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Reduced Hepatitis B Virus (HBV)-Specific CD4$^+$ T-Cell Responses in Human Immunodeficiency Virus Type 1-HBV-Coinfected Individuals Receiving HBV-Active Antiretroviral Therapy

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Functional hepatitis B virus (HBV)-specific T cells are significantly diminished in individuals chronically infected with HBV compared to individuals with self-limiting HBV infection or those on anti-HBV therapy. In individuals infected with human immunodeficiency virus type 1 (HIV-1), coinfection with HBV is associated with an increased risk of worsening liver function following antiviral therapy and of more rapid HBV disease progression. Total HBV-specific T-cell responses in subjects with diverse genetic backgrounds were characterized by using a library of 15-mer peptides overlapping by 11 amino acids and spanning all HBV proteins. The magnitude and breadth of CD4$^+$ and CD8$^+$ T-cell responses to HBV in peripheral blood were examined by flow cytometry to detect gamma interferon production following stimulation with HBV peptide pools. Chronic HBV carriers ($n = 34$) were studied, including individuals never treated for HBV infection ($n = 7$), HBV-infected individuals receiving anti-HBV therapy ($n = 13$), and HIV-1–HBV-coinfected individuals receiving anti-HBV therapy ($n = 14$). CD4$^+$ and CD8$^+$ HBV-specific T-cell responses were more frequently detected and the CD8$^+$ T-cell responses were of greater magnitude and breadth in subjects on anti-HBV treatment than in untreated chronic HBV carriers. There was a significant inverse correlation between detection of a HBV-specific T-cell response and HBV viral load. HBV-specific CD4$^+$ and CD8$^+$ T-cell responses were significantly (fivefold) reduced compared with HIV-specific responses. Although, the frequency and breadth of HBV-specific CD8$^+$ T-cell responses were comparable in the monoinfected and HIV-1–HBV-coinfected groups, HBV-specific CD4$^+$ T-cell responses were significantly reduced in HIV-1–HBV-coinfected individuals. Therefore, HIV-1 infection has a significant and specific effect on HBV-specific T-cell immunity.

There are 350 million individuals chronically infected with hepatitis B virus (HBV) worldwide (36, 42, 59). Each year 1 million to 1.5 million carriers die from HBV-related liver disease and liver cancer such as hepatocellular carcinoma (36, 48). In the United States, Europe, and Australia, approximately 6 to 7% of individuals infected with human immunodeficiency virus type 1 (HIV-1) are also coinfected with HBV (23, 41). Liver disease is now a major comorbidity in HIV-1-infected individuals (11, 66). Although hepatitis C virus (HCV)–HIV-1 coinfection is more common than HBV infection, liver failure occurs more frequently with persistent HBV infection (60, 63). Coinfection of HBV with HIV-1 leads to elevated HBV DNA levels, a lower rate of seroconversion to HBeAg, and lower alanine aminotransferase (ALT) levels than those in HBV monoinfection (21, 31). Spontaneous flares and HBeAg seroconversion occur in 5% of HBV-monoinfected individuals; however, in the setting of HIV-1–HBV coinfection, spontaneous flares or seroconversion to HBeAg is rare (18). In individuals infected with HIV-1 who subsequently acquire HBV infection, there is an increased risk of persistent chronic infection (32, 62). On the other hand, HBV does not appear to have a significant impact on AIDS progression or the rate of CD4$^+$ T-cell loss (41, 62).

Current treatments for HBV include nucleotide and nucleoside reverse transcriptase inhibitors (RTI) that also suppress HIV-1 replication. The most widely used anti-HBV nucleoside analog, lamivudine (2′-deoxy-3′-thiacytidine), inhibits both HIV-1 and HBV reverse transcriptases. An increase in HBV-specific CD4$^+$ and CD8$^+$ T-cell responses in both HBeAg-positive and -negative patients following the administration of lamivudine to HBV-infected individuals has been described (13, 14, 25, 44, 47). Long-term lamivudine therapy (more than 100 weeks) leads to HBeAg seroconversion in 27% of individuals but has also been associated with the frequent development of drug resistance (39, 40). Other nucleotide RTI, such as adefovir [9-(2-phosphonylmethoxyethyl)adenine] and tenofovir [9-(*R*)-(2-phosphonyl-methoxypropyl)adenine], and the nucleoside RTI entecavir (formerly BMS-200475, a carbocyclic 2′-deoxyguanosine analog) have also been shown to be effective against HBV (1, 6, 23). Tenofovir is often used in combination with lamivudine for effective treatment of HBV in HIV-1-infected individuals, as both agents are active against HIV-1 as well as HBV. Recent work demonstrates that tenofovir reduces the HBV viral load significantly in HIV-1–HBV-coinfected individuals infected with lamivudine-resistant HBV

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(53). As well as being effective in individuals with lamivudine-resistant HBV, tenofovir is also effective in individuals who fail alpha interferon (IFN-α) therapy (61). Initiation of antiretroviral therapy (ART) for HIV-1 can lead to severe acute hepatitis with or without clearance of HBV (16, 18). Better understanding of how the immune system responds to HBV following ART and how infection with HIV-1 alters this response is needed. Understanding the pathogenesis of HIV-1 and HBV interaction will allow for a rational approach to the management of coinfected individuals.

In persistent HBV infection there is a reduction in HBV-specific $CD4^+$ and $CD8^+$ T cells compared with those in individuals who successfully clear infection. HBeAg has been suggested to facilitate HBV persistence by depleting HBeAg- and HBcAg-specific Th1 $CD4^+$ T cells via fibroblast-associated (Fas)-mediated apoptosis (51). HBeAg crosses the placenta and therefore may establish tolerance to HBV in newborns, increasing the frequency of persistent HBV infection with vertical transmission. The imbalance of Th1/Th2 responses promotes suppression of HBeAg/HBcAg-specific $CD8^+$ T-cell responses and suppression of Th1 effector cells by production of anti-inflammatory cytokines such as interleukin-4 (IL-4) and IL-10 (30, 51, 52). A generalized $CD4^+$ T-cell hyporesponsiveness in individuals with chronic HBV infection has also been described, with lower responses to mitogens in untreated HBV-infected individuals than in HBV-negative controls. Following a reduction in HBV viral load after anti-HBV therapy, this hyporesponsiveness is reversed (13). This T-cell hyporesponsiveness may arise from decreased function in infected dendritic cells, which have reduced IFN-γ, tumor necrosis factor alpha, and IL-12 production and hence reduced stimulation of T-cell responses (5).

In individuals with persistent HBV infection, the HBV-specific T-cell response is significantly diminished when evaluated by proliferative responses to whole HBV antigens or defined epitopes in HLA-A2-positive chronic carriers (30, 45). In particular, in HLA-A2 HBeAg-positive chronic carriers, $CD8^+$ T cells that recognize an HBV core epitope (in region c18-27) are almost undetectable when measured by tetramers and have diminished ability to produce IFN-γ. HBV-specific $CD8^+$ T cells are also found in the liver, where they may cause an inflammatory response but are ineffective in clearing HBV infection (35, 45).

Examination of HBV-specific T cells by limiting-dilution assays (7, 56), proliferation assays (13, 29, 30, 35), tetramer staining (46, 58, 69), and cytokine production has been limited to responses against predicted HLA-A2 epitopes of core, surface, and polymerase proteins and/or whole-antigen stimulation (2, 46, 49, 64). In HIV-1 infection the dominant HIV-1-specific T-cell response is often to non-HLA-A2-associated peptides as well as to accessory proteins. Therefore, early studies using only HLA-A2 peptides or HIV-1 structural or envelope proteins did not accurately reflect the breadth and intensity of HIV-1-specific T-cell immunity (10). We therefore developed an overlapping peptide library which allows for detection of all HBV-specific $CD4^+$ and $CD8^+$ T-cell responses to all gene products to examine the effects of HIV-1 coinfection on the breadth and magnitude of HBV-specific immune responses.

