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DOI:  
[10.1086/589510](https://doi.org/10.1086/589510)

*Document Version*  
Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*  
Mizukoshi, E, Eisenbach, C, Edlin, B, Newton, K, Raghuraman, S, Weiler-Normann, C, Tobler, L, Busch, M, Carrington, M, McKeating, J, O'Brien, T & Rehermann, B 2008, 'Hepatitis C Virus (HCV)-Specific Immune Responses of Long-Term Injection Drug Users Frequently Exposed to HCV', *The Journal of Infectious Diseases*, vol. 198, no. 2, pp. 203-212. <https://doi.org/10.1086/589510>

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# Hepatitis C Virus (HCV)–Specific Immune Responses of Long-Term Injection Drug Users Frequently Exposed to HCV

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**Background.** Injection drug users (IDUs) who successfully clear hepatitis C virus (HCV) have a reduced risk of developing chronic reinfection, despite their continuing exposure to the virus. To identify immunological correlates for this apparent protection, we studied HCV-specific immune responses in long-term IDUs (duration, >10 years).

**Methods.** HCV-specific T cell responses were assessed in proliferation, enzyme-linked immunospot (ELISPOT), interferon (IFN)- $\gamma$  secretion, and cytotoxicity assays, whereas HCV-specific antibodies were assessed in enzyme immunoassays (EIAs), chemiluminescent assays, and in vitro neutralization assays.

**Results.** HCV-specific T cell proliferation and IFN- $\gamma$  production were more common in nonviremic EIA-positive IDUs (16 [94%] of 17 IDUs) than in viremic EIA-positive IDUs (9 [45%] of 20 IDUs) ( $P = .003$ ). They were also noted in 16 (62%) of 26 nonviremic EIA-negative IDUs. In contrast, 19 (90%) of 21 viremic IDUs displayed neutralizing antibodies (nAbs), compared with 9 (56%) of 16 nonviremic EIA-positive IDUs ( $P = .04$ ) and 0 of 24 nonviremic EIA-negative IDUs. Nonviremic IDUs with nAbs were older ( $P = .0115$ ) than those without nAbs, but these groups did not differ in terms of either injection drug use duration or HCV-specific T cell responses.

**Conclusion.** The reduced risk of HCV persistence in IDUs previously recovered from HCV infection correlated with T cell responses, and prolonged antigenic stimulation appears to be required to maintain humoral responses.

Injection drug users (IDUs) constitute the largest group of HCV-infected people in the United States and account for 42% of new infections [1]. After 10 years of injection drug

use, ~90% of IDUs test anti-HCV positive by means of standard EIA [2]; of these IDUs, 80% are persistently infected with HCV [2, 3]. The most common mode of HCV transmission is multiperson use of contaminated syringes and other injection-related paraphernalia [4].

More than 40,000 IDUs live in the San Francisco Bay area [5]. From 1986 through 2005, the Urban Health Study (UHS) recruited active IDUs from street settings in inner-city San Francisco Bay area neighborhoods, counseled them regarding reducing the risk of infection,

Received 6 July 2007; accepted 19 December 2007; electronically published XX May 2008.

Potential conflicts of interest: none reported.

Financial support: This research was supported in part by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIH), and the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH; in whole or in part with federal funds from the National Cancer Institute, NIH (contract N01-CO-12400); and by the Public Health Service (grants AI050798 [to J.M.K.] and R01-DA09532, R01-DA13245, and R01-DA16159 [to B.E.]) and the City and County of San Francisco Department of Public Health.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.

The Journal of Infectious Diseases 2008; 198:xxx

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0022-1899/2008/19802-00XX\$15.00

DOI: 10.1086/589510

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tested them for HIV antibodies, and referred them to appropriate medical and social services [6–8]. Prospective, semiannual sampling revealed that the prevalence of HCV antibody increased in association with the duration of injection drug use [2, 3, 9, 10], which likely reflects an effect of cumulative exposure. Interestingly, however, the incidence of new HCV infections, as measured by seroconversion, decreased in association with longer durations of injection drug use. This reduction in the incidence of HCV infection did not appear to be a chance phenomenon and was independent of risk behavior (B.R.E., unpublished data), suggesting that seronegative IDUs with a long duration of HCV exposure may have some degree of innate or acquired immunity.

In this context, 2 recent epidemiological studies demonstrated that IDUs who successfully cleared HCV in the past have a reduced risk of developing persistent HCV viremia, despite continued injection drug use and continued exposure to HCV [11, 12]. Subjects who had cleared a past HCV infection were 2 times [11] and 4 times [12] less likely to develop persistent HCV infection than were subjects without evidence of past HCV exposure. To identify potential immunological correlates of immune protection, we studied the cellular and humoral immune responses of a subgroup of IDUs in the UHS who reported injection drug use of >10 years' duration.

## SUBJECTS AND METHODS

### Study Population

The UHS was a serial, cross-sectional study that recruited IDUs in street settings in 6 inner-city San Francisco Bay Area neighborhoods to investigate factors affecting the health of these IDUs and to develop and evaluate interventions and policies to improve their health. A new cross-section of IDUs was recruited every 6 months, by use of targeted sampling methods [6, 13]. Eligibility for the study was based on the subjects having injected drugs in the past 30 days. After participating in the study once, IDUs were eligible to participate again as often as every 6 months, but they were not specifically recruited in follow-up [7, 13].

