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Research Article

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Identification of Immunodominant T Cell Epitopes of the Hepatitis B Virus Nucleocapsid Antigen

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Abstract

Several lines of experimental evidence suggest that inclusion of core sequences in the hepatitis B vaccine may represent a feasible strategy to increase the efficacy of the vaccination. In order to identify immunodominant core epitopes, peripheral blood T cells purified from 23 patients with acute hepatitis B and different HLA haplotypes were tested with a panel of 18 short synthetic peptides (15 to 20 amino acids [AA]) covering the entire core region. All patients except one showed a strong T cell proliferative response to a single immunodominant 20 amino acid sequence located within the aminoterminal half of the core molecule. Two additional important sequences were also identified at the aminoterminal end and within the carboxyterminal half of the core molecule. These sequences were able to induce significant levels of T cell proliferation in 69 and 73% of the patients studied, respectively. T cell response to these epitopes was HLA class II restricted. The observations that (*a*) polyclonal T cell lines produced by PBMC stimulation with native HBcAg were specifically reactive with the relevant peptides and that (*b*) polyclonal T cell lines produced with synthetic peptides could be restimulated with native HBcAg, provide evidence that AA sequences contained within the synthetic peptides represent real products of the intracellular processing of the native core molecule. In conclusion, the identification of immunodominant T cell epitopes within the core molecule provides the molecular basis for the design of alternative and hopefully more immunogenic vaccines. (*J. Clin. Invest.* 1991. 88:214–222.) Key words: synthetic peptides • helper T cells • polyclonal T cell lines • acute hepatitis • hepatitis B vaccine

Introduction

The cell-mediated immune events involved in liver damage and viral clearance during hepatitis B virus (HBV)[1] infection in man still remain largely unknown. A pathogenetic role in the clearance of infected hepatocytes has been suggested for nucleocapsid-specific cytolytic T cells based on the finding that autologous liver cells can be apparently lysed in vitro by peripheral blood T cells and that this phenomenon can be selectively blocked by anti-HBc monoclonal antibodies (1). The possibility that hepatitis B core antigen (HBcAg) represents an important sensitizing antigen for viral-specific T cells within the liver of patients with chronic HBV infection is also indicated by the recent observation that CD4+ and CD8+ T cells with HBcAg-specific helper and suppressor function respectively, are present in the infected liver at the site of cell injury and viral synthesis (2, 3). However, the in vitro HLA class II-restricted peripheral blood T cell response to nucleocapsid antigens in patients with chronic HBV infection is dramatically lower than that displayed by patients with acute HBV infection (4). This difference may have important pathogenetic implications because appearance of a detectable level of class II-restricted T cell sensitization to nucleocapsid antigens is temporally associated with the clearance of viral particles from sera of subjects with self-limited acute HBV infection (4). The demonstration that HBcAg-specific helper T cells can directly cooperate in vivo with envelope-specific B cells supporting the production of virus neutralizing anti-envelope antibodies (5) may provide an explanation of this temporal association.

If the development of an adequate immune response to HBV nucleocapsid antigens is actually important for HBV clearance, it follows that identification of immunodominant T cell epitopes within the core molecule could theoretically be useful for the design of more effective alternative vaccines against HBV infection and possibly to plan future strategies to manipulate the immune response to HBV in subjects who do not spontaneously clear the virus.

In an initial effort to characterize the molecular elements involved in the activation of the different T cell subsets during HBV infection in man, we have studied the proliferative response of peripheral blood T cells from a large group of patients with self-limited acute HBV infection and different genetic backgrounds to a panel of short synthetic peptides covering the entire HBV core region. Our data show that an immunodominant epitope for CD4+ T cells is present within the amino-terminal half of the core molecule (amino acid [AA] 50–69). In addition, two further important sequences are located, one at the aminoterminal end and the other within the carboxyterminal half of the core molecule.

Methods

Patients. 23 patients (6 females and 17 males) with acute hepatitis B were studied. The diagnosis was based on the finding of elevated values of serum glutamic pyruvic transaminase activity (at least 10 times the upper level of the normal), associated with the detection of IgM anti-HBc antibodies in the serum and the recent onset of jaundice and other typical symptoms of acute hepatitis. All patients recovered completely

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1. *Abbreviations used in this paper:* AA, amino acid; APC, antigen presenting cell; HBc, e, s Ag, hepatitis B core, e, surface antigen; HBV, HB virus; SI, stimulation index.

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from the illness, with normalization of transaminase and clearance of hepatitis B surface antigen (HBsAg) from the serum. Control experiments were performed on 20 healthy subjects with no evidence of previous exposure to HBV, i.e., they were negative for HBsAg, antibody to HBsAg (anti-HBs), and antibody to HBcAg (anti-HBc).

HBV antigens and synthetic peptides. A recombinant preparation of HBcAg was obtained from bacterial extracts of *Escherichia coli* K12 strain HB 101 harboring the recombinant plasmid carrying the HBcAg coding gene as described previously (6). Purity was ~ 90% as determined by scanning densitometry of Coomassie Blue stained SDS-polyacrylamide gel. Human cytoplasmic HBcAg (hHBcAg) was purified from the liver of a patient on dialysis as described (7). This core preparation had a high HBcAg activity specifically detected up to a dilution of $1:2^{11}$ in solid phase radioimmunoassay.

An HBcAg deletion mutant lacking the carboxyl-terminal 39 amino acid residues of the core molecule was produced in *E. coli* and provided by Biogen (Geneva, Switzerland). A detailed characterization of this recombinant antigen has recently been reported (8). Its purity was 99.8% and it is herein designated as rHBeAg.