MATERIALS AND METHODS

Patient population. Patients with chronic HBV (defined as having detectable HBsAg on two occasions more than 6 months apart) were recruited from the Royal Melbourne and Alfred Hospitals, Victoria, Australia. Participation was approved by the relevant hospital ethics committee, and signed consent was obtained. Eight uninfected healthy control subjects were also studied for comparison. Patients ($n = 34$) were classified into three groups: (i) individuals never treated with anti-HBV therapy ($n = 7$); (ii) HIV-1-uninfected, HBV-infected individuals currently or previously treated with one or more anti-HBV agents ($n = 13$); and (iii) HIV-1-infected, HBV-infected individuals currently or previously treated with one or more anti-HBV agents ($n = 14$).

Peptides. To examine total HBV-specific T-cell responses, an overlapping peptide library was made by using the consensus sequence for genotype A HBV (68). Genotype A was selected because this is the most common genotype in HIV-1–HBV-coinfected individuals in Australia (23). A total of 394 peptides consisting of 15 amino acids (aa) overlapping by 11 amino acids were designed across the full genome (Mimotopes, Clayton, Australia). Fifteen-mer peptides were chosen because these can be presented to and recognized by both $CD4^+$ and $CD8^+$ T cells (8, 28, 43). A total of 149 extra peptides were synthesized to cover regions of significant variation between genotype A and the three other main genotypes (B, C, and D) (12, 55) to facilitate the study of HBV-specific immunity in individuals infected with non-genotype A HBV, resulting in a total of 543 peptides. Ideally, a complete peptide library should be made for each genotype; however, given that this was impractical, the following approach was used for peptide design: when only one or two amino acids differed from genotype A, a single 15-mer peptide was synthesized with the variable region usually in the center of the peptide, while in regions of greater variability, a limited set of overlapping peptides was made. This allowed for responses to genotypes B to D to be more accurately measured. Each peptide was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, Mo.) to a final concentration of 100 or 50 $\mu g\ \mu l^{-1}$ (43). The 15-mer peptides were combined to create eight HBV peptide pools, according to the relevant protein, with each peptide represented at equal concentrations. The peptide pools therefore included precore/core (PC), X protein (X), three surface antigen pools (S1.1, S1.2, and S1.3), and three polymerase pools (Pol1, Pol2, and Pol3). The polymerase and surface antigen peptides were subdivided into three pools each so that the final DMSO concentration was lower than 1% of the total stimulation volume to reduce any toxicity of DMSO. Peptide pool S1.1 covered aa 1 to 131 of the genotype A pre-S1 protein and also the extra genotype B to D peptides corresponding to this same region, S1.2 covered aa 121 to 251, and S1.3 covered aa 241 to 400. Peptide pool Pol1 covered aa 1 to 256, Pol2 covered aa 246 to 496, and Pol3 covered aa 486 to 845 of the polymerase. The HBV peptide pools were made with 50 to 100 peptides in each pool, without significant inhibition of stimulation as previously described (43). The final peptide concentration used in stimulating peripheral blood mononuclear cells or whole blood was 1 $\mu g\ ml^{-1}$ for each peptide. Similar peptide pools were also made for HIV-1 peptides (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health), resulting in six peptide pools: for HIV-1 polymerase (HIV Pol1 and HIV Pol2), Gag (HIV GAG), and envelope (HIV ENV) and HIV-1 accessory proteins Rev, Tat, and Vpu (HIV RTV) and Vif, Vpr, and Nef (HIV VVN).

Measurement of antigen-specific T-cell responses by ICS. Intracellular cytokine staining (ICS) was performed as previously described (8, 9, 34, 43) with some minor modifications. Briefly, whole blood was collected in sodium heparin tubes and prepared for peptide stimulation either on the day of collection or after keeping blood at room temperature overnight. Round-bottomed 96-well plates were prepared with costimulatory molecules CD28 and CD49d monoclonal antibody together with peptides. DMSO with the same costimulatory molecules was used as a negative control, and pokeweed mitogen (Sigma) and staphylococcal enterotoxin B (Sigma) were used as positive controls. Each stimulation was performed in duplicate wells with 200 μl of whole blood. The final concentrations were 1 $\mu g\ ml^{-1}$ for each costimulatory molecule, 1 $\mu g\ ml^{-1}$ for each peptide in each pool, and 5 $\mu g\ ml^{-1}$ for each of the positive controls. The plates were incubated at 37°C for 2 h before brefeldin A (Sigma) was added to a final concentration of 10 $\mu g\ ml^{-1}$ and incubated for a further 5 h. The plates either were kept at 4°C overnight or were surface stained immediately with a combination of fluorescently labeled CD3-fluorescein isothiocyanate, CD4-phycoerythrin, and CD8-peridinin chlorophyll-a protein antibodies (BD Biosciences, San Jose, Calif.). Plates were incubated for 30 min in the dark at room temperature, and the contents were then transferred to separate fluorescence-activated cell sorter (FACS) tubes containing FACS lysing solution (BD Biosciences). The tubes were incubated for 10 min at room temperature and frozen at −80°C, or

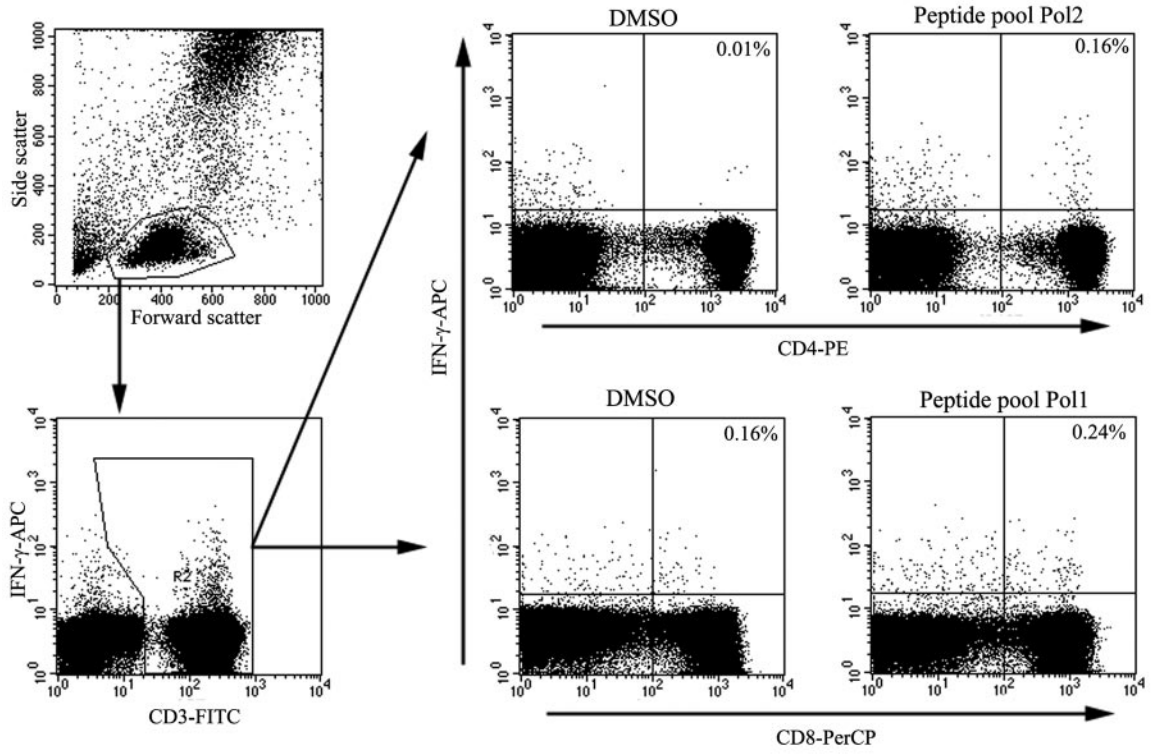

FIG. 1. Measurement of HBV-specific $CD4^+$ and $CD8^+$ T-cell responses by flow cytometry. Cells were gated initially on small lymphocytes (top left) and then on $CD3^+$ cells. The percentages of $CD4^+$ and $CD8^+$ T cells that produced IFN-γ were then examined. The response to DMSO was measured for each individual and subtracted from the responses found against the peptide pools. Therefore, the resulting $CD4^+$ and $CD8^+$ T-cell responses to peptide pools Pol2 and Pol1 are 0.15 and 0.08%, respectively. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll-a protein.

in some cases ICS was continued without freezing. Keeping plates overnight at 4°C prior to staining and freezing in FACS lysing solution has been previously shown to have no deleterious effects on detection of T-cell responses (54). The frozen tubes were thawed in a 37°C water bath and then washed with phosphate-buffered saline. The cells were resuspended in FACS permeabilizing solution (BD Biosciences) and incubated in the dark at room temperature for 10 min. The cells were washed once again and then stained intracellularly with anti-IFN-γ–allophycocyanin for 1 h in the dark at room temperature. The cells were washed and resuspended in 5% paraformaldehyde.