The research presented in the current article was conducted using epidemiological data for and blood samples obtained from UHS participants who had injected drugs for >10 years and who fulfilled one of the following criteria: they were (1) viremic, as determined by reverse-transcription polymerase chain reaction (RT-PCR) (sensitivity, 50 copies/mL) [14], and anti-HCV positive, as determined by 2 consecutive standard EIAs (HCV Version 3.0 ELISA Test System; Ortho-Clinical Diagnostics) performed  $\geq 6$  months apart; (2) nonviremic, as determined by RT-PCR, and EIA positive, as determined by 2 consecutive tests performed  $\geq 6$  months apart; or (3) nonviremic, as determined by RT-PCR, and EIA negative, as determined from results for 2 consecutive samples assessed  $\geq 6$  months apart by use of the HCV Version 3.0 ELISA Test System. HCV RNA was quanti-

tated using the Cobas HCV TaqMan assay (Roche). The study was limited to the first 66 HIV-negative IDUs who fulfilled these criteria and were willing to participate. Twenty-nine healthy, HCV RNA-negative, HCV EIA-negative blood donors at the National Institutes of Health (NIH) who did not have any history of hepatitis served as controls for the immunological assays.

All subjects provided written, informed consent for research testing, which was approved by the Committee on Human Subjects Research at the University of California, San Francisco, and the institutional review board of the National Cancer Institute, NIH. Participants were advised about reducing their risk behaviors; given pretest and posttest HIV, HBV, and HCV counseling; and referred to medical and social services, as needed. Risk behaviors and demographic data were collected by trained interviewers who used a detailed, standardized questionnaire administered at community-based field sites.

### Synthetic Proteins and Peptides

Recombinant HCV core, NS3, helicase, NS4, NS5A, and NS5B proteins (genotype 1, the predominant HCV genotype of the UHS participants) were purchased from Mikrogen. Fifteen-mer peptides that were synthesized according to the HCV genotype 1 H77 sequence and were overlapping by 10 amino acids (Mimotopes) were pooled to generate 1 HCV core pool (38 peptides), 3 HCV NS3 pools (each with 42 peptides), 1 HCV NS4A pool (12 peptides), and 2 HCV NS4B pools (each with 26 peptides). The HLA-A2-restricted CD8<sup>+</sup> T cell epitopes NS3<sub>1073–1081</sub> CVNGVC-WTV, Core<sub>35–44</sub> YLLPRRGPRRL, and Core<sub>131–140</sub> ADLMGYIPLV were synthesized at the Center for Biologics Evaluation and Research of the US Food and Drug Administration (FDA).

### Proliferation Assays

Blood samples were encoded so that the subject's identity was not revealed and obtained in acid citrate dextrose-anticoagulated tubes at community-based field sites and were shipped to the NIH overnight at ambient temperature. Peripheral blood mononuclear cells (PBMCs) were separated from 50 mL of blood, and proliferation assays were performed as described elsewhere [14]. Results were expressed as the stimulation index (counts per minute in the presence of antigen divided by counts per minute in the absence of antigen). The cutoff value for a significant response was a stimulation index of 3, which was equivalent to the mean plus 2 SDs proliferative response of 29 healthy, anti-HCV-negative NIH blood donors.

### IFN- $\gamma$ Enzyme-Linked Immunospot (ELISPOT) and Cytokine Secretion Assays

Duplicate cultures of  $3 \times 10^5$  freshly isolated PBMCs were stimulated with 1  $\mu$ g/mL HCV protein, 1  $\mu$ g/mL each HCV peptide in the respective pool, or 1  $\mu$ g/mL phytohemagglutinin (PHA; Murex Biotech) in RPMI 1640 containing 5% serum from donors with blood group AB and 2 mmol/L L-glutamine [15]. The

**Table 1. Characteristics of the injection drug users studied.**

Characteristic	Viremic EIA-positive subjects <sup>a</sup> ( <i>n</i> = 21)	Nonviremic subjects		<i>P</i> <sup>d</sup>
		EIA positive <sup>b</sup> ( <i>n</i> = 17)	EIA negative <sup>c</sup> ( <i>n</i> = 28)	
Age, median (IQR), years	51 (48.5–55)	51 (48.5–53.5)	44.5 (38–50)	.0016 <sup>e</sup> ; .0011 <sup>f</sup>
Duration of IDU, median (IQR), years	34 (29.5–35.5)	32 (29–34.5)	18 (12.5–28)	<.00011 <sup>e</sup> ; <.0001 <sup>f</sup>
Sex, no. (%) of male subjects	20 (95)	10 (59)	24 (86)	
Race				
White	7 (33)	7 (41)	12 (43)	
Black	14 (67)	7 (41)	15 (54)	
Other	0	3 (18)	1 (4)	
Needle sharing <sup>g</sup>				
Yes	2 (10)	5 (29)	4 (14)	
No	19 (90)	11 (65)	24 (86)	
Unknown	0	1	0	
Frequency of IDU				
In the past month				
Median (IQR)	8 (0–44)	1 (0–60)	12 (0–34.5)	
Unknown	0	0	1	
In the past 6 months				
Median (IQR)	48 (3–371.5)	323 (36–393)	77 (36.5–259)	
Unknown	0	1	0	
No. of sex partners, <sup>g</sup> median (IQR)	1 (0–1)	1 (0.5–1)	2 (1–4)	
Type of sexual contact				
Homosexual	0	0	1 (4)	
Bisexual	2 (10)	0	1 (4)	
Heterosexual	18 (86)	17 (100)	23 (82)	
Unknown	1	0	3	
Received money or drugs for sex	5 (24)	4 (24)	10 (36)	

**NOTE.** Data are no. or no. (%) of subjects, unless otherwise indicated. IDU, injection drug use; IQR, interquartile range;

<sup>a</sup> HCV RNA positive by reverse-transcriptase polymerase chain reaction (RT-PCR) and HCV antibody positive by EIA assay.

<sup>b</sup> HCV RNA negative by RT-PCR and HCV antibody positive by EIA assay.

<sup>c</sup> HCV RNA negative by RT-PCR and HCV antibody negative by EIA assay.

<sup>d</sup> By Mann-Whitney *U* test; only statistically significant findings with a 2-sided *P* value of <.05 are indicated.

<sup>e</sup> Viremic EIA-positive subjects vs. nonviremic EIA-negative subjects.

