixed, permeabilized and stained with anti-human IFN-γ-FITC antibody (clone 4S.B3; BD Biosciences) and with anti-human IL-10-PE antibody (clone JES3-9D7; Serotec) for 30 minutes at 4°C and washed again. At least 50,000 lymphocyte-gated events were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with Cellquest (BD Biosciences). Background staining was assessed with an isotype-matched control monoclonal antibody and subtracted from all values.

RESULTS

Presence of HBx-specific IFN-γ-secreting T cells in chronic HBV carriers

We analyzed the HBx-specific IFN-γ-secreting T-cell response by Elispot assays and the use of three peptide pools (A, B, C) covering the entire HBx sequence to stimulate PBMC from 52 chronic inactive carriers of HBV. HBx-specific IFN-γ-secreting T cells were found in 36 of the 52 patients studied (69%): 28 (78%) had T cells recognizing epitopes in the carboxy-terminal part of HBx (pool C), 17 (42%) had T cells specific for the central region of the protein (pool B) and 9 (25%) had T cells specific for epitopes in the amino-terminal part of the protein (pool A) (Figure 1A). The diversity of HBx-specific IFN-γ-secreting T-cell responses to pools A, B and C is shown in Figure 1B. No more than 10% of patients displayed specific responses to all three peptide pools.

Mapping of the IFN-γ-secreting T-cell response to individual pool C peptides

As most HBx-specific IFN-γ-secreting T cells recognized the carboxy-terminal part of the protein, we mapped the single epitopes targeted by T cells in this region. PBMC from the 28 patients with pool C-specific T cells were stimulated with the entire peptide pool C and with individual pool C peptides, and then tested in Elispot assays (Figure 2). T-cell activation following PBMC culture with the entire peptide pool resulted from specific stimulation with a single peptide, as 24 of the 28 patients (86%) had T cells specific for the x26 epitope (compare left and middle panels of Figure 2). Moreover, x26-specific T cells were the only T cells reactive against the domain of the protein covered by peptide

pool C in at least 13 of the 28 patients (46%), and an absence of T cells recognizing the x26 epitope was noted in only four of these patients (patients P62, P34, P53 and P38). We analyzed PBMC from 11 uninfected individuals to check the specificity of these responses. None had T cells responding to pool C or x26 peptides in Elispot assays after *in vitro* expansion (data not shown).

T cells specific for individual pool C peptides other than x26 were detected in 8 patients (Figure 2, right panel). In the absence of x26-specific T-cell reactivity, T cells recognized the x22 peptide (patients P62, P53 and P38). In 5 of the 24 patients with x26-specific T cells, weak reactivity to x21 to x25 and x27 was also observed (patients P31, P46, P59, P28 and P23, right panel, Figure 2). With the exception of patient P23, whose T cells were more strongly activated with x25 than with x26, specific T-cell reactivity was 5 to 10 times higher for x26 than for other pool C single peptides (compare middle and right panels).

In conclusion, IFN-γ-secreting T cells recognizing the carboxy-terminal domain of HBx targeted a single immunodominant epitope. As this epitope was recognized by T cells from a large number of patients expressing different HLA molecules, x26 peptide may be considered a promiscuous epitope.

HBx-derived peptides activated IFN-γ- and IL-10-secreting T cells in chronic HBV carriers

Even if HBx-specific IFN-γ-secreting T cells were found in PBMC from most of the studied patients, we asked whether IL-10 secretion can be detected in PBMC from IFN-γ-Elispot-negative patients. We quantified and compared IFN-γ and IL-10 secretion after the *in vitro* expansion of PBMC from 31 chronic HBV carriers with and without pool C-specific IFN-γ-secreting T-cell responses. In 13 of the 31 patients (42%), neither IFN-γ- nor IL-10-producing specific T cells were detected. No IL-10 secretion was observed in 11 patients (35%) with IFN-γ-secreting T-cell responses of various magnitudes (Figure 3A). Finally, only 7 of the 31 patients (23%) with generally strong IFN-γ-secreting T-cell responses had detectable numbers of specific IL-10-secreting T cells (Figure 3B). Except in patient P26, the frequency of IFN-γ-secreting T cells was always higher than that of IL-10-secreting T cells (Figure 3B). The IFN-γ-secreting T-cell response was around ten times higher than the IL-10-producing T-cell response in Elispot (range: 4.8 - 27.4 fold, median; 11.5).

