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Virus-Specific CD8$^+$ Lymphocytes Share the Same Effector-Memory Phenotype but Exhibit Functional Differences in Acute Hepatitis B and C

Simona Urbani,[1] Carolina Boni,[1] Gabriele Missale,[1] Gianfranco Elia,[1] Cristina Cavallo,[1] Marco Massari,[2] Giovanni Raimondo,[3] and Carlo Ferrari[1]*

Divisione Malattie Infettive ed Epatologia, Azienda Ospedaliera Universitaria di Parma, 43100 Parma,[1] Laboratory of Molecular Biology and Section of Hepatology Research, Department of Internal Medicine and Medical Therapy, University of Messina, Messina,[3] and Unità Operativa Malattie Infettive, Azienda Ospedaliera S. Maria Nuova di Reggio Emilia, 42100 Reggio Emilia,[2] Italy

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Hepatitis B and hepatitis C viruses (HBV and HCV) are both noncytopathic and can cause acute and chronic infections of the liver. Although they share tropism for the same organ, development of chronic hepatitis is much more frequent following HCV infection, suggesting different mechanisms of viral persistence. In this study, we show that circulating HBV- and HCV-specific tetramer-positive CD8 cells during the acute phase of hepatitis B and C belong almost entirely to an effector-memory subset (CCR7$^-$ CD45RA$^-$). Despite this phenotypic similarity, HBV- and HCV-specific CD8 cells show striking functional differences. HBV-specific tetramer-positive CD8 cells express high perforin content ex vivo, expand vigorously, and display efficient cytotoxic activity and gamma interferon (IFN-γ) production upon peptide stimulation. A comparable degree of functional efficiency is maintained after the resolution of hepatitis B. In contrast, HCV-specific CD8 cells in the acute phase of hepatitis C express significantly lower levels of perforin molecules ex vivo and show depressed CD8 function in terms of proliferation, lytic activity, and IFN-γ production, irrespective of the final outcome of the disease. This defect is transient, because HCV-specific CD8 cells can progressively improve their function in patients with self-limited hepatitis C, while the CD8 function remains persistently depressed in subjects with a chronic evolution.

Cytotoxic T lymphocytes (CTL) play a central role in the control of virus infections (15). In infections by noncytopathic viruses, they contribute to both virus elimination and pathology, because elimination of intracellular virus is achieved by the destruction of infected cells and by a cytokine-mediated suppression of viral-gene expression within host cells (6, 11). Therefore, characterization of the functional features of virus-specific CTL at the early stages of infections with noncytopathic viruses, including hepatitis B virus (HBV) and hepatitis C virus (HCV) that are able to chronically persist in the infected host, can provide important insights into the pathogenesis of viral clearance and persistence (4, 7).

The use of HLA class I tetramers together with phenotypic markers of activation, homing, and differentiation represents a powerful tool to analyze ex vivo virus-specific CD8 cells (1, 25). Four subsets of CD8 cells can be distinguished by staining them with antibodies to CCR7, a chemokine receptor involved in homing to secondary lymphoid organs and surface molecules associated with naive and memory T-cell subsets: naive CD45RA$^+$ CCR7$^+$ T cells, CD45RA$^-$ CCR7$^+$ central memory T cells, CD45RA$^-$ CCR7$^-$ effector-memory T cells, and CD45RA$^+$ CCR7$^-$ differentiated effector T cells (2, 12, 31). Perforin expression and gamma interferon (IFN-γ) secretion have been reported to be predominant functions of the more differentiated CCR7$^-$ subsets (2, 31).

Using tetramer technology to quantify virus-specific CD8 cells, the frequency of tetramer-positive lymphocytes has been shown to be high during the acute phase of both HBV (21) and HCV (19, 34) infections. However, the high frequency of circulating HBV-specific cells is associated with a favorable outcome of acute hepatitis B. In contrast, >70% of patients with acute hepatitis C develop chronic disease, despite the high frequency of CD8$^+$ HCV-specific cells detectable in their blood (19, 34).

With the aim of investigating the mechanisms underlying such different behavior, we compared prospectively the phenotypic and functional characteristics of tetramer-positive HBV- and HCV-specific CD8 cells during acute hepatitis B and C, when the pathogenetic events crucial for the outcome of infection are likely to occur.

MATERIALS AND METHODS

Patients. Five HLA-A0201-positive patients with acute hepatitis B and seven HLA-A0201-positive patients with acute hepatitis C enrolled at the Department of Infectious Diseases and Hepatology of the University Hospital of Parma were studied. The diagnosis of acute HCV infection was based on the following criteria: documented seroconversion to anti-HCV antibodies by recombinant immunoblotting assay (RIBA), levels of serum alanine aminotransferase (ALT) at least 10 times the upper limit of normal (50 U/liter), detection of HCV RNA, and exclusion of other possible causes of acute hepatitis (i.e., viruses, toxins, alcohol, autoimmunity, and metabolic factors). Three patients (patients 2C, 3C, and 4C) were asymptomatic and were diagnosed because of the detection of elevated ALT levels during the course of a laboratory screening in the absence of symptoms related to hepatitis.

* Corresponding author. Mailing address: Divisione Malattie Infettive ed Epatologia, Azienda Ospedaliera e Universitaria di Parma, Via Gramsci 14, 43100 Parma, Italy. Phone: 39/521/991056. Fax: 39/521/988706. E-mail: cafer@tin.it.

TABLE 1. Demographic, virologic, and biochemical characteristics of patients with acute HBV infection[a]

Patient	Age (yr)	Sex	1st time point			2nd time point			3rd time point			Outcome
			Wk from clinical presentation	AST/ALT	HBV DNA (copies/ml)	Wk from clinical presentation	HBV DNA (copies/ml)	AST/ALT	Wk from clinical presentation	HBV DNA (copies/ml)	AST/ALT	
1B	37	M	1	433/2,747	44,900	9	<200	25/59	24	<200	22/45	Self-limited
2B	46	M	2	26/76	<200	8	<200	18/25	28	<200[b]	10/13	Self-limited
3B	39	M	2	57/154	13,800	6	1,420	21/26	26	<200	21/29	Self-limited
4B	34	M	2	27/49	<200	9	<200	29/37	28	<200	25/17	Self-limited
5B	26	M	1	499/927	<200	13	<200	16/16	ND	ND	ND	Self-limited

[a] Patients were studied at three different time points of the follow-up. ND, not determined; M, male. The lower limit of detection for HBV DNA is 200 copies/ml.
[b] Viremia evaluated at a later time point relative to immunological analysis.

The diagnosis of acute HBV infection was based on elevated ALT levels (at least 10 times the upper limit of normal) and detection of hepatitis B surface antigen and immunoglobulin M anti-hepatitis B core antigen antibodies in the serum. All patients were negative for anti-human immunodeficiency virus type 1 (HIV-1) and HIV-2 antibodies and for other markers of viral or autoimmune hepatitis. All gave written informed consent before entering the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virological assessment. Hepatitis B surface antigen, anti-hepatitis B surface antigen, total and immunoglobulin M anti-hepatitis B core antigen, HBeAg, anti-HBe, anti-hepatitis D virus, anti-HCV, and anti-HIV-1 and -2 were determined by commercial enzyme immunoassay kits (Abbott Laboratories, North Chicago, Ill.; Ortho Diagnostic Systems, Raritan, N.J.; Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Anti-HCV antibodies were also analyzed by RIBA (RIBA II; Ortho Diagnostic Systems). Serum HBV DNA was quantified by PCR (Cobas Amplicor test; Roche Diagnostic, Basel, Switzerland); the lower limit of detection by this method is 200 copies/ml. Serum HCV RNA was analyzed by branched-DNA assay (System 340b DNA analyzer; Bayer Corp., Tarrytown, N.Y.) and was expressed as copies per milliliter of serum; the lower limit of detection by this method is 2,500 copies/ml.

Amplification of the HCV NS4B genomic region coding for the 1992-2000 peptide. RNA was extracted from sera of patients 2C, 3C, and 4C. The portion of the NS4B gene coding for the amino acid sequence 1992 to 2000 was amplified by reverse transcription-PCR as follows: after reverse transcription performed with an antisense primer (5′-CTACACCACGGGCCCCTGTAC-3′ for both genotypes 1a and 3), the cDNA was amplified with genotype-specific primers (5′-CTACACCACGGGCCCCTGTAC-3′ and 5′-GAGGGGGCAGTGCART GGATG-3′ for genotype 1a; 5′-CTACACCACGGGCCCCTGTAC-3′ and 5′-T GCTGAGTTCYCTAACYGTC-3′ for genotype 3). Nested PCR amplification was performed with the product of the first reaction and the inner primers (5′-CGGCTRATAGCCTTCGCCTCC-3′ and 5′-GGACGATGAGGATCGTC GGT-3′ for genotype 1a; 5′-CTTGYAGCGRCGATTGGCTRC-3′ and 5′-GG GTCCATGCGGCTRGCAGGG-3′ for genotype 3). The products of amplification were electrophoresed in an ethidium bromide-stained 2% agarose gel, cut out from the gel, and purified by a minicolumn system (QIAquick Gel Extraction kit; Qiagen, Hilden, Germany). The purified products were directly sequenced in both strands by the ABI PRISM 310 genetic sequence analyzer using an ABI PRISM dye terminator cycle-sequencing ready-reaction kit (Applera Europe B.V., AH Nieuwerker, The Netherlands).