<sup>f</sup> Nonviremic EIA-positive subjects vs. nonviremic EIA-negative subjects.

<sup>g</sup> In the past 6 months.

number of specific spots (i.e., the number of spots in the presence of antigen minus the number of spots in the absence of antigen) was determined using a KS ELISPOT Reader (Zeiss). A positive response was defined as a response greater than the mean plus 2 SDs of the IFN- $\gamma$  response of healthy NIH blood donors without a history of HCV infection. The protein-specific cutoff values were 7.8 spots (for HCV core), 21 spots (for NS3), 5 spots (for NS4A), and 13.4 spots (for NS4B).

IFN- $\gamma$  cytokine secretion assays were performed according to the instructions of the manufacturer (Miltenyi Biotec), with the use of  $2 \times 10^6$  PBMCs stimulated for 3 h with or without the respective peptide pool, or with 1  $\mu$ g/mL PHA in 500  $\mu$ L of RPMI 1640 (Cellgro) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100  $\mu$ g/mL streptomycin, 100 U/mL penicillin. Samples were analyzed on a FACScalibur flow cytometer with CellQuest software (version 3.3; Becton Dickinson).

### HLA Typing, Generation of HLA-A2-Restricted T Cell Lines, and Cytotoxicity Assay

HLA class I typing was performed by amplifying genomic DNA according to protocols recommended by the 13th International Histocompatibility Workshop [16]. Alleles were assigned according to the reaction patterns of the sequence-specific oligonucleotides probes, and ambiguities were resolved by sequencing.

PBMCs from HLA-A2-positive, exposed, uninfected subjects were stimulated with epitopes NS3<sub>1073–1081</sub> CVNGVCWTV, Core<sub>35–44</sub> YLLPRRGPR, and Core<sub>131–140</sub> ADLMGYIPLV, to generate T cell lines [14]. Cytotoxicity was assessed in a standard cytotoxicity assay using <sup>51</sup>Cr-labeled, peptide-pulsed JY-Epstein-Barr virus (EBV) cells as target cells [14]. Spontaneous release was <15% of maximum release.

**Table 2. Immune responses of viremic EIA-positive and nonviremic EIA-positive injection drug users, as stratified by age and race.**

Response	Viremic EIA-positive subjects, n/N (%) <sup>a</sup>	Nonviremic EIA-positive subjects n/N (%) <sup>a</sup>	OR (95% CI)	Exact <i>P</i>
T cell proliferation				
All subjects	8/21 (38)	13/17 (76)	5.28 (1.06–29.2)	.04
Adjusted <sup>b</sup> for age and race			4.76 (1.04–27.2)	.07
T cell IFN- $\gamma$ production				
All subjects	3/20 <sup>c</sup> (15)	13/17 (76)	18.4 (2.84–138)	.0004
Adjusted <sup>b</sup> for age and race			37.4 (4.79–981)	.0001
Any T cell response <sup>d</sup>				
All subjects	9/20 <sup>c</sup> (45)	16/17 (94)	19.6 (2.04–893)	.003
Adjusted <sup>b</sup> for age and race			19.9 (2.46–550)	.002
nAbs				
All subjects	19/21 (90)	9/16 <sup>c</sup> (56)	0.13 (0.01–0.95)	.04
Adjusted <sup>b</sup> for age and race			0.07 (0.002–0.60)	.01

**NOTE.** Statistical analyses were performed using StatXact software (version 7; Cytel). CI, confidence interval; IFN, interferon; nAbs, neutralizing antibodies; OR, odds ratio.

<sup>a</sup> No. of responders/total no. of subjects (% of subjects who were responders).

<sup>b</sup> OR combined over strata was estimated using exact methods with a mid-*P*-corrected CI. Analysis controlled for sex produced similar results.

<sup>c</sup> One subject was not tested.

<sup>d</sup> Proliferation or IFN- $\gamma$ .

### Analysis of Humoral Immune Responses

Whereas the HCV Version 3.0 ELISA Test System (Ortho-Clinical Diagnostics), along with HCV RNA testing, was used to classify subjects as viremic EIA-positive, nonviremic EIA-positive, and nonviremic EIA-negative groups (see above), 2 assays were used to further characterize the antibody responses of each group.

**Neutralizing antibodies (nAbs).** Plasma samples were screened at a final dilution of 1/100 for their ability to neutralize infection of human hepatoma Huh-7.5 cells by retroviral pseudotypes bearing either HCV genotype 1a (H77) glycoproteins or, as a specificity control, murine leukemia virus glycoproteins [17]. Heat-inactivated samples were mixed with virus at their appropriate dilution in 3% fetal bovine serum/DMEM plus 4  $\mu$ g/mL polybrene and were incubated for 1 h at 37°C. This virus-plasma mix was allowed to infect Huh-7.5 cells seeded in 96-well plates (8  $\times$  10<sup>3</sup> cells/well) for 6 h at 37°C. Unbound virus was removed, and the infected cells were incubated for a total of 72 h. Cells were lysed with cell buffer (Promega) and tested for luciferase activity [17]. The percentage of neutralization was determined by comparing pseudotype infectivity in the presence of test plasma in relation to infection in the presence of control plasma (without HCV antibodies) at the same dilution. Plasma samples that inhibited infection by >50% were considered to be neutralizing.

**Anti-HCV chemiluminescent assay.** Unlike traditional EIAs, the FDA-approved Vitros Immunodiagnostic Anti-HCV Assay (ECi; Ortho-Clinical Diagnostics) has a very broad, dynamic range for the detection of antibodies to recombinant

HCV core, NS3, NS4, and NS5 antigens, which, in part, is due to the fact that enhanced chemiluminescence is the positive signal generator [18, 19]. Samples with a signal/cutoff (S/CO) ratio of >1.00 were considered to have a positive result, and samples with an S/CO of <0.90 were considered to have a negative result. Samples with an S/CO ratio of >0.90 and <1.00 were retested in duplicate on the basis of the manufacturer's recommendations.