IL-10 secretion was observed in only two and one of the 24 studied patients after the activation of PBMC with peptide pools A and B, respectively. IFN-γ secretion by activated T cells was detected simultaneously in these three patients with IL-10-producing T cells. Peptide pool A activated 505 IFN-γ- and 170 IL-10-secreting T cells per million PBMC from patient P7 and 104 IFN-γ- and 313 IL-10-SFC per million PBMC from patient P27. Finally, 652 IFN-γ- and 79 IL-10-SFC pool B-specific T cells per million PBMC were detected for patient P41.

Overall, IL-10 secretion after T-cell activation with HBx-derived peptides was weak or undetectable. Studies focusing on the carboxy-terminal region showed that peptide pool C or x26 activated IFN-γ-production more efficiently than IL-10 production.

Phenotype of x26-specific T cells

We investigated whether the x26 epitope activated CD4+ or CD8+ T cells, by intracellular IFN-γ staining of PBMC stimulated *in vitro* with x26. The phenotype of CD3+ IFN-γ-producing x26-specific T cells from a representative patient (P30) is shown in Figure 4A. The promiscuous, immunodominant x26 peptide specifically stimulated CD4+ but not CD8+ T cells. This result was confirmed in 10 patients with x26-positive response.

The x26-specific T-cell response of patient P26 was further characterized by intracellular staining of both IFN-γ and IL-10, to identify more precisely the T cells producing either or both cytokines. We found that 7.66% CD4+ T cells produced IFN-γ and 0.73 % produced IL-10 (Figure 4B, left and central panels) However, most CD4+ T cells activated by x26 (6.95% of total CD4+ T cells) produced IFN-γ but not IL-10 (Figure 4B, right panel). Less than 10% of the x26-specific CD4+ T-cell population (0.73% of total CD4+ T cells) produced IL-10 together with IFN-γ. No activated T cells producing IL-10 only were observed.

This is consistent with the small number of IL-10-producing T cells identified by Elispot and highlights the more precise quantification of IFN-γ and IL-10 by intracellular staining than by Elispot assays when these cytokines are produced simultaneously (compare Figures 3B and 4B).

HLA class II restriction of x26 peptide

We used three experimental approaches to characterize the HLA class II-restriction of x26. After *in vitro* expansion, PBMC were incubated with anti-HLA-DR, anti-HLA-DQ or anti-HLA-DP antibodies and tested in Elispot assays. Prior incubation with anti-HLA-DR antibodies inhibited IFN-γ-secretion upon stimulation with x26 by at least 80%. No such effect was observed after the prior incubation of PBMC with anti-HLA-DP or anti-HLA-DQ antibodies, or with irrelevant control anti-HLA-A2 antibodies (Figure 5). The x26 epitope is therefore presented by HLA-DR molecules.

We assessed the capacity of x26 and of other pool C peptides to bind to various purified HLA-DR molecules. An HBcAg-derived peptide, c59-60, described as HBV promiscuous epitope (25) was tested in parallel (Table 1). High-affinity binding was observed for x25 peptide with at least 4 of the 10 HLA-DR molecules tested (DRB1*0301, DRB1*1101, DRB1*1301 and DRB1*1501). The x26 peptide could be presented by the HLA-DRB1*0101, HLA-DR01*0401, HLA-DRB1*1301 and HLA-DRB5*0101 molecules. DRB1*1301 molecules could bind x22, x23 and x27 peptides as well, while x22 also bound to DRB4*0101. In comparison, the HBcAg-derived promiscuous epitope exhibited a good affinity for DRB1*0301, DRB1*1301 and DRB1*1501.

Finally, we genotyped the HLA-DR molecules of 30 inactive HBV carriers with or without x26-specific T cells. For the HLA-DRB1 gene, the prevalence of alleles DRB1*0301 (9/30), DRB1*0401(9/30), DRB1*1101 (9/30) and DRB1*1301 (7/30) was high among the studied patients. In 16 patients, the presence or absence of HLA-DR alleles binding x26 (HLA-DRB1*0101, HLA-DR01*0401, HLA-DRB1*1301 and HLA-DRB5*0101) was found to be related to specific IFN-γ-secreting T-cell reactivity (Table 2).