Synthetic peptides, peptide-HLA class I tetramers, and antibodies. HLA-A2 synthetic peptides corresponding to HBV core 18-27, polymerase 575-583, envelope 335-343, and HCV NS3 1073-1081, NS3 1406-1415, and NS4B 1992-2000 were purchased from Chiron Mimotopes (Victoria, Australia).

Phycoerythrin-labeled tetrameric-peptide–HLA class I complexes were purchased from Proimmune Ltd. (Oxford, United Kingdom). HLA-A2 tetramers contained the HBV peptides core 18-27 (FLPSDFFPSV), polymerase 575-583 (FLLSLGIHL), and envelope 335-343 (WLSLLVPFV) and the HCV peptides NS3 1073-1081 (CINGVCWTV), NS3 1406-1415 (KLVALGINAV), and NS4B 1992-2000 (VLSDFKTWL).

Anti-CD8 (conjugated with quantum red or fluorescein isothiocyanate [FITC]) and anti-IFN-γ–FITC were purchased from Sigma Aldrich (St. Louis, Mo.). Anti-CD27 (FITC), anti-HLA-DR (FITC), and anti-CD8 conjugated with allophycocyanin (APC) antibodies were purchased from Becton Dickinson Immunocytometry Systems, San Jose, Calif. Anti-CD45RO (FITC), anti-CD28, -CCR5, -CD45RA, and -CD8 cytochrome (phycoerythrin-Cy5), and anti-perforin (FITC) were purchased from BD Pharmingen. The rat anti-CCR7 antibody was a kind gift of Martin Lipp (Max-Delbruck-Center, Berlin, Germany); goat anti-rat antibodies conjugated with FITC or APC were purchased from BD Pharmingen and Caltag Laboratories (Burlingame, Calif.), respectively.

Isolation of PBMC and in vitro expansion of HBV- and HCV-specific CTL. Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and resuspended in RPMI 1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 50 μg of gentamicin/ml, and 8% human serum (complete medium). For CTL expansion, PBMC were resuspended in 96-well plates at a concentration of 2×10^6/ml in complete medium, supplemented with interleukin-7 (IL-7) (5 ng/ml) and IL-12 (100 pg/ml), and stimulated with HBV or HCV peptides at 1 μM final concentration. Recombinant IL-2 (50 IU/ml) was added on day 4 of culture, and the cytotoxicity assay was performed on day 10.

Cell surface and intracellular staining. (i) Staining with tetramers and other surface markers. PBMC (10^6) were incubated for 30 min at 37°C with the phycoerythrin-labeled tetrameric complex in RPMI 1640 and 8% human serum.

TABLE 2. Demographic, virologic, and biochemical characteristics of patients with acute HCV infection[a]

Patient	Genotype	Age (yr)	Sex	1st time point			2nd time point			3rd time point			Outcome
				Wk from clinical presentation	AST/ALT	HCV RNA (copies/ml)	Wk from clinical presentation	HCV RNA (copies/ml)	AST/ALT	Wk from clinical presentation	HCV RNA (copies/ml)	AST/ALT	
1C	1b	58	F	1	177/875	<2,500	14	<2,500	19/17	25	<2,500	13/11	Self-limited
2C	3a	26	M	5	13/69	<2,500	16[b]	324,449	23/100	30	305,202	20/63	Chronic
3C	3a	25	M	1	123/356	<2,500	9	<2,500	23/31	29	<2,500	28/32	Self-limited
4C	1a	34	M	2	125/336	71,891	12	9,150	29/182	40	171,545	33/55	Chronic
5C	3a	27	F	1	1,120/2,190	ND	14[b]	ND	18/30	30	184,356[c]	233/388	Chronic
6C	1b	33	M	2	52/277	2,533	13	398,209	44/83	26[b]	2,263,736	44/145	Chronic
7C	3a	30	M	1	50/269	ND	16[b]	<2,500	32/27	80	<2,500	17/9	Self-limited

[a] Patients were studied at three different time points of the follow-up. ND, not determined; F, female; M, male. The lower limit of detection for HCV RNA is 2,500 copies/ml.
[b] No immunological analysis performed in parallel.
[c] Viremia evaluated at a later time point relative to immunological analysis.

TABLE 3. Frequency of HBV-specific CD8 cells detected ex vivo in peripheral-blood by tetramer staining[a]

Patient	HBV tetramer frequency ex vivo (%)		
	CORE 18–27	ENV 335–343	POL 575–583
1B	**0.6**	**0.7**	**0.06**
2B	**1.1**	**0.22**	**0.6**
3B	0.03	**0.12**	<0.01
4B	**0.43**	<0.01	<0.01
5B	**1.1**	**0.84**	ND

[a] ENV, envelope; POL, polymerase; ND, not determined. Significant values are in boldface.

After the cells were washed, staining was performed for 15 min in the dark, using a panel of FITC-, cytochrome-, or APC-conjugated antibodies. The cells were then washed and analyzed immediately on a Becton Dickinson flow cytometer (FACScalibur) using CellQuest software.

(ii) Perforin staining. Tetramer-stained cells were first incubated with anti-CD8 quantum red monoclonal antibody for 20 min at 4°C and then fixed with a fixing buffer (Caltag) for 15 min at room temperature, washed with phosphate-buffered saline–0.1% fetal calf serum, and permeabilized with a permeabilization buffer (Caltag) in the presence of anti-perforin–FITC for 15 min at room temperature. The cells were washed again and then analyzed by flow cytometry.

(iii) IFN-γ staining. Tetramer-stained cells were incubated in medium alone (control) or with HBV or HCV peptides (1 μM) for 1 h; brefeldin A (10 μg/ml) was added for an additional 4 h of incubation. After being washed, the cells were stained with anti-CD8 quantum red monoclonal antibody for 20 min at 4°C and then fixed and permeabilized as described above. The cells were finally stained with anti-IFN-γ–FITC for 15 min at room temperature, washed again, and analyzed by flow cytometry.

Chromium release assay. Cytotoxic activity was assessed by incubating peptide-stimulated PBMC with peptide-pulsed ^{51}Cr-labeled, HLA-A2-matched or -mismatched Epstein-Barr virus-transformed B cells as targets for 4 h in round-bottom 96-well plates. The percent specific lysis was calculated as described previously (27). Significant CTL responses to synthetic peptides were defined by testing a group of 10 healthy HCV- and HBV-seronegative subjects who never showed levels of specific lysis of >3%. In order to adopt very stringent criteria and avoid the risk of false-positive results, only levels of CTL lysis of ≥13% were considered significant.

Statistical analysis. The frequencies of HBV- and HCV-specific CTL line perforin expression ex vivo and after peptide stimulation detected in patients with acute hepatitis B and C were compared by Student's *t* test for unpaired data.

RESULTS

HBV- and HCV-specific CD8 cells in the acute stage of hepatitis B and C express similar phenotypes. The phenotypes of virus-specific CD8 cells were characterized in five and seven HLA-A2-positive patients with acute hepatitis B and C, respectively. All patients were studied between the first and second week after clinical presentation, with the exception of patient 2C, who was studied 3 weeks later (Tables 1, 2, 3, and 4). Patients 2B, 3B, 4B, and 6C showed a rapid decline of ALT levels that were higher than 1,000 IU/ml at the time of clinical presentation, 1 or 2 weeks before T-cell analysis. In line with previous reports of the incubation stage of hepatitis showing that the decline of viremia starts before the rise of ALT levels (34, 36), all HBV- and HCV-infected patients expressed low or undetectable levels of viremia at the time of immunological analysis (Tables 1 and 2).

TABLE 4. Frequency of HCV-specific CD8 cells detected ex vivo in peripheral blood by tetramer staining[a]

Patient	HCV tetramer frequency ex vivo (%)		
	NS3 1073–1081	NS3 1406–1415	NS4B 1992–2000
1C	**0.42**	**0.2**	<0.01
2C	<0.01	<0.01	**0.15**
3C	<0.01	<0.01	**1.21**
4C	**2**	<0.01	**3**
5C	<0.01	<0.01	**0.24**
6C	**0.4**	<0.01	<0.01
7C	<0.01	<0.01	**0.06**

[a] Significant values are in boldface.