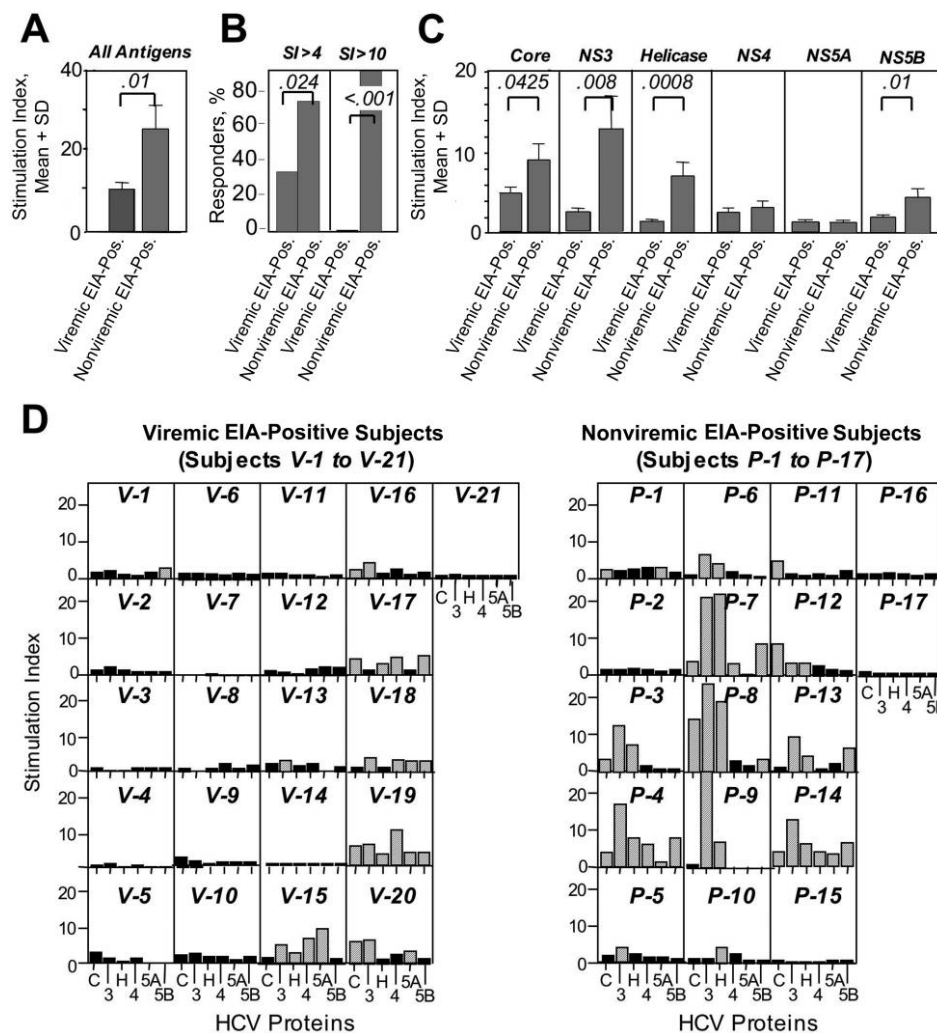
### Statistical Analysis

Fisher's exact test (2-tailed) was used to compare the frequency of immune responses in the 3 different IDU groups. Nonparametric Mann-Whitney tests and unpaired Student's *t* tests were used to compare subject age and the duration of injection drug use, as well as the strength of responses in different IDU groups. Stratified analysis and logistic regression models were used to compare the HCV-specific immune responses in HCV-infected subjects with those in HCV-recovered subjects while controlling for age (<50 years vs.  $\geq$ 50 years), duration of injection drug use, race (black vs. white or other), and sex. The odds ratio (OR) combined over strata was estimated using exact methods with a mid-*P*-corrected confidence interval. Analyses were performed using Prism software (version 3; GraphPad) and StatXact software (version 7; Cytel). A 2-sided *P* value of <.05 was considered to be statistically significant.

### RESULTS

On the basis of 2 consecutive tests performed  $\geq$ 6 months apart, 66 UHS participants were stratified into HCV-viremic EIA-positive,