18 peptides, 10–20 residues long, corresponding to the complete sequence of the core and precore region encoded polypeptides (subtype ayw), were synthesized by Multiple Peptide System (La Jolla, CA). Peptides were designed taking into account the amphipathic regions of the antigenic molecules (9) and the presence of consensus motifs based on Rothbard studies (10). Amino acid sequences of the synthetic peptides used in this study and indicated by the amino acid position from the NH_2-terminus of the core and precore derived polypeptides are as follows: 1–21, 20–34, 28–47, 38–54, 50–59, 50–64, 50–69, 61–80, 70–89, 82–101, 100–119, 117–131, 120–139, 131–145, 140–155, 155–169, 169–183, 20 precore-2 core (Fig. 1).

Isolation of peripheral blood lymphomononuclear cells. PBMC were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation. The T cells and non-T cells were separated by rosetting PBMC with 2-aminoethylisothiuronium bromide (AET; Sigma Chemical Co., St. Louis, MO) treated sheep erythrocyte (11). The E-rosette forming T cells were separated from the nonrosetting (non-T) cells by Ficoll-Hypaque gradient. Purity of the T and non-T cell fractions was evaluated by direct immunofluorescence with anti-CD3 monoclonal antibody after red cell osmotic lysis. The T cell population contained more than 95% CD3+ cells, whereas the nonrosetting fraction contained less than 3% of CD3+ cells.

Isolated cell populations were resuspended to 1×10^6 cells/ml in RPMI 1640 supplemented with 25 mM Hepes, 2 mM L-glutamine, 50 μg/ml gentamycin, and 10% human AB positive serum (complete medium).

Isolation of HBV nucleocapsid antigen-specific T cell lines. PBMC were cultured in round-bottomed wells of 96-well plates (Costar, Cambridge, MA) in the presence of different concentrations of HBcAg, HBeAg, or synthetic peptides. After 7 d, activated T cells were expanded by adding IL-2. Growing lines were restimulated after an additional 5 to 7 d with the appropriate HBV antigen plus irradiated (3,000 rad) autologous PBMC (5×10^5/ml) as antigen presenting cells (APC) in medium supplemented with 20 U/ml of rIL-2. From this point, colonies were restimulated every 7 d and were provided with supplementary IL-2-containing medium between restimulations to maintain the cell concentration between 3×10^5 and 1×10^6/ml.

Proliferation assays. Unfractionated PBMC (2×10^5/well) were incubated in 96-well microtiter plates (Costar) for 7 d at 37°C in an atmosphere of 5% CO_2 in air in the presence of different concentrations of each HBV antigen or synthetic peptide above described. In selected experiments, purified peripheral blood T cells (1×10^5) were incubated with autologous irradiated (3,000 rad) non-T cells (1×10^4) as APC.

For the study of polyclonal T cell lines, T cells were washed extensively to remove IL-2 and FCS. Subsequently, 5×10^4 cells/well were incubated for 3 d with 1×10^5 autologous irradiated (3,000 rad) PBMC as APC in complete medium in the presence of different concentrations of HBV antigens or synthetic peptides. All proliferation assays were performed in triplicate and [^{3}H]thymidine (0.5 μCi/well; sp act 2.0 Ci/mM; Amersham International, Amersham, UK) was added 18 h before harvesting. The results are expressed as the mean counts per minute of triplicate determinations. The stimulation index (SI) was calculated as the ratio between mean counts per minute obtained in the presence of antigen to that obtained in the absence of antigen.

HLA restriction of T cell activation. The HLA restriction of peptide recognition by T cells was determined using murine MAb to human HLA class I and class II antigens to inhibit T cell proliferation. The following MAb were used in this study: D1-12 (IgG2a) and E.31 (IgG2a) specific for DR molecules; BT3/4 (IgG1) and SPV-L3 (IgG2a) recognizing DQ molecules; B7/21 (IgG2a) specific for DP antigens; and W6/32 (IgG2a) anti-HLA A, B, C specific. The specificity of these anti-HLA antibodies has previously been described in detail (12–19).

D1-12, BT3/4, and B7/21 MAb were used as hybridoma culture supernatants at 1:5 final dilution. This dilution corresponds to an antibody concentration sufficient to give 50% of maximal binding on Raji lymphoblastoid cell lines and always corresponds to saturating antibody concentration for the APC used in our functional assays. Purified W6/32, E31, and SPV-L3 MAb were used at the concentration of 10 μg/ml.

Anti-HLA class I and anti-HLA class II MAb were incubated overnight with autologous PBMC as APC and the stimulatory peptide. APC were then washed, irradiated, and added to responder peptide-specific T cell lines. [^{3}H]thymidine incorporation was measured after 3 d of culture.

Results

PBMC proliferative response to HBV nucleocapsid synthetic peptides. Patients with acute hepatitis B were chosen for the study of the fine specificity of the T cell response to HBV nucleocapsid antigens since a strong level of T cell response to HBcAg and HBeAg is always detectable during the acute phase of HBV infection (4).

The complete panel of 18 synthetic peptide analogs of HBcAg and HBeAg used in this study is represented in Fig. 1.

