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Anergic TH1 Clones Specific for Hepatitis B Virus (HBV) Core Peptides Are Inhibitory to Other HBV Core-Specific $CD4^+$ T Cells In Vitro

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A strong and transient hepatitis B virus core (HBc)-specific $CD4^+$ T-cell response has been shown to be associated with viral elimination in acute self-limited hepatitis B but to be absent in chronic hepatitis B. So far, little is known about immunological mechanisms involved in the regulation of the HBc-specific $CD4^+$ T-cell response. We studied 28 patients with acute hepatitis B, and frequently a sudden decrease in the HBc-specific $CD4^+$ T-cell response was found between 4 and 8 weeks after disease onset. Thirty-two $CD4^+$ T-cell clones specific for amino acids 50 to 69, 81 to 105, 117 to 131, or 141 to 165 of HBc were isolated from a patient shortly before the peripheral blood mononuclear cell response to most HBc-derived peptides abruptly disappeared. TH1 clones, but not TH0 clones, could be anergized in vitro by stimulation with specific peptides even in the presence of costimulatory cells. Moreover, when anergic cells were mixed with responsive cells, the proliferation of HBc-specific TH1 or TH0 clones was inhibited antigen specifically by anergic cells. The unusual susceptibility of HBc-specific TH1 clones to anergy induction in vitro as well as their potential to inhibit other HBc-specific TH1 and TH0 clones suggests that anergy induction may be involved in the downregulation of the virus-specific immune response during acute hepatitis B in vivo.

Although chronic hepatitis B virus (HBV) infection is one of the most widespread chronic infectious diseases, its pathogenesis and the mechanisms of viral persistence are rather ill understood. The spectrum of infection ranges from fulminant hepatitis, acute self-limited hepatitis, and chronic hepatitis to a chronic carrier state. All of these may be explained by a decreasingly efficient antiviral immune response (3, 21, 37). Recently, a transient hepatitis B core (HBc)-specific $CD4^+$ T-lymphocyte response was found to be associated with viral elimination during acute hepatitis B (8, 14) as well as with recovery from chronic hepatitis B (6, 39). As an underlying mechanism, the induction of a cytotoxic-T-lymphocyte response against HBV-infected liver cells with subsequent viral clearance has been proposed. The importance of an HLA class II-restricted T-helper-cell response is also supported by the recently observed association between the HLA-DRB1 allele 1302 and self-limited acute hepatitis B in the Gambia (38). So far, little is known about the regulatory mechanisms controlling the development of HBc-specific $CD4^+$ T cells. When the acute HBV infection resolves, a dramatic decline in the HBc-specific $CD4^+$ T-cell response is frequently observed within the first 3 months of disease. In contrast to this astounding eclipse of HBc-specific $CD4^+$ T cells are preliminary data which demonstrate a potentially strong memory response several years after viral elimination (27). The identification of cellular and molecular factors underlying the dynamic kinetics of the HBc-specific $CD4^+$ immune response may be critical for a deeper understanding of chronic hepatitis B infection. As T-cell anergy has been proposed as a major mechanism for the maintenance of self-tolerance and regulation of the cellular immune response (2, 13, 18, 33, 34), we intended to study the role of T-cell anergy during the natural course of HBV infection. For this purpose, HBc-specific $CD4^+$ T-cell clones were isolated from a patient with self-limited acute hepatitis B just before the cellular response to HBc decreased abruptly. The aims of the present study were to characterize in detail HBc-specific $CD4^+$ T cells during this critical phase and to analyze whether and how anergy could be induced in these clones. The characteristic phenotype and function of these anergic T-cell clones are presented and correlated with in vivo immunological variables of the patient.

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MATERIALS AND METHODS

Patients. Twenty-six patients with acute hepatitis B and 23 patients with chronic hepatitis B were studied. Acute hepatitis B was diagnosed on the basis of elevated aminotransferases, positive hepatitis B surface antigen (HBsAg), HBV DNA, and anti-HBc immunoglobulin M (IgM), as determined by kits obtained from Abbott, Wiesbaden, Germany. All patients with chronic hepatitis B were positive for HBsAg, hepatitis B e antigen (HBeAg), and HBV DNA but negative for anti-HBc IgM. Two patients with acute hepatitis B developed fulminant disease; one received a liver transplant, and one died while awaiting transplantation. Twenty-four patients with acute hepatitis B recovered uneventfully, and no patient developed chronic disease. T-cell clones were isolated from two male patients who were studied weekly during the first 6 weeks of the disease and on three occasions during 7 and 15 months of follow-up, respectively.

Major histocompatibility complex typing. Typing for HLA-DRB1 alleles (low resolution) was performed by oligonucleotide hybridization with primers and oligonucleotides from the 11th International Histocompatibility Workshop (16) and a detection system described elsewhere (24).

HBV DNA sequencing. Five hundred microliters of serum was incubated with 0.5% sodium dodecyl sulfate–10 mM Tris-HCl (pH 7.5)–10 mM EDTA–10 mg of proteinase K per ml at 37°C overnight. Serum DNA was extracted twice with phenol-chloroform and precipitated with ethanol in the presence of 30 μg of tRNA. The pellet was dissolved in 100 μl of 10 mM Tris-HCl–1 mM EDTA. HBV DNA was amplified by using oligonucleotide primers located upstream of the pre-C initiation codon (5′-GTCAACGACCGACCTTGAGGC-3′) and downstream of the C gene stop codon (5′-CCCACCTTATGAGTCCAAGG-3′).

Symmetrical amplification and asymmetrical amplification were performed as previously described (30). The amplified DNA was directly sequenced by the dideoxynucleotide chain termination method using 5′ ^{32}P-labeled primers and a sequencing kit obtained from U.S. Biochemicals, Denver, Colo.

Recombinant protein and synthetic peptides. Recombinant HBV core protein (rHBc) obtained from bacterial extracts of *Escherichia coli* K-12 strain HB101 was purchased from Biogen, Geneva, Switzerland (26). Nine overlapping 25-mer peptides covering the entire HBV core sequence (amino acids [aa] 1 to 25, 20 to 45, 40 to 65, 60 to 85, 80 to 105, 101 to 125, 121 to 145, 141 to 165, and 161 to 183) and two immunodominant peptides described previously (aa 50 to 69 and 117 to 131) (8) were synthesized by Multiple Peptide Systems, San Diego, Calif. Truncated peptide variants were synthesized by Centocor, Malvern, Pa. All peptides were purified to more than 95% by high-pressure liquid chromatography.

PBMC proliferation assay. Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Isopaque gradients purchased from Pharmacia, Uppsala, Sweden, and washed four times in phosphate-buffered saline (PBS). PBMC (5×10^4 per well) were incubated in 96-well U-bottom plates from Costar, Cambridge, Mass., for 5 days in the presence of rHBc (1, 2, and 5 μg/ml) or peptides (10 μg/ml) in 150 μl of RPMI 1640 medium, obtained from Gibco, Grand Island, N.Y., containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% human AB serum. The cell cultures were labeled by incubation for 16 h with 2 μCi of [^{3}H]thymidine (specific activity, 80 mCi/mmol; Amersham). The cells were collected and washed on filters from Dunn, Asbach, Germany, with a cell harvester (Skatron, Sterling, Va.), and the amount of radiolabel incorporated into DNA was estimated by a beta counter (LKB/Pharmacia, Uppsala, Sweden). Triplicate cultures were assayed routinely, and the results were expressed in mean counts per minute. The stimulation index (SI) was calculated as the ratio between the counts per minute obtained in the presence of antigen and those obtained without antigen. An SI of >3 was considered significant. By separation experiments and fluorescence-activated cell sorter (FACS) analyses, it could be demonstrated that proliferation was confined to the CD4$^+$ T-cell subset (14).