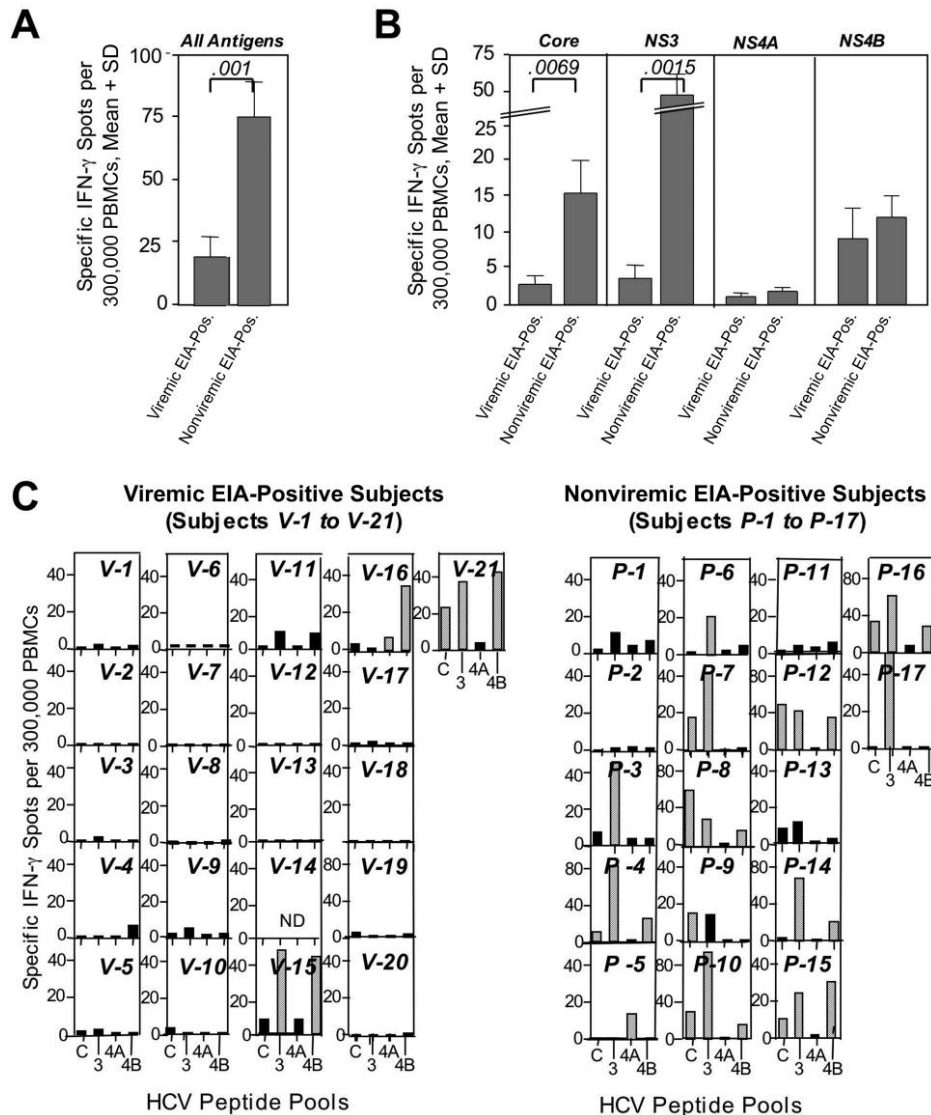




**Figure 1.** Hepatitis C virus (HCV)-specific proliferative T cell response to recombinant HCV proteins. *A–C*, Strength of the HCV-specific proliferative T cell response to all (*A* and *B*) or individual HCV proteins (*C*). The mean (+1 SD) of the antigen-specific response is indicated for viremic EIA-positive subjects and nonviremic EIA-positive subjects, respectively. *D*, Response pattern of individual subjects. The stimulation index indicates proliferation in the presence of antigen relative to proliferation in the absence of antigen. Hatched bars denote positive responses (defined as responses greater than the mean stimulation index [+2 SDs] of the response of healthy blood donors). C, HCV core protein; 3, HCV NS3 protein; H, HCV helicase protein; 4, HCV NS4 protein; 5A, HCV NS5A protein; 5B, HCV NS5B protein; Pos., positive; SI, stimulation index.

nonviremic EIA-positive, and nonviremic EIA-negative groups. Viremic participants had a median HCV titer of  $3.39 \times 10^6$  IU/mL (interquartile range [IQR],  $1.34 \times 10^6$ – $1.17 \times 10^7$ ), and the majority was infected with HCV genotype 1. Viremic EIA-positive participants were comparable to nonviremic EIA-positive participants, in terms of age and duration of injection drug use (table 1). In contrast, nonviremic EIA-negative IDUs were, on average, 6.5 years younger (*P* = .0011) than other UHS participants and reported a ~15-year shorter duration of injection drug use (*P* < .0001). Most viremic EIA-positive IDUs were male and black, consistent with the overall trend observed among UHS participants [3]. Approximately two-thirds injected drugs less than once a day, and they did not share syringes. Most were heterosexual and had few sex partners (table 1).

**HCV-specific T cell proliferation in viremic EIA-positive and nonviremic EIA-positive IDUs.** HCV-specific T cells were first evaluated for their proliferative capacity in response to HCV core, NS3, NS4, NS5A, and NS5B proteins. HCV-specific T cell proliferation was more common among nonviremic EIA-positive IDUs (76%) than among viremic EIA-positive IDUs (38%) (OR, 5.28; *P* = .04). Analyses that controlled for the effects of age, race, and sex produced consistent results (table 2). Compared with viremic EIA-positive IDUs, nonviremic EIA-positive IDUs had a higher mean stimulation index (25.4 vs. 10.5; *P* = .01) (figure 1A) and were more likely to have a stimulation index value >10 (figure 1B). Among the individual HCV proteins, the greatest difference in the strength of the response between groups was observed for the HCV core protein and the



**Figure 2.** T cell-mediated interferon (IFN)- $\gamma$  response to pools of overlapping hepatitis C virus (HCV) peptides. *A* and *B*, Frequency of HCV-specific IFN- $\gamma$ -producing T cells in response to all tested pools of HCV peptides (*A*) and to peptide pools corresponding to individual HCV antigens (*B*). *C*, Response pattern of individual subjects. Positive responses (hatched bars) were defined as responses greater than the mean stimulation index ( $\pm 2$  SDs) of the response of healthy blood donors. HCV-specific IFN- $\gamma$ -producing T cells were detected in 3 of 20 viremic EIA-positive injection drug users (left panels, note that subject V-14 was not tested) and in 13 of 17 nonviremic EIA-positive injection drug users (right panels). C, HCV core peptide pool; 3, HCV NS3 peptide pool; 4A, HCV NS4A peptide pool; 4B, HCV NS4A peptide pool. Peptides covering helicase were included in the pool of HCV-NS3-specific peptides, because helicase is a part of NS3. ND, not done; Pos., positive.

nonstructural HCV proteins NS3 and NS5B (figure 1C and 1D). The differential response to HCV helicase, which is part of the NS3 protein, confirmed the NS3-specific results (mean stimulation index, 7.1 vs. 1.4;  $P = .0008$ ) (figure 1C).