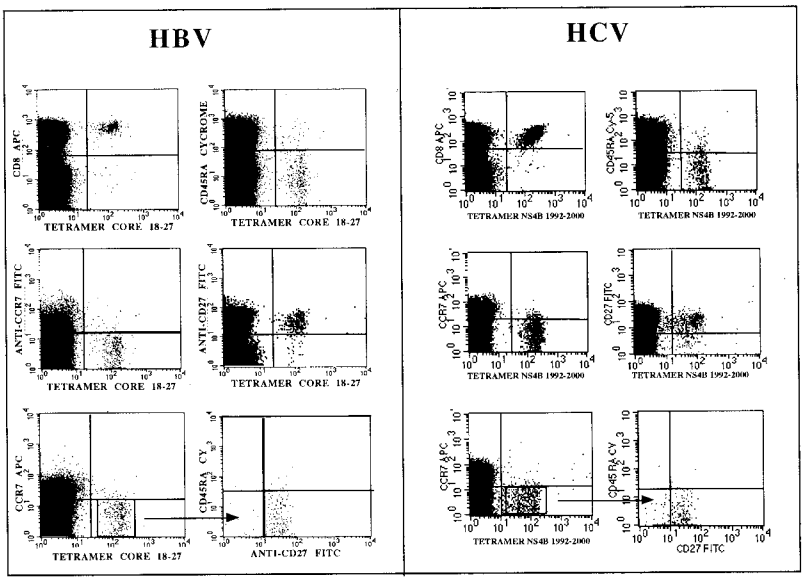
HLA-peptide tetramers were used to directly quantify ex vivo circulating CD8$^+$ cells specific for three HBV (core 18-27, envelope 335-343, and polymerase 575-583) and three HCV (NS3 1073-1081, NS3 1406-1415, and NS4B 1992-2000) epitopes known to be frequently recognized in acute hepatitis (Tables 3 and 4). Elevated frequencies of tetramer-positive cells were detectable in both HBV- and HCV-infected patients. However, the maximal frequency of HBV tetramer-positive cells was 1.1% of circulating CD8$^+$ lymphocytes (patients 2B and 5B), while in two of six patients with acute HCV infection, the frequency of HCV-specific CD8 cells exceeded 1.2% of the total CD8 population, reaching values of 3% with tetramer NS4B 1992-2000 in patient 4C (Table 4).

The stage of differentiation of tetramer-positive cells was studied by staining CD8 cells simultaneously with antibodies to CCR7, CD45RA, and CD27 molecules (Fig. 1A). As shown in Fig. 1B, the great majority of HBV- and HCV-specific cells were CCR7 negative; most CCR7$^-$ cells did not express CD45RA. The CCR7$^-$ CD45RA$^-$ subset was also predominantly CD27$^+$ (Fig. 1C); this phenotype was expressed by CD8 cells of all epitope specificities tested, showing that most HBV and HCV tetramer-positive cells belong to an effector-memory subset. The analysis of HBV- and HCV-specific cells during the follow-up (from the acute phase to approximately 6 months after onset) indicated that tetramer-positive cells were still predominantly CCR7$^-$ CD45RA$^-$ (Fig. 2) and CD27$^+$ (data not shown). A different phenotype was observed in patient 5B with acute HBV infection, whose tetramer-positive CD8 cells were predominantly CD45RA$^+$ and CD27$^+$ during both the acute phase of infection and the follow-up. These cells may belong to a more differentiated effector type that still has to switch off the CD27 molecule.

HBV and HCV tetramer-positive CD8 cells were further characterized with other surface markers. During the acute stage, they were homogeneously HLA-DR$^+$ (92 to 98%), CCR5$^-$ (96 to 100%), and CD45RO$^+$ (95 to 100%); CD28 expression ranged between 40 and 50% of tetramer-positive CD8 cells (Fig. 3). With the exception of HLA-DR, which became progressively negative, none of the surface markers changed during the follow-up (data not shown).

Perforin expression is different in HBV- and HCV-specific CD8 cells ex vivo in the acute phase of hepatitis B and C. Perforins are molecules produced by cytotoxic lymphocytes that are stored within cytoplasmic granules and that are involved in target cell death by osmotic lysis or apoptosis. Virus-specific CTL were analyzed in both groups of patients for their perforin contents ex vivo. Tetramer-positive cells specific for HCV showed a significantly lower perforin content ex vivo than HBV-specific CD8 cells during the acute phase of infec-

A



B

% TETRAMER +/CCR7-				% TETRAMER +/CCR7-			
	CORE 18-27	ENV 335-343	POL 575-583		NS3 1073-1081	NS3 1406-1415	NS4B 1992-2000
Pt				**Pt**			
1B	93%	91%	84%	**1C**	98%	91%	(*)
2B	96%	98%	99%	**2C**	(*)	(*)	91%
3B	78%	99%	(*)	**3C**	(*)	(*)	92%
4B	93%	(*)	(*)	**4C**	96%	(*)	94%
5B	98%	95%	NT	**5C**	(*)	(*)	74%
				6C	80%	(*)	(*)
				7C	NT	NT	NT

% TETRAMER+ CCR7-/CD45RA-				% TETRAMER+ CCR7-/CD45RA-			
	CORE 18-27	ENV 335-343	POL 575-583		NS3 1073-1081	NS3 1406-1415	NS4B 1992-2000
Pt				**Pt**			
1B	85%	97%	95%	**1C**	100%	98%	(*)
2B	86%	89%	94%	**2C**	(*)	(*)	92%
3B	66%	99%	(*)	**3C**	(*)	(*)	97%
4B	64%	(*)	(*)	**4C**	85%	(*)	88%
5B	20%	10%	NT	**5C**	(*)	(*)	85%
				6C	97%	(*)	(*)
				7C	NT	NT	NT

C

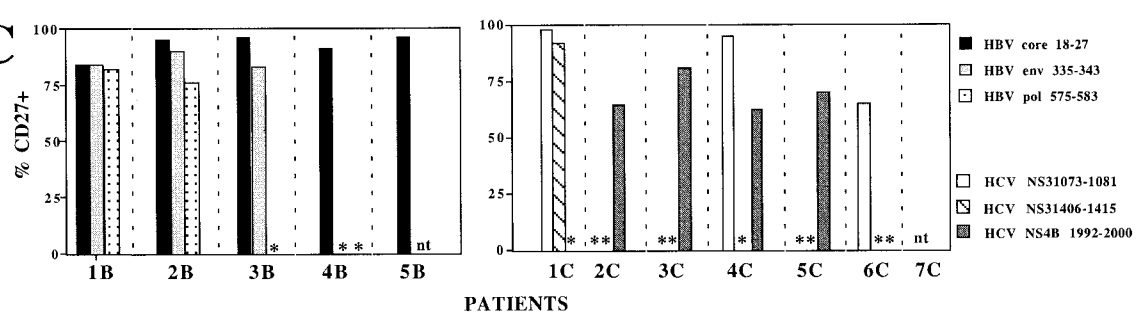


FIG. 1. Phenotypic analysis of HBV- and HCV-specific CTL. (A) Representative dot plot analyses of HBV and HCV tetramer-positive cells stained with anti-CD8, anti-CCR7, anti-CD27, and anti-CD45RA monoclonal antibodies. (B) Percentages of $CCR7^-$ (top) and $CD45RA^-$ $CCR7^-$ (bottom) cells among HBV tetramer-positive CD8 lymphocytes specific for core 18-27, envelope 335-343, and polymerase 575-583 epitopes and among HCV tetramer-positive CD8 cells specific for NS3 1073-1081, NS3 1406-1415, and NS4B 1992-2000 epitopes in each patient (Pt) with acute HBV and HCV infection. (C) Percentages of $CD27^+$ cells among $CCR7^-$ $CD45RA^-$ tetramer-positive CD8 cells in each patient with acute HBV and HCV infection. (*), frequency of tetramer-positive cells was <0.01% of $CD8^+$ cells; NT, not tested.

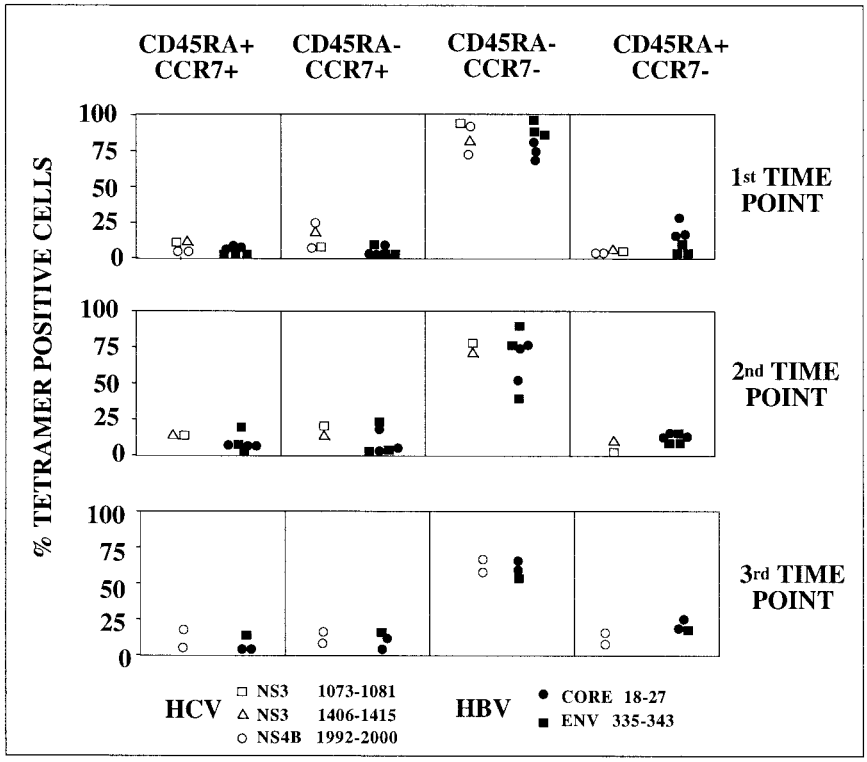


FIG. 2. Peripheral-blood distribution of CD8 differentiation phenotypes during and after the acute stage of hepatitis B and C. The surface expression of CD8, CD45RA, and CCR7 was tested at three sequential time points as indicated in Tables 1 and 2. Each symbol represents the percentage of tetramer-positive cells expressing the indicated phenotypic markers.