Flow cytometry analysis. All data was acquired on a FACSCalibur or FACScan instrument within 8 h of staining and were analyzed by using CellQuest (BD Biosciences). Cells were gated initially on lymphocytes as determined by forward and side scatter, and, where possible, data from at least 100,000 small lymphocytes were collected. Activated T cells down regulate surface expression of CD3, CD4, and CD8 due to internalization of these molecules (8, 67). The reduced expression of CD3, CD4, and CD8 can be accounted for by the use of intracellular staining (8, 43). Alternatively, an inverted P gate adapted from that described by Betts et al. (8) was used to include T cells with both high and low CD3 expression. The inverted P gate was set by using blood stimulated with pokeweed mitogen and staphylococcal enterotoxin B and was used for all subsequent analyses for the particular individual (Fig. 1). For most samples, 10,000 to 20,000 $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells were collected for analysis. Antigen-specific cells are expressed as the percentage of IFN-γ-producing $CD8^+$ or $CD4^+$ T cells.

Quantification of HBV viral load. HBV viral load was determined with the HBV Digene Hybrid Capture II microplate assay (Roche, Branchburg, N.J.) in accordance with the manufacturer's instructions. For the Digene assay, the lower detection limit was 0.5 pg (equivalent to 1.42×10^5 copies/ml). If HBV viral load was undetectable by Digene assay, the Versant HBV DNA 3.0 assay (Bayer HealthCare-Diagnostics, Tarrytown, N.Y.) was used in accordance with the manufacturer's instructions. The limit of detection for this assay was 2,000 copies/ml.

Sequencing of precore/core promoter mutations. HBV DNA was extracted from 200 μl of serum, using the QIAamp DNA Minikit (QIAGEN, Chatsworth, Calif.) according to the manufacturer's instructions. The catalytic domain of the reverse transcriptase/polymerase and the basal core promoter/precore regions were sequenced as previously described (4, 22). Briefly, the polymerase was amplified by using internal primers Seq2 (5′-TTG GCC AAA ATT CGC AGT C-3′) (nucleotide [nt] 2122 to 2138) and OS2 (5′-TCT CTG ACA TAC TTT CCA AT-3′) (nt 2798 to 2817). The HBV basal core promoter/precore regions were amplified by using the same PCR and sequencing conditions but with the sense primer 3026 (5′-GCT GAC GCA ACC CCC ACT ACT-3′) (nt 3007 to 3027) and the antisense primer PC2 (5′-GGC AAA AAC GAG AGT AAC TC-3′) (nt 540 to 559). A heminested second-round PCR used the sense primer PC5 (5′-TTG CAT GGA GAC CAC CGT GA-3′) (nt 204 to 223). The specific amplified products were purified with PCR purification columns (MO BIO Laboratories Inc., La Jolla, Calif.) and directly sequenced by using a Big Dye Terminator cycle sequencing ready reaction kit (Perkin-Elmer Cetus, Norwalk, Conn.). DNA sequences were compared by using MacVector (Oxford Scientific).

Statistical analysis. Statistical analysis was performed with SPSS for Windows version 11.5.0 (LEAD Technologies, Inc.). Comparisons of viral loads and ALT levels between two groups were done with the Mann-Whitney U test. Differences in magnitudes of responses between two groups were examined with Student's *t* test. Nominal values were compared by using the Fisher exact test if the sample contained a subpopulation that was less than 4 or by using the χ^2 test if all subpopulation sizes were not less than 4. Correlations were examined by using Spearman's rho test for nonparametric values.

RESULTS

Patient characteristics and viral load. Demographic and clinical details of the individuals studied ($n = 34$) are summarized in Tables 1 and 2. All individuals recruited were HBsAg

TABLE 1. Clinical characteristics of individuals studied by using the ICS technique

Group (*n*) and patient	ALT (U/liter)[a]	HBV viral load (copies/ml)[b]	HBsAg	HBeAg/ HBeAb[c]	CD4 count, cells/μl (%)	HIV viral load (copies/ml)[d]	Duration of HBV therapy (mo)	Duration of ART (mo)[f]
Therapy-naive chronic HBV carriers (7)								
TN1	118	6.93×10^8	+	+/−	NA[h]	NA	NA	NA
TN2	41	3.81×10^4	+	−/+	NA	NA	NA	NA
TN3	30	$<2.0 \times 10^3$	+	UA[g]/UA	NA	NA	NA	NA
TN4	56	6.47×10^8	+	+/−	NA	NA	NA	NA
TN5	19	3.67×10^8	+	+/−	NA	NA	NA	NA
TN6	20	2.97×10^4	+	−/+	NA	NA	NA	NA
TN7	78	$<2.0 \times 10^3$	+	−/+	NA	NA	NA	NA
HBV-monoinfected individuals on therapy (13)								
TH1	132	5.60×10^5	+	−/+	NA	NA	2.0	NA
TH2	44	$<2.0 \times 10^3$	+	−/−	NA	NA	5.5[e]	NA
TH3	14	$<2.0 \times 10^3$	+	−/−	NA	NA	7.5	NA
TH4	16	$<2.0 \times 10^3$	+	−/+	NA	NA	8.0	NA
TH5	35	UA	+	−/+	NA	NA	8.0	NA
TH6	17	1.13×10^4	+	−/+	NA	NA	10.0[e]	NA
TH7	31	1.15×10^8	+	+/−	NA	NA	17.5	NA
TH8	31	4.66×10^8	+	+/−	NA	NA	19.0	NA
TH9	25	UA	+	−/+	NA	NA	23.0	NA
TH10	121	UA	+	UA/UA	NA	NA	32.5	NA
TH11	47	$<2.0 \times 10^3$	+	UA/UA	NA	NA	37.0	NA
TH12	34	$<2.0 \times 10^3$	+	UA/UA	NA	NA	46.0	NA
TH13	19	$<2.0 \times 10^3$	+	−/+	NA	NA	46.5	NA
HIV-HBV-coinfected individuals on therapy (14)								
TC1	114	2.52×10^5	+	−/−	76 (6)	6.99×10^3	1.0	1.0
TC2	136	$<2.0 \times 10^3$	+	+/−	490 (13)	6.99×10^3	2.0	13.0
TC3	25	$<2.0 \times 10^3$	+	+/−	200 (22.6)	<50	2.5	1.5
TC4	73	$<2.0 \times 10^3$	+	+/−	528 (33)	<50	4.5	3.0
TC5	14	$<2.0 \times 10^3$	+	−/+	1,155 (35)	<50	5.0	29.0
TC6	70	$<2.0 \times 10^3$	+	UA/UA	92 (14)	<50	6.0[e]	50.5
TC7	18	$<2.0 \times 10^3$	+	−/+	292 (17)	<50	6.0	6.0
TC8	29	2.69×10^6	+	+/−	932 (34)	<50	10.0	10.0
TC9	45	1.96×10^6	+	+/−	197 (8)	<50	23.5	23.0
TC10	41	$<2.0 \times 10^3$	+	UA/UA	593 (19)	400	37.5	36.5
TC11	59	UA	+	UA/UA	308 (18.5)	1.63×10^4	47.0	47.0
TC12	64	$<2.0 \times 10^3$	+	−/+	418 (17)	250	49.0	42.0
TC13	28	$<2.0 \times 10^3$	+	+/−	390 (38)	<400	55.5	55.5
TC14	33	UA	+	+/−	310 (22.4)	400	60.0	60.0

[a] The normal range is 0 to 40 U/liter.
[b] Measured by HBV Digene Hybrid Capture II microplate assay and Bayer Versant HBV DNA 3.0 assay.
[c] HBeAg and HBeAb status measured within 1 year before sample analysis.
[d] Measured by Roche Amplicor kit reverse transcription-PCR.
[e] Currently off therapy, but previous therapy duration is recorded.
[f] ART is defined as therapy with at least three anti-HIV agents.
[g] UA, unavailable.
[h] NA, not applicable.

positive for at least 6 months prior to analysis. Overall there was no statistical difference between the two groups as defined by the HBeAg and HBeAb status within the preceding 12 months of sample collection. There was a trend towards an increase in HBeAg persistence in HIV-1–HBV-coinfected individuals ($P = 0.05$; odds ratio [OR] = 0.198; confidence interval [CI] = 0.037 to 1.045). This trend has previously been documented in studies examining the effects of HIV-1 on the natural history of HBV, which suggested that coinfected individuals have a lower rate of loss of HBeAg (23, 31). ALT levels at the time of study were not significantly different between the two groups.