**IFN- $\gamma$  production of HCV-specific T cells in viremic EIA-positive IDUs and in nonviremic EIA-positive IDUs.** To comprehensively analyze the IFN- $\gamma$  response of both CD4 $^{+}$  and CD8 $^{+}$  T cells in the context of the HLA haplotype of each subject, we performed ex vivo ELISPOT assays with pools of overlapping HCV peptides (figure 2). Consistent with the results of the proliferation assays, HCV-specific IFN- $\gamma$  responses were more

common in nonviremic EIA-positive IDUs (13 [76%] of 17 IDUs) than in viremic EIA-positive IDUs (3 [15%] of 23 IDUs) (OR, 18.4;  $P = .0004$ ) (table 2), and this result was similar when adjusted for age, race, and sex. Moreover, nonviremic EIA-positive IDUs displayed a higher mean frequency of IFN- $\gamma$ -producing T cells in response to all tested HCV peptides (74.8 IFN- $\gamma$ -producing cells/300,000 PBMCs) than did viremic EIA-positive IDUs (19.3 IFN- $\gamma$ -producing cells/300,000 PBMCs) ( $P = .001$ ) (figure 2A). When peptide pools of individual HCV proteins were considered, the greatest differences between the nonviremic EIA-positive group and the viremic EIA-

**Table 3. Comparison of nonviremic EIA-positive injection drug users with or without in vitro neutralizing antibodies (nAbs).**

Characteristic	Nonviremic EIA-positive IDUs		<i>P</i> <sup>a</sup>
	With nAbs ( <i>n</i> = 9)	Without nAbs ( <i>n</i> = 7)	
Age, median (IQR), years	53 (51.0–54.5)	50 (45.0–51.0)	.0115
Duration of IDU, median (IQR), years	32 (31.5–37.0)	30 (24.0–34.0)	NS
Frequency of IDU, median (IQR)			
In the past month	1 (0–65)	1 (0–87.5) <sup>b</sup>	NS
In the past 6 months	355 (54–391) <sup>c</sup>	150 (12.5–830)	NS
No. of sex partners, median (IQR)	1 (0–65)	1 (0–4.5)	NS
HCV Abs, median (IQR), ECi score	30.5 (28.5–30.8) <sup>c</sup>	15 (3.6–29.8)	.0093
HCV-specific T cell proliferation			
No. (%) of subjects	8 (89)	4 (57)	NS
Response strength, median (IQR), SI	13.4 (9.5–30.2)	11.8 (5.1–72.2)	NS
HCV-specific T cell IFN- $\gamma$ production			
Subjects with T cell IFN- $\gamma$ production, no. (%)	6 (67)	6 (86)	NS
Response strength, median (IQR), ELISPOTS/300,000 PBMCs	65 (918.5–130.5)	68 (29.5–111.5)	NS

**NOTE.** Abs, antibodies; ECi, Vitros Immunodiagnostic Anti-HCV Assay (Ortho-Clinical Diagnostics); ELISPOT, enzyme-linked immunospots; HCV, hepatitis C virus; IDU, injection drug use; IFN, interferon; IQR, interquartile range; NS, not significant; PBMCs, peripheral blood mononuclear cells; SI, stimulation index.

<sup>a</sup> By Mann-Whitney *U* test. One subject was not tested for neutralizing antibodies.

<sup>b</sup> Data are shown for 6 subjects; 1 subject was not tested.

<sup>c</sup> Data are shown for 8 subjects; 1 subject was not tested.

positive group were observed for HCV core peptides (15.2 vs. 2.8 HCV core-specific IFN- $\gamma$ -producing cells/300,000 PBMCs; *P* = .0069) and HCV NS3 peptides (44.7 vs. 5.6 HCV NS3-specific IFN- $\gamma$ -producing cells/300,000 PBMCs; *P* = .0015) (figure 2B). These were the same antigens that yielded the greatest differential response in the proliferation assay (figure 2C). Moreover, most subjects with IFN- $\gamma$  responses also displayed proliferative responses (subjects P-3 to P-10, P-12, and P-14 and subjects V-15 and V-16, respectively) (figure 1D and figure 2C).

Combining the data for T cell proliferation and IFN- $\gamma$  production, we found that evidence of any T cell response to HCV was more common among nonviremic EIA-positive IDUs (94%) than among viremic EIA-positive IDUs (45%) (OR, 19.6; *P* = .003) (table 2). Analyses that controlled for the effects of age, race, and sex produced consistent results.