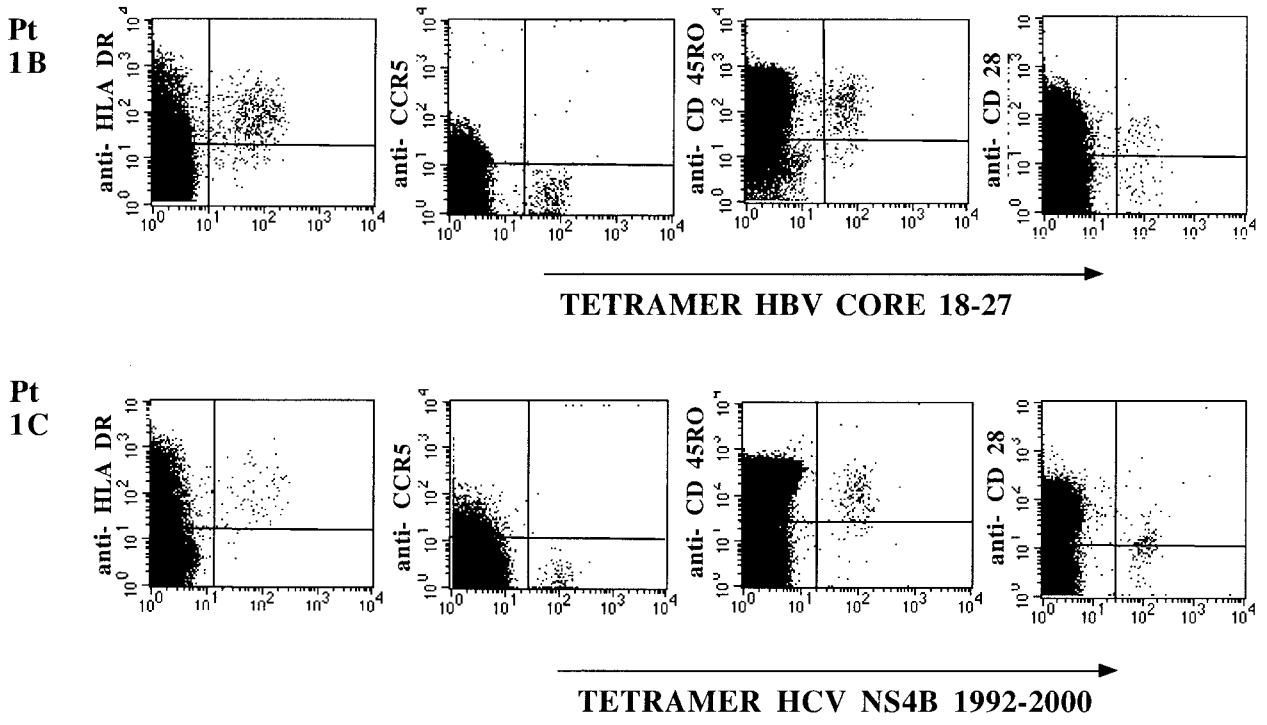


FIG. 3. Representative dot plot analyses of HBV core 18-27 and HCV NS4B 1992-2000 tetramer-positive cells stained with anti-HLA-DR, anti-CCR5, anti-CD45RO, and anti-CD28 monoclonal antibodies. Pt, patient.

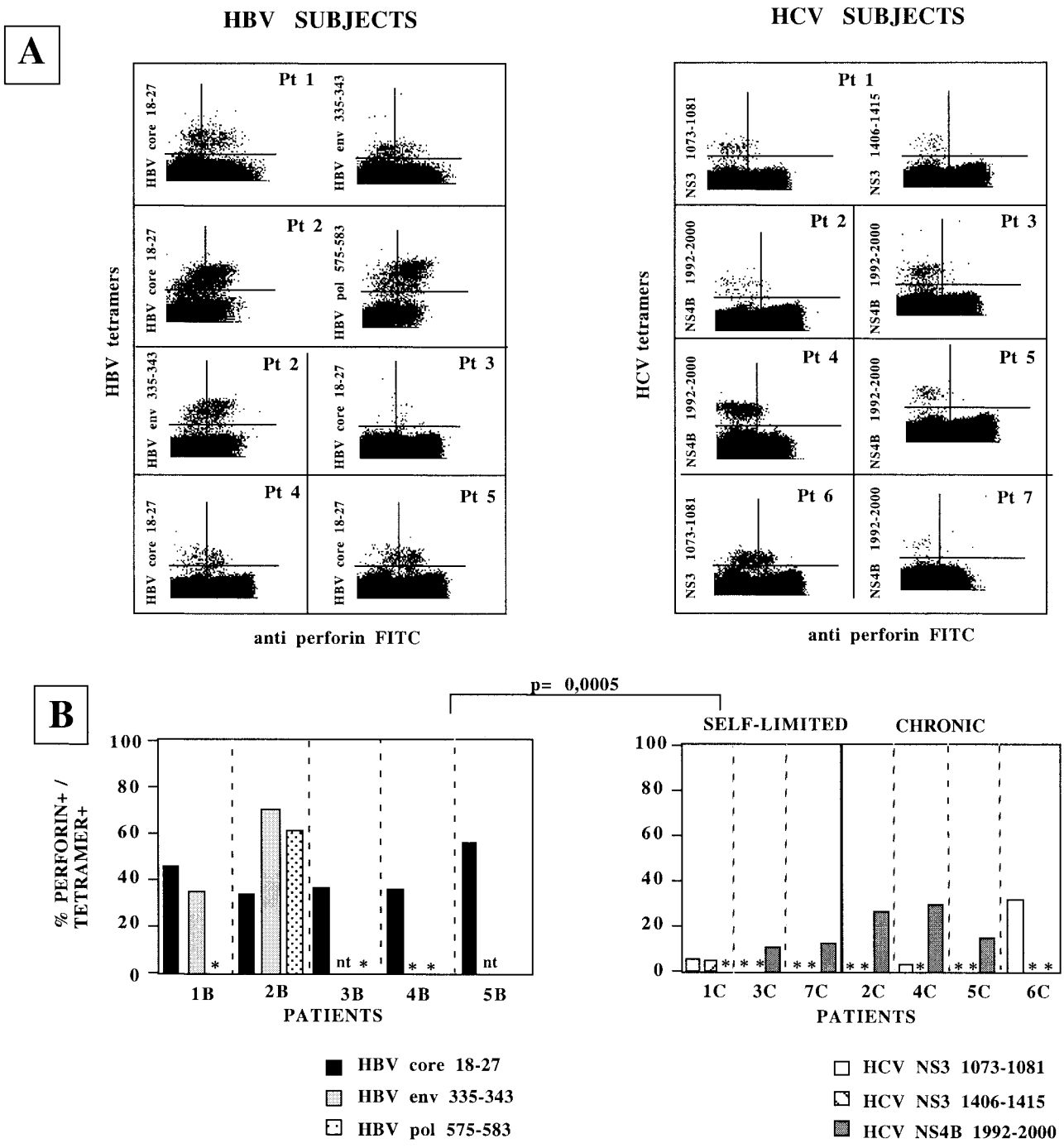


FIG. 4. Analysis of the perforin contents of HBV- and HCV-specific CD8 cells ex vivo. (A) Dot plot analysis of perforin expression by HBV- and HCV-specific CD8 cells stained with anti-perforin monoclonal antibodies. Pt, patient. (B) Percentages of perforin-positive cells assessed ex vivo among the overall population of HBV- or HCV-specific CD8 lymphocytes. PBMC from patients with acute HBV and HCV infection were tested with HBV core 18-27, envelope (env) 335-343, and polymerase (pol) 575-583 and with HCV NS3 1073-1081, NS3 1406-1415, and NS4B 1992-2000 tetramers, respectively. The outcome of the disease (self-limited or chronic evolution) is indicated. *, frequency of tetramer-positive cells was <0.01% of $CD8^+$ cells; nt, not tested. The percentages of perforin-positive cells in the two groups of patients were significantly different by Student's *t* test.

tion (Fig. 4). Low intracellular perforin content was observed irrespective of the outcome of hepatitis C.