Stimulation of PBMC with the panel of partially overlapping peptides revealed that all acute hepatitis patients responded to at least one T cell epitope (Table I). It is remarkable that all except one (patient 12 of Table I) showed a significant

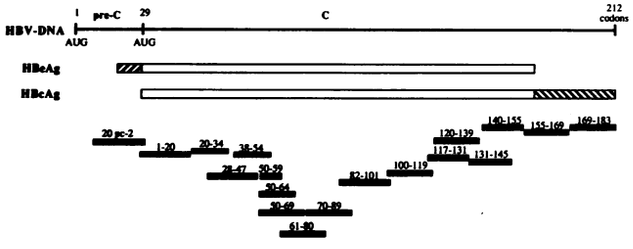

Figure 1. Schematic representation of the synthetic peptides sequences, the HBcAg and HBeAg molecules, the HBV core open reading frame. HBcAg and HBeAg are both encoded by the core open reading frame (40), which contains two in-frame translation initiation codons separated by an 87-bp region, designated precore. HBcAg is synthesized when the second initiation codon is used, whereas the e protein is probably generated by the cleavage of a precursor polypeptide that is translated from the entire core open reading frame (41–43). Production of HBeAg results from removal of a signal peptide at the amino end of this precursor polypeptide by a signal peptidase and from cleavage of the arginine-rich carboxy end by trypsin-like proteases (44). The hatched portions of the HBcAg and HBeAg molecules denote amino acid sequences that are not shared by core and e.

Table I. PBMC Response to HBcAg Synthetic Peptides in 23 Patients with Acute Hepatitis B

Patients' HLA	HBc-peptides															HBcAg	HBeAg
	1–20	20–34	28–47	50–59	50–69	70–89	82–101	100–119	117–131	120–139	131–145	140–155	155–169	169–183	20pc–2c		
1. DR1, 7, w53 DQw1, w2																	
1 μg/ml	—	—	—	n.t.	—	—	—	n.t.	n.t.	—	n.t.	—	—	—	—	9.5	4.8
10	11.1	—	—	n.t.	—	—	—	n.t.	n.t	—	n.t.	—	—	—	—		
100	14.3	—	4.7	n.t.	4.2	—	—	n.t.	n.t.	3.6	n.t.	—	—	—	—		
2. Drw6, w52 DQw1, w3																	
1 μg/ml	—	—	—	n.t.	5.2	—	—	—	n.t.	—	n.t.	—	—	—	—	11.6	8.4
10	—	—	—	n.t.	5.4	—	—	—	n.t.	—	n.t.	—	—	—	—		
100	—	—	—	n.t.	4.6	—	—	—	n.t.	—	n.t.	—	—	—	—		
3. DR5, w6, w52																	
1 μg/ml	—	—	—	n.t.	3.6	—	3.5	—	n.t.	—	n.t.	—	—	—	—	6.8	11.3
10	—	—	—	n.t.	5.4	—	3.9	—	n.t.	—	n.t.	—	—	—	—		
100	—	—	—	n.t.	4.9	—	4.6	5.3	n.t.	—	n.t.	—	—	—	—		
4. DR5, 7, w52, w53, DQw2, w3																	
1 μg/ml	3.1	—	—	n.t.	16.2	—	—	—	—	—	n.t.	—	—	—	—	9.5	7.4
10	5.0	—	4.8	n.t.	11.8	—	—	—	6.3	—	n.t.	—	—	—	—		
100	6.1	—	9.3	n.t.	8.7	—	—	—	n.t.	—	n.t.	—	—	—	—		
5. DR2, 5, w52 DQw1, w3																	
1 μg/ml	—	—	—	n.t.	3.3	—	—	—	n.t.	—	n.t.	—	—	—	—	13.3	7.7
10	3.6	—	—	n.t.	3.3	—	—	—	n.t.	—	n.t.	—	—	—	—		
100	4.0	—	6.0	n.t.	5.0	—	—	—	n.t.	4.9	n.t.	—	—	—	—		
6. DR3, 7, w52, w53, DQw2, w3																	
1 μg/ml	6.4	—	7.7	n.t.	4.2	—	—	—	n.t.	—	n.t.	—	—	—	—	16.4	12.6
10	16.4	—	16.1	n.t.	6.8	—	—	—	n.t.	—	n.t.	—	—	—	—		
100	n.t.	—	n.t.	n.t.	11.6	—	—	—	n.t.	3.3	n.t.	—	—	—	—		
7. DR3, 5, w52 DQw2, w3																	
1 μg/ml	4.2	—	—	n.t.	3.0	—	—	—	—	—	—	—	—	—	—	18.2	14.7
10	4.0	—	—	n.t.	4.0	—	—	—	—	—	—	—	—	—	—		
100	5.1	—	4.9	n.t.	7.5	—	—	5.6	—	—	—	—	—	—	—		
8. DR5, w52 DQw3																	
1 μg/ml	—	—	—	—	21.6	—	—	4.1	7	—	—	—	—	—	—	45.6	16.6
10	20.8	—	—	—	34.1	—	—	6.5	7.8	4.0	—	—	—	—	8.6		
100	n.t.	—	—	—	40.4	—	—	6.1	7.4	4.3	—	—	—	—	3.2		
9. DR7, w53 DQw2																	
1 μg/ml	6.4	—	—	—	4.7	—	—	—	18.6	—	—	—	—	—	—	26.1	30.1
10	5.4	3.1	5.3	—	13.1	—	—	—	19.0	—	—	—	—	—	—		
100	9.2	6.9	5.8	—	13.1	—	—	—	5.2	—	—	—	—	—	—		
10. DR7, w8, w53, w52, DQw2																	
1 μg/ml	6.4	—	—	n.t.	9.1	—	—	—	7.2	—	—	—	—	—	—	5.9	6.5
10	6.2	—	4.9	n.t.	7.9	—	—	—	12.1	—	—	—	—	—	—		
100	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.		
11. DRw6, w11, w52, DQw1																	
1 μg/ml	—	—	—	n.t.	3.9	—	—	—	n.t.	—	n.t.	—	—	—	—	2.8	3.8
10	—	—	—	n.t.	7.0	—	—	—	n.t.	—	n.t.	—	—	—	—		
100	—	—	—	n.t.	5.6	—	—	—	n.t.	—	n.t.	—	—	—	—		