There was no significant difference in the HBV viral load between HBV-monoinfected and HIV-1–HBV-coinfected patients receiving anti-HBV therapy. Sequencing for precore/core promoter mutations for individuals on treatment was limited to those with detectable HBV DNA by nested PCR. Where sequence data were obtained ($n = 13$), 61.8% of the individuals were positive for the A1762T and G1754A and/or for the G1896A mutation, each of which is associated with reduced or lack of HBeAg synthesis. Infection with a precore mutant was significantly reduced in HIV-1–HBV-coinfected individuals ($P = 0.032$; OR = 0.036; CI = 0.002 to 0.741).

The extent of immune suppression in the HIV-1–HBV-coinfected individuals varied, and the mean CD4 count was 423 ± 304 (range of 76 to 1,155) cells μl^{-1}. A total of 78.6% of the

TABLE 2. Summary of clinical data for patients examined by using the ICS technique

Parameter	Value for group	
	HBV monoinfected, on therapy	HIV-HBV coinfected on therapy
No. of patients	13	14
No. positive/total (% positive)		
HBsAg	13/13 (100)	14/14 (100)
Current HBeAg	2/6 (33.3)	7/12 (58.3)
Ever HBeAg	5/13 (38.5)	10/13 (76.9)
Current HBeAb	3/6 (50.0)	3/12 (25.0)
Ever HBeAb	9/13 (69.2)	4/13 (30.8)
Precore mutant[a]	7/8 (87.5)	1/5 (20.0)
HBV viral load (copies/ml)		
Mean ± SD	$5.82 \times 10^7 \pm 1.48 \times 10^8$	$4.10 \times 10^5 \pm 9.11 \times 10^5$
Range	2,000–4.66×10^8	2,000–2.69×10^6
No. with undetectable viral load/total (% with undetectable viral load)	6/10 (60.0)	9/12 (75.0)
ALT (U/liter)		
Mean ± SD	43.5 ± 38.3	53.5 ± 35.9
Range	14–132	14–136
Current or recent therapy duration (mo)		
Mean ± SD	20.2 ± 15.6	22.1 ± 22.6
Range	2–46.5	1–60
No. receiving treatment/total (% receiving treatment)		
Lamivudine	10/13 (76.9)	14/14 (100)
Adefovir	2/13 (15.4)	(0)
Entecavir	1/13 (7.7)	(0)
Tenofovir	(0)	8/14 (57.1)
CD4 count (cells/μl)		
Mean ± SD	NA[b]	423 ± 304
CD4 nadir (cells/μl)		
Mean ± SD	NA[b]	227 ± 142
HIV-1 viral load Mean (copies/ml ± SD)		
Mean ± SD	NA[b]	$2.29 \times 10^3 \pm 4.72 \times 10^3$

[a] Precore could be assessed only for individuals with detectable HBV-DNA by PCR, which included only a subset of individuals in the study.
[b] NA, not applicable.

HIV-1–HBV-coinfected individuals had an HIV-1 viral load equal to or below 400 copies ml^{-1}, with a mean HIV (plus or minus standard deviation) viral load of $2.29 \times 10^3 \pm 4.72 \times 10^3$ (range of 50 to 1.63×10^4) copies ml^{-1}. All HIV-1–HBV-coinfected patients were on ART, and the mean duration on ART was 27.0 ± 21.5 (range of 1 to 60) months. In all cases, ART included an anti-HBV active agent such as lamivudine ($n = 6$) or both lamivudine and tenofovir ($n = 8$).

There was no significant difference in duration of anti-HBV therapy between the monoinfected and HIV-1–HBV-coinfected individuals. For those individuals who were off therapy at sample time but had previously been on either IFN-α, adefovir, or lamivudine ($n = 3$), the duration of their last treatment period was included in the analysis. HBV-monoinfected individuals were treated with either lamivudine ($n = 9$), lamivudine and entecavir ($n = 1$), adefovir ($n = 2$), or IFN-α ($n = 1$).

$CD4^+$ and $CD8^+$ T-cell responses as measured by intracellular cytokine staining. HBV-specific T-cell responses were analyzed with fresh blood by using the ICS assay. A response was calculated as the percentage of $CD4^+$ or $CD8^+$ T cells that produced IFN-γ above background levels. Background IFN-γ production was determined for each patient by stimulation of whole blood with DMSO and the same combination of costimulatory molecules. The background level of nonspecific T-cell responses to HBV peptides was determined with blood from HIV-1- and HBV-uninfected volunteers ($n = 8$). The overall means plus two standard deviations of all responses to HBV peptide pools and to HIV-1 peptide pools were both 0.03% IFN-γ-producing $CD4^+$ or $CD8^+$ T cells above background. A conservative cutoff level for positive responses of 0.05% was therefore used and is consistent with the published literature (8, 28).

IFN-γ production from $CD4^+$ T cells following stimulation with all HBV peptide pools was not observed in any of the seven untreated HBV-monoinfected individuals (data not shown). In contrast, there was a significantly higher number of $CD4^+$ T-cell responses in HBV-monoinfected individuals receiving anti-HBV therapy, with 6 of the 13 having detectable HBV-specific $CD4^+$ T cells ($P = 0.046$; OR = 0.467; CI =

