MAJOR ARTICLE

Clearance of Hepatitis C Viremia Associated with Cellular Immunity in the Absence of Seroconversion in the Hepatitis C Incidence and Transmission in Prisons Study Cohort

Jeffrey J. Post,[1] Yong Pan,[8] Anthony J. Freeman,[9] Charles E. Harvey,[9] Peter A. White,[2,8] Patricia Palladinetti,[1] Paul S. Haber,[5] George Marinos,[9] Michael H. Levy,[6] John M. Kaldor,[7] Kate A. Dolan,[3] Rosemary A. Ffrench,[4,10] Andrew R. Lloyd,[1] William D. Rawlinson,[1,2,8] and the Hepatitis C Incidence and Transmission in Prisons Study (HITS) Group[a]

[1]Department of Pathology, School of Medical Sciences, [2]School of Biochemistry and Biomedical Sciences, [3]National Drug and Alcohol Research Centre, and [4]School of Women's and Children's Health, University of New South Wales, and [5]Drug and Alcohol Department, Royal Prince Alfred Hospital, Sydney, [6]Corrections Health Service, Matraville, [7]National Centre in HIV Epidemiology and Clinical Research, Darlinghurst, and [8]Virology Division, Department of Microbiology, South Eastern Area Laboratory Services, [9]Viral Hepatitis Research Unit, Gastrointestinal and Liver Unit, Prince of Wales Hospital, and [10]Department of Immunology and Infectious Diseases, Sydney Children's Hospital, Randwick, Australia

Understanding the earliest virological and immunological events in acute hepatitis C virus (HCV) infection may provide insight into the determinants of protective immunity. Four cases of HCV viremia with subsequent viral clearance, but without biochemical hepatitis or anti-HCV seroconversion, are reported from a prospective cohort study of prison inmates. Two of the subjects who developed sustained viremia were assessed for production of interferon (IFN)–γ, by use of the enzyme-linked immunospot (ELISPOT) method and by assessment of HCV cytotoxic T lymphocyte (CTL) activity, CD4 lymphocyte proliferative responses, HCV load, and genotype. After 2–6 months of viremia, all 4 subjects cleared serum HCV RNA. Specific cellular responses were detected in both of the subjects who were assessed, and production of IFN-γ was demonstrated in one subject. All subjects had weak, but consistent, serological reactivity against HCV nonstructural proteins on immunoblot testing, despite repeatedly nonreactive HCV ELISA tests. These cases highlight the potential for cellular immune responses against HCV to facilitate viral clearance, responses that may model those required for effective HCV vaccination.

The natural history of primary hepatitis C virus (HCV) infection has previously been defined in posttransfusion hepatitis [1–3], in cases of HCV infection coming to clinical attention [4–6], and in cross-sectional studies of individuals positive for anti-HCV antibodies [7, 8]. On the basis of these data, persistence of HCV infection is thought to occur in up to 86% of infected individuals. However, long-term follow-up studies have suggested that ~50% of persons infected with HCV may clear the virus [9–13]. Studies based on serological or biochemical case definitions inevitably underestimate the rate of viral clearance if viremia without seroconversion or

Received 6 July 2003; accepted 3 November 2003; electronically published 26 April 2004.

Reprints or correspondence: Dr. Jeffrey J. Post, Inflammation Research Unit, Dept. of Pathology, School of Medical Sciences, University of New South Wales, Sydney, NSW 2052, Australia (j.post@unsw.edu.au).

The Journal of Infectious Diseases 2004;189:1846–55
© 2004 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2004/18910-0008$15.00

Presented in part: Annual Conference of the Australasian Society for Infectious Diseases, Leura, New South Wales, Australia, 15–19 2000 (abstract 26); Annual Conference of the Australasian Society for HIV Medicine, Melbourne, Victoria, Australia, 4–7 October 2001 (abstract 50); 3rd Australasian Hepatitis C Conference, Melbourne, Victoria, Australia, 24–27 March 2002 (oral abstract 170); 11th International Symposium on Viral Hepatitis and Liver Disease, Sydney, Australia, 6–10 April 2003 (abstract session WF-6, presentation 5).

Financial support: National Health and Medical Research Council project (grant 990657); New South Wales Department of Health; National Health and Medical Research Council (Medical Postgraduate Research Scholarships to J.J.P. and A.J.F. and Dora Lush Postgraduate Research Scholarship to C.E.H.).

[a] Study group members are listed after the text.

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

biochemical hepatitis occurs. Furthermore, cross-sectional studies may overestimate the proportion of HCV infections that progress to chronicity, because complete seroreversion after clearance of HCV infection is also well recognized [14–16].

The immune response against HCV is characterized by the generation of HCV-specific antibodies, cytotoxic T lymphocytes (CTLs), and CD4 lymphocytes and by production of interferon (IFN)–γ [17]. The importance of each of these components of the immune response, with regard to clearance of HCV infection, is not clear, because all have been demonstrated in individuals with chronic infection [17–20]. However, in individuals with acute HCV infection, viral clearance has been associated with strong multispecific CD4 lymphocyte and CTL responses [4, 21–26]. In a limited number of cases, the generation of IFN-γ from CD8 T lymphocytes has been associated with a reduction in the HCV load [27]. Given that there is no preventative vaccine and that the treatment of chronic HCV infection is imperfect [28], a better understanding of the effective host immune response in individuals with acute HCV infection is required.

Prisoners are at risk for HCV infection, with a high prevalence of HCV recorded among individuals entering prison [29–33], and transmission of HCV in prison is well documented [33–36]. The present report details the outcomes for 4 subjects who developed HCV infection without seroconversion and who were identified in the Hepatitis C Incidence and Transmission in Prisons Study (HITS), a prospective cohort study of Australian prison inmates. All case patients eliminated HCV viremia without biochemical or clinically apparent hepatitis. This newly recognized phenomenon challenges the current understanding of the natural history of primary HCV infection in high-risk populations.

SUBJECTS, MATERIALS, AND METHODS

Subjects. Adult male prison inmates >18 years of age were eligible for inclusion in the HITS cohort if they were negative for anti-HCV antibodies, were able to give informed consent, and were incarcerated full time in New South Wales, Australia. Subjects were excluded from the cohort if they were severely mentally ill, if they were forensic inmates (i.e., they were not able to stand trial by reason of mental illness), or if they had a prior positive or indeterminate result of testing for the presence of anti-HCV antibody. The subjects who were enrolled in the study were prospectively followed, at monthly intervals, with a structured interview and sampling of blood for detection of HCV RNA by reverse-transcriptase polymerase chain reaction (RT-PCR).