Table I. (Continued)

Patients' HLA	HBc-peptides 1–20	20–34	28–47	50–59	50–69	70–89	82–101	100–119	117–131	120–139	131–145	140–155	155–169	169–183	20pc–2c	HBcAg	HBeAg
12. DR3, w11, w52, DQw2, w3																	
1 μg/ml	5.6	—	—	n.t.	—	—	—	—	n.t.	—	n.t.	—	—	—	—	8.9	2.4
10	13.5	—	—	n.t.	—	—	—	—	n.t.	—	n.t.	—	—	—	—		
100	n.t.	—	—	n.t.	—	—	—	—	n.t.	—	n.t.	—	—	—			
13. DR5, 10, w52 DQw1, w3																	
1 μg/ml	—	—	—	—	14.3	—	—	—	—	—	—	—	—	—	—	15.5	6.1
10	—	—	—	—	30.4	—	—	—	—	—	—	—	—	—	—		
100	—	—	—	—	46.8	—	3.7	—	—	—	—	—	—	—	—		
14. DR2, w6, w52, DQw1																	
1 μg/ml	6.3	—	—	—	7.5	—	—	—	9.3	—	—	—	—	—	—	13.2	26.4
10	8.1	—	7.5	—	8.3	—	—	—	14.9	—	—	—	—	—	—		
100	9.3	—	6.8	—	15.4	—	—	—	15.1	—	—	—	—	—	—		
15. DR3, 5, w52 DQw2, w3																	
1 μg/ml	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6.7	4.9
10	3.6	—	—	—	3.2	—	—	—	—	—	—	—	—	—	—		
100	4.0	—	—	—	4.1	—	—	—	—	—	—	—	—	—	—		
16. DR4, w53 DQw3																	
1 μg/ml	—	—	—	n.t.	14.4	—	—	—	6.9	—	—	—	—	—	—	9.8	6.7
10	—	—	—	n.t.	10.9	3.6	—	—	7.3	—	—	—	—	—	—		
100	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.		
17. DR3, w52 DQw2																	
1 μg/ml	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	7.2	5.2
10	4.7	—	—	—	4.0	—	—	—	—	—	—	—	—	—	—		
100	3.2	—	—	—	4.9	3.1	—	—	—	—	—	—	—	—	—		
18. DR3, w6, w52 DQw1, w2																	
1 μg/ml	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6.1	4.5
10	—	—	—	—	3.1	—	—	—	—	—	—	—	—	—	—		
100	—	—	—	—	5.1	—	—	—	—	3.7	—	—	—	—	—		
19. DR5, w6, w52 DQw1, w3																	
1 μg/ml	—	—	—	—	8.9	—	—	—	—	—	—	—	—	—	—	13.6	11.5
10	4.3	—	—	—	17.6	—	—	—	3.9	—	—	—	—	—	—		
100	6.2	6.9	—	—	n.t.	—	—	—	4.1	—	—	—	—	—	—		
20. DR3, w52 DQw2																	
1 μg/ml	—	—	—	—	—	—	—	—	4.9	—	—	—	—	—	—	11.2	7.8
10	7.3	—	—	—	4.8	3.1	4.6	5.6	7.2	3.7	—	—	—	—	4.2		
100	6.2	—	—	—	6.7	5.2	n.t.	n.t.	11.3	n.t.	—	—	—	—	n.t.		
21. DR1, 2 DQw1																	
1 μg/ml	—	—	8.4	n.t.	6.0	—	4.2	—	3.7	—	—	—	—	—	—	8.9	15.1
10	—	—	10.7	n.t.	9.1	n.t.	5.1	3.7	4.3	—	—	—	—	10.0	—		
100	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.		
22. DR5, w6, w52 DQw1, w3																	
1 μg/ml	—	—	—	—	15.4	—	—	—	—	—	—	—	—	—	—	14.4	7.0
10	—	—	—	—	21.7	—	—	—	—	—	—	—	—	—	—		
100	3.4	—	—	—	11.1	—	—	—	17.7	n.t.	n.t.	n.t.	5.2	n.t.	5.0		

Table I. (Continued)

Patients' HLA	HBc-peptides 1–20	20–34	28–47	50–59	50–69	70–89	82–101	100–119	117–131	120–139	131–145	140–155	155–169	169–183	20pc–2c	HBcAg	HBeAg
23. DR3, w52 DQw2																	
1 μg/ml	—	—	—	—	3.6	—	—	—	4.5	—	—	—	—	—	—	8.5	12.5
10	9.7	—	4.8	—	3.3	—	—	3.4	4.6	—	—	—	—	—	—		
100	12.1	—	6.6	—	6.0	5.4	—	7.5	5.6	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.		