Generation of T-cell clones and specificity testing. Two million PBMC were stimulated with 3 μg of rHBc per ml in 96-well U-bottom plates as described above. On day 6, recombinant interleukin-2 (IL-2) was added to a final concentration of 15 U/ml (kindly provided by Boehringer, Mannheim, Germany). On day 10, cells were counted and plated at 75, 150, and 225 cells per well on a 96-well plate. Cells were allowed to sediment for 4 h, and the cell numbers in individual wells were counted. A well containing close to 150 cells was chosen, and the cells of this well were redistributed at 0.5 cells per well in the presence of 3×10^4 autologous, irradiated PBMC per well, 15 U of IL-2 per ml, and 2 μg of phytohemagglutin (PHA) (HA16; Murex Diagnostics, Dartford, United Kingdom) per ml. After 3 to 5 weeks, growing clones were tested for specificity to rHBc and rHBc-specific clones were subsequently tested for peptide specificity. For this, 1×10^4 to 5×10^4 clone cells were added to 3×10^4 autologous, irradiated PBMC with and without 2 μg of rHBc per ml or 10 μg of peptide per ml and cultured for 5 days. The proliferation assay was performed as described for PBMC. For expansion, T-cell clones were stimulated every 3 to 5 weeks with irradiated, autologous or allogeneic PBMC, 15 U of IL-2 per ml, and 2 μg of PHA per ml.

FACS analyses. Triple immunofluorescence staining was performed on T-cell clones with the following combinations of conjugated antibodies: CD3 (MT301-fluorescein isothiocyanate; kindly provided by E. P. Rieber, Institute for Immunology, Munich, Germany), CD4 (Leu-3a-PE; Becton Dickinson, Hamburg, Germany), CD8 (3B5-TRI-Color; Medac, Hamburg, Germany), CD25 (IL-2R1–fluorescein isothiocyanate; Coulter, Hialeah, Fla.), HLA-DR (L243-PE; Becton Dickinson), and CD4 (S3,5-TRI-Color; Medac). FACS analysis was performed with a FACScan from Becton Dickinson, as described previously (11).

Major histocompatibility complex restriction. T-cell clones were stimulated in the presence of irradiated, autologous PBMC with rHBc or specific peptides and with 10 μl of anti-DR (catalog no. 7730), anti-DP (catalog no. 7450), or anti-DQ (catalog no. 7360; obtained from Becton Dickinson). Proliferation assays were performed as described above. For confirmation and identification of the exact HLA allele, the following well-characterized (45) B-lymphoblastoid cell lines (kindly provided by D. Schendel, Institute for Immunology) were used as antigen-presenting cells (APC) in proliferation assays: TEM (DRA*0101, DRB1*1401, DQA1*0101, DQB1*05031, DPA1*01, and DPB1*0401), AMALA (DRA*0102, DRB1*1402, DQA1*0501, DQB1*0301, DPA1*01, and DPB1*0402), CB6B (DRA*0101, DRB1*1301, DQA1*0103, DQB1*0603, DPA1*02021, and DPB1*1901), HO301 (DRA*0102, DRB1*1302, DQA1*0102, DQB1*0605, DPA1*0201, and DPB1*0501), and WT47 (DRA*0102, DRB1*1302, DQA1*0102, DQB1*0604, DPA1*01, and DPB1*1601) (15). In parallel with proliferation assays, HLA class II restriction was determined by FACS analysis. HBc-specific CD4$^+$ T-cell clones were incubated with homozygous lymphoblastoid cell lines carrying one of the patient HLA class II alleles. After stimulation with rHBc for 20 h, stimulation was measured as the increase in CD25 expression in the CD4$^+$ T-cell population. Specific stimulation could be blocked by monoclonal HLA class II antibodies.

Lymphokine assays. rHBc-specific T-cell clones were stimulated (10^5 cells per 100 μl) with a combination of anti-CD2 (clones 6G4 and 4B2) and anti-CD28 (clone 15E8) monoclonal antibodies (1:4,000) and 1 ng of phorbol myristate acetate (Sigma, St. Louis, Mo.) per ml or with specific peptides (10 μg/ml) in the presence of HLA-matched lymphoblastoid cell lines at a ratio of 1:1. Supernatants were collected after 24 h and stored at −80°C. The secretion of IL-4, IL-5, and gamma interferon was measured by sandwich enzyme-linked immunosorbent assay (ELISA) techniques previously described (20, 28, 40). Transforming growth factor β1 (TGF-β1) was measured by a commercial ELISA (Genzyme). The sensitivities of these assays were 50 to 200 pg/ml for IL-4, 2,500 to 3,000 pg/ml for IL-5, and 100 pg/ml for gamma interferon and TGF-β1. IL-2 was measured by a standard CTLL assay (10).

Anergy induction. The T-cell clones used for anergy induction typically 3 to 5 weeks after the last stimulation with PHA were CD25 negative, as determined by FACS analysis. The following regimens were used for anergy induction: 10^4 clone cells per well were stimulated with specific peptides at various concentrations (0.01, 0.1, 1.0, 3, and 10 μg/ml) either alone or in the presence of irradiated, autologous PBMC (3×10^4 per well) and the presence or absence of exogenous IL-2 (15 U/ml). To exclude autopresentation of peptide by the T-cell clone, stimulation was also performed with irradiated, autologous PBMC that had been preincubated with peptide overnight and had then been washed extensively. In every instance, clone cells were stimulated in parallel with PHA. All experiments were performed in the presence of 15 U of IL-2 per ml. The expression of T-cell-receptor components (CD3 and CD4) and activation markers was determined by FACS analysis at least weekly. Proliferation tests and inhibition experiments were performed between days 21 and 28.

Inhibition experiments. Specific inhibition assays were essentially performed as previously described (19). In brief, peptide-pulsed (10 μg/ml for 2 h and then washed extensively with PBS), irradiated, autologous PBMC were used as APC. Responsive clone cells (3×10^3 per well) were cultered with peptide-pulsed APC either alone or together with anergic clone cells at ratios of 1:1 and 1:3. Routinely, the tests were performed in the absence of exogenous IL-2; in some experiments, IL-2 was added to a final concentration of 15 U/ml. Proliferation, measured as [^{3}H]thymidine incorporation, was determined after 5 days. The data presented here are representative of at least three independently performed experiments.

Cytokines and cytokine antibodies. Recombinant IL-10 was purchased from PharMingen, San Diego, Calif., and used at concentrations of 3 to 30 U/ml. Recombinant TGF-β1 from Boehringer was used at 1 to 5 ng/ml. Neutralizing IL-10 antibody (B-T10; IgG2b) was the generous gift of J. Wijdenes (Dia-Clone, Besancon, France) and was used at 10 μg/ml. Neutralizing TGF-β1/2/3 antibody (code 1835-01; IgG1; Genzyme) was used at 50 μg/ml.

RESULTS

HBc-specific CD4$^+$ T-cell response during acute and chronic hepatitis B. The HBc-specific CD4$^+$ T-cell response was studied in 28 patients with acute hepatitis B during the acute phase of the disease and in 23 patients with chronic hepatitis B. For most patients, the time course of the T-cell response against HBc was analyzed in a longitudinal fashion. Two patients with acute hepatitis B developed fulminant disease, while the other twenty-six recovered uneventfully. Of this group, 23 of 28 patients responded with a HBc-specific CD4$^+$ T-cell reaction which was strongest in the 2 patients with fulminant disease (Fig. 1). In four of five patients who were longitudinally studied for more than 3 months after disease onset, the T-cell response declined and disappeared almost completely shortly after the clinical resolution of disease (Fig. 2). Mapping the specificity of the PBMC response with overlapping synthetic peptides revealed that the reactions to individual epitopes were also lost at about this time (Fig. 2B and C). In order to study the mechanism of this downregulation in more detail, we isolated a total of 32 HBc-specific CD4$^+$ T-cell clones taken from an individual during viral elimination just 1 and 2 weeks before the recognition of most peptide epitopes was lost. This patient could be monitored in detail over 6 weeks from the onset of clinical symptoms until HBV DNA was no longer detectable in serum and HBs antibodies appeared (Fig. 2C). HBc-specific proliferative T cells were present throughout this period; they then gradually decreased and became undetectable for more than 20 months. Two peaks of reactivity, the first coinciding with the loss of HBsAg and seroconversion to anti-HBs (day 14) and the second 6 weeks after the onset of acute disease, were discerned. During the first peak, peptides consisting of aa 50 to 69, 61 to 85, 81 to 105, and 117 to 131 were recognized. Only 7 days later, the response to the core