The structured interview recorded demographic data, potential risk factors for bloodborne communicable diseases (including injection drug use [IDU], tattooing, body piercing, sexual activity, medical interventions, and physical violence), and symptoms potentially associated with HCV infection. Subjects who were negative for anti-HCV antibodies and serum HCV RNA at baseline but who subsequently had HCV viremia detected were defined as case patients with incident HCV (i.e., "incident patients"). Incident patients underwent clinical assessment by a physician and were followed, where possible, on a weekly basis for 6 weeks, every 2 weeks until week 24, and, then, at ~3-month intervals thereafter. Individuals who were negative for anti-HCV antibodies but positive for serum HCV RNA at enrollment were excluded from the cohort, but they were followed monthly until viral clearance or seroconversion was documented. Control subjects for the immunological assays were selected from outside the prison population; they reported no risk factors for HCV infection and were negative for anti-HCV antibodies and serum HCV RNA.

The present study was approved by the human research ethics committees of the University of New South Wales (Sydney, New South Wales, Australia), the Corrections Health Service (Matraville, New South Wales, Australia), and the Department of Corrective Services (Sydney, New South Wales, Australia). All participants provided written, informed consent.

Samples. Blood samples were transported at room temperature. Samples obtained at screening were stored at 4°C on arrival at the laboratory, before undergoing separation, which was usually performed within 6 h of the time that the samples were obtained. This has been demonstrated to have minimal effect on the quantitation of the HCV load in serum [37, 38]. All subsequent serum samples obtained from prospectively identified case patients were separated and frozen on arrival at the laboratory and were received within 4 h of the time that they were obtained. Archival serum samples stored at −20°C were obtained with the individual case patients' permission and were tested for HCV RNA, alanine aminotransferase (ALT), and anti-HCV antibody, to screen for HCV infection that may have occurred before enrollment in the HITS cohort.

Serologic testing. Serum samples obtained at baseline were analyzed using a third-generation HCV ELISA (Murex anti-HCV III; Murex Biotech), and subjects with positive results were excluded from additional follow-up. Supplemental testing of serum samples obtained subsequently from incident patients was performed using Innotest HCV Ab III (Innogenetics) and IMx HCV antibody assay (version 3.0; Abbott Diagnostics). HCV immunoblot assays were performed using Wellcozyme HCV Western Blot (Murex Biotech).

Virologic testing. Serum samples were separated, and aliquots were stored at −80°C and were thawed once, immediately before use. Total RNA was extracted either from 100 μL of serum (for the qualitative RT-PCR assay) or 200 μL of serum (for the quantitative RT-PCR assay), by use of a modified method of Chomczynski [39]. The qualitative RT-PCR result and the HCV

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

genotype were determined by amplifying and sequencing the 5′-untranslated region (5′-UTR), respectively [39].

Real-time quantitative RT-PCR was performed using SYBR Green I detection of products in a LightCycler, as described elsewhere [40]. Nested RT-PCR was used, with an initial 1-step RT-PCR and 15 cycles of PCR. The HCV load was then measured by real-time PCR performed with the use of inner primers hep21b and hep22 [39]. Reaction mixtures contained 2 μL of Fast Start DNA Master SYBR Green I, 4 mmol/L $MgCl_2$, 0.5 μmol/L each primer, and 2 μL of template in a total volume of 20 μL. After initial incubation was performed for 10 min at 95°C, 40 cycles of LightCycler PCR were performed for 15 s at 95°C, annealing was performed for 5 s at 60°C, and extension was performed for 10 s at 72°C. Fluorescence was monitored at 530 nm, and the specificity of the signal was checked by a melting-curve analysis. A standard curve was calculated using a series of 10-fold dilutions of quantified plasmid DNA that contained cloned 5′-UTR PCR product. The limit of detection of the virus load assay was 10 copies/reaction tube, which was equivalent to 500 copies/mL of serum.

ELISPOT assay for HCV-specific IFN-γ–producing effector cells. Peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation and were cryopreserved. Autologous Epstein-Barr virus (EBV)–immortalized B lymphoblastoid cell lines (BLCLs) were established [41] and were infected with recombinant vaccinia virus (vv) constructs that encoded HCV genotype 1a proteins as antigen-presenting cells. The entire HCV-H genotype 1a polyprotein was encoded in 2 overlapping constructs (Core-nonstructural [NS] 2 [aa 1–966] and NS2–NS5 [aa 827–3011], a gift from C. E. Rice, Washington University, St. Louis, MO) [42]. ELISPOT assays for the production of IFN-γ were performed according to standard procedures [43, 44]. Controls included vv-Lac (National Institute of Health AIDS Reagent program), PBMCs cocultured with uninfected BLCLs, and PBMCs cultured either in media alone or with phytohemagglutinin (PHA; 10 μg/mL). Spots were counted using an ELISPOT analyzer (AutoImmun). Results represent the mean of duplicate wells, expressed as IFN-γ spot-forming units (sfu)/10^6 cells. In all assays designated as valid, 2×10^5 PBMCs demonstrated >500 sfu in response to PHA, the number of spot-forming units reduced with reducing cell numbers, and the intra-assay variance was low (designated by an SEM that was <20%). The number of HCV-specific spot-forming units was calculated using the following formula: number of spots $\times$ (vv-HCV − vv-Lac).

In vitro restimulation and chromium-release assay for HCV-specific CTL activity. In vitro restimulation and use of a standard 6-day chromium-release assay for HCV-specific CTL activity were performed as described elsewhere [45]. Chromium-labeled BLCLs that were infected with recombinant vv-HCV constructs were used as the target cell (T) population, with 10^3 "hot" targets/well. Non–chromium-labeled "cold" targets infected with vv-Lac were used to inhibit reactivity to EBV and vv (ratio of cold targets to hot targets, 50:1). Effector cells (E) from bulk culture were serially diluted from 10^5 cells/well (initial E:T, 100:1), in a 96-well U-bottomed microtiter plate. The percentage of specific lysis was calculated using the following formula: (release in assay − spontaneous release)/(maximum release − spontaneous release) $\times$ 100. In all assays designated as being valid, labeling of 0.1–1 count/min/cell was achieved, spontaneous release was <20% of maximum release, specific lysis reduced with a reducing E:T cell ratio, and the SEM was <10%. Chromium-labeled targets that were infected with vv-Lac were used to evaluate background reactivity against EBV and vv antigens.

HCV-specific CD4 lymphocyte proliferative responses. HCV recombinant antigens derived from genotype 1a (Chiron) were used [20]. These antigens included c22 (aa 2–120), c100-3 (aa 1569–1931), c200 (aa 1192–1931), and NS5 (aa 2054–2995). Recombinant superoxide dismutase and yeast extracts were used as controls for nonspecific proliferation.