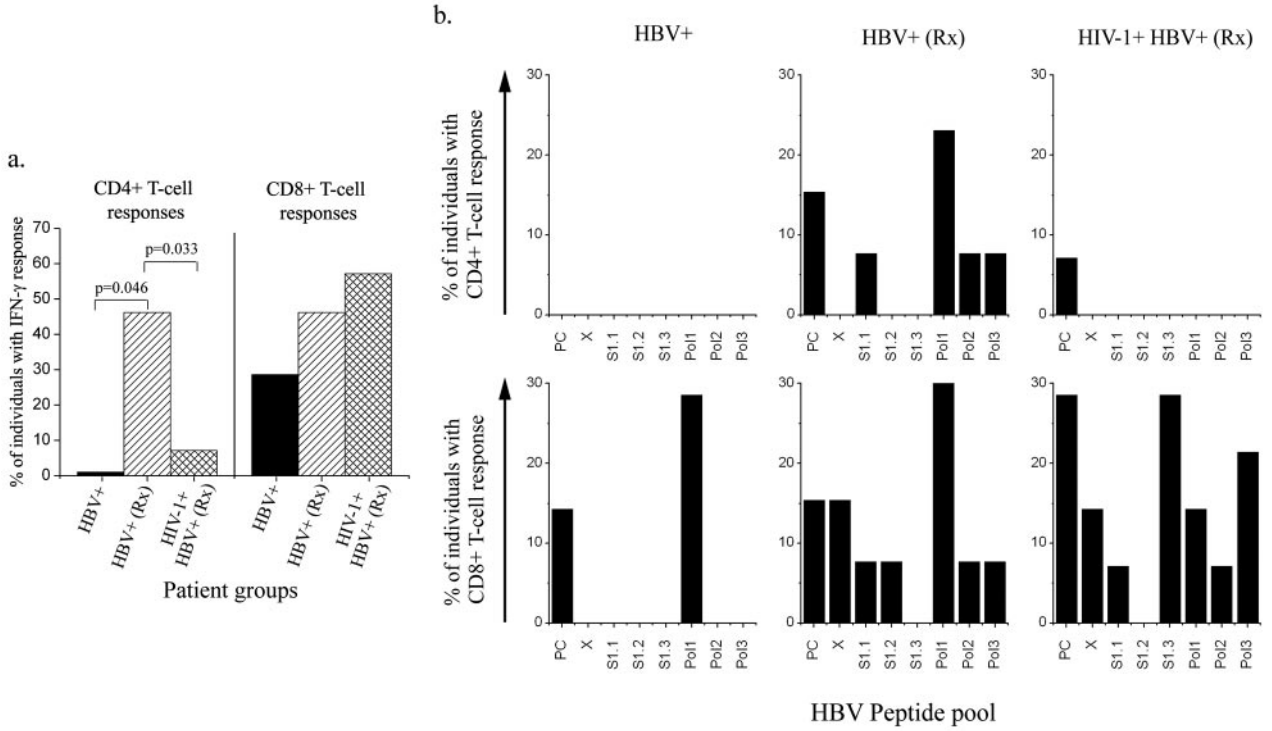

FIG. 2. Frequencies of HBV-specific $CD4^+$ or $CD8^+$ T-cell responses in each clinical group. (Rx), individuals receiving anti-HBV therapy. (a) Percentage of individuals in each group with $CD4^+$ (left panel) or $CD8^+$ (right panel) T-cell responses to at least one HBV peptide pool. Significantly more HBV-monoinfected individuals receiving anti-HBV therapy had HBV-specific $CD4^+$ T-cell responses then either those not receiving anti-HBV therapy ($P = 0.046$) or the HIV-1–HBV-coinfected subjects ($P = 0.033$) (Fisher exact test). No significant difference in the frequency of HBV-specific $CD8^+$ T-cell responses was observed between the three groups. (b) Percentage of individuals in each group who had a $CD4^+$ (top panels) or $CD8^+$ (bottom panels) T-cell response to each of the peptide pools. The breadth and frequency of $CD8^+$ T-cell responses were similar in the HBV-monoinfected and HIV-1–HBV-coinfected subjects receiving anti-HBV therapy despite the difference in $CD4^+$ T-cell responses.

0.272 to 0.802) (Fig. 2a). These HBV-specific $CD4^+$ T cells responded to several of the HBV peptide pools, with responses to peptide pool Pol1 most frequently detected (Fig. 2b). IFN-γ production in response to one peptide pool was present in four subjects, and two individuals responded to more than one pool (TH4 to the S1.1 and Pol1 pools and TH10 to the Pol1 and Pol2 pools) (Fig. 3a). These data confirm previous reports (assessing proliferation to HBV antigen and peptide stimulation) that anti-HBV treatment can lead to the reactivation of HBV-specific $CD4^+$ T-cell responses (13). Although the HIV-1–HBV-coinfected individuals were also on anti-HBV therapy, only 1 of the 14 subjects had a detectable $CD4^+$ T-cell response to one HBV peptide pool (Fig. 4a). Compared with HBV-monoinfected individuals receiving anti-HBV treatment, there were significantly lower number of HIV-1–HBV-coinfected individuals with an HBV-specific $CD4^+$ T-cell response ($P = 0.033$; OR = 0.90; CI = 0.009 to 0.902) (Fig. 2a). In individuals with detectable IFN-γ production by $CD4^+$ T cells, the duration of anti-HBV therapy was 15.2 ± 14.6 months (mean ± SD; range, 2 to 37 months).

Overall, in six of the seven individuals in whom IFN-γ-producing HBV-specific $CD4^+$ T cells were identified, IFN-γ-producing $CD8^+$ T-cell responses to at least one HBV peptide pool were observed. HBV-specific $CD8^+$ T-cell responses were again rare in untreated individuals (two of seven), with responses to PC and Pol1 only (data not shown). In 6 of 13 HBV-monoinfected individuals receiving anti-HBV therapy, HBV-specific $CD8^+$ T cells were detected in response to at least one peptide pool, with 3 individuals demonstrating a positive response to more than one peptide pool, including S1.1 and Pol1 (TH4); X, S1.2, Pol1, and Pol2 (TH10); and X, Pol1, and Pol3 (TH11) (Fig. 3b). Again, Pol1 was the most immunogenic of the HBV peptide pools (Fig. 2b). In 8 of the 14 HIV-1–HBV-coinfected individuals receiving anti-HBV treatment, HBV-specific $CD8^+$ T cells were observed in response to stimulation with at least one peptide pool. Of these eight, $CD8^+$ T cells from one individual responded to two pools, those from one individual responded to three pools, and those from two individuals responded to four pools (Fig. 4b). The magnitude of the HBV-specific $CD8^+$ T-cell response for individual TC13 was significantly higher then that for the rest of the group, with a response of 2.72% to Pol3, which was more than 22-fold higher than the next-highest measured response against Pol3 in this study. No correlation was found between anti-HBV treatment duration and magnitude of $CD8^+$ T-cell responses. In individuals with a detectable HBV-specific $CD8^+$ T-cell response, the duration of anti-HBV therapy was 16.7 ± 19.0 months (mean ± standard deviation) (range, 1 to 55.5 months).

For three individuals, we were able to obtain samples from more than one time point up to 7 months following the initiation of anti-HBV therapy. At each time point, ICS was used

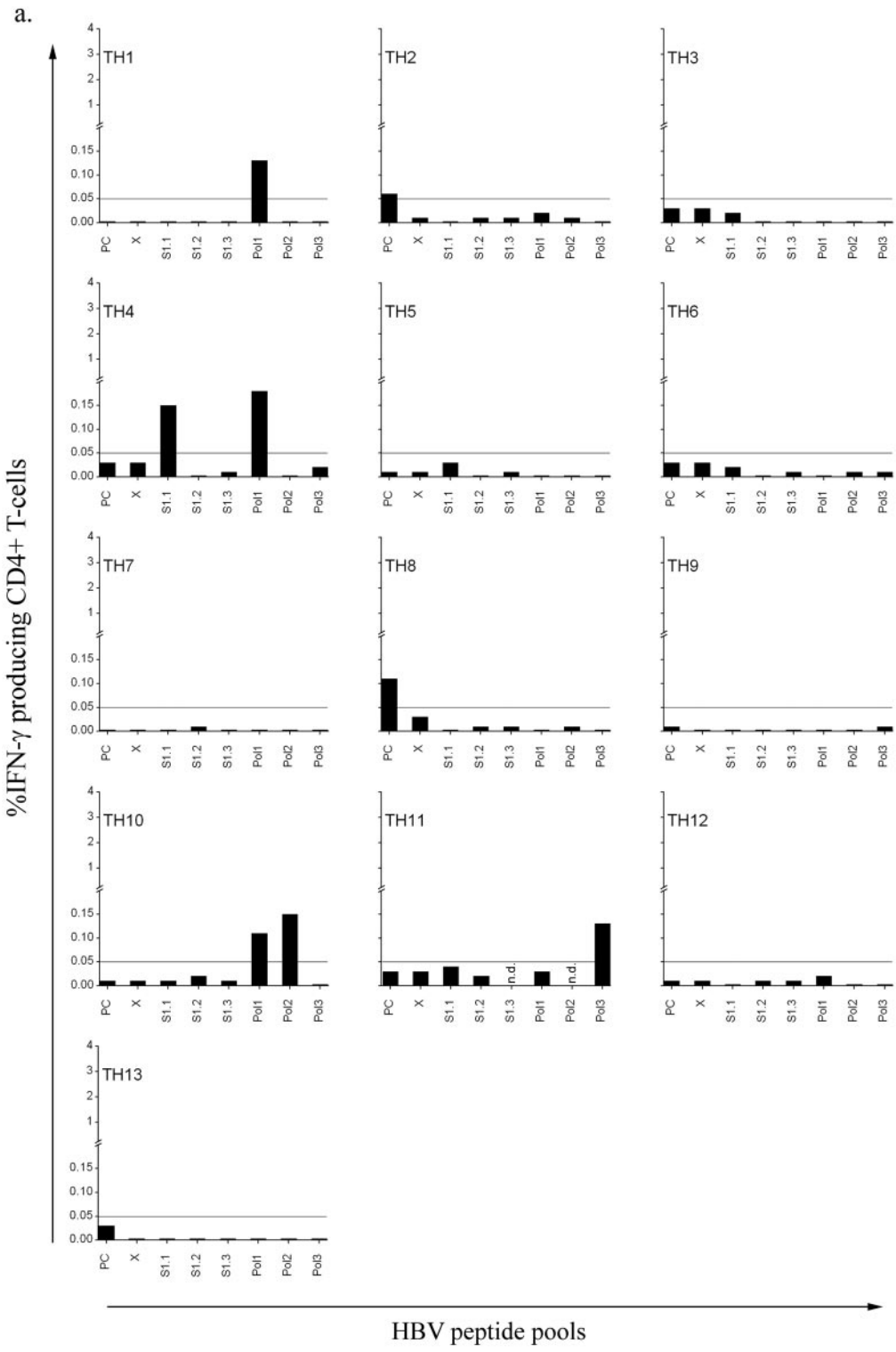

FIG. 3. Intracellular IFN-γ production as a measure of HBV-specific T-cell responses in HBV-monoinfected treated individuals (n = 13). $CD4^+$ T-cell responses (a) and $CD8^+$ T-cell responses (b) to stimulation with an HBV peptide pool of 1 μg ml^{-1} for each peptide are shown. A response was considered positive if more the 0.05% of $CD4^+$ or $CD8^+$ T cells produced IFN-γ. n.d., not done.