Recognition of viral mutants by x26-specific T cells

Some HBx mutations in basic core promoter mutant viruses concern the x26 epitope. An analysis of sequences from 40 cloned HBV genomes published in GenBank (http://www.ncbi.nlm.nih.gov) shows that the frequent codon 130 and 131 (K130M and V131I) mutations were present in 12 of the 40 sequences. Codon 127 (I127T) mutation occurred in 4 of the 40 HBx sequences. We therefore evaluated the recognition of viral sequence variants by x26-specific T cells. For PBMC stimulation *in*

vitro, we used x26 peptide, covering the wild-type epitope, separately or mixed with the peptides V2 and V3, corresponding to viral mutant sequences. No activation of x26-specific T cells by the variant peptides V2 and V3 was found in 10 of 13 patients with x26-specific T-cell responses (Figure 6). When the three peptides were used for in vitro expansion (Figure 6 right panel), markedly fewer cells were recalled in only three patients in Elispot assay with the variant peptides. None of 12 patients with x26-negative T-cell responses in IFN-γ Elispot assays had T-cell responses to variant peptides (not shown).

We investigated whether mutant peptides activated IL-10- rather than IFN-γ-secreting T cells, by assessing cytokine secretion after the stimulation of PBMC with V2 and V3. No IL-10-secreting T cells were found in 15 studied patients with (n=6) or without (n=9) x26-specific IFN-γ-secreting T cells. In conclusion, viral mutant sequences activated T cells specific for the x26 epitope much less efficiently and no cross-recognition of variant sequences by x26 specific-T cells was found.

Sequencing of HBV viral DNA from patients

We next investigate if HBV genome from patients may have viral mutations affecting the x26 epitope. Sequencing of the x26 encoding HBV DNA region could be performed only on three samples (P48, P59, P46) with HBV viral load exceeding $2x10^4$ copies /ml and detectable HBV DNA after nested PCR (see online figure). For patients P48 and P59, A-T and G-A mutations at nucleotides 1762 and 1764 were found in HBV genome, changing amino acid in the HBx protein at positions K130M and V131I. Clinical data from patient P48 shows that this patient was infected at birth with an HBeAg-negative mutant (see online Table). X26 T-cell response was found negative in this patient (see Table 2). In contrast, in patient P59 carrying a virus with BCP mutation, x26-specific T cells were detected in PBMC taken at the time of DNA sequencing (Figure 2). Finally, the amplified virus from patient P46 with x26-specific T-cell response (Figure 2) showed a wild type BCP sequence.

This indicates that HBV variants with BCP mutations can be found in some of our patients at the inactive carrier state of the disease.

DISCUSSION

HBV-specific CD4+ T cells play an important role in HBV infection, secreting the Th1 cytokines that downregulate HBV replication (27) and by contributing to the induction and maintenance of efficient CD8+ T-cell and B-cell responses (28). CD4+ T-cell epitopes have been identified in the core protein (25), HBe antigen (29), envelope (30) and polymerase proteins (31). Immune responses to HBx protein are poorly documented, with only one study dealing with CD4+ T-cell responses (20). In an attempt to define more accurately the breadth and epitope specificity of T-cell responses to HBx protein, we carried out a systematic analysis of T cells from 52 chronic HBV carriers. Following *in vitro* stimulation with pools of peptides covering the HBx sequence, IFN-γ-secreting T cells specific for HBx were detected in 69% of patients, and in 67% (24/36) of them CD4+ T cells could be defined. This contrasts with the low prevalence of the Th cell responses against structural HBV proteins usually detected during chronic HBV infection. In this study, using the core 50-69 peptide (25, 32) for stimulation of PBMC and our cutured Elispot assay, IFN-γ-secreting T-cell response was found in 3 out of 8 patients, but with a ten fold lower number of specific T cells (data not shown). Previous studies have found that HLA-class II-restricted nucleocapsid antigen-specific Th cell responses are only detectable transiently during hepatitis exacerbation (14, 33). The CD4+ T-cell response to envelope proteins is markedly reduced during chronic HBV infection (1, 34). The high prevalence of IFN-γ-secreting HBx-specific T cells reported here may be due to the protein itself or to the clinical status of the patients, i.e. HBV carriers in the inactive stage of the disease with less than 100,000 HBV copies/ml. The presence of HBx-specific CD4+ T cells during chronic infection may reflect T-cell activation due to the release of HBx protein by apoptotic hepatocytes during viral replication. The persistence of HBx-specific T cells could be related to the small amounts of HBx produced by infected hepatocytes, preventing the deletion or anergy of specific T cells occurring with other highly expressed viral antigens, such as HBsAg or HBcAg. The impact of viral load on antiviral T-cell responses has been characterized in mouse models of chronic infection and in humans (35). T-cell responses to HBV antigens are detected more easily in patients with less than 10^7 copies/ml or after

successful antiviral treatment (36, 37). Consistent with these data, we found x26-specific T cells after stimulation of PBMC from only 1 of 13 patients with chronic active hepatitis and $>10^5$ copies/ml (data not shown).