Thawed PBMCs were washed 3 times in medium (RPMI 1640; Gibco Laboratories) that contained 5% pooled human AB serum ($RPMI^{AB}$) and then were stained with the intracellular fluorescent dye 5-(and -6)–carboxy fluorescein diacetate succinimidyl ester (CFSE) [46]. Proliferation assays were performed in 96-well U-bottomed microtiter plates. PBMCs (2×10^5 cells) were added to each well in 0.2 mL of $RPMI^{AB}$ and were cultured in the presence and absence of HCV recombinant proteins at final concentrations of 0.1, 1, and 10 μg/mL. PHA and tetanus toxoid were used as positive control and recall antigen, respectively. Cells were harvested on day 6, were washed, and were stained for CD3 allophycocyanin and CD4 peridinin chlorophyll protein (Becton Dickinson) for 15 min at room temperature. The cells were washed and fixed in 0.5 mL of 2% paraformaldehyde, and proliferative responses were analyzed using flow cytometry.

Specific fluorescence intensity was determined using a FACS-Calibur flow cytometer (Becton Dickinson) with the use of Cell Quest software. Lymphocytes and blast cells were first gated according to forward and side-scatter parameters and, secondly, by CD3 and CD4 expression. Proliferation was detected by a decrease in CFSE fluorescence relative to unstimulated cells. Proliferation was considered to be positive when the simulation index (i.e., the percentage of $CD3^+/CD4^+$ cells proliferating in the presence of antigen divided by the percentage of $CD3^+/CD4^+$ cells proliferating in the absence of antigen) was $\geqslant$3. Samples obtained from 10 uninfected control subjects without risk factors for HCV infection were used to determine background reactivity in cellular assays.

Timeline. For the purpose of analysis, week 0 (i.e., the putative onset of viremia) was designated as the midpoint between the date that the last negative result of testing for HCV

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

RNA was obtained and either the date that the subsequent positive sample was obtained, for incident patients, or the date of the enrollment visit, for patients who had viremia at baseline. The week number for archival serum samples (i.e., the samples obtained before week 0 or study enrollment) was designated with a minus sign.

Statistics. Data were analyzed using Stata (version 7.0; Stata). Data are presented as the mean ± SD.

RESULTS

Case patients. In the main phase of the study, 193 subjects were enrolled up to August 2002; of these subjects, 33 were excluded from the study because they were anti-HCV positive at baseline. One case patient with clinically apparent hepatitis and anti-HCV seroconversion was identified in the prison during the pilot phase of the present study and is not included in the report [34]. Forty-three subjects were not followed (1 had no baseline result, 1 was too dangerous, 28 were released to freedom before the first follow-up visit occurred, 11 were not yet due for follow-up at the time of this analysis, and 2 revoked consent before the first follow-up visit). Therefore, 117 subjects had ⩾1 follow-up visit. Three individuals fulfilled the definition of an incident patient (case patients 1 and 2, and a third subject who had detectable viremia at a single time point only and who was not analyzed in detail), as did 1 additional subject who was anti-HCV seronegative, yet viremic, at enrollment (case patient 3). The incident patients did not experience any symptoms that were potentially related to hepatitis, and they had serum levels of ALT that were persistently in the range considered to be normal, both during the period of viremia and in archival samples (which were available for case patient 1 at weeks −45, −15, and −4 and for case patient 2 at weeks −239 and −114). For case patient 1, the ALT level range was 10–28 U/L (normal range, 10–63 U/L) at all time points, except week 19, when it increased to 43 U/L. For case patient 2, the ALT level range was 7–31 U/L, with all measurements <20 U/L, except at weeks 20 and 33. Case patient 3 had the ALT level measured on 3 occasions while he had viremia, and the ALT level range was 12–18 U/L.

Case patient 1 reported a long history of IDU. Case patients 2 and 3 reported drug use, but not IDU. Case patient 2 had been in several fights that involved blood-to-blood contact within the prison system, and, at a subsequent physical examination, he was noted to have injection marks in the cubital fossae, consistent with undisclosed IDU. Case patient 3 reported prior tattooing and body piercing in the baseline interview. None of the case patients had clinical evidence of immunodeficiency, although case patient 1 had type 1 diabetes mellitus. Case patients 1–3 had detectable anti–hepatitis B virus surface antibody (case patient 1 also had anti–hepatitis B core antibodies that indicated natural infection; case patients 2 and 3 had serological responses to hepatitis B virus immunization), and they were also anti-HIV negative.

Virologic findings. HCV viremia was detected in >1 sample consecutively obtained from case patients 1–3, who had subsequent clearance of viremia in a pattern suggestive of primary infection. The HCV genotypes (3a, 1a, and 1a) did not change on testing of successive samples (figure 1). The peak virus load of the 2 incident patients was 40,000 and 27,000 HCV RNA copies/mL, occurring at weeks 10 and 6, respectively. In comparison, in a case patient with acute HCV associated with seroconversion, the peak virus load, which was tested using the same methodology, was 1.4×10^7 HCV RNA copies/mL. Archival serum samples were HCV RNA negative at week −45, for case patient 1, and at week −160, for case patient 2.

Serologic findings. All case patients were found to be anti-HCV negative in the prospective phase of the study, by use of third-generation HCV ELISAs. All ratios of optical density to the cutoff value were <0.30. All assays were valid, according to the manufacturer's instructions, with positive and negative control samples as provided. The same assay kit provided positive results for other samples from patients with chronic HCV infection. Archival serum samples from both incident patients were also documented to be anti-HCV negative (for case patient 1, at weeks −45, −15, and −4; for case patient 2, at weeks −239, −169, −160, −114, and −84). However, HCV immunoblot analysis revealed weak antibody responses against nonstructural viral proteins at weeks 10, 40, 50, and −45, for case patient 1, at weeks 6, 33, 46, and −160, for case patient 2, and at week 53, for case patient 3 (figure 2). These indeterminate responses did not change during viremia, during follow-up, or in comparison with archival samples (data not shown). Rheumatoid factor was measured at <20 IU/mL for case patient 1 at week 40 and for case patient 2 at week 33.