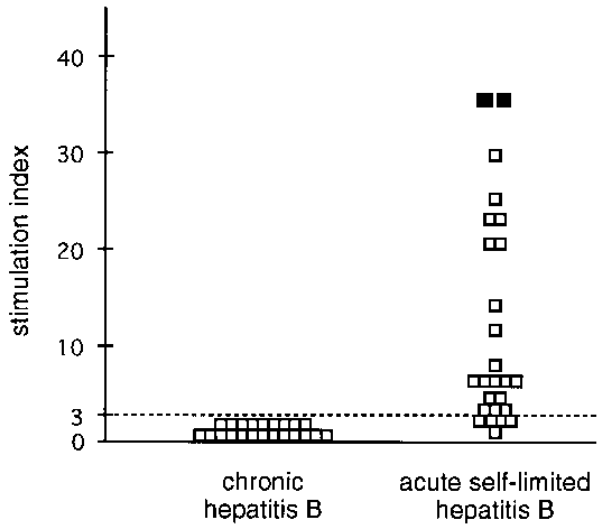


FIG. 1. Proliferative response of PBMC to rHBc. Comparisons of best PBMC responses to rHBc in sequential proliferation assays of patients with chronic hepatitis B and acute self-limited hepatitis B. Solid squares, patients with acute fulminant disease. The dotted line (SI = 3) stands for the level of significance.

protein was diminished and there was no detectable response to any of the previously recognized peptides. During the second peak of reactivity (days 33 and 36), responses to aa 50 to 69 and 61 to 85 returned and additional epitopes, aa 41 to 65 and 141 to 165, were recognized.

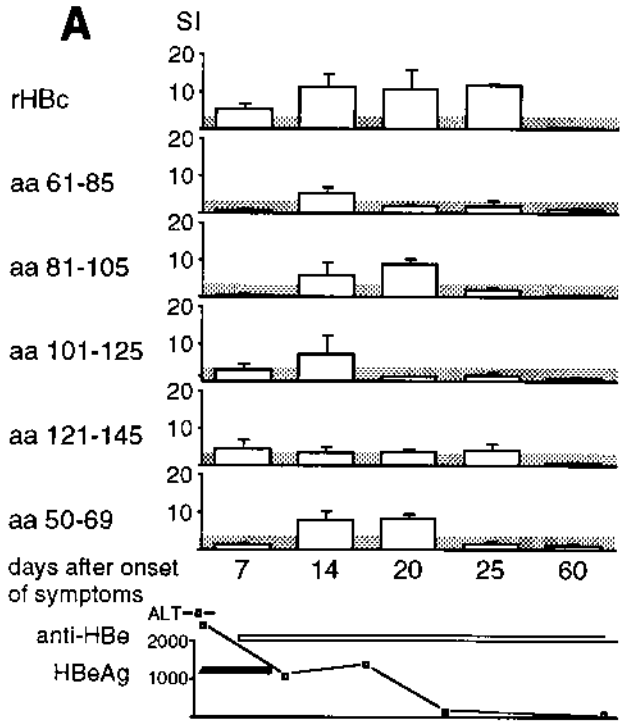
Induction of anergy in HBc-specific TH1 clones. HBc-specific $CD4^+$ T-cell clones were isolated on days 6 and 14, and the specificities of 19 different clones were determined. Ten clones were specific for aa 81 to 105, five were specific for aa 141 to 165, three were specific for aa 50 to 69, and one was specific for aa 117 to 131. By using monoclonal anti-HLA class II antibodies, all clones could be shown to be HLA-DR restricted. For the identification of individual restriction elements, B-lymphoblastoid cell lines homozygous for one of the patient HLA-DR alleles were used as APC. T-cell clones specific for aa 50 to 69, 81 to 105, or 141 to 165 recognized the peptide bound to HLA-DRA*0101/DRB1*1302 (data not shown). Fine mapping of the T-cell epitopes using a series of N and C terminally truncated peptides (5a) revealed aa 52 to 62, 93 to 103, and 145 to 155 as the minimal epitopes required for T-cell recognition. Lymphokine production of individual T-cell clones was measured by stimulation either with specific peptides presented by HLA-matched lymphoblastoid cell lines or with monoclonal antibodies to CD2, CD3, and CD28 without any accessory APC. Regardless of the mode of stimulation, the lymphokine profiles observed for individual clones belonged to either the TH1 or TH0 pattern (Table 1). The majority of HBc-specific $CD4^+$ T-cell clones were specific for aa 81 to 105, corresponding to a strong PBMC response to the very same peptide on day 14. One week later (day 20), however, the PBMC response to this epitope was undetectable; in fact, the entire HBc-specific PBMC response, including the responses to other peptides, was also reduced. While responsiveness to peptides consisting of aa 50 to 69 and 141 to 165 gradually returned over time, the response to aa 81 to 105 could not be recovered. To study their presumably increased susceptibilities to anergy induction, we investigated three clones specific for peptide aa 81 to 105 (G9 [TH0] and G40 and G42 [both TH1]) and three clones specific for peptide aa 141 to 165 (G61 [TH0] and G27 and G41 [both TH1]). The various protocols used are demonstrated in Fig. 3A and B. Anergy, as defined by the absence of a proliferative response to a specific antigen, could be induced in all TH1 clones with specific peptides in the absence or presence of APC. Anergy could also be induced when the peptide was presented by peptide-pulsed APC and was not prevented by the presence of IL-2 (15 U/ml). No anergy was induced when an irrelevant peptide was used for stimulation. Significant cell death occurred within the first 48 h of anergy induction and was highest in the absence of APC (up to 80%). Cell numbers remained constant or increased slightly thereafter. TH0 clones, in contrast, could not be anergized by any of the protocols. During anergy induction, an initial intense proliferation was observed. The anergic state was reached within 24 h and lasted for at least 6 weeks.

The sensitivity of HBc-specific $CD4^+$ T cells to anergy induction was subsequently studied in another patient with self-limited acute hepatitis B. In that individual, HLA-DR7-restricted $CD4^+$ T-cell clones specific for aa 61 to 85 of HBc could be isolated. The functional characteristics of these clones have been published previously (14). Again, HBc-specific T-cell clones could be rendered anergic by stimulation with specific peptides in the presence of autologous APC (Fig. 3A and B). As with the experiments described above, stimulation of these clones also led to an initial intense proliferation which subsequently resulted in a refractory state.

Influence of truncated peptide variants on anergy induction. From the study of naturally processed antigens (41) eluted from purified HLA class II molecules, it is known that for an individual antigenic peptide sequence, nested sets of peptides that have various amino- and carboxy-terminal extensions are presented to T cells. We cannot exclude the possibility that the peptides used for anergy induction in our study were, at least to some extent, processed to truncated variants, which could potentially induce anergy or act as partial T-cell activators (36). We therefore compared the stimulatory and anergy-inducing potential of the 25-mer peptide consisting of aa 141 to 165 with those of the truncated variants aa 145 to 158 (containing the minimal epitope aa 145 to 155), 146 to 159, and 145 to 154. There were no differences between the stimulatory capacities of peptides 145 to 158 and 141 to 165, whereas peptides 146 to 159 and 145 to 154 were at least 100-fold-less potent to induce T-cell activation (Fig. 4A). The preincubation of specific T-cell clones with truncated peptide variants did not induce anergy (Fig. 4B).

Characterization of anergic T-cell clones. Phenotypically, anergic cells were not different from PHA-stimulated clone cells with regard to the expression of CD3, CD4, and high-affinity IL-2 receptor (CD25) for the first 2 weeks after stimulation. After 4 weeks, however, anergic cells showed higher levels of expression of CD25, CD3, and CD4 compared with those of responsive cells that had been stimulated with PHA (Fig. 5). From about 24 h after initial stimulation, anergic cells did not respond to a further antigenic stimulus but could be expanded by exogenous IL-2 (Fig. 6A). The cultivation of anergic cells for up to 8 weeks in 50 U of IL-2 per ml did not restore reactivity (data not shown). To exclude the possibility that the peptide consisting of aa 81 to 105 represented a so-called altered peptide ligand (36), the HBc sequence of the infecting strain was determined. The deduced amino acid sequence was identical to that of the synthetic peptide used (data not shown).