In contrast to the high prevalence of IFN-γ-secreting HBx-specific T cells, IL-10-secreting T cells were detected in very few of the studied patients, always in the presence of IFN-γ production. This is consistent with previous reports of IL-12-induced IFN-γ/IL-10-secreting T cells generated in response to chronic infection (38, 39). Regulatory T cells specific for HBx, producing IL-10, are therefore unlikely to exist in our group of patients (40). This contrasts with HCV persistent infection, which is associated with enhanced IL-10 production in response to non-structural HCV antigens such as NS3, IL-10-producing T cells in the liver and weak CD4+ Th 1 reactivity in the periphery (16, 41).

We observed a high prevalence of peripheral CD4+ T-cell responses principally targeting the C-terminal part of HBx. This finding is in agreement with a previous report characterizing T-cell clones recognizing peptides within this domain of HBx (20). Remarkably, most of our patients had IFN-γ-secreting CD4+ T cells recognizing a previously unidentified single peptide, HBx 126-140 referred here to x26. In addition, x26 peptide was immunogenic in the context of multiple HLA-class II molecules and may therefore be considered a promiscuous epitope. Nevertheless, some patients with x26-specific T cells lack the HLA-DR molecules that bound the peptide with high affinity *in vitro*. Other class II molecules not tested here are therefore probably able to bind x26. The partial correlation between the HLA-DR-restriction of the peptides and the pattern of DR alleles from different donors is consistent with published findings (31, 42).

Chronic HBV infection evolves from an initial HBeAg-positive phase, through HBe seroconversion to an HBeAg-negative phase in which replication levels are lower. This process is characterized by a progressive switch from viral quasi-species dominated by “wild-type” variants lacking precore or core promoter mutations, to viral quasi-species in which precore or core promoter variants predominate (43, 44). But it was recently shown that precore and core promoter mutations exist in a substantial proportion of patients before HBeAg clearance (22). What role do x26-specific IFN-γ-secreting T cells play against both wild-type and mutated x26-derived sequences? The effector properties of x26-

specific T cells may constitute an immune system pressure against which these mutant viral variants are selected.

The lack of recognition of variant HBx peptides, corresponding to BCP mutant sequences, by x26-specfic T cells could not be explained by a decreased binding capacity of these sequences to HLA-DR molecules, as shown by comparative binding studies with the x26, V2 and V3 peptides and purified class II molecules (data not shown). Although core promoter mutations may appear early in HBV infection (8), the possibility of initial infection with the mutant virus cannot be excluded. This is the case for patient P48 who was infected at birth with an HBeAg-negative virus, and lacked for x26-specific T-cell response. On the other hand, a wild-type sequence virus was found in patient P46 concomitantly with detectable x26-specific T cells. However, for other patients such as P59 who were initially infected with a "wild-type" variant lacking core promoter mutations, x26-specific memory T cells may exist and can be detected in our assay despite the presence of a core promoter variant viral quasi-species at the time of blood collection. Our study shows that HBV strains with mutations affecting the immunodominant HBx epitope are likely to induce weaker T-cell responses, favoring the accumulation of such mutant strains.

Mutations resulting in HBx protein truncation have also been associated with low levels of HBV replication and decreases in hepatitis activity in anti-HBe antibody-positive HBV carriers (45). Thus, at least some of the studied chronic carriers may produce truncated HBx. The presence of BCP mutants or truncated HBx mutants might account for the lack of T-cell reactivity specific for the wild-type x26 peptide in some of our patients. Therefore, x26 and variant peptides could be used for the immunomonitoring of HBV sequence changes during disease progression. This hypothesis should be tested in longitudinal studies in groups of patients differing in clinical status.