HBV- and HCV-specific CD8 cells in the acute phase of hepatitis B and C show different degrees of functional efficiency. PBMC from both groups of patients with acute hepatitis B and C were stained with tetramers after 10 days of peptide stimulation, and the capacity for expansion was assessed by comparing the frequency of HBV- and HCV-specific

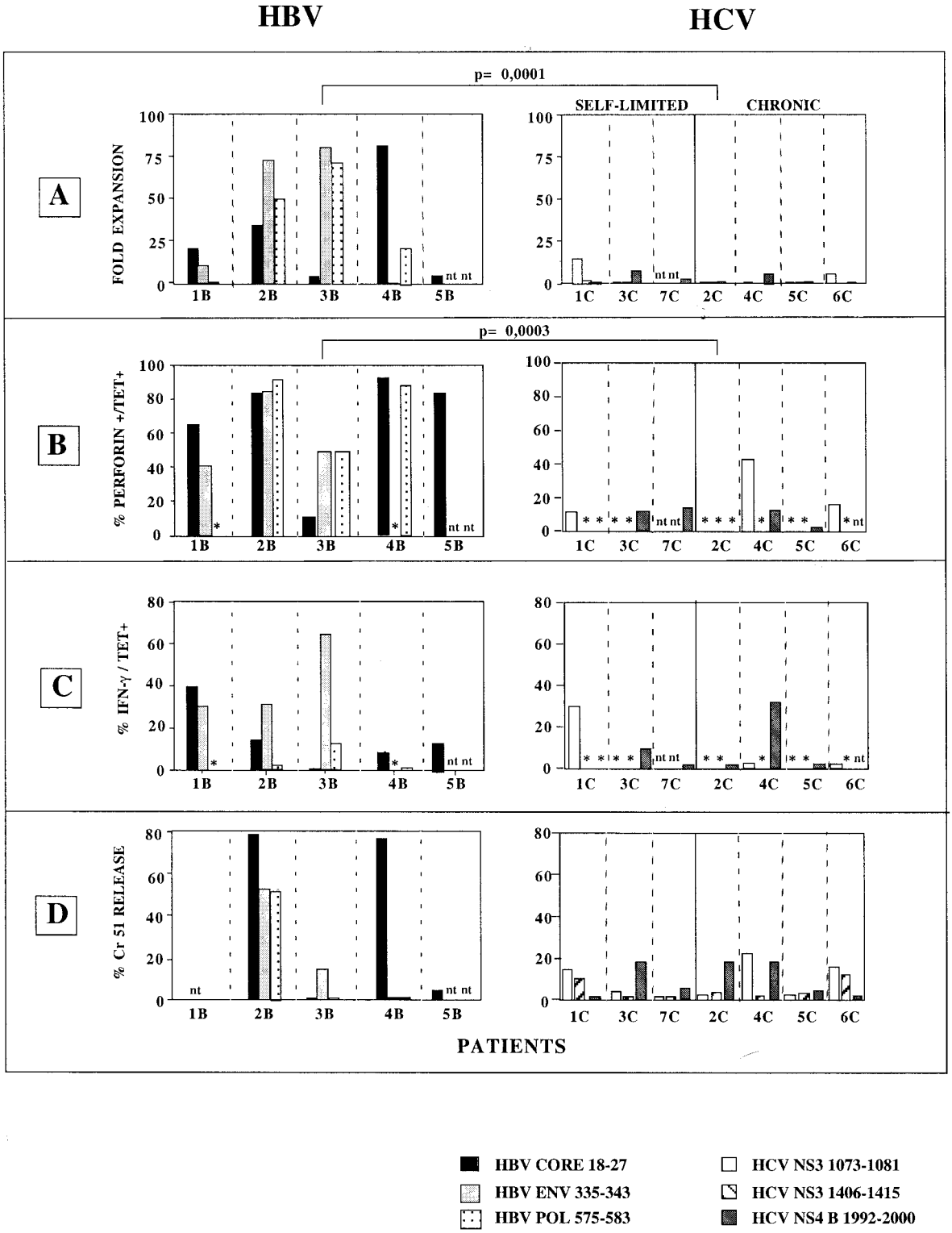
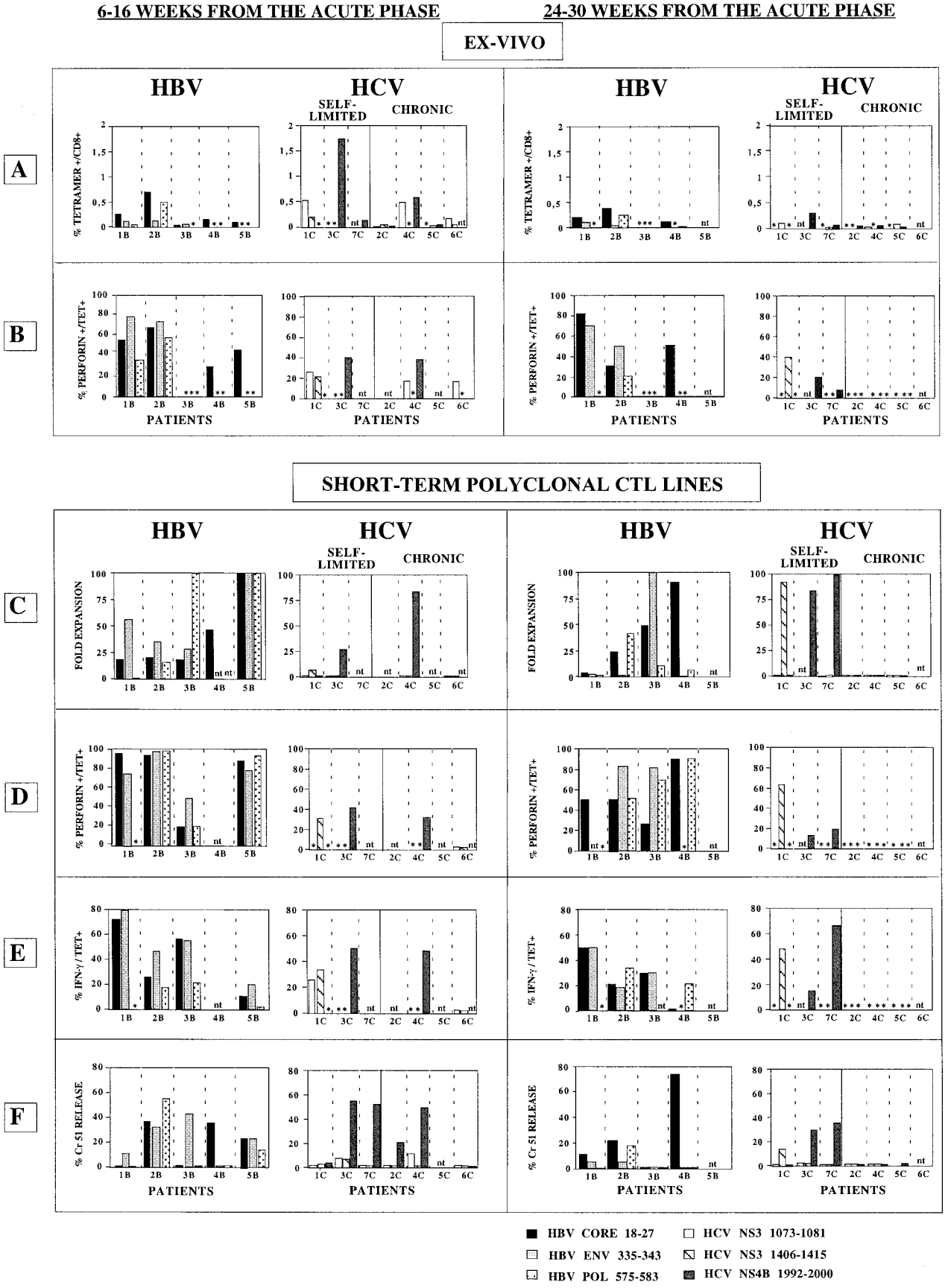


FIG. 5. (A) Capacity for expansion after 10 days of peptide stimulation of HBV and HCV tetramer-positive cells. PBMC from patients with acute HBV and HCV infection were stimulated with HBV core 18-27, envelope (ENV) 335-343, and polymerase (POL) 575-583 and with HCV NS3 1073-1081, NS3 1406-1415, and NS4B 1992-2000 peptides, respectively. Each bar represents the expansion after 10 days of stimulation. The capacity for expansion in the two groups were significantly different by Student's *t* test. (B) Analysis of the perforin contents of HBV- and HCV-specific CD8 cells from short-term polyclonal CTL lines. Each bar represents the percentage of perforin-positive cells after 10 days of peptide stimulation among the overall population of HBV- or HCV-specific CD8 lymphocytes. (C and D) Functional characterization of HBV- and HCV-specific short-term CTL lines assessed as IFN-γ production (C) and cytolytic activity (D). Virus-specific CD8 cells were analyzed after 10 days of peptide stimulation in patients with acute HBV and HCV infection. *, frequency of tetramer-positive cells was <0.01% of $CD8^+$ cells; nt, not tested.