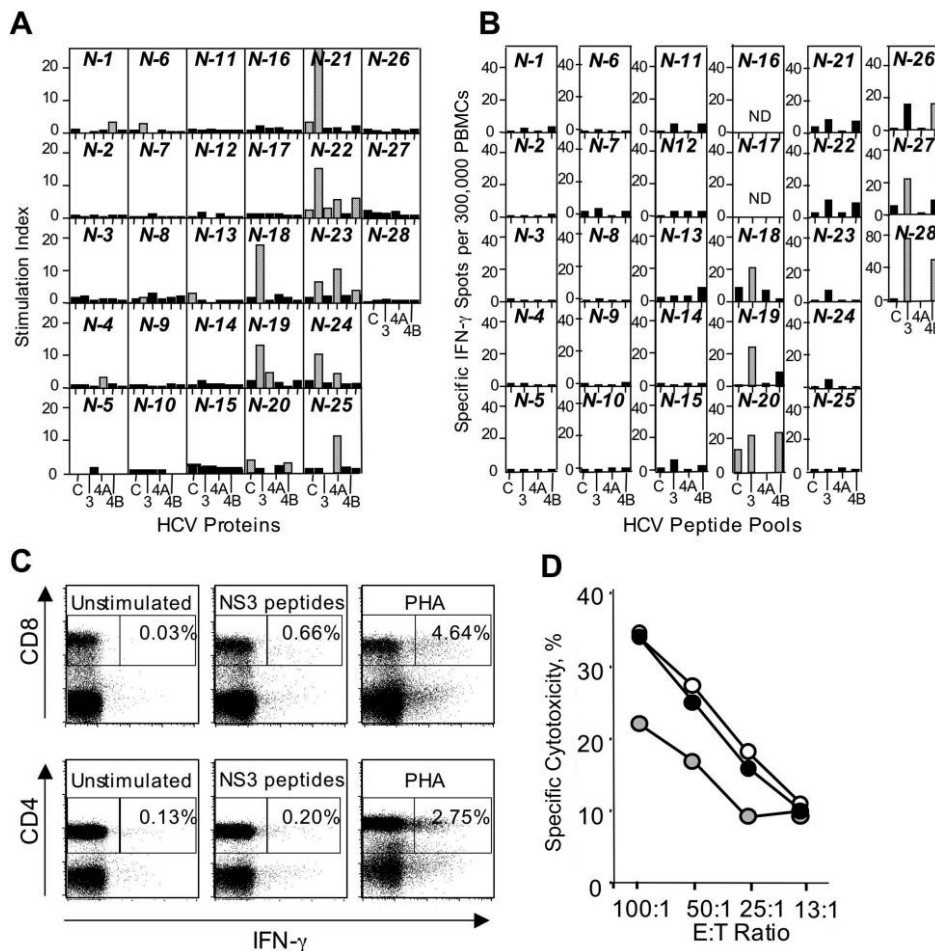
**HCV-specific nAbs in viremic EIA-positive and in nonviremic EIA-positive IDUs.** Whereas antibodies to structural and nonstructural proteins of HCV, as detected in standard EIAs, have been the standard diagnostic criteria for current or past HCV infection for nearly 2 decades [20] and were used in the present study to classify the subject groups, 2 recent assays were used to further characterize the humoral immune response. HCV pseudoparticles were used to assess in vitro antibody-mediated inhibition of virus cell entry [17, 21, 22]. nAbs were detected in 19 (90%) of 21 viremic EIA-positive subjects, compared with 9 (56%) of 16 nonviremic EIA-positive subjects (OR, 0.130; *P* = .04) (table 2). Whereas the majority (11 [58%] of 19) of viremic EIA-positive subjects with nAbs displayed a very high

nAb titer (>9000), only 3 nonviremic EIA-positive subjects had a titer of >9000, and the average titer for the remaining 6 recovered subjects with nAbs was 2000. Interestingly, nonviremic EIA-positive subjects with nAb responses also had stronger antibody responses to core, NS3, NS4, and NS5, as determined in the anti-HCV chemiluminescent assay (median ECi score, 30.5 vs. 15; *P* = .0093), and were slightly older than nonviremic EIA-positive subjects without nAb responses (median age, 53 years vs. 50 years; *P* = .0115) (table 3). However, both subgroups were relatively small and did not significantly differ in terms of duration of injection drug use, frequency of injection drug use, or prevalence or strength of HCV-specific T cell responses (table 3).

**HCV-specific immune responses of nonviremic EIA-negative IDUs.** HCV-specific T cell proliferation was detectable in 13 (46%) of 28 [23] nonviremic EIA-negative IDUs (figure 3A). Six of these subjects also displayed IFN- $\gamma$  responses to overlapping HCV peptides (figure 3B), which were mostly mediated by CD8<sup>+</sup> T cells (figure 3C). As an independent confirmation of these ex vivo responses, we expanded HCV-specific CD8<sup>+</sup> T cells in vitro from PBMCs and established T cell lines. As shown in figure 3D, representative CD8<sup>+</sup> T cell lines from nonviremic EIA-negative IDUs exhibited cytotoxic effector function against HLA-matched, peptide-pulsed EBV B cell lines.

Overall, the response pattern varied widely among individual subjects. Whereas subjects N-18 to N-20 displayed both proliferative and IFN- $\gamma$  responses, subjects N-1, N-4, N-6, N-8, N-13, N-21, and N-21 to N-25 tested positive solely in T cell proliferation assays, and subjects N-26 to N-28 tested positive solely in T





**Figure 3.** Characterization of hepatitis C virus (HCV)-specific T cell responses of nonviremic EIA-negative subjects. *A* and *B*, HCV-specific proliferative T cell responses to HCV proteins were detected in 13 of 28 exposed, uninfected subjects (*A*), and HCV-specific interferon (IFN)- $\gamma$ -producing T cells in response to pools of overlapping HCV peptides were detected in 6 of 26 exposed, uninfected subjects (*B*). Note that IFN- $\gamma$ -producing T cell responses were not tested for subjects N-16 and N-17. The stimulation index (SI) in panel *A* denotes proliferation in the presence of antigen relative to proliferation in the absence of antigen. Positive responses (*hatched bars*) were defined as responses greater than the mean stimulation index ( $+2$  SDs) of the response of healthy blood donors. C, HCV core peptide pool; 3, HCV NS3 peptide pool; 4A, HCV NS4A peptide pool; 4B, HCV NS4A peptide pool. *C*, Both CD4 $^{+}$  and CD8 $^{+}$  T cells of exposed, uninfected subjects produce IFN- $\gamma$  in response to overlapping peptides. Representative data are shown. *D*, HCV-specific T cell lines from HLA-A2–positive, nonviremic EIA-negative subjects lyse HLA-matched B cell lines pulsed with the HLA-A2–restricted HCV peptides NS3<sub>1073–1081</sub> CVNGVCWTV (*open circles*; subject 8), Core<sub>35–44</sub> YLLPRRGPRLL (*filled circles*; subject 8), and Core<sub>131–140</sub> ADLMGYIPLV (*hatched circles*; subject 29), respectively.

cell IFN- $\gamma$  assays (figure 3*A* and 3*B*). Statistical comparisons between the nonviremic EIA-negative participants who had T cell responses and those who did not were limited by the small number of subjects, but it may be noteworthy that all 4 subjects who indicated that they shared needles with others during the 6 months before analysis tested positive for T cell responses, compared with 12 of 24 subjects who did not share needles ( $P = .11$ ) (data not shown). In contrast to the high prevalence of T cell responses, nAb responses were not displayed by any nonviremic EIA-negative subject.