Anergic TH1 clones act as antigen-specific suppressor cells. A striking feature of the time course of PBMC responses to HBc and HBc-derived peptides was the reduction in the HBc-specific response during the third week of acute hepatitis,



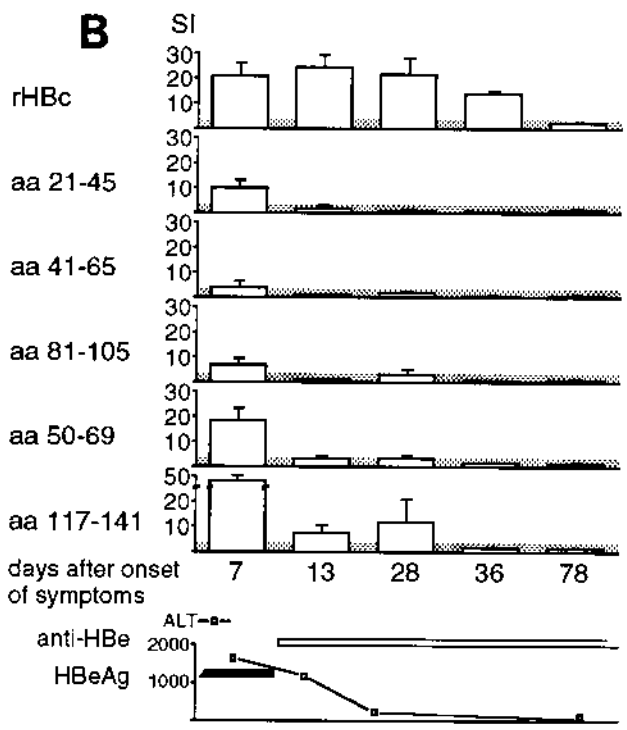
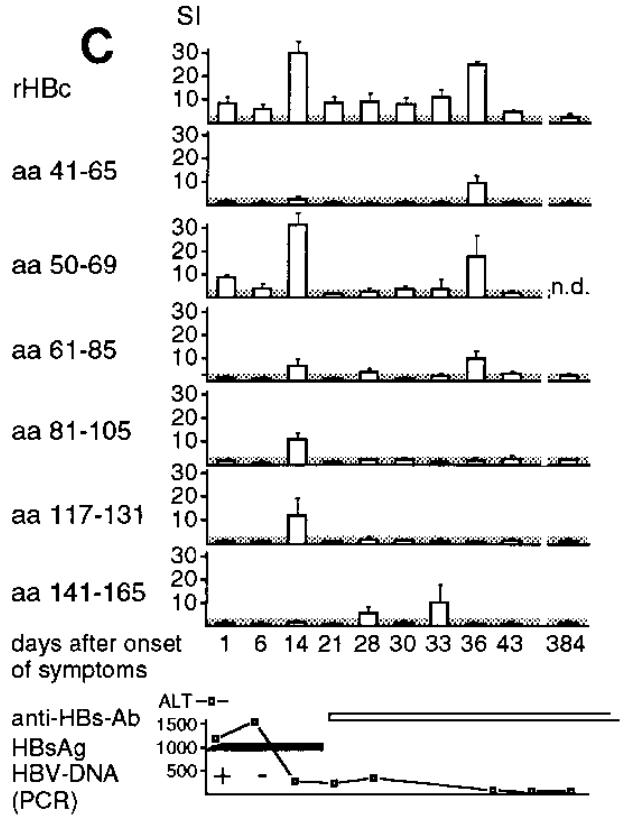


FIG. 2. Serial measurements of the PBMC responses to HBc and HBV core peptides in three patients (A through C) with acute self-limited hepatitis B during viral elimination and loss of HBeAg or HBsAg, respectively. Hatched areas represent nonsignificant SIs (<3.0). Error bars are also shown. Ab, antibody; n.d., not determined.



affecting all epitopes simultaneously, although several HBc-specific TH0 clones were resistant to anergy induction in vitro. Because it has recently been shown that anergic TH1 clones can act as antigen-specific suppressor cells (19), we tested whether anergic TH1 clones specific for the peptide consisting of aa 81 to 105 could inhibit the response of reactive TH1 and TH0 clones specific for the same peptide or different peptides of HBc. Responsive clone cells were stimulated with peptide-pulsed, irradiated, autologous PBMC either alone or mixed with anergic cells at a ratio of 1:1 or 1:3. The strong antigen-specific proliferation of responsive cells could be markedly inhibited by the addition of anergic cells derived from the same $CD4^+$ T-cell clone (Fig. 6B). A similar inhibitory effect was observed when responsive cells from another clone specific for the same peptide epitope were tested (Fig. 6C). The addition of IL-2 at a final concentration of 15 U/ml largely reversed this inhibition (Fig. 6D).

To study the effects of anergic TH1 clones on TH0 clones and HBc-specific clones with different peptide specificities, we added anergic clone G40 (specific for aa 81 to 105) to responsive clone G21 (TH0; specific for aa 141 to 165). To provide naturally processed ligands for both clones, we used rHBc as the stimulating antigen. Again, significant inhibition of antigen-specific proliferation was observed, albeit to a lesser extent than that observed when anergic and responsive clones had the same peptide specificity (Fig. 6E). To study the relevance of antigen recognition by the anergic clone, we repeated the latter experiment but used the peptide consisting of aa 141 to 165 instead of rHBc as the stimulatory antigen. Thus, the specific antigen for responsive clone G21 was present but the antigen for anergic clone G40 was not. Under those conditions, no inhibition could be observed (Fig. 6F).

In order to elucidate whether the inhibitory effects of anergic TH1 cells could be mediated by suppressive cytokines, we tested the susceptibilities of our responsive clones to inhibition

TABLE 1. Lymphokine production of rHBc-specific CD4$^+$ T-lymphocyte clones

Clone (type)	IL-4 (ng/ml)	IL-5 (ng/ml)	Gamma interferon (ng/ml)
aa 50–69 specific, G25 (TH0)	<0.2	3.4	1.6
aa 81–105 specific			
G9 (TH0)	5.0	22.1	12.5
G12 (TH0)	1.3	ND[a]	2.1
G17 (TH0)	<0.1	7.4	0.2
G40 (TH1)	0.21	<3.0	11.5
G42 (TH1)	0.21	<3.0	8.5
G64 (TH1)	<0.05	<3.0	21.6
aa 117–131 specific, G16 (TH1)	<0.1	<3.0	0.44
aa 141–165 specific			
G21 (TH0)	0.3	<3.0	0.48
G27 (TH1)	<0.05	<3.0	19.2
G41 (TH1)	0.5	<3.0	38.8
G61 (TH0)	3.3	4.1	35.5
G77 (TH1)	<0.05	<3.0	3.7

[a] ND, not determined.

by recombinant IL-10 and TGF-β1. Whereas IL-10 did not lead to a significant reduction in proliferation after antigen-specific stimulation, TGF-β1 induced a maximum of about 65% inhibition (Fig. 7A). Measurements of TGF-β1 in the supernatants of antigen-stimulated responsive and anergic clones revealed no differences in the amount of TGF-β1 secreted (from 1,383 to 1,520 pg/ml), and the addition of anti-TGF-β increased antigen-specific proliferation of responsive and anergic clone cells by 23 and 50%, respectively. Most importantly, however, when responsive and anergic cells were mixed in the presence of neutralizing antibodies to either IL-10 or TGF-β, no reversal of the inhibitory effects of anergic cells was observed (Fig. 7B).