PBMC (2×10^5/well) were cultured in the presence of the indicated peptide concentrations and proliferation was measured as [^{3}H]thymidine incorporation. Results are expressed as stimulation index which is the ratio between mean counts per minute obtained in the presence of antigen and those obtained in the absence of antigen. The proliferative response induced by 1 μg/ml of hHBcAg and rHBeAg is also indicated. Stimulation indexes higher than three were considered as significant values of proliferative response. Values of ^{3}H-TdR incorporation by PBMC in the absence of antigen or peptides were usually less than 2,000 cpm. No significant levels of PBMC proliferation to core peptides were observed in the normal control population. —, No proliferative responses at any of the tested concentrations. n.t., Not tested.

level of T cell response to the 50–69 sequence irrespective of the HLA haplotype. Concentrations of this peptide as low as 0.01 μg/ml were stimulatory for PBMC of several patients (data not shown).

Two additional important sequences were also identified at the amino-terminal end and within the carboxy-terminal half of the core molecule. The 1–20 sequence was recognized by 16 of 23 patients (69%) and the 117–131 sequence was able to induce significant levels of PBMC proliferation in 11 of 15 patients studied (73%). Minor T cell sites which were stimulatory for a few patients at high peptide concentrations were amino acid sequences 20–34, 28–47, 70–89, 82–101, 100–119, 140–155, 169–183, 20pc-2c.

On a weight basis, the whole core protein was more efficient than the synthetic peptides in the stimulation of the T cell response in most of the patients (Table I), and the difference between HBcAg and peptides was even higher on a molar basis because 1 μg/ml of HBcAg corresponds to 0.045 μM, 1 μg/ml of peptide 1–20 to 0.385 μM, 1 μg/ml of peptide 50–69 to 0.382 μM, and 1 μg/ml of peptide 117–131 to 0.502 μM.

This weaker peptide-induced proliferation with respect to that to native HBcAg suggests that multiple distinct epitopes are responsible for the proliferative response to HBcAg in a single individual or that the processing of native HBcAg yields peptides that are related to but not identical with the synthetic peptides, thereby inducing cross-reactive T cells with higher affinity for the endogenously processed determinants.

Fractionation experiments performed with purified populations of T and non-T cells showed that unfractionated PBMC and purified T cells (cocultured with autologous irradiated non-T cells as APC) express similar dose-response curves when stimulated with the core peptide analog 50–69 (data not shown).

The observation that peptides corresponding to core sequences 38–54, 50–59, and 50–64 were not stimulatory for T cells from five different patients responsive to peptide 50–69 (Fig. 2) suggests that critical residues required for T cell activation are likely contained, at least for these patients, within the COOH-terminal end of the peptide. This is apparently confirmed in two subjects by the significant level of T cell stimulation induced by peptide 61–80 (Fig. 2). Further examination of this likelihood is in progress, at the clonal level, using truncated and substituted analogue peptides.

Most of the patients who showed a significant proliferative response to the 117–131 sequence did not recognize the 120–139 and 100–119 sequences, suggesting that T cell recognition of the 117–131 peptide is probably focussed on its NH_2-terminal portion. In contrast, in two patients, all three peptides (117–131, 100–119, 120–139) were simultaneously recognized (patients 8 and 20; Table I), implying that more than one T cell site is probably contained in the core region ranging from residue 100 to 139.

T cell response to the relevant core peptide analogues is temporally associated with the T cell response to the native HBcAg molecule. 10 patients were studied prospectically during the symptomatic period and during convalescence for PBMC proliferation in response to native nucleocapsid antigens and synthetic peptide analogues. The presence of a significant proliferative response to the core peptides was always asso-

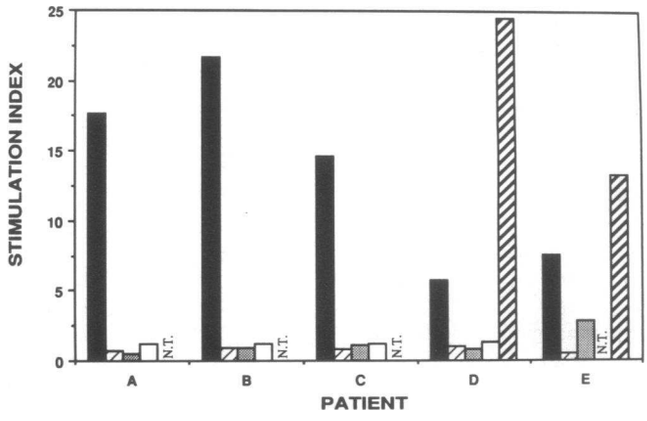

Figure 2. PBMC proliferative response to peptide 50–69 (■), 50–59 (▨), 50–64 (□), 38–54 (□), and 61–80 (▨) in five representative patients with acute hepatitis B. PBMC were stimulated with 10 μg/ml of peptide and [^{3}H]thymidine incorporation was measured after 7 d of culture. ^{3}H-TdR incorporation by PBMC in the absence of peptide was 2949, 1075, 389, 527, and 970 cpm for patients A, B, C, D, and E, respectively. N. T., not tested.