Cellular immune response. In control subjects who were free of risk factors for HCV infection, the ELISPOT assay for production of IFN-γ yielded results of 50 ± 150 HCV-specific sfu/10^6 PBMCs. The ratio of spot-forming units produced in the presence of the vv-HCV constructs to spot-forming units produced in the presence of vv-Lac was 1.0 ± 0.5. Accordingly, study samples were considered to be positive if >350 HCV-specific sfu/10^6 PBMCs were produced (mean of control subjects + 2 SD) and if the number of spot-forming units in the presence of the vv-HCV constructs exceeded the number of spot-forming units in the presence of vv-Lac by a factor of at least 2. The background lysis of vv-Lac–infected targets in the CTL assay was 8% ± 6%, at an E:T ratio of 100:1. HCV-specific lysis from subjects free of risk factors for HCV infection was 13% ± 5%. Study samples were considered to be positive if lysis was >12% (+2 SD) above background lysis and >23% with regard to absolute percentage (mean of control subjects

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

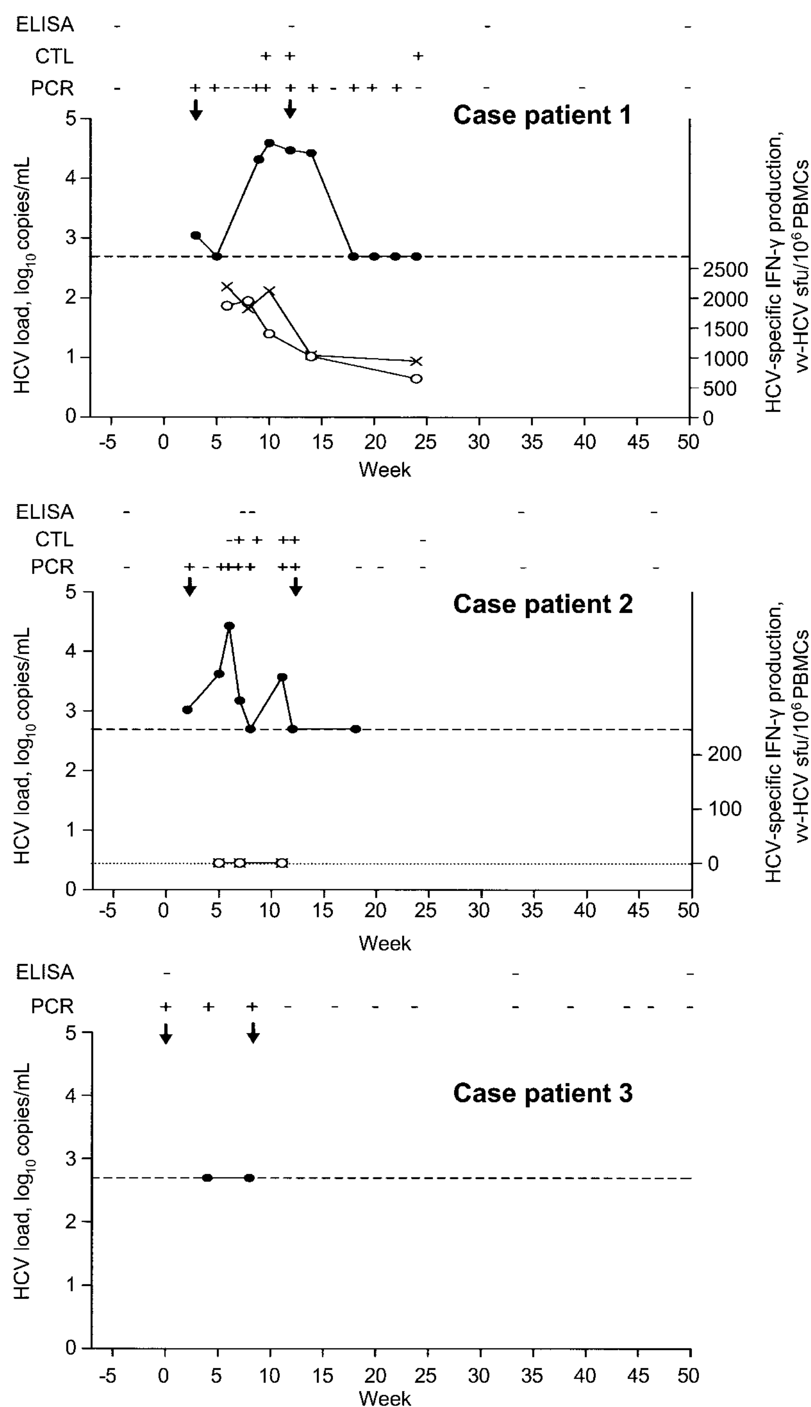

Figure 1. Details of 3 cases of acute hepatitis C virus (HCV) infection. The HCV genotypes were 3a, for case patient 1, and 1a, for case patients 2 and 3. HCV-specific interferon (IFN)–γ production against core-NS2 (○) and NS2–NS5 (×) constructs are shown as the no. of spot-forming units (sfu) of HCV-specific IFN-γ production, as measured by ELISPOT assay. Samples were considered to be positive if >350 HCV-specific sfu/10^6 peripheral blood mononuclear cells (PBMCs) were produced, and if the no. of spot-forming units in the presence of the vaccinia virus (vv)–HCV constructs exceeded the no. of spot-forming units in the presence of vv-Lac by a factor of at least 2. Results for which the difference between the HCV-specific and nonspecific responses was <0 were designated as 0. Cytotoxic T lymphocyte (CTL) responses are shown to be positive if a response was detected against either construct. Samples were considered to be positive if lysis was >12% above background lysis and >23% with regard to absolute percentage. ELISA, anti-HCV antibody assay by third-generation ELISA, as described in Subjects, Materials, and Methods; PCR, detection of serum HCV RNA, as determined by an in-house qualitative reverse-transcriptase polymerase chain reaction (PCR) assay, as described in Subjects, Materials, and Methods. ●, HCV load; *dashed line,* the cutoff of the virus load assay (500 HCV RNA copies/mL); *arrows,* time points when HCV genotyping was undertaken.

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

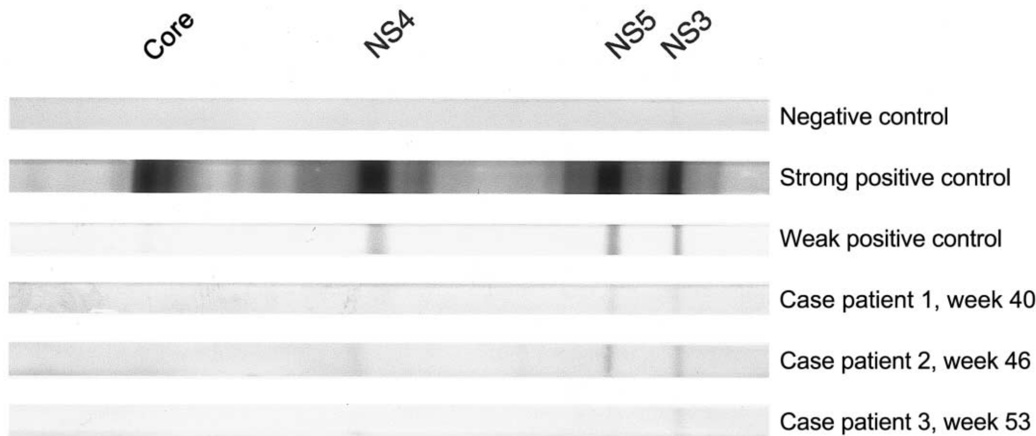

Figure 2. Representative samples of hepatitis C virus (HCV) immunoblot assays. Wellcozyme HCV Western Blot (Murex Biotech) was performed according to the manufacturer's instructions. Indeterminate antibody responses (responses weaker than that of the weak positive control) against nonstructural regions of HCV were demonstrated for all 3 case patients. The intensity of bands was similar at all time points studied (weeks 10, 40, 50, and −45 for case patient 1; weeks 6, 33, 46, and −160 for case patient 2; and week 53 for case patient 3; data not shown).