Is the observed inhibition caused by anergic cells or by a cellular contaminant? One interpretation of our data would be that anergy induction leads to apoptosis of the specific clone and that subsequently a contaminating, nonspecific cell population could expand, which would be inhibitory via the secretion of antiproliferative cytokines. To exclude that possibility, the following lines of evidence can be proffered: (i) antibodies to the inhibitory cytokines IL-10 and TGF-β did not reverse the suppressive effects of anergic cells; (ii) the isolation of T-cell-receptor mRNA from clones yielded a single Vβ-chain

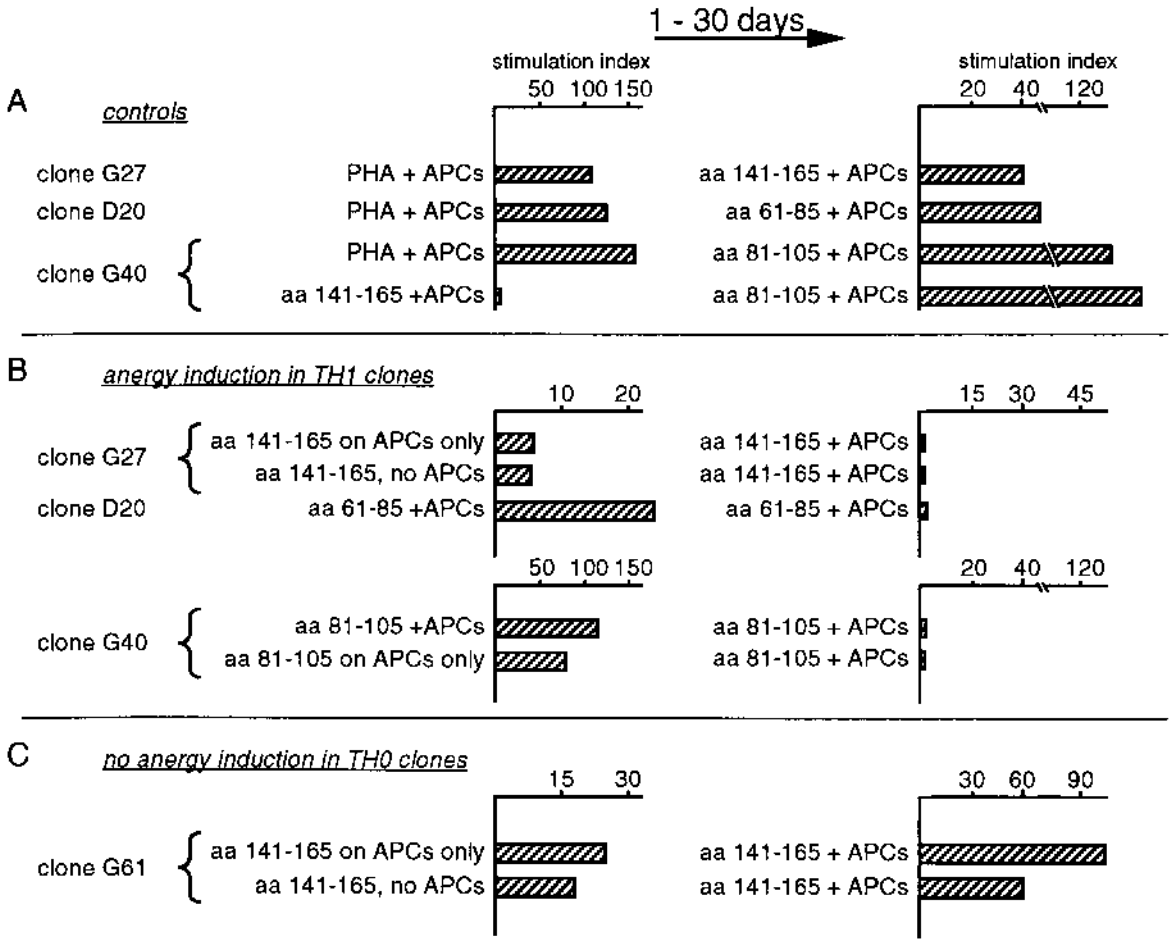


FIG. 3. Anergy induction in TH1 clones G40, G27, and D20 is not prevented by accessory cells or IL-2 and is accompanied by initial proliferation. The first column shows first stimulation or anergy induction; the second column shows the results of restimulation. (A) Stimulation with PHA leads to strong proliferation, and responsiveness to the specific peptide is maintained. Stimulation with an irrelevant peptide (when clone G40, which is specific for aa 81 to 105, is stimulated with the peptide consisting of aa 141 to 165) induces neither proliferation nor anergy. (B) Stimulation of TH1 clones (G40, G27, and D20) with specific peptides (aa 81 to 105, 141 to 165, and 61 to 85, respectively) in the presence or absence of accessory cells results in intense proliferation. Restimulation after 1 to 30 days with specific peptides in the presence of APC, however, reveals anergy. (C) No anergy is induced in TH0 clone G61.

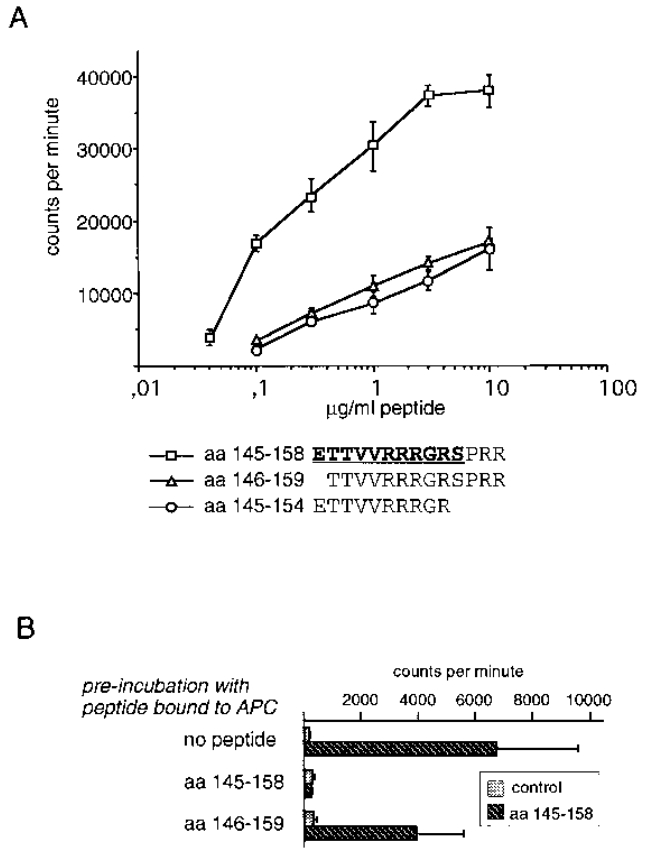


FIG. 4. (A) Stimulatory capacities of truncated peptide variants of epitope aa 145 to 155. Peptide aa 145 to 158, which contains the complete minimal epitope (aa 145 to 155; underlined), is at least 100-fold more potent in stimulating clone G27 compared with the amino- and carboxy-terminally truncated variants aa 146 to 159 and 145 to 154. (B) Preincubation of clone G27 with a truncated peptide variant (aa 146 to 159; 10 µg/ml) does not induce anergy. Error bars are also shown.

by Vβ-family-specific PCR (8a); (iii) the stimulation of clones with specific peptide and subsequent FACS analysis demonstrated that 100% of $CD4^+$ T cells were activated; (iv) weekly FACS analyses of anergized clone cells demonstrated that activated $CD4^+$ T cells slowly returned to a state of residual low-level CD25 expression (Fig. 5) (no evidence for the outgrowth of a previously unstimulated cell population could be found); and (v) inhibition experiments mixing anergic clone G40 (specific for aa 81 to 105) and responsive clone G21 (specific for aa 141 to 165) revealed that this inhibition depended on the presence of the antigen for anergic clone G40, while no inhibition was observed with the peptide consisting of aa 141 to 165 alone.