CD8 cells detected after stimulation with the frequency observed ex vivo. The ex vivo frequency of CD8 cells specific for HBV core, envelope, and polymerase epitopes ranged from 0.03 to 1.1% of total $CD8^+$ cells (Table 3); following specific peptide stimulation, these cells were able to expand vigorously 10 to 75 times the initial values (Fig. 5A). With the exception of the polymerase epitope in patient 1B, the core epitope in patient 3B, and the envelope epitope in patient 4B, all peptides tested induced expansion of HBV-specific CD8 cells in vitro. In contrast to HBV-specific cells, HCV tetramer-positive CD8 cells expanded very poorly (five to eight times the initial values) or not at all after peptide stimulation (Fig. 5A), although they were detectable at high frequencies ex vivo. When the overall capacities of expansion of HBV- and HCV-specific CTL upon peptide stimulation were compared, a statistically significant difference was observed (Fig. 5A).

Peptide stimulation increased the expression of perforin molecules in HBV-specific cells, whereas the perforin content of HCV tetramer-positive CD8 cells did not increase substantially following specific stimuli. As shown in Fig. 5B, >40% of tetramer-positive cells derived from the different T-cell lines specific for the three HBV epitopes were perforin positive after peptide stimulation, with the exception of the core 18-27-specific CTL line of patient 3. In contrast, all HCV-specific CTL lines were defective in perforin production, since perforin-positive cells never exceeded 20% of tetramer-positive lymphocytes, with the exception of the NS3 1073-1081-specific CTL line of patient 4. Therefore, the perforin content in HBV-specific CTL was significantly higher than in HCV-specific CTL, not only ex vivo but also after peptide stimulation (Fig. 5B).

As a likely result of the better expansion capacity of HBV-specific CD8 cells, higher levels of cytolytic activity, measured by the chromium release assay, were detected in HBV-specific than in HCV-specific T-cell lines (Fig. 5C and D).

HCV-specific cells from patients with self-limited but not from those with chronically evolving hepatitis C acquire functional efficiency after the acute phase of infection. The clinical features and the immunological response of patients with HBV and HCV infection were followed over time. In particular, all individuals were tested at two sequential time points during the follow-up, 6 to 16 weeks (second time point) and 24 to 30 weeks (third time point) after clinical presentation, with the exception of the third time point in patients 4C and 7C, who were studied at 40 and 80 weeks, respectively (Table 2). All patients with acute HBV infection resolved the hepatitis, with transaminase normalization and rapid decline of viremia that was already undetectable after 6 to 13 weeks from the acute phase, with the exception of patient 3B. In contrast, four patients with acute HCV infection (patients 2C, 4C, 5C, and 6C) showed a chronic evolution, while three patients (patients 1C, 3C, and 7C) recovered from the hepatitis with ALT normalization and decline of viremia to undetectable levels (Table 2).

During the follow-up period, the frequencies of tetramer-positive cells tested ex vivo decreased or became completely negative as a function of time in all patients with acute hepatitis B and C (Fig. 6A).

While in patients with HBV infection HBV-specific cells expressed similar perforin contents (Fig. 6B) and similar functional activities upon peptide stimulation during the follow-up compared to the acute phase of infection (Fig. 6C, D, and E), patients with acute hepatitis C showed different functional behaviors in relation to the different outcomes of the disease. In both self-limited and chronically evolving HCV infections, the ex vivo frequency of tetramer-positive cells declined progressively. However, CD8 cells from patients with chronic evolution remained functionally inefficient as in the acute phase of infection, with the exception of patient 4C, who displayed a high frequency of tetramer-positive cells and a strong functional activation upon peptide stimulation that was still detectable 3 months after infection. HCV-specific cells from this patient became undetectable and unable to expand upon peptide stimulation 40 weeks after onset, even if the sequence of the epitope NS4B 1992-2000 did not change during the follow-up. In contrast to cells from patients with chronic evolution, HCV-specific cells from patients 1C, 3C, and 7C, who resolved the hepatitis, progressively improved their capacity to proliferate upon peptide stimulation to levels comparable to those of HBV-specific T cells of patients with hepatitis B. Although ex vivo CD8 frequencies were within the same range of magnitudes 24 to 30 weeks after the acute phase in groups of HCV patients with self-limited hepatitis and those with chronic evolution of disease, expansion was very vigorous (75- to 100-fold) in patients who recovered but was totally absent in patients who did not. For example, NS3 1406-1415-specific CD8 cells had the same ex vivo frequency in patients 1C (self-limited evolution) and 5C (chronic evolution), but they were able to expand 90-fold in the former but not at all in the latter. Recovery of expansion efficiency was associated with a functional improvement in terms of IFN-γ production, lytic activity, and perforin expression (Fig. 6C to F).

Can depressed CTL function in acute hepatitis C be explained by the use of peptides with inappropriate amino acid sequences? The epitope NS4B 1992-2000, corresponding to a viral region that is variable across the different genotypes, was sequenced to assess whether the peptide and the tetramer used for the study corresponded to the sequence of the prevalent infecting virus population and whether the low T-cell expansion capacity was related to the use of an analogue peptide containing amino acid variations. Patients 2C, 3C, and 4C were

FIG. 6. Follow-up analysis of HBV- and HCV-specific CD8 cell frequency and function. The analyses were performed at two different time points (6 to 16 weeks and 24 to 30 weeks after clinical presentation). (A and B) Frequencies and perforin contents of tetramer-positive HBV- and HCV-specific CD8 cells ex vivo. (C, D, E, and F) Capacities for expansion, perforin expression, IFN-γ production, and cytolytic activity after 10 days of stimulation with peptide. (C) Each bar represents the percentage of tetramer-positive cells calculated based on the total number of CD8 cells. (D and E) Each bar represents the percentage of perforin-positive and IFN-γ positive cells, respectively, among the overall population of HBV or HCV tetramer-positive CD8 lymphocytes. (F) The bars represent the percentages of specific lysis expressed by CD8 cells against peptide-pulsed target cells. *, frequency of tetramer-positive cells was <0.01% of $CD8^+$ cells; nt, not tested.

selected because their ex vivo CD8 frequencies ranged from low to very high levels and the capacity of expansion of the CD8 cells was lower than that observed in HBV-infected patients with comparable ex vivo frequencies of HBV-specific CD8 cells. No amino acid differences from the sequences of the peptides used for T-cell analysis were detected in the acute phase, showing that the ex vivo frequency and peptide stimulation ability were not lower in these HCV-infected patients because of the use of inappropriate peptides. In addition, the NS4B 1992-2000 epitopes of patients 2C and 4C, with chronic evolution, were sequenced at two different time points to assess the emergence of viral mutations during the follow-up, but no amino acid changes were observed. This indicates that the loss of reactivity against this epitope in patient 4C is not due to the effects of viral mutations.

DISCUSSION

HBV and HCV are both hepatotropic viruses that can cause acute and chronic liver diseases. The liver damage during HBV and HCV infections is believed to be mostly immune mediated (4, 7). However, acute HBV infections are generally self-limited, whereas HCV infection is usually followed by a chronic evolution (26). Therefore, comparative analyses of the phenotypic and functional features of HBV- and HCV-specific T cells during acute hepatitis B and C may provide important insights into the pathogenetic mechanisms which are involved in viral clearance and persistence in these two infections.

During the acute phase of self-limited HBV infection, a strong multispecific T-cell response is mounted and directed against many epitopes located within structural and nonstructural regions of the virus (8, 14, 24, 27, 29). Since acute symptomatic HBV infections are almost exclusively self-limited, no data are available about the features of the early T-cell responses preceding chronic evolution. Also, only limited information is available about the early cell-mediated immune events during the acute phase of HCV infection, given the asymptomatic nature of hepatitis C, which makes its diagnosis difficult. Recovery has been reported to be associated with strong, multispecific, and Th1-oriented HLA class II-restricted T-cell responses, mostly directed against nonstructural HCV proteins, which are undetectable or much weaker in patients with chronic evolution (5, 16, 23, 35). Similar observations have been made about CD8 responses, but the numbers of patients and studies are still too limited to draw definitive conclusions (10, 20, 34). The recent introduction of tetramer technology has allowed the quantification and functional characterization of virus-specific $CD8^+$ cells ex vivo (13, 19, 32). Several studies indicate that during the acute phase of hepatitis C, the frequency of tetramer-positive cells is high (9, 19, 20, 34). By applying this technology, important information about the early immune events occurring during the incubation phase of HCV infection has been obtained from subjects infected after accidental needle stick exposure (13). Interestingly, the appearance of HCV-specific CD8 cells in peripheral blood by tetramer staining followed the detection of viremia by 1 month or more. In the single patient with self-limited hepatitis, these cells were initially unable to produce IFN-γ but acquired the capacity to secrete this cytokine concurrently with a sharp decline in viremia and resolution of the disease (34). A similar transient failure of CD8 cells to secrete IFN-γ was observed by Lechner et al. (20) in a patient with self-limited hepatitis C, but long-lasting persistence of a poor antiviral cytokine response has also been reported (9).