+ 2 SD). The range of magnitude of the positive HCV-specific responses was 25%–34% specific lysis. The background stimulation index (SI) in the proliferation assay, in the presence of superoxide dismutase, was 1.0 ± 0.6. The SI for control subjects free of risk factors for HCV infection was 1.6 ± 0.7. Accordingly, study samples were considered to be positive if the SI was greater than 1.4 (+2 SD) above background lysis and was >3.0 (mean of control subjects + 2 SD).

The cellular immune response of case patient 1 featured consistently positive results of ELISPOT assays for production of IFN-γ against both vv-HCV constructs during the period of detectable viremia. These responses progressively decreased during viral clearance (figure 1). Between weeks 6 and 24, the number of HCV-specific sfu/10^6 PBMCs decreased from 1870 to 650 sfu/10^6 PBMCs and from 2190 to 940 sfu/10^6 PBMCs in the vv-Core-NS2 and vv-NS2-NS5 wells, respectively. Case patient 1 also demonstrated HCV-specific CTL activity against the vv-NS2-NS5 construct, but not against vv-Core-NS2. HCV-specific CD4 lymphocyte responses were detected against core, c200, and NS5 (figure 3).

In contrast, case patient 2 demonstrated no HCV-specific IFN-γ ELISPOT responses. CTL activity was initially undetectable at week 6, but it became consistently positive between weeks 7 and 12 and then became undetectable at week 24. Strong HCV-specific CD4 proliferative responses were detected at all time points tested (figure 3). Unlike case patient 1, case patient 2 also had CD4 proliferative responses against c100, and the peak response against c100 correlated with the resolution of viremia (at week 12), with a shift in the dose-response curve, such that the response at this time point occurred at a 10-fold lower concentration of antigen. HCV-specific CD4 lymphocyte responses persisted beyond clearance of viremia in both case patients.

DISCUSSION

This is the first report to prospectively study high-risk subjects for the development of acute HCV infection by use of a nucleic-acid detection system with frequent blood sampling. There are no other reports that have demonstrated sustained viremia without seroconversion or biochemical hepatitis with subsequent viral clearance. HCV viremia without seroconversion has been recognized in immunocompetent individuals, but these reports are limited by the use of less sensitive serological assays or a limited duration of follow-up [47–55]. This phenomenon has also been reported in HIV-infected individuals and in subjects with hypogammaglobulinemia [56, 57]. Of importance, no previous studies have demonstrated sustained HCV viremia without seroconversion, by use of a sensitive third-generation ELISA in a high-risk population. The findings indicate that transient viremia (viremia lasting weeks to months) without anti-HCV seroconversion may be unrecognized in populations at high risk for HCV infection. Therefore, a potentially unrecognized, but transiently infectious, population with HCV infection may exist. These cases would not be detected clinically, biochemically, or serologically, and, therefore, anti-HCV antibody–negative blood samples from high-risk individuals should be considered to be potentially infectious. Seroconversion may be abrogated in case patients with acute HCV infection treated with IFN-α [58, 59], but none of the case patients who we have described received this treatment. It is possible that the phenomenon reported in the present study represents very low-level chronic viremia without hepatitis or seroconversion. Such

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

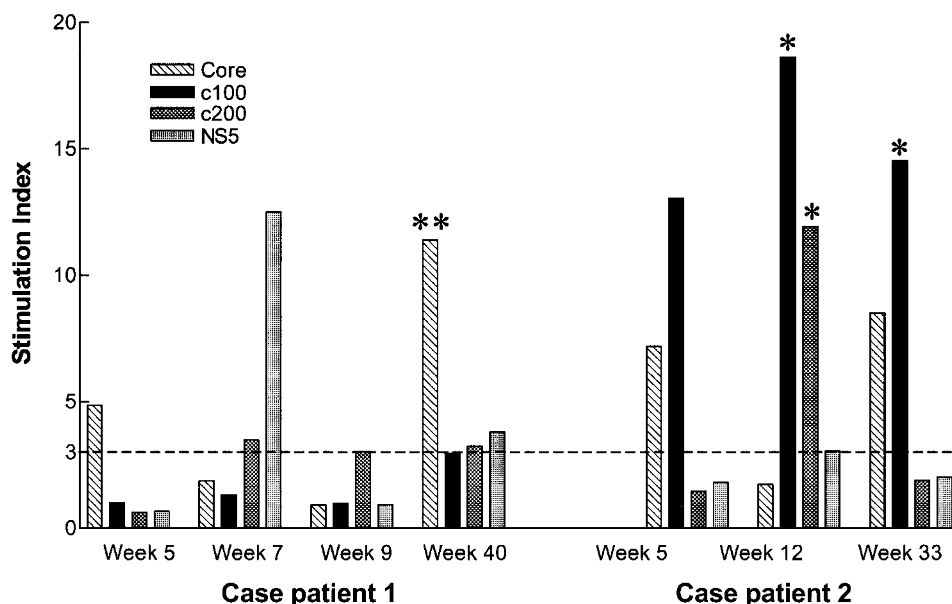

Figure 3. Hepatitis C virus (HCV)–specific proliferative responses, over time, for case patient 1 and case patient 2. CD4 proliferation assays were performed as outlined in Subjects, Materials, and Methods. Peripheral blood mononuclear cells from multiple time points were cultured in the presence or absence of HCV antigens. Each antigen was tested at 3 different concentrations (10, 1, and 0.1 mg/mL). Samples were harvested at day 6, and the percentage of proliferating $CD3^+$ $CD4^+$ T lymphocytes was determined by flow cytometry. The peak response for each antigen over time is shown. The stimulation index (SI) was defined as the percentage of $CD4^+$ T cells proliferating in the presence of antigen divided by the percentage of $CD4^+$ T cells proliferating in the absence of antigen. SI $\geqslant 3$ was considered to be positive as shown by the dashed line. *, The peak proliferative response was observed at an antigen concentration 10-fold less than that noted at week 5; **, the peak proliferative response was observed at an antigen concentration 100-fold less than that noted at week 5.

a phenomenon has not been reported in immunocompetent hosts, and it is less likely, given the pattern of viremia and the presence of documented risk exposures occurring before the onset of viremia in 2 of the case patients.