DISCUSSION

The consistent association of an HBc-specific $CD4^+$ T-cell response with subsequent viral clearance in patients with hepatitis B allows the inference that $CD4^+$ T cells play a pivotal role in antiviral immune defense (8, 14, 39). This study con-

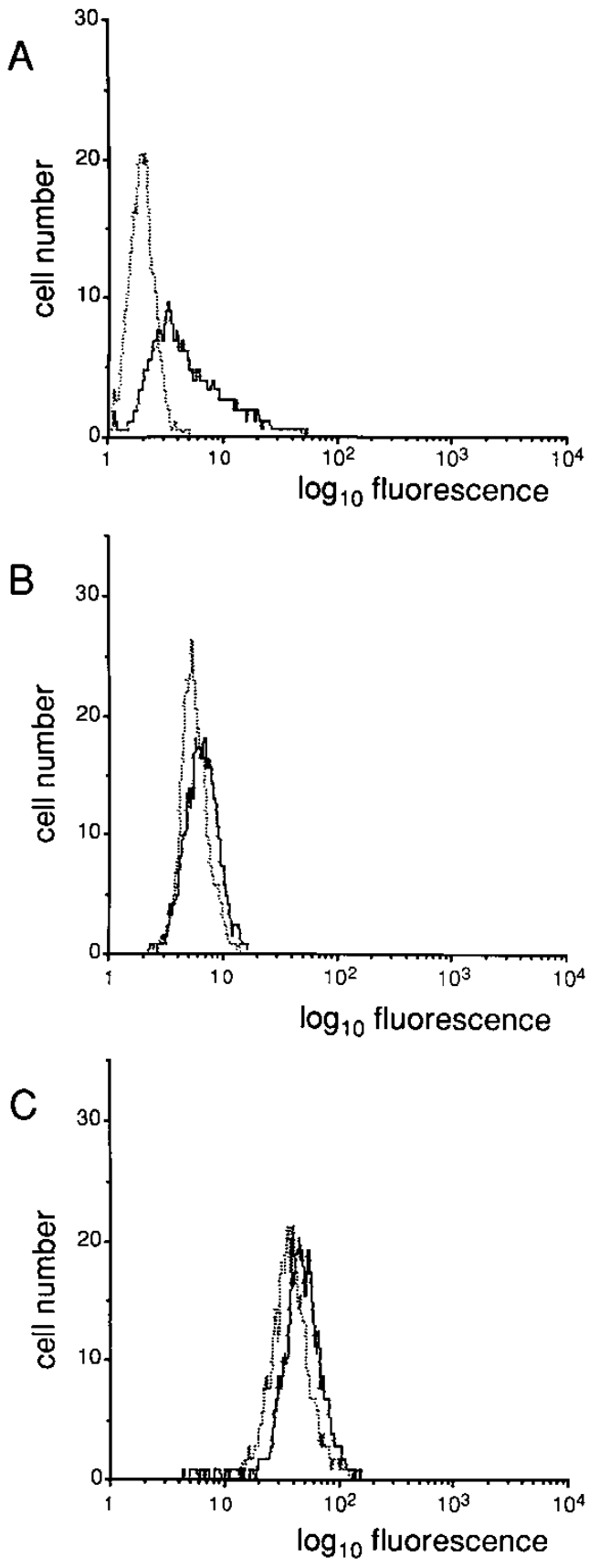


FIG. 5. FACS analyses of anergic clone G42 (solid lines) and responsive clone G42 (dashed lines). Three weeks after stimulation with peptide or PHA, anergic cells exhibit considerably stronger expression of CD25 (A) and slightly stronger expression of both CD3 (B) and CD4 (C).

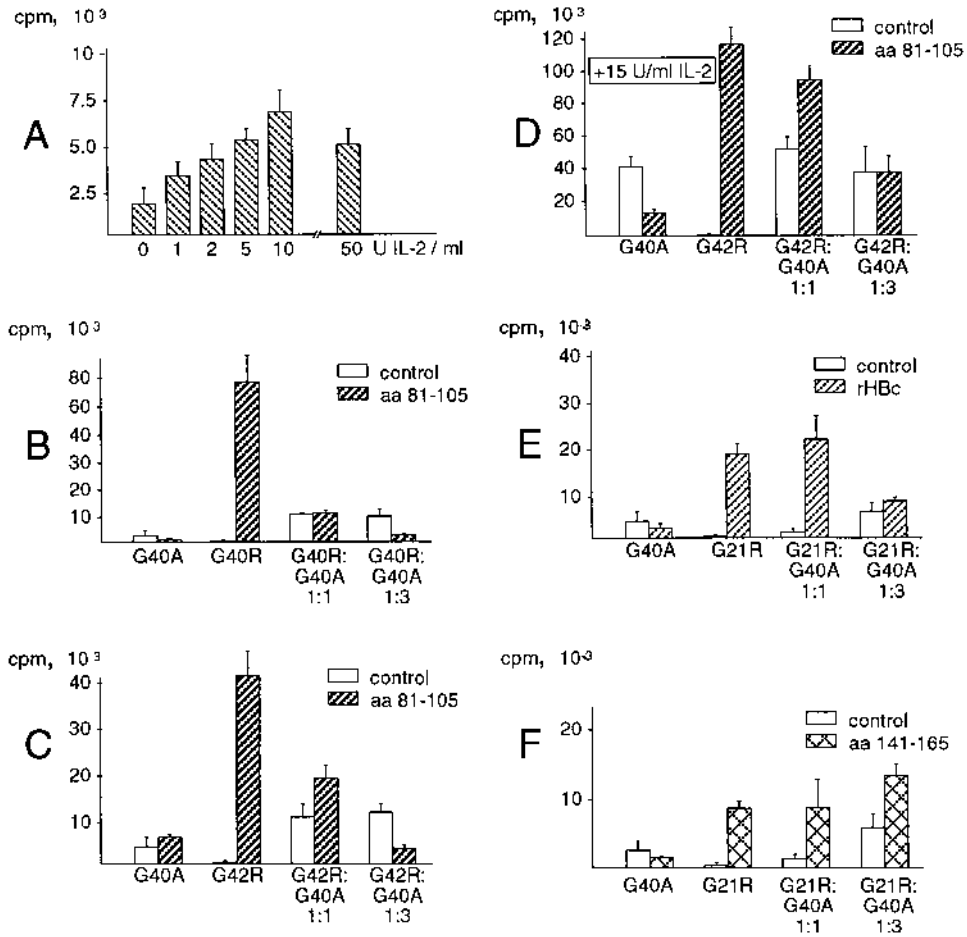


FIG. 6. Anergic clone G40 causes antigen-specific inhibition of proliferation of responding HBc-specific clones. (A) Anergic cells proliferated in response to exogenous IL-2. (B) Anergic clone G40 (G40A) inhibited the proliferation of responsive clone G40 (G40R) when the clone cells were mixed at ratios of 1:1 and 1:3. (C) Another clone specific for the same peptide aa 81 to 105 (G42) was also suppressed by anergic clone G40A. (D) Inhibition can be partially reversed by the addition of exogenous IL-2 (15 U/ml). (E) An HBc-specific clone recognizing aa 141 to 165 was also inhibited by G40A when stimulation was performed with rHBc. (F) There was no inhibition when peptide aa 141 to 165 was used as the antigen. Error bars are also shown.

firms and extends previous work demonstrating that the HBc-specific $CD4^+$ T-cell response is polyclonal within an individual patient and directed against multiple epitopes. The isolation of HBc-specific $CD4^+$ T-cell clones yields predominantly clones with a TH1 or TH0 lymphokine profile.

It is intriguing, however, why the HBc-specific $CD4^+$ T-cell response declines regularly in peripheral blood within the first 3 months of acute hepatitis B. The preliminary demonstration of a strong $CD4^+$ T-cell memory to HBc several years after successful viral clearance (27) does not necessarily contradict our findings but asks for an explanation as to why the T-cell response is so often transiently downregulated in the late acute phase. We cannot fully exclude the possibility that HBc-specific $CD4^+$ T cells are recruited and retained in the liver and therefore are absent in the periphery. It is unlikely, however, because liver inflammation and viral load, as reflected by HBV DNA and HBeAg in serum, are already waning in that late acute phase in patients with resolving acute hepatitis B (Fig. 2). The following factors may be involved in this remarkable decline in the HBc-specific $CD4^+$ T-cell response: (i) the disappearance of viral antigens and thus removal of the antigenic stimulus; (ii) T-cell exhaustion, which has been reported for virus-specific $CD8^+$ T lymphocytes in murine lymphocytic choriomeningitis virus infection in which a high virus load leads to complete induction of specific $CD8^+$ T cells followed by cell death and chronic infection (22); (iii) the generation of virus-specific T cells secreting suppressive cytokines; and (iv) the induction of clonal anergy as a transitional state towards deletion (42). In the present study, we tried to determine whether T-cell clonal anergy is involved in termination of the immune response during that particular phase of acute HBV infection. To this end, susceptibility to anergy induction in vitro was studied in HBc-specific $CD4^+$ T-cell clones isolated from two patients just before their HBc-specific PBMC responses declined. The induction of anergy in these clones is remarkable in the following respects. (i) In contrast to the general notion that anergy induction depends on isolated stimulation of the T-cell receptor in the absence of costimulatory second signals (18, 34), in the described system anergy cannot be prevented by functional APC. (ii) In the induction protocols followed here, an initial strong proliferation is a characteristic phase prior to development of the anergic state. This stands in contrast to previous reports claiming the absence of proliferation as a necessary step for the induction of anergy (4, 9, 12, 23, 25, 29, 43) but is similar to various in vivo models of anergy induction in which the initial expansion of the anergic T cells is followed by a state of nonresponsiveness (31, 32, 42). (iii) Anergy could not be reversed by culturing anergic cells in the presence of