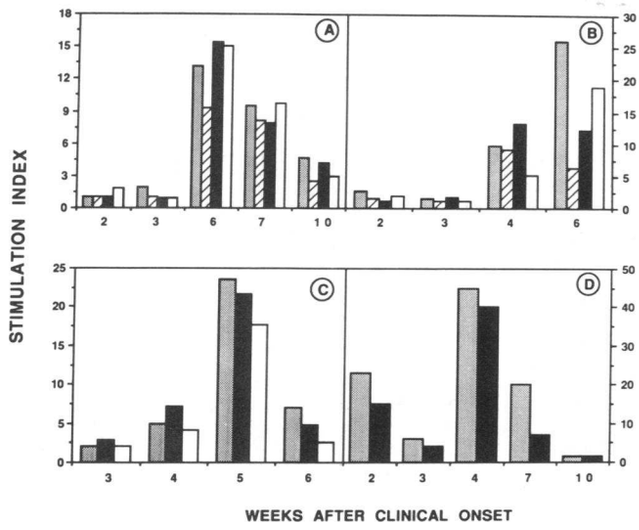

Figure 3. Serial determinations of the PBMC proliferative response to HBcAg (□) and to the relevant core peptides (▨, peptide 1–20; ■, peptide 50–69; □ peptide 117–131) at different times during the course of the acute HBV infection in four representative patients. Data correspond to antigen concentrations that gave maximum SI. Mean values of SI±SD with control PBMC were: HBcAg = 1.5±0.5; peptide 1–20 = 1.1±0.51; peptide 50–69 = 1.4±0.8; peptide 117–131 = 1.1±0.7. ^{3}H-TdR incorporation in the negative control cultures was usually lower than 2,000 cpm. Patients represented in panel A, B, C, and D correspond to patients 14, 9, 22, and 8 of Table I, respectively.

ciated with the detection of a significant response to the native core protein, suggesting that amino acid sequences contained within these peptides probably represent the products of the nucleocapsid processing involved in the activation of core-specific T cells (Fig. 3). In three of these patients the T cell responsiveness to peptide 50–69 was only transient because it became undetectable when T cells were still able to respond to the native HBcAg (data not shown). This implies that for a minority of subjects the amino acid sequence 50–69 does not represent the major T cell epitope within the core molecule.

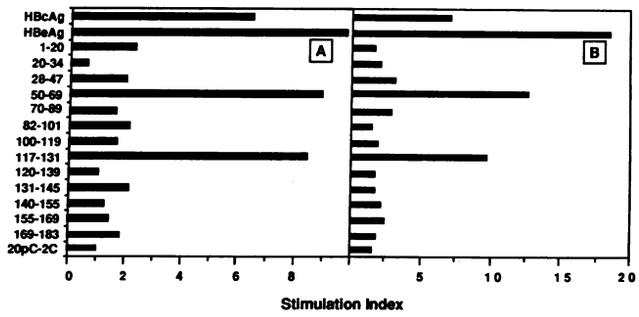

Figure 4. Proliferative response to 1 μg/ml of HBcAg, HBeAg, and synthetic peptides of two representative polyclonal T cell lines produced by PBMC stimulation (patient 14 of Table I) either with HBcAg (*A*) or with HBeAg (*B*). For the production of HBV nucleocapsid-specific T cell lines see Methods. Values of ^{3}H-TdR incorporation in the absence of antigen were 386 cpm for the T cell line represented in *A* and 506 cpm for the T cell line in *B*.

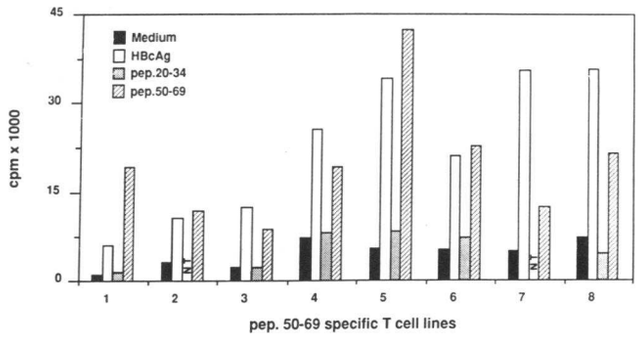

Figure 5. Proliferative response to 1 μg/ml of HBcAg, peptide 50–69 and an irrelevant peptide (20–34) of eight polyclonal T cell lines produced from different acute patients by PBMC stimulation with peptide 50–69. The concentration of peptide 50–69 used for the generation of the polyclonal T cell lines was 0.1 μg/ml. *pep.*, Peptide.

Amino acid sequences identified by the stimulatory peptides represent immunodominant core epitopes. To further investigate whether the stimulatory core peptides actually contain amino acid sequences of the core molecule recognized by nucleocapsid-specific T cells, HBcAg- and HBeAg-specific polyclonal T cell lines were produced from five patients by PBMC stimulation with native antigen and IL-2. T cell lines specifically reactive to HBcAg and HBeAg, but not to other HBV antigens (envelope antigens; data not shown), only displayed significant levels of proliferation in the presence of the relevant peptides recognized by autologous PBMC (Fig. 4). In the reciprocal experiment, polyclonal T cell lines produced by PBMC stimulation with the relevant synthetic peptides were able to recognize the native core molecule (Figs. 5 and 6), confirming that the stimulatory amino acid sequences represented by the synthetic peptides are actually available after processing of the native core molecule. T cell recognition of core peptides was HLA class II restricted because peptide-induced proliferation of polyclonal T cell lines was significantly inhibited by anti-HLA class II monoclonal antibodies, but not by an anti-HLA

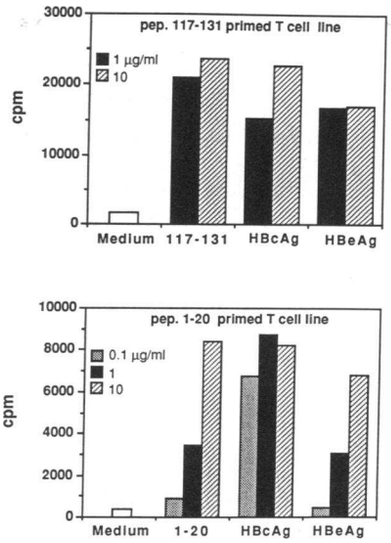

Figure 6. Peptide 1–20 and peptide 117–131 specific T cell lines can recognize the whole HBcAg and HBeAg molecules. T cell lines were produced from patient 23 of Table I as described in Methods and tested with different concentrations of HBcAg, HBeAg, and the relevant sensitizing peptide. *pep.*, Peptide.