The immunological features of this phenotype include a broad cellular immune response with little evidence of an HCV-specific humoral response. These observations support the hypothesis that cellular immune responses are important in the clearance of HCV infection [15, 21, 24, 26, 27, 60]. Evidence for a critical role of the immune response in viral clearance may be inferred from the temporal associations of response and clearance.

In this regard, CTL activity developed during viremia in case patient 2 and then resolved in parallel with the resolution of viremia. Similarly, case patient 1 had detectable CTL responses during viremia. Unfortunately, fresh PBMCs were not available at earlier and later time points, to further delineate the possible role of CTLs in viral clearance in this subject. A similar analysis of IFN-γ production and viral clearance revealed that ELISPOT responses were strong and that they decreased in concert with the decrease in the HCV load in case patient 1. Conversely, case patient 2 was able to clear HCV viremia without apparent production of IFN-γ from CD8 T cells (at least during periods of detectable viremia). This finding contrasts with the findings of Thimme et al. [27], who found that the presence of cells producing IFN-γ coincided closely with control of viral replication in cases of acute HCV infection with biochemical hepatitis. In the present study, both case patients demonstrated multispecific CD4 lymphocyte responses. However, in case patient 1, these responses were not sustained during viremia, whereas, in case patient 2, the response against nonstructural proteins was consistently present and peaked in concert with viral clearance. This may be noteworthy, because demonstration of CD4 lymphocyte responses against nonstructural regions of the virus has been a feature of previous reports of viral clearance [4, 18, 20, 22, 24, 25, 27, 61]. Taken together, the findings of these studies suggest that multiple effector mechanisms are associated with resolution of HCV viremia. The high rate of clearance in the present study (4 of 4 subjects), compared with the rate of clearance in the study of Thimme et al. (1 of 5 subjects), may indicate a bias toward an efficient cellular, rather than humoral, immune response. Therefore, additional studies of comparable case patients without seroconversion may better define the determinants of viral clearance. Differences in the populations studied (prison inmates vs. health care workers) may also influence the outcome, particularly if prior and, perhaps, repeated exposure to HCV with viral clearance is important in the difference between the outcomes in these 2 studies. Recent data from the chimpanzee model of HCV infection support the validity of this phenotype [62].

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

The indeterminate serological responses, on HCV immunoblot analysis, for the case patients described in the present study were detected in serum samples obtained before the onset of viremia and did not alter during viremia or follow-up. This finding suggests that these responses did not contribute to viral clearance but, rather, may reflect prior exposure and seroreversion after previous resolved HCV infection [14–16]. Similar patterns of serological responses against nonstructural antigens were detected in aviremic chimpanzees that were able to clear a subsequent viral challenge [63]. The absence of significant titers of rheumatoid factor in these case patients suggests that HCV-associated cryoglobulinemia is unlikely to be the cause of the low virus titers seen or the inability to detect seroconversion with sensitive serological assays [64]. The HCV loads in the present report are lower than those found in other reports of acute HCV infection [50, 65–68]. This pattern of a low peak virus load and weak antibody production is similar to that seen in chimpanzees that clear HCV infection when reinfected [69, 70]. The recent study by Mehta et al. [71] also found lower peak HCV loads in subjects who were positive for anti-HCV antibody and developed recurrent HCV viremia. Of importance, cellular immunity was not studied in subjects in that report.

The current estimates of viral clearance in subjects with HCV infection are likely to be underestimated, because the case patients with the phenotype reported in the present study would not be detected by HCV ELISA, by the presence of biochemical hepatitis, or by clinical presentation. Cross-sectional studies that have used an RT-PCR–based method of case ascertainment [52, 72] may also have failed to detect such cases, because the duration of viremia in the present study was 8–23 weeks. Similarly, prospective studies that assess subjects at 6 monthly intervals may not detect transient episodes of viremia. The natural history of cases of acute HCV infection defined by RT-PCR may also be more benign than that of cases defined by the presence of the anti-HCV antibody. This may be one of the reasons why such a high response rate was seen in a recent uncontrolled trial of IFN-α therapy in subjects with RT-PCR–defined acute HCV infection [73]. It should be noted that the subjects in that study differed from the case patients described in the present study, because they had biochemical hepatitis. Controlled studies of such interventions are still warranted.

The present study indicates that selected high-risk subjects can mount an effective immune response against HCV infection in the absence of antibody production and that they may have a higher likelihood of viral clearance in that setting. It is quite possible that the subjects described in the present study have been previously infected and have successfully cleared primary HCV infection, and that the responses detected in this study represent polarized type 1 cellular immunity following from earlier exposure. These findings may represent a form fruste of the immune responses likely to be required from an effective HCV vaccine.

HEPATITIS C INCIDENCE AND TRANSMISSION IN PRISONS STUDY (HITS) GROUP MEMBERS

In addition to the authors of the present study, the members of the HITS group are as follows: Marian Gray, Deanne Wood, and Susan Harper (Corrections Health Service, Matraville, New South Wales, Australia); Jenean Spencer (National Centre in HIV Epidemiology and Clinical Research, University of New South Wales, Sydney, New South Wales, Australia); Sophie DeKantzow and Joanne Micallef (Virology Research Laboratory, Department of Microbiology, South Eastern Area Laboratory Services, Randwick, New South Wales, Australia); and Jenny Gates (National Drug and Alcohol Research Centre, University of New South Wales, Sydney, Australia). R.A.F., P.A.W., A.R.L., and W.D.R. are principal investigators of the Australian Centre for Hepatitis Virology.

Acknowledgments

We are indebted to Charles E. Rice of Washington University (St. Louis, MO) for providing the vaccinia–hepatitis C virus (HCV) constructs, Michael Houghton (Chiron) for providing the recombinant HCV antigens, L. Ross Whybin for technical assistance, the subjects for their participation, and the staff of Corrections Health Service (Matraville, New South Wales, Australia) for their support in developing and maintaining this cohort.