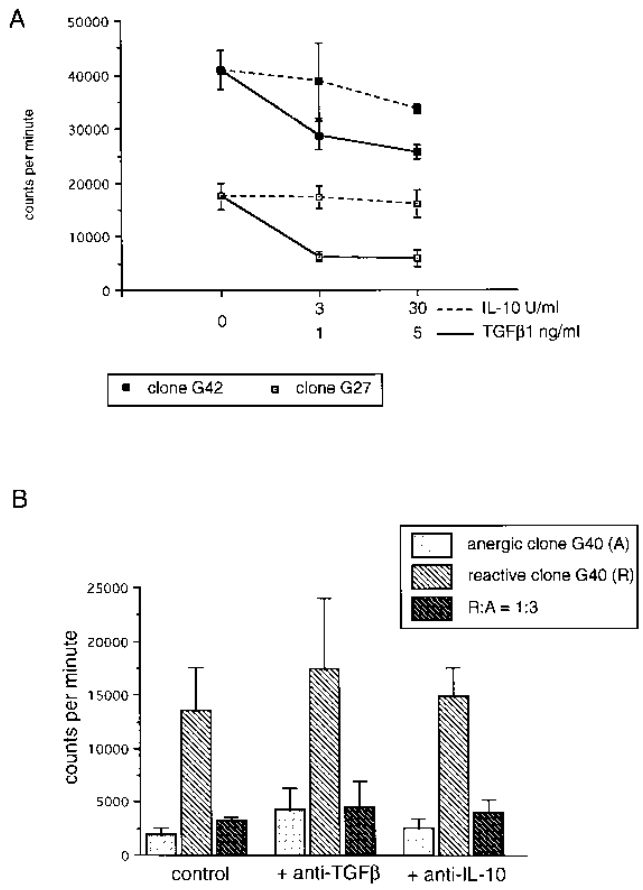


FIG. 7. (A) Antigen-specific proliferation of HBc-specific $CD4^+$ T-cell clones G27 and G42 was not significantly inhibited by recombinant IL-10. Recombinant TGF-β1, however, caused up to 65% inhibition of antigen-specific proliferation. (B) Coculture of anergic clone G40A with responsive clone G40R led to significant suppression of proliferation. The addition (+) of neutralizing TGF-β antibodies induced a moderate increase in the antigen-specific proliferation of anergic clone cells as well as responsive clone cells. The suppressive effects of anergic cells, however, cannot be reversed by the presence of neutralizing antibody to IL-10 (10 μg/ml) or TGF-β (50 μg/ml).

IL-2, which again contrasts with several in vitro studies of anergy (4, 7) but has been observed in in vivo models of anergy (31). Some of the differences from other in vitro models of anergy may be explained by the fact that usually T cell clones were expanded by restimulation every 1 to 2 weeks with antigen in the presence of APC (23, 43). Since that mode of restimulation induced anergy in our T-cell clones, we would not have been able to propagate these clones and would have missed detecting that population of HBc-specific TH1 cells. In conclusion, our observations support the concept that anergy is not a single, clearly defined state; anergic clones seem to display very distinct characteristics but have in common the inability to produce IL-2 upon antigen-specific stimulation (1).

Since viral mutations can affect T-cell recognition of a specific epitope in several ways, including anergy induction (17, 36) and T-cell-receptor antagonism (35), we determined the sequence of the infecting viral strain and found complete identity to the peptide sequence used in our in vitro studies. We also found no evidence that additional processing of peptides by APC could lead to truncated variants with the ability to induce anergy.

From the data presented here, it can be speculated that HBc-specific TH1 clones may also be susceptible to anergy induction in vivo. However, since many HBc-specific $CD4^+$ T-cell clones were of the TH0 type and resistant to anergy induction in vitro, anergy alone cannot satisfactorily explain the complete loss of the PBMC response to several HBc epitopes. Recently, it has been demonstrated that anergic TH1 clones can act as antigen-specific suppressor cells in vitro (19). In that study, the capacity of anergic TH1 cells to inhibit antigen-specific proliferation of the identical, responsive clone was explained by competition for locally produced IL-2 as well as for presented epitopes on APC. In our study, anergic HBc-specific TH1 clones were potent antigen-specific inhibitors of the proliferation of responsive HBc-specific TH1 and TH0 clones. The inhibitory effect strictly depended on the presence of the specific antigen for the anergic clone. Neutralizing antibodies to the inhibitory cytokines IL-10 (5) and TGF-β (44) did not reverse the inhibition, arguing against a major role for these cytokines in the suppressive effects of anergic TH1 clones. Instead, since exogenous IL-2 could largely abolish the inhibitory effects of anergic cells, it seems more likely that local competition for IL-2 in the vicinity of the APC is the key mechanism of inhibition.

The in vivo relevance of this mechanism has yet to be proven. Nevertheless it is rather suggestive of an active, antigen-specific immunoregulatory role for anergic TH1 clones. Between fulminant hepatitis on the one hand and chronic hepatitis on the other, careful balancing of the antiviral immune response seems to be required to achieve viral clearance while limiting hepatocellular injury. The induction of T-cell clonal anergy in HBc-specific TH1 cells may be among others a potent immunoregulatory mechanism of the HBc-specific $CD4^+$ T-cell response.

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REFERENCES

1. **Arnold, B., G. Schönrich, and G. J. Hämmerling.** 1993. Multiple levels of peripheral tolerance. Immunol. Today **14:**12–14.
2. **Boussiotis, V. A., G. J. Freeman, J. D. Griffin, G. S. Gray, J. G. Gribben, and L. M. Nadler.** 1994. CD2 is involved in maintenance and reversal of human alloantigen-specific clonal anergy. J. Exp. Med. **180:**1665–1673.
3. **Chisari, F. V., and C. Ferrari.** 1995. Hepatitis B virus immunopathogenesis. Annu. Rev. Immunol. **13:**29–60.
4. **De Silva, D. R., K. B. Urdahl, and M. K. Jenkins.** 1991. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. J. Immunol. **147:**3261–3267.
5. **De Waal Malefyt, R., H. Yssel, and J. E. De Vries.** 1993. Direct effects of IL-10 on subsets of human $CD4^+$ T cell clones and resting T cells: specific inhibition of IL-2 production and proliferation. J. Immunol. **150:**4754–4765.

5a. **Diepolder, H. M., et al.** Submitted for publication.

6. **Diepolder, H. M., M. C. Jung, R. M. Hoffmann, R. Zachoval, F. M. Zwiebel, C. Korherr, G. Paumgartner, G. Riethmüller, and G. R. Pape.** 1994. Immune mechanisms during interferon-alpha (IFN) induced virus elimination in chronic HBV and HCV infection: analysis of virus-specific CD4+ T-lymphocytes. J. Hepatol. **21**(Suppl. 1)**:**S24. (Abstract.)
7. **Essery, G., M. Feldmann, and J. R. Lamb.** 1988. Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. Immunology **64:**413–417.
8. **Ferrari, C., A. Bertoletti, A. Penna, A. Cavalli, G. Missale, M. Pilli, P. Fowler, T. Giuberti, F. V. Chisari, and F. Fiaccadori.** 1991. Identification of immunodominant T cell epitopes of the hepatitis B virus nucleocapsid antigen. J. Clin. Invest. **88:**214–222.

8a. **Gerlach, T.** Unpublished data.