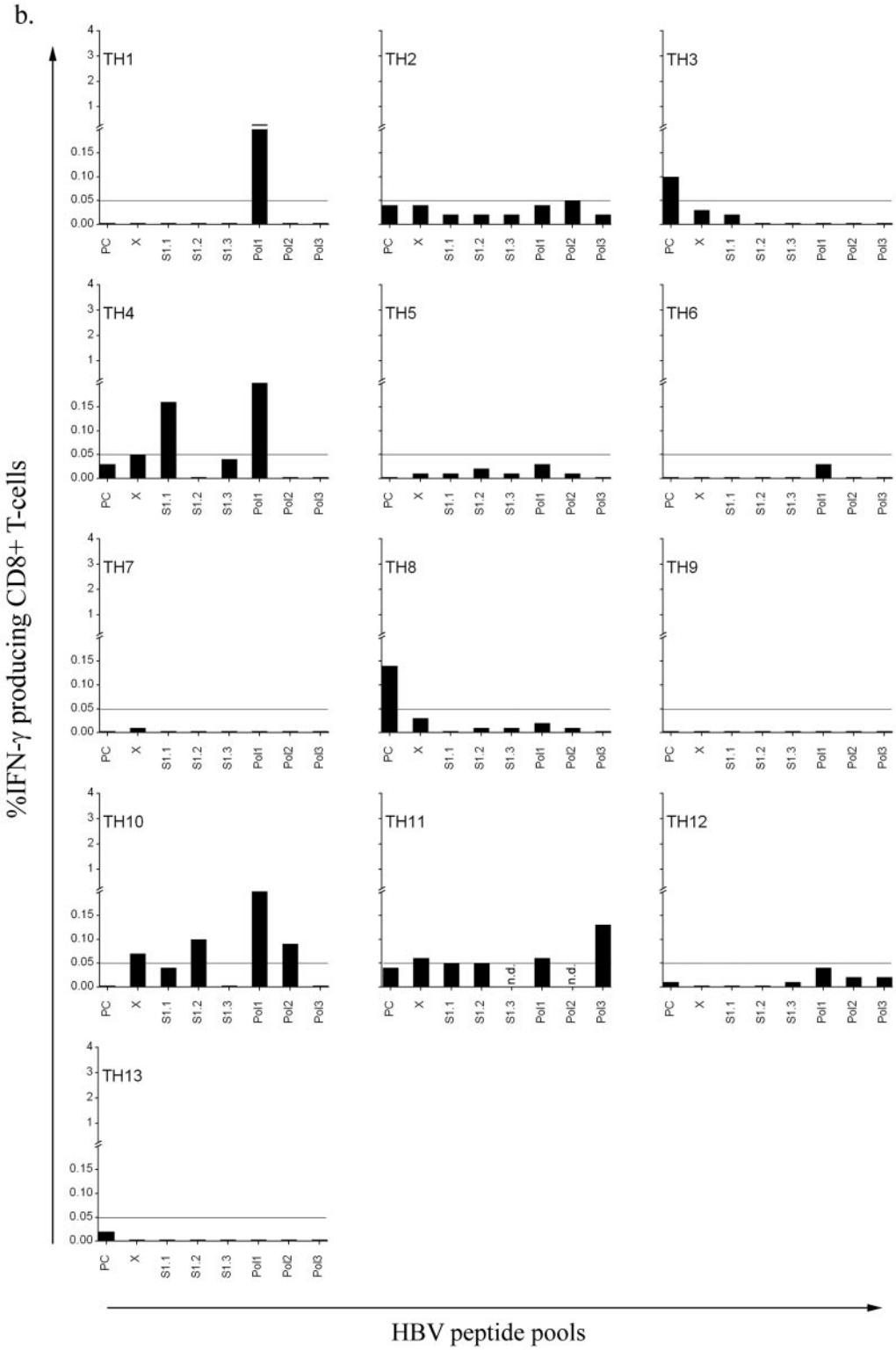

FIG. 3—*Continued*.

to quantify IFN-γ production following stimulation with HBV peptides. In two individuals, we found an initial increase in the number of HBV-specific IFN-γ-producing T cells following initiation of therapy; however, after 7 months on therapy, these responses were greatly diminished or in some cases disappeared (data not shown). During this period there was no seroconversion or clearance of HBV in any of the three indi-