References

1. Barrera JM, Bruguera M, Ercilla MG, et al. Persistent hepatitis C viremia after acute self-limiting posttransfusion hepatitis C. Hepatology **1995**;21:639–44.
2. Seeff LB, Hollinger FB, Alter HJ, et al. Long-term mortality and morbidity of transfusion-associated non-A, non-B, and type C hepatitis: a National Heart, Lung, and Blood Institute collaborative study. Hepatology **2001**; 33:455–63.
3. Freeman AJ, Dore GJ, Law MG, et al. Estimating progression to cirrhosis in chronic hepatitis C virus infection. Hepatology **2001**; 34:809–16.
4. Gerlach JT, Diepolder HM, Jung MC, et al. Recurrence of hepatitis C virus after loss of virus-specific $CD4^+$ T-cell response in acute hepatitis C. Gastroenterology **1999**; 117:933–41.
5. Amoroso P, Rapicetta M, Tosti ME, et al. Correlation between virus genotype and chronicity rate in acute hepatitis C. J Hepatol **1998**; 28: 939–44.
6. Giuberti T, Marin MG, Ferrari C, et al. Hepatitis C virus viremia following clinical resolution of acute hepatitis C. J Hepatol **1994**; 20:666–71.
7. Alter MJ, Kruszon-Moran D, Nainan OV, et al. The prevalence of hepatitis C virus infection in the United States, 1988 through **1994**. New Engl J Med **1999**; 341:556–62.
8. Conry-Cantilena C, VanRaden M, Gibble J, et al. Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection. New Engl J Med **1996**; 334:1691–6.
9. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

contaminated anti-D immune globulin. Irish Hepatology Research Group. New Engl J Med **1999**; 340:1228–33.
10. Rodger AJ, Roberts S, Lanigan A, Bowden S, Brown T, Crofts N. Assessment of long-term outcomes of community-acquired hepatitis C infection in a cohort with sera stored from 1971 to 1975. Hepatology **2000**; 32:582–7.
11. Vogt M, Lang T, Frosner G, et al. Prevalence and clinical outcome of hepatitis C infection in children who underwent cardiac surgery before the implementation of blood-donor screening. New Engl J Med **1999**; 341:866–70.
12. Grellier L, Brown D, Power J, Dusheiko G. Absence of anti-envelope antibodies and clearance of hepatitis C virus in a cohort of Irish women infected in 1977. J Viral Hepat **1997**; 4:379–81.
13. Wiese M, Berr F, Lafrenz M, Porst H, Oesen U. Low frequency of cirrhosis in a hepatitis C (genotype 1b) single-source outbreak in Germany: a 20-year multicenter study. Hepatology **2000**; 32:91–6.
14. Lefrere JJ, Guiramand S, Lefrere F, et al. Full or partial seroreversion in patients infected by hepatitis C virus. J Infect Dis **1997**; 175:316–22.
15. Takaki A, Wiese M, Maertens G, et al. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat Med **2000**; 6:578–82.
16. Kondili LA, Chionne P, Costantino A, et al. Infection rate and spontaneous seroreversion of anti-hepatitis C virus during the natural course of hepatitis C virus infection in the general population. Gut **2002**; 50: 693–6.
17. Freeman AJ, Marinos G, Ffrench RA, Lloyd AR. Immunopathogenesis of hepatitis C virus infection. Immunol Cell Biol **2001**; 79:515–36.
18. Diepolder HM, Zachoval R, Hoffmann RM, Jung MC, Gerlach T, Pape GR. The role of hepatitis C virus specific $CD4^+$ T lymphocytes in acute and chronic hepatitis C. J Mol Med **1996**; 74:583–8.
19. Koziel MJ, Walker BD. Characteristics of the intrahepatic cytotoxic T lymphocyte response in chronic hepatitis C virus infection. Springer Semin Immunopathol **1997**; 19:69–83.
20. Botarelli P, Brunetto MR, Minutello MA, et al. T-lymphocyte response to hepatitis C virus in different clinical courses of infection. Gastroenterology **1993**; 104:580–7.
21. Gruner NH, Gerlach TJ, Jung MC, et al. Association of hepatitis C virus–specific $CD8^+$ T cells with viral clearance in acute hepatitis C. J Infect Dis **2000**; 181:1528–36.
22. Diepolder HM, Gerlach JT, Zachoval R, et al. Immunodominant CD4+ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. J Virol **1997**; 71:6011–9.
23. Rehermann B. Cellular immune response to the hepatitis C virus. J Viral Hepat **1999**; 6:31–5.
24. Pape GR, Gerlach TJ, Diepolder HM, Gruner N, Jung M, Santantonio T. Role of the specific T-cell response for clearance and control of hepatitis C virus. J Viral Hepat **1999**; 6:36–40.
25. Koziel MJ, Wong DK, Dudley D, Houghton M, Walker BD. Hepatitis C virus–specific cytolytic T lymphocyte and T helper cell responses in seronegative persons. J Infect Dis **1997**; 176:859–66.
26. Diepolder HM, Zachoval R, Hoffmann RM, et al. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet **1995**; 346:1006–7.
27. Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med **2001**; 194:1395–406.
28. Lauer GM, Walker BD. Hepatitis C virus infection. New Engl J Med **2001**; 345:41–52.
29. Butler TG, Dolan KA, Ferson MJ, McGuinness LM, Brown PR, Robertson PW. Hepatitis B and C in New South Wales prisons: prevalence and risk factors. Med J Aust **1997**; 166:127–30.
30. Reindollar RW. Hepatitis C and the correctional population. Am J Med **1999**; 107:100–3S.
31. Ruiz JD, Molitor F, Sun RK, et al. Prevalence and correlates of hepatitis C virus infection among inmates entering the California correctional system. West J Med **1999**; 170:156–60.
32. Spaulding A, Greene C, Davidson K, Schneidermann M, Rich J. Hepatitis C in state correctional facilities. Prev Med **1999**; 28:92–100.
33. Vlahov D, Nelson KE, Quinn TC, Kendig N. Prevalence and incidence of hepatitis C virus infection among male prison inmates in Maryland. Eur J Epidemiol **1993**; 9:566–9.
34. Post JJ, Dolan KA, Whybin LR, Carter IW, Haber PS, Lloyd AR. Acute hepatitis C virus infection in an Australian prison inmate: tattooing as a possible transmission route. Med J Aust **2001**; 174:183–4.
35. Haber PS, Parsons SJ, Harper SE, White PA, Rawlinson WD, Lloyd AR. Transmission of hepatitis C within Australian prisons. Med J Aust **1999**; 171:31–3.
36. Tsang TH, Horowitz E, Vugia DJ. Transmission of hepatitis C through tattooing in a United States prison. Am J Gastroenterol **2001**; 96:1304–5.
37. Gessoni G, Barin P, Frigato A, et al. The stability of hepatitis C virus RNA after storage at +4 degrees C. J Viral Hepat **2000**; 7:283–6.
38. Halfon P, Khiri H, Gerolami V, et al. Impact of various handling and storage conditions on quantitative detection of hepatitis C virus RNA. J Hepatol **1996**; 25:307–11.
39. White PA, Zhai X, Carter I, Zhao Y, Rawlinson WD. Simplified hepatitis C virus genotyping by heteroduplex mobility analysis. J Clin Microbiol **2000**; 38:477–82.
40. White PA, Pan Y, Freeman AJ, et al. Quantification of hepatitis C virus in human liver and serum samples by using LightCycler reverse transcriptase PCR. J Clin Microbiol **2002**; 40:4346–8.
41. Wong DK, Dudley DD, Afdhal NH, et al. Liver-derived CTL in hepatitis C virus infection: breadth and specificity of responses in a cohort of persons with chronic infection. J Immunol **1998**; 160:1479–88.
42. Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM. Expression and identification of hepatitis C virus polyprotein cleavage products. J Virol **1993**; 67:1385–95.
43. Currier JR, Kuta EG, Turk E, et al. A panel of MHC class I restricted viral peptides for use as a quality control for vaccine trial ELISPOT assays. J Immunol Methods **2002**; 260:157–72.
44. Larsson M, Jin X, Ramratnam B, et al. A recombinant vaccinia virus based ELISPOT assay detects high frequencies of Pol-specific CD8 T cells in HIV-1–positive individuals. AIDS **1999**; 13:767–77.
45. Freeman AJ, Pan Y, Harvey CE, et al. The presence of an intrahepatic cytotoxic T lymphocyte response is associated with low viral load in patients with chronic hepatitis C virus infection. J Hepatol **2003**; 38: 349–56.
46. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods **1994**; 171:131–7.
47. Alter MJ, Margolis HS, Krawczynski K, et al. The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic non-A, non-B Hepatitis Study Team. New Engl J Med **1992**; 327:1899–905.
48. Thomas DL, Vlahov D, Solomon L, et al. Correlates of hepatitis C virus infections among injection drug users. Medicine (Baltimore) **1995**; 74: 212–20.
49. Esteban JI, Gonzalez A, Hernandez JM, et al. Evaluation of antibodies to hepatitis C virus in a study of transfusion-associated hepatitis. New Engl J Med **1990**; 323:1107–12.
50. Arai Y, Noda K, Enomoto N, et al. A prospective study of hepatitis C virus infection after needlestick accidents. Liver **1996**; 16:331–4.
51. Mitsui T, Iwano K, Masuko K, et al. Hepatitis C virus infection in medical personnel after needlestick accident. Hepatology **1992**; 16:1109–14.
52. Crofts N, Hopper JL, Bowden DS, Breschkin AM, Milner R, Locarnini SA. Hepatitis C virus infection among a cohort of Victorian injecting drug users. Med J Aust **1993**; 159:237–41.
53. Schwartz JM, Sharon F, Larson AM, Kowdley KV, Carithers RL. Viremia following occupational exposure to hepatitis C is often transient [abstract 1356]. Hepatology **1999**; 30:499A.
54. Hanuka N, Sikuler E, Tovbin D, et al. Hepatitis C virus infection in renal failure patients in the absence of anti-hepatitis C virus antibodies. J Viral Hepat **2002**; 9:141–5.
55. Durand F, Beauplet A, Marcellin P. Evidence of hepatitis C virus viremia