## DISCUSSION

Despite the high prevalence of HCV exposure and the high overall rate of HCV persistence [2, 4, 9, 10, 23–25], it was recently

demonstrated that IDUs who have successfully cleared HCV infection in the past have a reduced risk of developing viremia on reexposure to the virus [11, 12].

Studying subjects with long-term injection drug use (duration,  $>10$  years), we observed that nonviremic EIA-positive IDUs demonstrated more frequent and stronger HCV-specific T cell responses than did viremic EIA-positive IDUs. This correlation held true for both proliferation and IFN- $\gamma$  production of HCV-specific T cells and was noted for responses to both recombinant HCV proteins and overlapping peptides. The greatest difference in the strength of the response between the groups was observed in association with HCV core and NS3. The frequency and strength of the T cell responses of these (presumably) frequently reexposed IDUs were comparable to those previously

observed in subjects who had been exposed to HCV only once in their lifetime [14, 26–30].

In contrast to subjects with HCV-specific T cell responses, subjects with nAbs were more frequent in the group of viremic EIA-positive IDUs than in the group of nonviremic EIA-positive IDUs. The high frequency of nAbs is consistent with what has been noted in the literature, which describes the development of such responses late in infection [21, 31, 32]. However, these antibodies may be ineffective in clearing autologous virus at the time of sampling, because of the emergence of viral escape variants [31]. The presence of nAbs in approximately one-half of the nonviremic EIA-positive IDUs in our study differs from previous reports, which typically did not detect nAbs in patients who had cleared HCV [21, 32–34]. Indeed, prospective studies demonstrated that HCV-specific antibody titers decrease and that these antibodies can become undetectable after spontaneous [14, 35] and therapy-induced clearance of HCV [36]. Our results may be attributable to the fact that the nonviremic EIA-positive IDUs have likely been frequently reexposed to HCV because of continuing injection drug use, whereas cohorts in published reports have consisted predominantly of individuals who had single exposures to the virus [21, 34]. These findings suggest that continued, long-term exposure to HCV antigens is required to maintain humoral immune response.

Nonviremic EIA-negative IDUs exposed to HCV did not display any nAb responses, but they frequently mounted T cell responses with detectable proliferation, IFN- $\gamma$  production, and cytotoxicity against structural and nonstructural HCV antigens. These subjects may have either lost all HCV-specific antibodies after clearance of a previous HCV infection [14, 33] or may have never mounted antibody responses [37]. The presence of CD8<sup>+</sup> T cells that target HCV nonstructural sequences is evidence that HCV RNA translation and/or replication must have occurred at least briefly, because CD8 T cell responses against epitopes in nonstructural proteins are either directly primed by virus-infected cells or cross-primed by cells that have engulfed virus-infected cells [38].

Our report on HCV-specific T cell responses in nonviremic EIA-negative IDUs differs from that noted in a previous study, which did not detect T cell responses in 8 IDUs who remained HCV EIA negative during 1 year of follow-up, despite the fact that they had injection drug use patterns associated with a high risk for HCV transmission [39]. The different results may be explained by differences in study populations and immunological techniques. The published report analyzed subjects who had recently started injecting drugs [39], and their cumulative exposure to HCV was therefore lower than that of the subjects with long-term injection drug use (duration, >10 years) in our study. Moreover, the published report was based on an *in vitro* T cell expansion technique performed with vaccinia virus-infected antigen-presenting cells [39], which is less sensitive than the *in vivo* IFN- $\gamma$  ELISPOT technique used with overlapping HCV

peptides in our study. At the same time, it should be acknowledged that the observations in our study are restricted to HCV genotype 1, because both the antigens used for immunological assays and the major genotype in the UHS cohort are of genotype 1.

Our results are consistent with the detection of HCV-specific T cell responses by IFN- $\gamma$  ELISPOT analysis in 20% of nonviremic EIA-negative family members of HCV-viremic patients [40, 41], and they are also reminiscent of HIV-specific T cell responses in prostitutes who remained HIV-negative despite having continued high-risk exposure [42]. In that report, a break from sex work for  $\sim$ 1 year was associated with a loss of HIV-specific CD8<sup>+</sup> T cell responses and a higher risk of HIV viremia and seroconversion [43]. These results, along with the epidemiological finding that HCV-nonviremic EIA-positive IDUs have a reduced risk of acquiring persistent infection [11, 12], suggest T cell-mediated immunity. Indeed, when chimpanzees that have recovered from previous HCV infection are rechallenged with homologous or heterologous strains of HCV [44–46], HCV-specific T cells are detected within 1–2 weeks, and HCV is cleared without evident liver injury. Depletion of either CD4<sup>+</sup> [45] or CD8<sup>+</sup> T cells [47] before rechallenge with virus abrogates protective immunity. Thus, HCV-specific T cell responses may contribute to the apparent immune protection of some IDUs [11, 12]. In contrast, the highest prevalence and titer of nAbs were found in the group of viremic EIA-positive IDUs and in a subgroup of nonviremic EIA-positive IDUs, who were older and who had a longer IDU history than those without nAbs. A protective role of nAb responses immediately after reexposure should not be excluded, however, because a recent study suggests that nAbs persist for very short periods (1–6 months) after infection [14, 33]. A prospective study has now been initiated to further assess the temporal relationship of risk behavior to HCV-specific T cell and nAb responses.

## Acknowledgments

We thank the Urban Health Study (UHS) staff for conducting the field operations; the UHS study participants, without whom this study would not have been possible; Joni Trenbeath and H. Alter (Clinical Center, National Institutes of Health), for HCV RNA quantitation; and Elizabeth Wright, (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health), for help with statistical analysis.

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