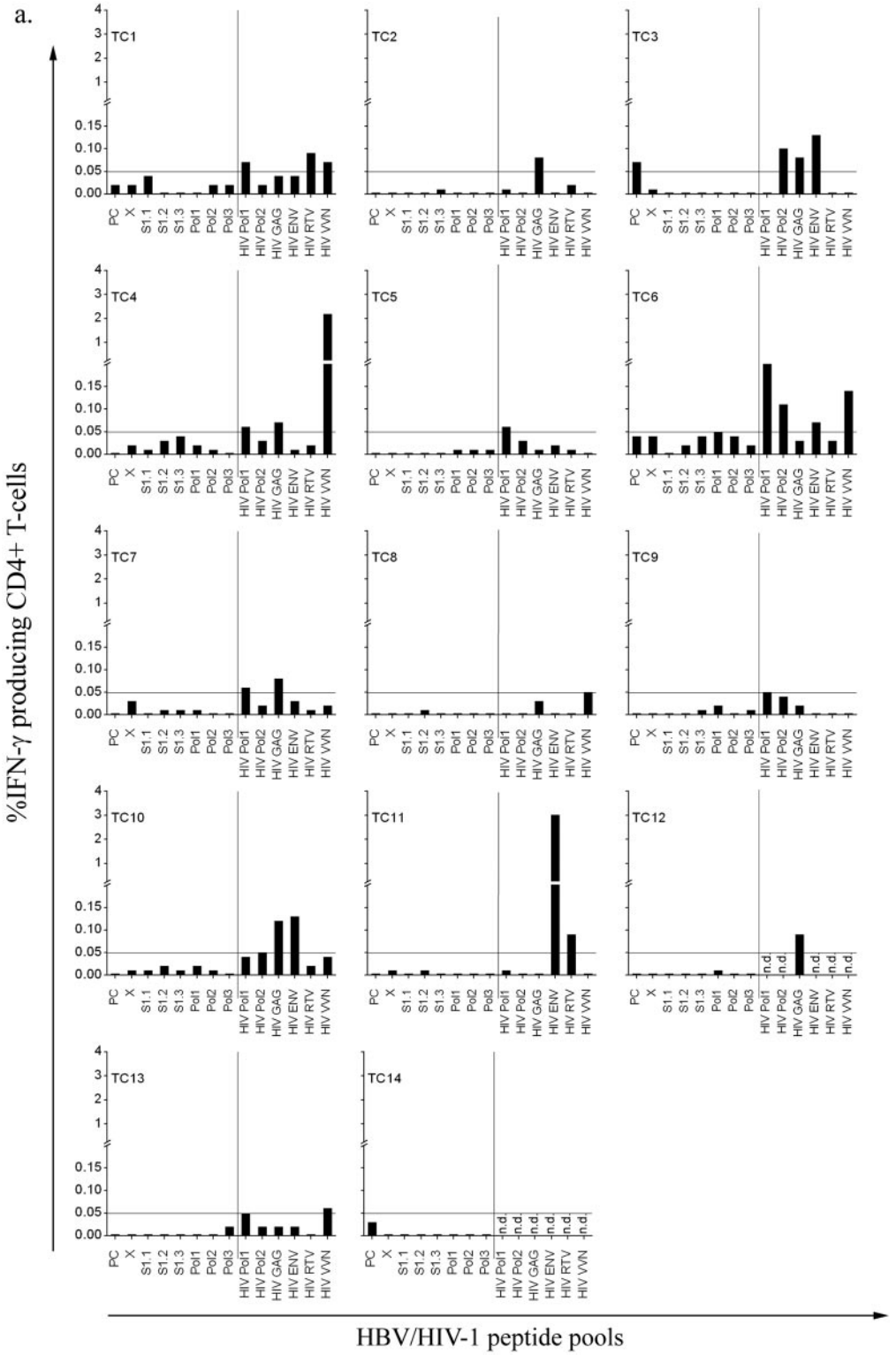

FIG. 4. Intracellular IFN-γ production as a measure of HBV-specific T-cell responses in HIV-1–HBV-coinfected treated individuals ($n = 14$). $CD4^+$ T-cell responses (a) and $CD8^+$ T-cell responses (b) to stimulation with an HBV peptide pool of 1 μg ml^{-1} for each peptide are shown to the left of the vertical bars, and those to stimulation with an HIV peptide pool of 1 μg ml^{-1} for each peptide are shown to the right of the vertical bars. A response was considered positive if more the 0.05% of $CD4^+$ or $CD8^+$ T cells produced IFN-γ. n.d., not done.

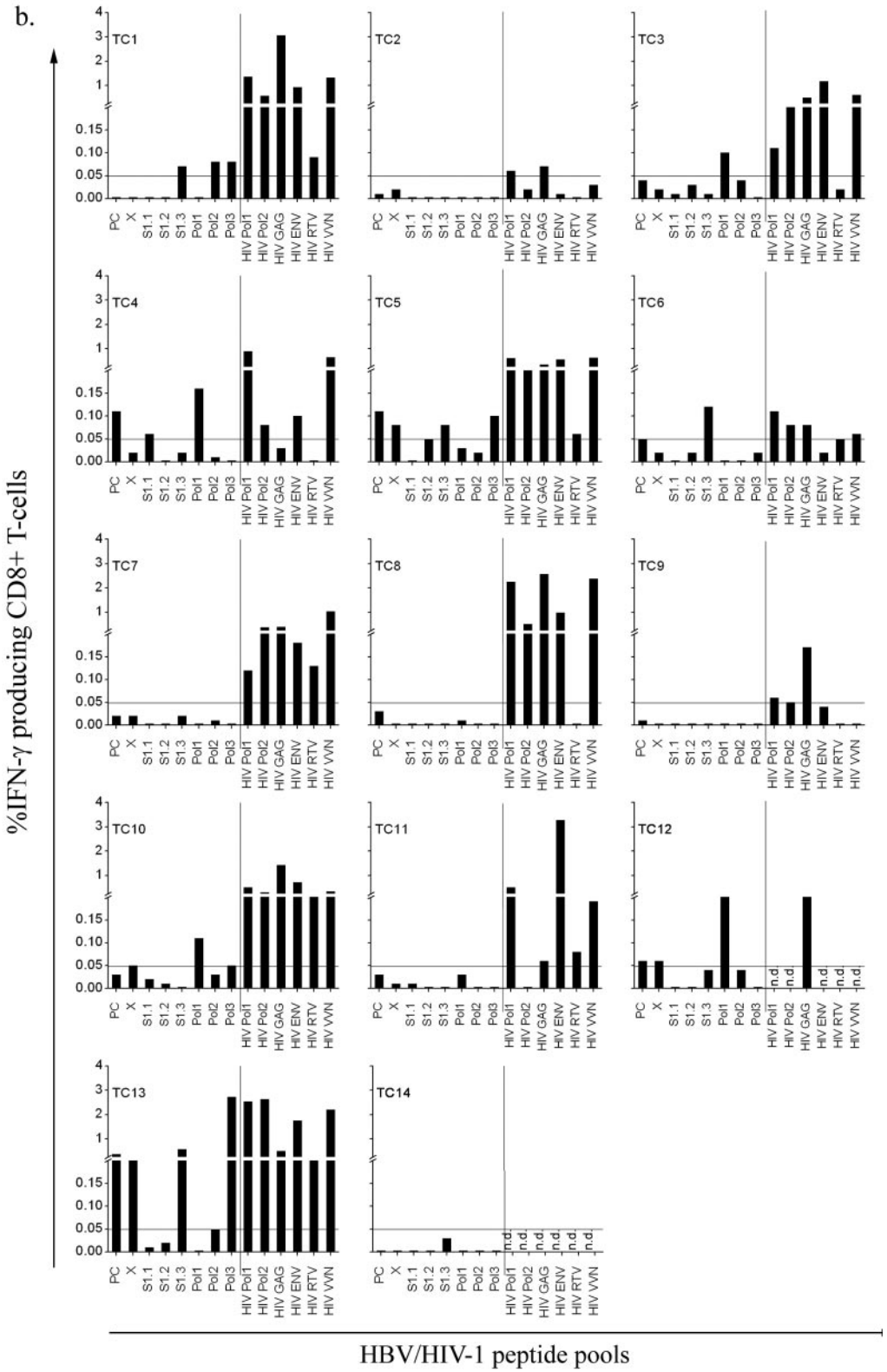

FIG. 4—*Continued*.

viduals. These limited studies raise the possibility that HBV-specific T-cell responses fluctuate or perhaps reduce over time.

General T-cell responses to mitogen. IFN-γ production following stimulation with pokeweed mitogen and staphylococcal enterotoxin B was used as positive controls (data not shown). IFN-γ production from $CD4^+$ and $CD8^+$ T cells was observed from all 34 individuals tested. General $CD4^+$ T-cell hyporesponsiveness in persistent HBV infection, as suggested in a previous study (13), was not observed; however, the magnitudes of mitogen-induced $CD8^+$ T-cell responses were significantly higher in HBV-monoinfected treated individuals (17.84% ± 8.03%) than in untreated individuals (9.75% ± 7.55%) ($P = 0.037$ by t test). Interestingly, this difference was not observed between the HIV-1–HBV-coinfected group (14.07% ± 7.97%) and the untreated group.

T-cell responses to HIV-1 peptide pools. IFN-γ production following stimulation with HIV-1 peptides in HIV-1–HBV-coinfected individuals was assessed to determine if there was a global reduction in T-cell responsiveness and to compare responses to two different but persistent viral infections (Fig. 4). All HIV-1–HBV-coinfected individuals tested had detectable $CD4^+$ and $CD8^+$ T-cell responses to at least one HIV-1 peptide pool. The magnitude of the HIV-1-specific $CD8^+$ T-cell responses was significantly higher than that of the HIV-1-specific $CD4^+$ T-cell responses ($P = 0.028$ by paired t test). The proportion of IFN-γ^+ $CD8^+$ T cells following stimulation with the HIV-1 peptide pools was significantly greater (fivefold) than that following stimulation with HBV peptide pools in this same coinfected group ($P = 0.016$ by paired t test). Only one HIV-1–HBV-coinfected individual had an HBV-specific $CD4^+$ T-cell response, so statistical analysis could not be performed. In summary, HBV-specific T-cell responses were significantly diminished compared to those against HIV-1 in HIV-1–HBV-coinfected individuals.

Association of HBV-specific responses, HBV viral load, and degree of immunosuppression. We next asked whether there was an association between the detection of HBV-specific T-cell responses and HBV viral load independent of therapy status and coinfection. Five individuals did not have an HBV viral load measured at sample time and so were not included in this analysis. HBV viral loads were compared between individuals in whom $CD4^+$ and/or $CD8^+$ HBV-specific T cells to at least one peptide pool were detected (responders; $n = 16$) and those in whom no response was detected (nonresponders; $n = 13$). There were significantly higher HBV viral loads in the nonresponders compared to the responders ($P = 0.035$ by Mann-Whitney U test), with responders having a larger portion of individuals with undetectable HBV viral loads (<2,000 copies/ml) ($P = 0.027$; OR = 0.144; CI = 0.027 to 0.774 [Fisher's exact test]). However, there was no significant correlation between the magnitude of either $CD4^+$ or $CD8^+$ T-cell responses and the HBV viral load (by Spearman's rho test). Therefore, effective suppression of HBV replication by anti-HBV therapy is a factor in recovery of HBV-specific T-cell responses. In the coinfected individuals, the duration of ART, the HIV-1 viral load, the CD4 count at sample time, and the CD4 nadir were not significantly different between responders ($n = 8$) and nonresponders ($n = 6$).