The aim of our study was to compare phenotypic and functional features of HBV- and HCV-specific CD8 cells in the acute stage of hepatitis, when the most relevant pathogenetic immune events are likely to take place. Moreover, a longitudinal analysis of the T-cell response was performed to assess whether different outcomes of the disease are correlated with different features of the immune response. The study was focused on CD8 responses, not only in consideration of the crucial role of these cells in the control of viral infections but also because the availability of HLA-peptide tetramers allows us to define ex vivo the frequencies, the differentiation stages, and the functions of virus-specific CD8 subsets. Only circulating T cells were analyzed because liver biopsies cannot be performed during acute hepatitis for ethical reasons.

By staining virus-specific circulating $CD8^+$ cells of patients with acute hepatitis B and C with three HBV and three HCV tetramers, elevated frequencies of virus-specific $CD8^+$ cells were detected in the acute phases of both infections. However, a higher frequency was observed in the blood of subjects with acute hepatitis C. HBV- and HCV-specific CD8 cells showed the same phenotype. Both were predominantly $CCR7^-$, $CD45RA^-$, and $CD27^+$, indicating that most circulating tetramer-positive HBV- and HCV-specific CD8 cells belong, in the acute stage of infection, to a memory-effector subset (2). The analysis of other activation and differentiation markers indicated a homogeneous expression of HLA-DR and CD45RO but variable expression of the costimulatory molecule CD28. Therefore, phenotypic analysis of virus-specific CD8 cells in the acute stage of HBV and HCV infections indicates that the large majority of these cells belong to a preterminally differentiated memory-effector subset in both viral infections. No significant differences were observed during the follow-up in comparison with the earlier stages of disease, with the exception of HLA-DR, which became progressively negative after the acute phase.

Several studies of chronic HIV infection have tried to correlate the inefficient immune protection with a particular distribution of different T-cell subsets, suggesting that different CTL phenotypes correlate with different clinical outcomes (2). Here, we show that the same phenotype of HBV- and HCV-specific CD8 cells is associated with striking functional differences. First, HBV- and HCV-specific $CD8^+$ T cells were characterized for the ability to expand upon peptide stimulation. Although the frequencies of both HBV and HCV tetramer-positive cells were high ex vivo during the acute phase of hepatitis B and C, the abilities to expand after peptide stimulation were significantly different, since HBV-specific CTL were able to proliferate much more efficiently than HCV-specific T cells. This suggests that phenotypic characterization is not sufficient to define functional differences.

To more deeply investigate the functional features of virus-specific CD8 cells, we then compared the expression of perforin ex vivo and after peptide stimulation in the two groups of patients with acute hepatitis B and C. A significant difference was also observed in relation to this parameter, since perforin expression was significantly reduced in HCV-specific com-

pared to HBV-specific $CD8^+$ T cells derived from the acute stage of hepatitis B and C. The perforin content is an expression of the lytic potential of CTL. Therefore, a reduced perforin expression in HCV-specific T cells suggests that HBV- and HCV-specific CTL, although similar in phenotype, could have different efficiencies in killing virus-infected cells. HCV-specific CD8 cells actually showed poorer lytic activity upon peptide stimulation than HBV-specific CD8 cells, but no conclusions can be drawn regarding their intrinsic killing efficiencies because in the absence of expansion, cytolytic activity is expected to be low or negative.

Even if the sampling time points cannot be perfectly equivalent in patients with hepatitis B and C with respect to the course of infection, several findings support the interpretation that HBV- and HCV-specific CD8 cells in the acute phase of hepatitis are functionally different. First, levels of viremia were similarly low in both infections at the time of T-cell analysis, making it unlikely that the reported functional difference was related to different viral loads. In addition, this functional difference does not seem to be related to different stages of T-cell differentiation, because HBV- and HCV-specific CD8 cells in all patients were homogeneously $CD45RA^-$ $CCR7^-$ $CD27^+$. The only exception were CD8 cells of a single HBV-infected patient, which were mostly $CD45RA^+$ and showed a weaker proliferative activity upon exposure to the specific antigen but contained high levels of perforin, consistent with a more differentiated effector cell type. Moreover, the functional defect of HCV-specific CD8 cells cannot be explained (at least in patients 2C, 3C, and 4C for the response to NS4B 1992-2000) by the use of inappropriate synthetic peptides with amino acid variations (with respect to the sequence of the infecting virus responsible for T-cell priming) located in critical positions within the epitope and causing suboptimal T-cell activation in vitro.

Although viremia was low at the time of T-cell analysis, the depressed CTL activity in acute hepatitis C may be the result of T-cell exhaustion due to preexisting high levels of virus. Additional studies are needed to clarify whether the failure of CD8 cells to expand in vitro is due to lack of activation, to induction of activation-induced cell death, or to other mechanisms. A direct effect of HCV or HCV gene products on the CD8 function may also contribute to the depth and duration of this "anergic" condition (30). Indeed, immunomodulatory activities of HCV proteins have recently been reported (3, 22, 28, 33, 38, 39), including the interference of HCV core with proliferative and cytolytic T-cell activities through its interaction with the receptor of the C1q component of the complement cascade (17, 18, 37).

A second important finding of our study is the different behavior of the CD8 response after the acute phase of hepatitis C in patients with resolving or persistent infection. Indeed, patients with self-limited hepatitis C showed a progressive improvement of the CD8 function. Interestingly, perforin expression remained low in two of the three patients with self-limited evolution despite the recovery of efficient capacity for expansion of their CD8 cells. In contrast, CD8 cells from HCV-positive patients with chronic evolution of hepatitis remained unable to expand following peptide stimulation; as a result of this, no cytolytic activity was detectable, and tetramer-positive cells were generally too few to allow reliable evaluation of perforin content and IFN-γ expression. The only exception was patient 4C, who showed an early CD8-mediated response (with concurrent but transient disappearance of HCV RNA from the serum) that was not sustained over time. As expected, because of the self-limited nature of all HBV infections studied, the HBV-specific CTL response also remained functionally efficient during the follow-up. Persistence of a depressed CD8 function in patients with a chronic evolution of hepatitis does not necessarily represent a mechanism of virus persistence but may rather be a consequence of it. On the other hand, recovery of the CD8 function in self-limited infection is likely to be a consequence of successful virus control rather than a cause of it.

In conclusion, this study provides a picture of the phenotypic and functional features of virus-specific CTL present in the peripheral blood of patients acutely infected by two noncytopathic viruses, HBV and HCV, that share a common tropism for the liver but have different capacities to persist and to cause chronic liver damage. Acute HBV infection generally has a self-limited evolution and is associated with the presence in the blood of high frequencies of HBV-specific effector-memory CD8 cells, most of which express perforin molecules and are able to proliferate vigorously, to mount efficient CTL activity, and to produce IFN-γ. Conversely, acute HCV infection is generally asymptomatic, has a more frequent chronic evolution, and is associated with the presence in the circulation of high frequencies of virus-specific CD8 cells with phenotypes identical to those detectable in acute HBV infection. These cells have a very poor perforin content ex vivo and a significantly lower capacity to expand and subsequently to kill target cells. This defect is only transient in patients with acute self-limited HCV infection, because viral clearance is associated with recovery of the T-cell function. In contrast, the CD8 function remains persistently depressed in patients with chronic evolution of hepatitis C.