In addition, the immunogenicity, the promiscuous HLA-DR binding and the efficient activation of specific IFN-γ-secreting T cells of this newly described HBx epitope suggest that it is a potential candidate for use in therapeutic vaccines for patients with chronic HBV infection.

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REFERENCES

1. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995;13:29-60.

2.Ganem D, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. N Engl J Med 2004;350:1118-1129.

3.Bouchard MJ, Schneider RJ. The enigmatic X gene of hepatitis B virus. J Virol 2004;78:12725-12734.

4.Murakami S. Hepatitis B virus X protein: a multifunctional viral regulator. J Gastroenterol 2001;36:651-660.

5.Baptista M, Kramvis A, Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology 1999;29:946-953.

6.Poussin K, Dienes H, Sirma H, Urban S, Beaugrand M, Franco D, Schirmacher P, et al. Expression of mutated hepatitis B virus X genes in human hepatocellular carcinomas. Int J Cancer 1999;80:497-505.

7.Kuang SY, Jackson PE, Wang JB, Lu PX, Munoz A, Qian GS, Kensler TW, et al. Specific mutations of hepatitis B virus in plasma predict liver cancer development. Proc Natl Acad Sci U S A 2004;101:3575-3580.

8.Leon B, Taylor L, Vargas M, Luftig RB, Albertazzi F, Herrero L, Visona K. HBx M130K and V131I (T-A) mutations in HBV genotype F during a follow-up study in chronic carriers. Virol J 2005;2:60.

9.Iavarone M, Trabut JB, Delpuech O, Carnot F, Colombo M, Kremsdorf D, Brechot C, et al. Characterisation of hepatitis B virus X protein mutants in tumour and non-tumour liver cells using laser capture microdissection. J Hepatol 2003;39:253-261.

10.Stuyver L, De Gendt S, Cadranel JF, Van Geyt C, Van Reybroeck G, Dorent R, Gandjbachkh I, et al. Three cases of severe subfulminant hepatitis in heart-transplanted patients after nosocomial transmission of a mutant hepatitis B virus. Hepatology 1999;29:1876-1883.

11.Sirma H, Giannini C, Poussin K, Paterlini P, Kremsdorf D, Brechot C. Hepatitis B virus X mutants, present in hepatocellular carcinoma tissue abrogate both the antiproliferative and transactivation effects of HBx. Oncogene 1999;18:4848-4859.

12.Bertoni R, Sidney J, Fowler P, Chesnut RW, Chisari FV, Sette A. Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. J Clin Invest 1997;100:503-513.

13. Chisari FV. Cytotoxic T cells and viral hepatitis. J Clin Invest 1997;99:1472-1477.

14.Maini MK, Boni C, Ogg GS, King AS, Reignat S, Lee CK, Larrubia JR, et al. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology 1999;117:1386-1396.

15.Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, Moss B, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995;181:1047-1058.

16.Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005;5:215-229.

17.Bertoletti A, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? Curr Opin Immunol 2000;12:403-408.

18.Chung MK, Yoon H, Min SS, Lee HG, Kim YJ, Lee TG, Lim JS, et al. Induction of cytotoxic T lymphocytes with peptides in vitro: identification of candidate T-cell epitopes in hepatitis B virus X antigen. J Immunother 1999;22:279-287.

19.Hwang YK, Kim NK, Park JM, Lee K, Han WK, Kim HI, Cheong HS. HLA-A2 1 restricted peptides from the HBx antigen induce specific CTL responses in vitro and in vivo. Vaccine 2002;20:3770-3777.

20.Jung MC, Stemler M, Weimer T, Spengler U, Dohrmann J, Hoffmann R, Eichenlaub D, et al. Immune response of peripheral blood mononuclear cells to HBx-antigen of hepatitis B virus. Hepatology 1991;13:637-643.

21.Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000--summary of a workshop. Gastroenterology 2001;120:1828-1853.

22.Yuan HJ, Ka-Ho Wong D, Doutreloigne J, Sablon E, Lai CL, Yuen MF. Precore and core promoter mutations at the time of HBeAg seroclearance in Chinese patients with chronic hepatitis B. J Infect 2006.

23.Texier C, Pouvelle-Moratille S, Busson M, Charron D, Menez A, Maillere B. Complementarity and redundancy of the binding specificity of HLA-DRB1, -DRB3, -DRB4 and -DRB5 molecules. Eur J Immunol 2001;31:1837-1846.