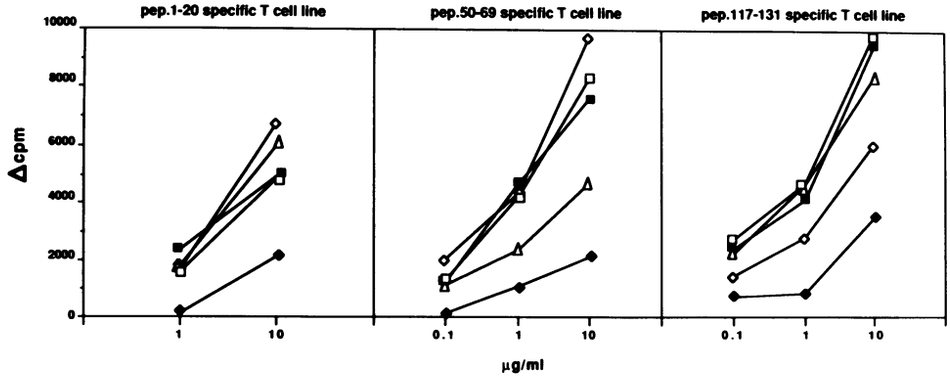

Figure 7. HLA restriction of peptide-induced T cell proliferation. Autologous APC were incubated overnight without (□) or with anti-HLA class I (W6/32, ■) and anti-HLA class II (D1-12 anti-DR, ♦) (B7/21 anti-DP, △) (BT 3/4 anti-DQ, ◇) monoclonal antibodies and synthetic peptides. After washing and irradiation, APC were added to T cells from three representative peptide-primed polyclonal T cell lines (1–20, 50–69, and 117–131 specific) and [^{3}H]thymidine incorporation was measured 3 d later. A 50–69 specific T cell line was also tested with different anti-HLA DR (E.31) and anti-HLA DQ (SVP-L3) MAb with similar results (data not shown). *pep.*, Peptide.

class I monoclonal antibody (Fig. 7). In addition, phenotypic analysis of 14 polyclonal T cell lines produced by PBMC stimulation with 1–20, 50–69, and 117–131 core sequences showed that T cell lines were almost exclusively CD4+ (more than 80%).

Discussion

It is well recognized that antienvelope antibodies can neutralize viral particles and, thereby, can confer protection against HBV infection (20). Based on this evidence, vaccines consisting of HBsAg purified from plasma of chronic HBsAg carriers or produced by recombinant DNA technology have been developed and proved to be highly effective in preventing HBV infection (21, 22). Though a protective antibody response is usually induced by the currently licensed HBsAg vaccines in more than 90% of healthy persons, the proportion of nonresponders is much higher among hemodialysis patients and other immunocompromised persons. For effective immunity these patients require an increased number of doses and a larger amount of immunogen (23, 24).

In an attempt to produce vaccines with enhanced immunogenic potential, inclusion of preS sequences of the HBV envelope in the HBsAg-based vaccine has been proposed (25–28). The HBV nucleocapsid antigen also represents a good candidate for the development of more immunogenic anti-HB vaccines for several reasons. First, HBcAg is a very powerful T cell immunogen (29). Second, HBcAg-specific helper T cells can support antienvelope antibody production by HBV envelope-specific B cells (5) and, thus, may play a central role in viral clearance. Third, HBcAg is an important sensitizing antigen for intrahepatic T cells during chronic HBV infection (2, 3) and it is thought to represent a target structure for the immune-mediated injury of infected hepatocytes (1). Fourth, immunization of chimpanzees and woodchucks with nucleocapsid antigen (HBcAg or WHcAg) provides complete or partial protection against HBV and WHV infection (30–33). Fifth, HBcAg can function as a carrier and adjuvant for other antigens (34, 35). Sixth, HBcAg is a T cell-dependent and -independent B cell immunogen (29).

To identify the amino acid sequences within the core molecule that are involved in the activation of nucleocapsid-specific T cells during HBV infection in man and to explore the feasibility of a synthetic HB vaccine including relevant core epitopes, we have analyzed the fine specificity of the T cell response to HBcAg using a panel of short synthetic peptides covering the entire HBV core region.

Analysis of patients with acute HBV infection and different HLA haplotypes reveals that several sequences within the core molecule can induce significant levels of T cell response in HBcAg-sensitized individuals, as previously reported in the mouse system (36). In addition, more than one peptide fragment is usually recognized by T cells of individual patients.

However, the most relevant finding of our study is the identification of an immunodominant amino acid sequence (residues 50–69) which was recognized by all but one patient (95%). Remarkably, concentrations of peptide as low as 10 ng/ml are sufficient to elicit a significant in vitro T cell response in several subjects (not shown).

Data derived from T cell stimulation with truncated and overlapping analogues of the 50–69 peptide show that the critical residues involved in T cell activation are likely located within the COOH-terminal region of the sequence. Interestingly, residues 64–67 (ELMT) correspond to the sequence motif, described by Rothbard et al., as being common to several T cell epitopes (10).