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REFERENCES

1. **Altman, J. D., P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer Williams, J. I. Bell, A. J. McMichael, and M. M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science **274:**94–96.
2. **Champagne, P., G. S. Ogg, A. S. King, C. Knabenhans, K. Ellefsen, M. Nobile, V. Appay, G. P. Rizzardi, S. Fleury, M. Lipp, R. Forster, S. Rowland-Jones, R. P. Sekaly, A. J. McMichael, and G. Pantaleo.** 2001. Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature **410:**106–111.
3. **Chen, C. M., L. R. You, L. H. Hwang, and Y. H. Lee.** 1997. Direct interaction of hepatitis C virus core protein with the cellular lymphotoxin-β receptor modulates the signal pathway of lymphotoxin-β receptor. J. Virol. **71:**9417–9426.
4. **Chisari, F. V., and C. Ferrari.** 1995. Hepatitis B virus immunopathogenesis. Annu. Rev. Immunol. **13:**29–60.
5. **Diepolder, H., R. Zachoval, R. Hoffman, E. Wierenga, T. Santorino, M. Jung, D. Eichenlaub, and G. Pape.** 1995. Possible mechanism involving T lymphocyte response to NS3 in viral clearance in acute HCV infection. Lancet **346:**1006–1007.
6. **Doherty, P. C., W. Allan, and M. Eichelberger.** 1995. Roles of αβ and γδ T cell subsets in viral immunity. Annu. Rev. Immunol. **10:**123–151.
7. **Ferrari, C., and F. V Chisari.** 2001. Immune mechanisms of viral clearance and disease pathogenesis during viral hepatitis, p. 763–782. *In* I. M. Arias, J. L. Boyer, F. V. Chisari, N. Fausto, D. Schachter, and D. A. Shafritz (ed.),

The liver: biology and pathobiology. Lippincott Williams & Wilkins, Philadelphia, Pa.
8. **Ferrari, C., A. Penna, A. Bertoletti, A. Valli, A. Degli Antoni, T. Giuberti, A. Cavalli, M. A. Petit, and F. Fiaccadori.** 1990. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J. Immunol. **145:**3442–3449.
9. **Gruener, N. H., F. Lechner, M. C. Jung, H. Diepolder, T. Gerlach, G. Lauer, B. Walker, J. Sullivan, R. Phillips, G. R. Pape, and P. Klenerman.** 2001. Sustained dysfunction of antiviral $CD8^+$ T lymphocytes after infection with hepatitis C virus. J. Virol. **75:**5550–5558.
10. **Gruener, N. H., J. T. Gerlach, M. C. Jung, H. M. Diepolder, C. A. Schirren, W. W. Schraut, R. Hoffmann, R. Zachoval, T. Santantonio, M. Cucchiarini, A. Cerny, and G. R. Pape.** 2000. Association of hepatitis C virus-specific CD8+ T cells with viral clearance in acute hepatitis C. J. Infect. Dis. **117:** 933–941.
11. **Guidotti, L. G., and F. V. Chisari.** 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol. **19:**65–91.
12. **Hamann, D., M. T. L. Roos, and R. A. W. Van Lier.** 1999. Faces and phases of human CD8+ T-cell development. Immunol. Today **20:**177–180.
13. **He, X. S., B. Rehermann, F. X. Lopez-Labrador, J. Boisvert, R. Cheung, J. Mumm, H. Wedemeyer, M. Berenguer, T. L. Wright, M. M. Davis, and H. B. Greenenberg.** 1999. Quantitative analysis of hepatitis C virus-specific CD8+ T cells in peripheral blood and liver using peptide-MHC tetramers. Proc. Natl. Acad. Sci. USA **96:**5692–5697.
14. **Jung, M. C., U. Spengler, W. Schraut, R. Hoffmann, R. Zachoval, J. Eisenburg, D. Eichenlaub, G. Riethmuller, G. Paumgartner, and H. W. Ziegler-Heitbrock.** 1991. Hepatitis B virus antigen-specific T-cell activation in patients with acute and chronic hepatitis B. J. Hepatol. **13:**310–317.
15. **Kagi, D., B. Lederman, K. Burki, R. M. Zinkernagel, and H. Hengartner.** 1996. Molecular mechanism of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu. Rev. Immunol. **14:**20–25.
16. **Kamal, S. M., J. W. Rasenack, L. Bianchi, A. Al Tawil, K. El Sayed Khalifa, T. Peter, H. Mansour, W. Ezzat, and M. Koziel.** 2001. Acute hepatitis C without and with schistosomiasis correlation with hepatitis C-specific CD4+ T-cell and cytokine response. Gastroenterology **121:**646–656.
17. **Kittlesen, D. J., K. A. Chianese-Bullock, Z. Q. Yao, T. J. Braciale, and Y. S. Hahn.** 2000. Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. J. Clin. Investig. **106:**1239–1249.
18. **Large, M. K., D. J. Kittlesen, and Y. S. Hahn.** 1999. Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. J. Immunol. **162:**1239–1241.
19. **Lechner, F., N. H. Gruener, S. Urbani, J. Uggeri, T. Santantonio, A. R. Kammer, A. Cerny, R. Phillips, C. Ferrari, G. R. Pape, and P. Klenerman.** 2000. CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. Eur. J. Immunol. **30:**2479–2487.
20. **Lechner, F., D. K. H. Wong, P. R. Dunbar, R. Chapman, R. T. Chung, P. Dohenwend, G. Robbins, R. Phillips, P. Klenerman, and B. D. Walker.** 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. J. Exp. Med. **191:**1499–1512.
21. **Maini, M. K., C. Boni, G. S. Ogg, A. S. King, S. Reignat, C. K. Lee, J. R. Larrubia, G. J. Webster, A. J. McMichael, C. Ferrari, R. Williams, D. Vergani, and A. Bertoletti.** 1999. Direct ex vivo analysis of hepatitis B virus-specific CD8+ T cells associated with the control of infection. Gastroenterology **117:**1386–1396.
22. **Matsumoto, M., T. Y. Hsieh, N. Zhu, T. Van Arsdale, S. B. Hwang, K. S. Jeng, A. E. Gorbalenya, S. Y. Lo, J. H. Ou, C. F. Ware, and M. M. Lai.** 1997. Hepatitis C virus core protein interacts with the cytoplasmic tail of lymphotoxin-B receptor. J. Virol. **71:**1301–1305.
23. **Missale, G., R. Bertoni, V. Lamonaca, A. Valli, M. Massari, C. Mori, M. Rumi, M. Houghton, F. Fiaccadori, and C. Ferrari.** 1996. Different clinical behaviors of acute HCV infection are associated with different vigor of the antiviral T cell response. J. Clin. Investig. **98:**706–714.
24. **Nayersina, R., P. Fowler, S. Guilhot, G. Missale, A. Cerny, H. J. Schlicht, A. Vitiello, R. Chesnut, J. L. Person, and A. G. Redeker.** 1993. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J. Immunol. **150:**4659–4671.
25. **Ogg, G. S., and A. J. McMichael.** 1998. HLA-peptide tetrameric complex. Curr. Opin. Immunol. **10:**393–396.
26. **Orland, J. R., T. Wright, and S. Cooper.** 2001. Acute hepatitis C. Hepatology **33:**321–327.
27. **Penna, A., F. V. Chisari, A. Bertoletti, G. Missale, P. Fowler, T. Giuberti, F. Fiaccadori, and C. Ferrari.** 1991. Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J. Exp. Med. **174:**1565–1570.
28. **Ray, R. B., K. Meyer, R. Steele, A. Shrivastava, B. B. Aggarwal, and R. Ray.** 1998. Inhibition of tumor necrosis factor (TNFα)-mediated apoptosis by hepatitis C virus core protein. J. Biol. Chem. **273:**2256–2259.
29. **Rehermann, B., P. Fowler, J. Sidney, J. Person, A. Redeker, M. Brown, B. Moss, A. Sette, and F. V. Chisari.** 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J. Exp. Med. **181:**1047–1058.
30. **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.
31. **Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia.** 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature **401:**708–712.
32. **Sobao, Y., H. Tomiyama, S. Nakamura, S. Hisahiko, K. Tanaka, and M. Takiguchi.** 2001. Visual demonstration of hepatitis C virus-specific memory CD8+ T-cell expansion in patients with acute hepatitis C. Hepatology **33:** 287–294.
33. **Taylor, D. R., S. T. Shi, P. R. Romano, G. N. Barber, and M. M. Lai.** 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science **285:**107–110.
34. **Thimme, R., D. Oldach, K. M. Chang, C. Steiger, S. C. Ray, and F. V. Chisari.** 2001. Determinant of viral clearance and persistence during acute hepatitis C virus infection. J. Exp. Med. **19:**1395–1406.
35. **Tsai, S. L., Y. F. Liaw, M. H. Chen, C. Y. Huang, and G. C. Kuo**. 1997. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology **25:**449–458.
36. **Webster, G. J., S. Reignat, M. K. Maini, S. A. Whalley, G. S. Ogg, A. King, D. Brown, P. L. Amlot, R. Williams, D. Vergani, G. M. Dusheiko, and A. Bertoletti.** 2000. Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology **32:**1117–1124.
37. **Yao, Z. Q., D. T. Nguyen, A. I. Hiotellis, and Y. S. Hahn.** 2001. Hepatitis C virus core protein inhibits human T lymphocyte response by a complement-dependent regulatory pathway. J. Immunol. **167:**5264–5272.
38. **You, L. R., C. M. Chen, and Y. H. Wu Lee.** 1999. Hepatitis C virus core protein enhances NF-κB signal pathway triggering by lymphotoxin-β receptor ligand and tumor necrosis factor alpha. J. Virol. **73:**1672–1681.
39. **Zhu, N., A. Khoshnan, A. Schneider, M. Matsumoto, G. Dennert, C. Ware, and M. M. Lai.** 1998. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. J. Virol. **72:**3691–3697.