9. **Gilbert, K. M., K. D. Hoang, and W. O. Weigle.** 1990. TH1 and TH2 clones differ in their response to a tolerogenic signal. J. Immunol. **144:**2063–2071.
10. **Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith.** 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol. **120:**2027–2032.

11. **Gruber, R., C. Reiter, and G. Riethmüller.** 1993. Triple immunofluorescence flow cytometry, using whole blood, of $CD4^+$ and $CD8^+$ lymphocytes expressing CD45RO and CD45RA. J. Immunol. Methods **163:**173–179.
12. **Jenkins, M. K.** 1992. The role of cell division in the induction of clonal anergy. Immunol. Today **13:**69–73.
13. **Johnson, J. G., and M. K. Jenkins.** 1994. The role of anergy in peripheral T cell unresponsiveness. Life Sci. **55:**1767–1780.
14. **Jung, M.-C., H. M. Diepolder, U. Spengler, E. A. Wierenga, R. Zachoval, R. M. Hoffmann, D. Eichenlaub, G. Frösner, H. Will, and G. R. Pape.** 1995. 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. **69:**3358–3368.
15. **Kimura, A., R. P. Dong, H. Harada, and T. Sasazuki.** 1992. DNA typing of HLA class II genes in B-lymphoblastoid cell lines homozygous for HLA. Tissue Antigens **40:**5–12.
16. **Kimura, A., and T. Sasazuki.** 1992. Eleventh International Histocompatibility Workshop reference protocol for the HLA DNA typing technique, p. 397–419. *In* K. Tsuji, M. Aizawa, and T. Sasazuki (ed.), HLA 1991. Oxford University Press, Oxford.
17. **Lamb, J. R., J. A. Higgins, C. Hetzel, J. D. Hayball, R. A. Lake, and R. E. O'Hehir.** 1995. The effects of changes at peptide residues contacting MHC class II T-cell receptor on antigen recognition and human Th0 cell effector function. Immunology **85:**447–454.
18. **Lamb, J. R., B. J. Skidmore, N. Green, J. M. Chiller, and M. Feldmann.** 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. J. Exp. Med. **157:**1434–1447.
19. **Lombardi, G., S. Sidhu, R. Batchelor, and R. Lechler.** 1994. Anergic T cells as suppressor cells in vitro. Science **264:**1587–1589.
20. **McNamee, L. A., D. I. Fattah, T. J. Baker, S. K. Bains, and P. H. Hissey.** 1991. Production, characterization and use of monoclonal antibodies to human interleukin 5 in an enzyme-linked immunosorbent assay. J. Immunol. Methods **141:**81–88.
21. **Milich, D. R.** 1991. Immune response to hepatitis B virus proteins: relevance of the murine model. Semin. Liver Dis. **11:**93–112.
22. **Moskophidis, D., F. Lechner, H. Pircher, and R. Zinkernagel.** 1993. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector cells. Nature (London) **362:**758–761.
23. **Mueller, D. L., M. K. Jenkins, L. Chiodetti, and R. H. Schwartz.** 1990. An intracellular calcium increase and protein kinase C activation fail to initiate T cell proliferation in the absence of a costimulatory signal. J. Immunol. **144:**3701–3709.
24. **Nevinny-Stickel, C., M. P. Bettinotti, A. Andreas, M. Hinzpeter, K. Mühlegger, G. Schmitz, and E. D. Albert.** 1991. Nonradioactive HLA class II typing using polymerase chain reaction and digoxigenin-11-2′-3′-dideoxy-uridinetriphosphate labeled oligonucleotide probes. Hum. Immunol. **31:**7–13.
25. **O'Hehir, R. E., R. A. Lake, T. J. Schall, H. Yssel, E. Panagiotopoulou, and J. R. Lamb.** 1996. Regulation of cytokine and chemokine transcription in a human TH_2 type T-cell clone during the induction phase of anergy. Clin. Exp. Allergy **26:**20–27.
26. **Pasek, M., T. Goto, W. Gilbert, B. Zink, P. Schaller, G. Mackay, G. Leadbetter, and K. Murray.** 1979. Hepatitis B virus genes and their expression in E. coli. Nature (London) **282:**575–579.
27. **Penna, A., A. Cavalli, M. Artini, M. Levrero, A. Bertoletti, F. Fiaccadori, and C. Ferrari.** 1995. Long-lasting HLA class II restricted anti-viral responses following self-limited acute hepatitis B. J. Hepatol. **23**(Suppl. 1)**:**73. (Abstract.)
28. **Pouw-Kraan, T., I. Rensink, and L. Aarden.** 1992. Interleukin (IL)-4 production by human T cells: differential regulation of IL-4 vs. IL-2 production. Eur. J. Immunol. **22:**1237–1241.
29. **Quill, H., and R. H. Schwartz.** 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. J. Immunol. **138:**3704–3712.
30. **Raimondo, G., R. Schneider, M. Stemler, V. Smedile, G. Rodino, and H. Will.** 1990. A new hepatitis B virus variant in a chronic carrier with multiple episodes of viral reactivation and acute hepatitis. Virology **179:**64–68.
31. **Rammensee, H.-G., R. Kroschewski, and B. Frangoulis.** 1989. Clonal anergy induced in mature Vβ6+ T lymphocytes on immunizing MLs-1b mice with Mls-1a expressing cells. Nature (London) **339:**541–544.
32. **Ramsdell, F., T. Lantz, and B. J. Fowlkes.** 1989. A nondeletional mechanism of thymic self tolerance. Science **246:**1038–1041.
33. **Rocha, B., A. Grandien, and A. A. Freitas.** 1995. Anergy and exhaustion are independent mechanisms of peripheral T cell tolerance. J. Exp. Med. **181:**993–1003.
34. **Schwartz, R. H.** 1990. A cell culture model for T lymphocyte clonal anergy. Science **248:**1349–1356.
35. **Sette, A., J. Alexander, J. Ruppert, K. Snoke, A. Franco, G. Ishioka, and H. M. Grey.** 1994. Antigen analogs/MHC complexes as specific T cell receptor antagonists. Annu. Rev. Immunol. **12:**413–431.
36. **Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen.** 1993. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. Nature (London) **363:**156–159.
37. **Sylvan, S. P.** 1991. Cellular immune response to hepatitis B virus antigens in man. Liver **11:**1–23.
38. **Thursz, M. R., D. Kwiatkowski, C. E. M. Allsopp, B. M. Greenwood, H. C. Thomas, and A. V. S. Hill.** 1995. Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. N. Engl. J. Med. **332:**1065–1069.
39. **Tsai, S.-L., P. J. Chen, M. Y. Lai, P. M. Yang, J.-L. Sung, J.-H. Huang, L. H. Hwang, T.-H. Chang, and D. S. Chen.** 1992. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. J. Clin. Invest. **89:**87–96.
40. **Van der Meide, P. H., M. Dubbeld, and H. Schellekens.** 1985. Monoclonal antibodies to human interferon-gamma and their use in a sensitive solid phase ELISA. J. Immunol. Methods **79:**293–305.
41. **Vignali, D. A. A., R. G. Urban, R. M. Chicz, and J. L. Strominger.** 1993. Minute quantities of a single immunodominant foreign epitope are presented as large nested sets by major histocompatibility complex class II molecules. Eur. J. Immunol. **23:**1602–1607.
42. **Webb, S., C. Morris, and J. Sprent.** 1990. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. Cell **63:**1249–1256.
43. **Williams, M. E., A. H. Lichtman, and A. K. Abbas.** 1990. Anti-CD3 antibody induces unresponsiveness to IL-2 in TH1 clones but not in TH2 clones. J. Immunol. **144:**1208–1214.
44. **Yamamoto, H., M. Hirayama, C. Genyea, and J. Kaplan.** 1994. TGF-β mediates natural suppressor activity of IL-2-activated lymphocytes. J. Immunol. **152:**3842–3847.
45. **Yang, S. Y., E. Milford, U. Hämmerling, and B. Dupont.** 1989. Description of the reference panel of B-lymphoblastoid cell lines for factors of the HLA system: the B-cell line panel designed for the 10th International Histocompatibility Workshop, p. 11–19. In B. Dupont (ed.), Immunobiology of HLA, vol. I. Histocompatibility testing 1987. Springer Verlag, New York.