24.Texier C, Pouvelle S, Busson M, Herve M, Charron D, Menez A, Maillere B. HLA-DR restricted peptide candidates for bee venom immunotherapy. J Immunol 2000;164:3177-3184.

25.Ferrari C, Bertoletti A, Penna A, Cavalli A, Valli A, Missale G, Pilli M, et al. Identification of immunodominant T cell epitopes of the hepatitis B virus nucleocapsid antigen. J Clin Invest 1991;88:214-222.

26.Mancini-Bourgine M, Fontaine H, Scott-Algara D, Pol S, Brechot C, Michel ML. Induction or expansion of T-cell responses by a hepatitis B DNA vaccine administered to chronic HBV carriers. Hepatology 2004;40:874-882.

27.Franco A, Guidotti L, Hobbs MV, Pasquetto V, Chisari FV. Pathogenic effector function of CD4-positive T helper 1 cells in hepatitis B virus transgenic mice. J. Immunol. 1997;159:2001-2008.

28.Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. J Exp Med 1998;188:2199-2204.

29.Jung MC, Diepolder HM, Spengler U, Wierenga EA, Zachoval R, Hoffmann RM, Eichenlaub D, et al. 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. J Virol 1995;69:3358-3368.

30.Celis E, Ou D, Otvos L, Jr. Recognition of hepatitis B surface antigen by human T lymphocytes. Proliferative and cytotoxic responses to a major antigenic determinant defined by synthetic peptides. J Immunol 1988;140:1808-1815.

31.Mizukoshi E, Sidney J, Livingston B, Ghany M, Hoofnagle JH, Sette A, Rehermann B. Cellular immune responses to the hepatitis B virus polymerase. J Immunol 2004;173:5863-5871.

32.Torre F, Cramp M, Owsianka A, Dornan E, Marsden H, Carman W, Williams R, et al. Direct evidence that naturally occurring mutations within hepatitis B core epitope alter CD4+ T-cell reactivity. J Med Virol 2004;72:370-376.

33.Tsai SL, Chen PJ, Lai MY, Yang PM, Sung JL, Huang JH, Hwang LH, et al. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen seroconversion. J Clin Invest 1992;89:87-96.

34.Bocher WO, Herzog-Hauff S, Schlaak J, Meyer zum Buschenfeld KH, Lohr HF. Kinetics of hepatitis B surface antigen-specific immune responses in acute and chronic hepatitis B or after HBs vaccination: stimulation of the in vitro antibody response by interferon gamma. Hepatology 1999;29:238-244.

35.Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol 2003;77:4911-4927.

36.Webster GJ, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, Williams R, et al. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 2004;78:5707-5719.

37.Boni C, Penna A, Ogg GS, Bertoletti A, Pilli M, Cavallo C, Cavalli A, et al. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. Hepatology 2001;33:963-971.

38.Pohl-Koppe A, Balashov KE, Steere AC, Logigian EL, Hafler DA. Identification of a T cell subset capable of both IFN-gamma and IL-10 secretion in patients with chronic Borrelia burgdorferi infection. J Immunol 1998;160:1804-1810.

39.Vingerhoets J, Michielsen P, Vanham G, Bosmans E, Paulij W, Ramon A, Pelckmans P, et al. HBV-specific lymphoproliferative and cytokine responses in patients with chronic hepatitis B. J Hepatol 1998;28:8-16.

40.Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, Roncarolo MG. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737-742.

41.Accapezzato D, Francavilla V, Paroli M, Casciaro M, Chircu LV, Cividini A, Abrignani S, et al. Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. J Clin Invest 2004;113:963-972.

42.Bauer T, Weinberger K, Jilg W. Variants of two major T cell epitopes within the hepatitis B surface antigen are not recognized by specific T helper cells of vaccinated individuals. Hepatology 2002;35:455-465.

43.Yuen MF, Sablon E, Yuan HJ, Hui CK, Wong DK, Doutreloigne J, Wong BC, et al. Relationship between the development of precore and core promoter mutations and hepatitis B e antigen seroconversion in patients with chronic hepatitis B virus. J Infect Dis 2002;186:1335-1338.

44.Yim HJ, Lok AS. Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. Hepatology 2006;43:S173-181.