DISCUSSION

Using a novel HBV peptide library that covers the whole HBV genome and analysis of T-cell responses by measuring IFN-γ production, we demonstrated that HBV-specific T-cell responses were greater in both HBV-monoinfected and HIV-1–HBV-coinfected individuals on therapy than in chronic HBV carriers not on treatment. T-cell responses to HBV X and Pol1 peptides, which have not been previously reported, were frequently detected, suggesting that previous methods may have underestimated the breadth and magnitude of HBV-specific T-cell responses. Coinfection with HIV-1 was associated with a reduction of HBV-specific $CD4^+$ responses despite clearly detectable HIV-1-specific $CD4^+$ and $CD8^+$ T-cell responses. Interestingly, despite the low-level HBV-specific $CD4^+$ T-cell responses in HIV-1–HBV-coinfected individuals, HBV-specific $CD8^+$ T-cell responses were similar to those seen in HBV-monoinfected individuals.

Using overlapping peptide libraries covering all HBV proteins and the ICS technique, we were able to successfully detect HBV-specific $CD4^+$ and $CD8^+$ T-cell production of IFN-γ. The frequency and magnitude of responses to polymerase peptides were more readily observed than those of responses to precore/core-derived peptides. Previous studies of acute HBV infection of HLA-A2-positive individuals demonstrated maximal response to precore/core (45, 46). It is possible that treatment of chronic HBV infection may effect the hierarchy of peptides recognized, as described for chronic lymphocytic choriomeningitis virus infections (71), and, as shown previously, the initiation of therapy may not lead to the reconstitution of all HBV-specific T cells evenly (15). We also detected $CD8^+$ T-cell responses to X protein, which have not previously been documented. These results highlight the increased information obtained by using overlapping peptides that cover the whole genome to assess HBV-specific T-cell responses.

The detection of HBV-specific T-cell responses in untreated individuals was markedly reduced compared with that in treated individuals, either HBV monoinfected or HIV-1–HBV coinfected. Boni et al. have previously described the appearance of HBV-specific T-cell responses following lamivudine therapy (13–15). In these studies the T-cell responses were measured by $CD4^+$ T-cell proliferation and/or $CD8^+$ T-cell cytolytic activity. These responses were induced by core and e antigens or peptides (13, 14) and also by previously defined HLA-A2 epitopes in the envelope and polymerase proteins (15). In one study the precore/core-specific IFN-γ producing T cells were also examined by ICS. Both our study and these previous studies (13–15) draw the same conclusion that anti-HBV therapy is associated with the detection of HBV-specific T-cell responses. The proliferative and cytolytic responses following therapy were more frequent than the IFN-γ production responses we observed. This discrepancy in T-cell responsiveness opens the possibility that although HBV-specific T-cell activity increases following anti-HBV therapy, the response may not be fully functional or equivalent to that seen following successful clearance of acute HBV infection. This may explain why long-term clearance of virus following anti-HBV therapy is rarely observed (35).

One possible explanation for the appearance of HBV-spe-

cific T cells following antiviral therapy is the reduction in HBV viral load (13, 38). The reduction in HBV viral load and HBV antigen load may lead to a loss of HBV anergy (51). We found that individuals with either a $CD4^+$ or a $CD8^+$ HBV-specific T-cell response had a lower serum HBV viral loads than those with no responses. An alternative explanation is that HBV-specific T cells migrate from the liver to the periphery, as recently suggested by studies using HBV-specific tetramers (47). Although HBV-specific $CD8^+$ T cells may not originate from the liver, they may traffic to the liver, which acts as a "killing field" for activated $CD8^+$ T cells that are usually involved in immune tolerance of oral antigens (24, 50).

In addition to the reconstitution of HBV-specific T-cell responses in individuals on anti-HBV therapy, the overall $CD8^+$ T-cell responses to mitogen stimulation were also significantly higher in the HBV-monoinfected treated group than in the untreated group. This supports the hypothesis proposed by Boni et al. that persistent HBV infection is associated with a broad T-cell hyporesponsiveness (13). However, their subsequent data suggest that this proliferative increase is not long-lasting (14). Clearly, this needs to be further analyzed before conclusive results can be obtained. Any HBV-associated suppression of T-cell activity may be important in the setting of coinfections with other viruses. Interestingly, the number of IFN-γ^+ $CD8^+$ T cells following mitogen stimulation in the HIV-1–HBV-coinfected group was not significantly greater than that in the untreated group, most likely due to immune suppression secondary to HIV-1 infection itself (57).

Overall the magnitude of the HIV-1-specific T-cell response was higher than that of the HBV-specific T-cell response. This may be due to compartmentalization of HBV infection to the liver hepatocytes, which have reduced HLA expression (33), while HIV-1 infects $CD4^+$ T cells (26) and therefore HIV-1 antigens may be more readily presented and recognized. In addition, functional HBV-specific T cells are likely to localize to the liver, as has previously been shown (45, 47).

While compartmentalization may account for the overall lower magnitude of HBV-specific T-cell responses compared to HIV-1-specific responses, it does not explain why there was a drop in the frequency of HBV-specific $CD4^+$ T-cell responses in HIV-1–HBV-coinfected individuals, while the frequency of HBV-specific $CD8^+$ T-cell responses remained comparable to that found in HBV-monoinfected treated individuals. HIV-1 infection is characterized by a reduction in the quantity and function of $CD4^+$ T cells; however, HIV-1-specific $CD4^+$ T-cell responses were clearly detectable. Similar findings have also been described for persistent HCV infection, where the frequency of HCV-specific $CD4^+$ T cells is also significantly reduced in individuals with HIV-1 coinfection (37). An alternative explanation for the absence of HBV-specific $CD4^+$ T cells in HIV-1–HBV-coinfected individuals could be infection of HBV-specific $CD4^+$ T cells with HIV-1 as they start to proliferate, differentiate, and become activated, given that HIV-1-specific $CD4^+$ T cells are preferentially infected with HIV-1 (27). We think this is an unlikely explanation for the absence of HBV-specific $CD4^+$ T cells in HIV-1–HBV coinfection, given that robust restoration of $CD4^+$ T cells to other organisms such as cytomegalovirus occurs following ART (3, 65). Nevertheless, HIV-1 coinfection impairs the generation of HBV-specific $CD4^+$ T-cell responses and may account for the increased risk of a persistent HBV infection when HBV infection occurs following HIV-1 infection (32, 62). Previous studies of chronic lymphocytic choriomeningitis virus infections show that $CD4^+$ T-cell help is essential in maintaining $CD8^+$ T-cell activity as well as for the production of neutralizing antibodies (20, 72). Therefore the ability to regenerate HBV-specific $CD4^+$ T-cell responses in the setting of HIV-1–HBV coinfection may be crucial for regulation of HBV-specific $CD8^+$ T-cell responses and the development of immune restoration hepatitis. Our study was not designed to address this, but a longitudinal study examining individuals prior to and following HBV-active ART would be of great interest.

Interestingly the specificity of HBV-specific $CD8^+$ T-cell responses differed between HBV monoinfection and HIV-1–HBV coinfection. The S1.3 and PC peptide pools were most commonly recognized by $CD8^+$ T cells in the coinfected individuals, while peptide pool Pol1 was most commonly recognized in HBV-monoinfected individuals. It is possible that coinfection with HIV-1 alters the T-cell immunodominance for HBV. Altered recognition of virus-specific epitopes between heterologous viruses has been demonstrated in virus coinfection in animal models and may be an important evolutionary mechanism of viral persistence (17, 19, 70).

HIV-1–HBV coinfection has a significant effect on HBV-specific $CD4^+$ T-cell function in individuals receiving anti-HBV and anti-HIV-1 therapy. A prospective longitudinal study further analyzing T-cell production of IFN-γ and changes in the HBV-specific intrahepatic T cells will be needed to fully characterize the effects of HBV-active ART on HBV-specific immunity.

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