A significant proportion of T cells from different patients were also able to respond to two additional peptides corresponding to amino acids 1–20 and 117–131 of the core molecule (69 and 73% of the analyzed patients, respectively). The single patient who did not recognize peptide 50–69 displayed a strong T cell response to peptide 1–20 (patient 12, Table I). This suggests that peptides 1–20 and 50–69 are capable of inducing significant T cell responses in all HBcAg-sensitized patients irrespective of their genetic background.

Our results partially differ from those reported by Milich et al. (36) who showed that the fine specificity of the murine T cell response to HBcAg is much more dependent on the MHC haplotype of the responder strain. In addition, the immunodominant T cell epitope located within residues 50–69 was not identified and also the murine T cell epitope represented by peptide 120–140 in Milich's study is probably contiguous but distinct from the dominant epitope for human T cells identified by peptide 117–131.

Although these synthetic peptides efficiently induce T cell activation in vitro, they need not represent the HBcAg frag-

ments generated by the intracellular processing of the core molecule that are recognized by HBcAg-specific T cells during natural infection. Three additional lines of evidence, however, show that peptides 1–20, 50–69, and 117–131 do contain amino acid residues generated by HBcAg processing relevant to the activation of a HBcAg-specific T cell response. First, the time of appearance of a significant T cell response to core peptides during the course of HBV infection is always associated with the time of appearance of the response to the native core antigen. Second, antigen-specific T cell lines produced by PBMC stimulation with HBcAg or HBeAg can be restimulated not only with the native nucleocapsid proteins but also with the relevant peptide analogs recognized by autologous PBMC. Finally, peptide-primed polyclonal T cell lines selected by PBMC stimulation with a single core peptide can react with the whole core protein. Collectively, these results demonstrate that immunodominant AA sequences generated by the processing of the native core molecule are contained within the synthetic peptides 1–20, 50–69, and 117–131.

Amino acid sequences 1–20, 50–69, and 117–131 appear to preferentially activate CD4+ T cells which recognize peptide fragments in the context of HLA class II molecules as shown by blocking experiments with anti-HLA monoclonal antibodies and phenotypic analysis of peptide-primed polyclonal T cell lines. Due to the limited life time and the poor growth capacity of the polyclonal T cell lines, identification of the specific HLA molecules capable of associating with peptide 50–69 was not possible. However, the observation that this peptide is recognized by most HBcAg-responsive patients suggests that it can efficiently bind to many different HLA alleles, as already reported for other peptides in different systems (37–39). In addition, the simultaneous inhibition of the proliferative response to the relevant core peptides obtained with anti-DR, anti-DP, and anti-DQ monoclonal antibodies probably means that different subsets of HLA class II molecules can serve as restriction elements for a single peptide. Clonal analysis of the T cell response to peptide 50–69 in different patients is required to address these important questions.

Peptides 1–20, 50–69, and 117–131 are not recognized by anti-HBc antibodies. This is shown by experiments in which the core peptides used to study the fine specificity of the T cell response were also used as solid phase antigens to detect anti-HBc antibodies in sera of six patients with acute and four with chronic HBV infection by an enzyme-linked immunosorbent assay (data not shown). In addition, competition experiments were performed to test the ability of the core synthetic peptides to inhibit the binding of anti-HBc positive sera to HBcAg-coated plates (data not shown). No specific binding between peptides and anti-HBc antibodies from patients at different stages of HBV infection was observed, suggesting either that B cell recognition of the core molecule is focussed on linear amino acid sequences which are missing in our panel of peptides or that antibody recognition of HBcAg is mostly conformational.

An important biological issue is whether HBcAg and HBeAg are recognized by T cells as distinct antigenic entities. The T cell response to HBeAg during acute HBV infection was usually less vigorous than the T cell response to HBcAg (Table I, Fig. 6). This may be related to the particulate nature of HBcAg (8), which could be more efficiently internalized by the APC, as suggested by the higher concentrations of HBeAg often required to induce levels of T cell activation similar to those induced by HBcAg (Fig. 6, lower panel). In addition, it could also be a reflection of a different efficiency of the intracellular processing because HBeAg and HBcAg are conformationally different even though they share a large portion of their linear sequence. Finally, it is also possible that T cell epitopes are present within the carboxyterminal region of HBcAg (which is not present in the circulating HBeAg) as indicated by T cell recognition of peptide 169–183 in a proportion of patients (Table I).

However, the observation that the immunodominant T cell epitopes identified in this study are located in the portion of the nucleoprotein molecule common to both HBcAg and HBeAg confirms that for human T cells as well, core and e antigens are cross-reactive, as already suggested for murine T cells (36).

In conclusion, our study indicates the existence of an immunodominant T cell epitope (AA 50–69) within the core molecule that is recognized by more than 95% of patients with acute HBV infection and different HLA haplotypes. Two additional important T cell recognition sites were also identified at the aminoterminal end and within the carboxyterminal half of the core molecule. Taken together, these data show that two short amino acid sequences of the core molecule can induce T cell responses in 100% of patients with acute HBV infection and different genetic backgrounds. These T cell epitopes physically linked to HBV envelope particles might be exploited to enhance the immunogenicity of the existing HBV envelope-based vaccines and to try to overcome tolerance to HBsAg particles in nonresponder vaccine recipients. In addition, the immunodominant T cell epitopes identified within the core molecule represent a good candidate for the design of alternative totally synthetic vaccines. Whether this finding may also be useful for therapeutic strategies directed to manipulate the immune response to HBV in subjects with chronic HBV infection remains to be investigated.

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