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022

without detectable antibody to hepatitis C virus in a blood donor. Ann Intern Med **2000**; 133:74–5.
56. George SL, Gebhardt J, Klinzman D, et al. Hepatitis C virus viremia in HIV-infected individuals with negative HCV antibody tests. J Acquir Immune Defic Syndr **2002**; 31:154–62.
57. Rossi G, Tucci A, Cariani E, Ravaggi A, Rossini A, Radaeli E. Outbreak of hepatitis C virus infection in patients with hematologic disorders treated with intravenous immunoglobulins: different prognosis according to the immune status. Blood **1997**; 90:1309–14.
58. Morand P, Dutertre N, Minazzi H, et al. Lack of seroconversion in a health care worker after polymerase chain reaction–documented acute hepatitis C resulting from a needlestick injury. Clin Infect Dis **2001**; 33:727–9.
59. Genesca J, Esteban JI, Quer J, et al. Hepatitis C virus markers in patients with acute post-transfusion hepatitis treated with interferon alfa-2b. Gut **1993**; 34(Suppl 2):S62–3.
60. Missale G, Bertoni R, Lamonaca V, et al. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J Clin Invest **1996**; 98:706–14.
61. Chang KM, Thimme R, Melpolder JJ, et al. Differential $CD4^+$ and $CD8^+$ T-cell responsiveness in hepatitis C virus infection. Hepatology **2001**; 33:267–76.
62. Shata MT, Tricoche N, Perkus M, et al. Exposure to low infective doses of HCV induces cellular immune responses without consistently detectable viremia or seroconversion in chimpanzees. Virology **2003**; 314: 601–16.
63. Major ME, Mihalik K, Puig M, et al. Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge. J Virol **2002**; 76:6586–95.
64. Antonescu C, Mayerat C, Mantegani A, Frei PC, Spertini F, Tissot JD. Hepatitis C virus (HCV) infection: serum rheumatoid factor activity and HCV genotype correlate with cryoglobulin clonality. Blood **1998**; 92:3486–7.
65. Chu CM, Lin SM, Hsieh SY, et al. Etiology of sporadic acute viral hepatitis in Taiwan: the role of hepatitis C virus, hepatitis E virus and GB virus-C/hepatitis G virus in an endemic area of hepatitis A and B. J Med Virol **1999**; 58:154–9.
66. Beld M, Penning M, McMorrow M, Gorgels J, van den Hoek A, Goudsmit J. Different hepatitis C virus (HCV) RNA load profiles following seroconversion among injecting drug users without correlation with HCV genotype and serum alanine aminotransferase levels. J Clin Microbiol **1998**; 36:872–7.
67. Yamaguchi K, Tanaka E, Higashi K, et al. Adaptation of hepatitis C virus for persistent infection in patients with acute hepatitis. Gastroenterology **1994**; 106:1344–8.
68. Bourliere M, Halfon P, Quentin Y, et al. Covert transmission of hepatitis C virus during bloody fisticuffs. Gastroenterology **2000**; 119:507–11.
69. Bassett SE, Guerra B, Brasky K, et al. Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. Hepatology **2001**; 33:1479–87.
70. Cooper S, Erickson AL, Adams EJ, et al. Analysis of a successful immune response against hepatitis C virus. Immunity **1999**; 10:439–49.
71. Mehta SH, Cox A, Hoover DR, et al. Protection against persistence of hepatitis C. Lancet **2002**; 359:1478–83.
72. Fabrizi F, Lunghi G, Guarnori I, et al. Virological characteristics of hepatitis C virus infection in chronic hemodialysis patients: a cross-sectional study. Clin Nephrol **1995**; 44:49–55.
73. Jaeckel E, Cornberg M, Wedemeyer H, et al. Treatment of acute hepatitis C with interferon alfa-2b. New Engl J Med **2001**; 345:1452–57.

Downloaded from https://academic.oup.com/jid/article/189/10/1846/2191510 by guest on 16 August 2022