45.Fukuda R, Nguyen XT, Ishimura N, Ishihara S, Chowdhury A, Kohge N, Akagi S, et al. X gene and precore region mutations in the hepatitis B virus genome in persons positive for antibody to hepatitis B e antigen: comparison between asymptomatic "healthy" carriers and patients with severe chronic active hepatitis. J Infect Dis 1995;172:1191-1197.

Legends to Figures

Figure 1. Presence and diversity of HBx-specific IFN-γ-secreting T cells in 52 patients with chronic HBV infection. PBMCs were stimulated *in vitro* with HBx-derived 15-mer peptides covering the whole HBx sequence, divided into pools A, B and C. IFN-γ-secreting T cells were determined by Elispot, using the same peptide pools. The proportion of patients testing positive is indicated on the top of each column. A) Percentage of patients with undetectable HBx-specific T cells (white column) and with HBx-specific T cells activated with each peptide pool (gray columns); B) diversity of recognition of regions within the HBx protein by HBx-specific T cells (gray striped columns).

Figure 2. IFN-γ-secreting T cells upon stimulation with peptide pool C and mapping of the T-cell response to single peptides. Number of IFN-γ-secreting T cells determined by Elispot and expressed as the number of specific spot-forming cells (SFC)/10^6 PBMCs after *in vitro* stimulation with peptide pool C (left panel), individual peptide x26 (middle panel), and single peptides or groups of peptides from pool C with the exception of x26 peptide (right panel). In the right panel, the number of SFC is indicated on each bar. The scale of the right panel differs from that of the left and central panels. ND: not done.

Figure 3. IFN-γ- and IL-10-secreting T cells determined by Elispot assays after *in vitro* stimulation of PBMC with the entire peptide pool C or x26 alone. Number of IFN-γ- or IL-10 spot-forming cells (SFC)/10^6 PBMC (black and gray columns, respectively) of 11 patients with only IFN-γ-secreting T cells (A panel) and 7 patients with both IFN-γ- and IL-10-secreting T cells (B panel). Number of SFC is indicated on the top of each column. The cutoff points for Elispot assays are 62 IFN-γ- and 40 IL-10-SFC/10^6 PBMC.

Figure 4. Phenotype of x26-specific T cells after *in vitro* expansion from PBMC with x26. A) Percentages of IFN-γ-secreting CD3+ CD4+ (left panel) or CD3+ CD8+ (right panel) specific T cells are shown, B) within the CD4+ T-cell population, the percentages of x26-specific CD4+ T cells secreting IFN-γ (left panel), IL-10 (central panel) and IL-10 and/or IFN-γ (right panel) are shown.

Figure 5. Anti-MHC class II antibody-mediated inhibition of IFN-γ secretion by x26-specific T cells. PBMC expanded *in vitro* with peptide x26 were first incubated with anti-class II HLA antibodies — anti-HLA-DR, anti-

HLA-DQ or anti-HLA-DP — or with an irrelevant antibody (anti-HLA-A2). PBMC were then tested in Elispot assays as described in Materials and Methods. Results obtained with three representative patients are shown.

Figure 6. Recognition of viral mutants by x26-specific T cells. PBMC from 13 patients with known x26-specific IFN-γ-producing T cells were expanded in vitro separately with x26 peptide (left panel) or with a mixture of x26, V2 and V3 peptides (right panel). IFN-γ-secretion after the activation with each of the three peptides in Elispot assays is shown.

Figure online. Amino acid sequence of x26 region.

Legends to Tables

Table (available online). Clinical, virological and immunological characteristics of patients included in this study.

Table 1. Binding of HBx-derived peptides to immunopurified class II HLA molecules. Data are expressed as relative activity: ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide. The relative activities of pool C peptides and an HBcAg-derived peptide (c50-69) are shown. Boldface indicates relative binding affinity below 100 and corresponds to good binders.

Table 2. Comparative analysis of patients' HLA-DR genotypes, x26-binding HLA-DR molecules and x26-specific IFN-γ-secreting T-cell responses. Brackets for DRB1 genotype indicate that heterozygosity could not be confirmed with the current assay. (#) HLA-DR molecules binding x26, as shown in Table 1 for DRB1*0101, DRB1*0401, DRB1*1301 and DRB5*0101. (##) determined by Elispot assays and expressed as ranges of IFN-γ-SFC per million PBMC: + 100-500; ++ 500-1000; +++ 1000-2000 and ++++ >2000.