Multispecific T cell response and negative HCV RNA tests during acute HCV infection are early prognostic factors of spontaneous clearance


Background/Aims: Hepatitis C virus (HCV) infection results in a high frequency of chronic disease. The aim of this study was to identify early prognostic markers of disease resolution by performing a comprehensive analysis of viral and host factors during the natural course of acute HCV infection.

Methods: The clinical course of acute hepatitis C was determined in 34 consecutive patients. Epidemiological and virological parameters, as well as cell mediated immunity (CMI) and distribution of human leukocyte antigens (HLA) alleles were analysed.

Results: Ten out of 34 patients experienced self-limiting infection, with most resolving patients showing fast kinetics of viral clearance: at least one negative HCV RNA test during this phase predicted a favourable outcome. Among other clinical epidemiological parameters measured, the self-limiting course was significantly associated with higher median peak bilirubin levels at the onset of disease, and with the female sex, but only the latter parameter was independently associated after multivariate analysis. No significant differences between self-limiting or chronic course were observed for the distribution of DRB1 and DQB1 alleles. HCV specific T cell response was more frequently detected during acute HCV infection, than in patients with chronic HCV disease. A significantly broader T cell response was found in patients with self-limiting infection than in those with chronic evolving acute hepatitis C.

Conclusion: The results suggest that host related factors, in particular sex and CMI, play a crucial role in the spontaneous clearance of this virus. Most importantly, a negative HCV RNA test and broad CMI within the first month after onset of the symptoms represent very efficacious predictors of viral clearance and could thus be used as criteria in selecting candidates for early antiviral treatment.

Acute hepatitis C virus (HCV) infection is usually a mild and asymptomatic disease, yet it is characterised by a high rate of chronic evolution, approaching 90% according to some studies. Nevertheless, complete recovery from acute HCV infection has been reported to occur in up to 30–50% of cases with community acquired symptomatic acute hepatitis C. It is still not clear whether this high rate of chronicity reflects differences in the viral or host characteristics. The host immunogenetic background and, particularly, the characteristics of the host immune response in the initial phase of the infection are emerging as key factors in determining disease evolution. Data from several groups have shown that virus specific T helper cell and cytotoxic T lymphocyte (CTL) responses are associated with control of acute HCV viremia and clearance of the virus in the chimpanzee model of infection as well as in humans. However, T cell responses have been studied using a limited set of previously mapped peptides in a small number of patients.

Understanding the virus host interactions, which enable a proportion of patients with acute infection to clear HCV, is probably a key to the development of more effective treatment and prevention strategies. To this end, we enrolled a large cohort of HCV acutely infected individuals and evaluated clinical epidemiological parameters, distribution of human leukocyte antigens (HLA) class II alleles, breadth and magnitude of CD4+ and CD8+ T cell responses in patients with acute hepatitis C in both those who resolve the infection and those who develop chronic infection. Subjects with already established chronic HCV infection and healthy bone marrow donors were also included in the analysis to better define the role of cellular mediated immunity (CMI) and of the HLA class II polymorphism.

PATIENTS AND METHODS

Study population
The study cohort included 34 consecutive patients with acute hepatitis C (seven women, 27 men, median age 30 years, range 20–61 years), referred to infectious disease clinics or attending drug dependency units, between March 1999 and August 2001. Diagnosis of acute HCV infection was based on (1) high levels of serum alanine aminotransferase (ALT) at least 10 times above the upper limit of normal; (2) seroconversion assessed by third generation enzyme linked immunosorbent assay, or anti-HCV positivity at the time of diagnosis; (3) presence of HCV RNA in at least the first month after onset of the symptoms represent very efficacious predictors of viral clearance and could thus be used as criteria in selecting candidates for early antiviral treatment.

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; CMI, cell mediated immunity; CTL, cytotoxic T lymphocyte; DMSO, dimethyl sulfoxide; ELISPOT, enzyme linked immunosorbent; HCV, hepatitis C virus; HLA, human leukocyte antigen; IFN, interferon; IVDU, intravenous drug use; MEIA, microparticle enzyme immunoassay; NPV, negative predictive value; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PPV, positive predictive value; RIBA, recombinant immunoassay; SFC, spot forming cells.
negative test in the previous 12 months were also diagnosed as having acute hepatitis C on the basis of their clinical state, anamnestic characteristics, and a retrospective diagnostic confirmation consisting in a change in the serological profile, with an increasing number of reactivities against further HCV antigens\textsuperscript{15} \textsuperscript{16} \textsuperscript{19} by recombinant immunoblot assay (RIBA). Only one patient, who showed anti-HCV and HCV RNA positive tests, had normal ALT values (20 U/L) at enrolment in the study. Slightly altered ALT values were observed corresponding to new positive HCV RNA detections during the follow up. This patient was diagnosed as having acute HCV infection because he was symptomatic (but not jaundiced), had an anti-HCV negative test 10 months before the disease onset, and reported a recognised risk factor for the acquisition of the infection in the two months before the disease onset. Furthermore in the RIBA test performed on follow up sera, a progressive, increasing number of reactivities against the HCV antigens were detected in this patient. Alternative causes of acute hepatitis, such as other viral infections, autoimmunity, alcohol, drugs, and toxins were excluded. Patients with concomitant immunological disorders or with HIV coinfection were also excluded from the study.

Patients were scheduled to undergo a medical visit and to take a blood sample for biochemical, virological, and immunological assessment at the onset of the disease (month 0: start of the observation) and after 1, 3, 6, 12 months, and at four month intervals for a total of 24 months or until the patients eventually underwent antiviral therapy because of progression to chronic hepatitis. All observations reported in this study refer to the natural history of the infection (that is, in the absence of therapy) unless otherwise specified.

Self-limiting HCV infection was defined as normalisation of serum ALT levels in association with clearance of serum HCV RNA within six months after the onset of the disease. At least two successive negative HCV RNA measurements 6–12 months apart from the first negative result was used as further criterion for the diagnosis of self-limiting HCV infection. Patients who showed detectable HCV RNA in serum for more than six months with or without persistent increased ALT levels were considered to have developed a chronic infection.

A cohort of 56 patients with histologically confirmed chronic HCV related liver disease was used to compare the profile and the strength of the T cell immune response in late chronic versus acute infection. Blood samples from 40 healthy unrelated bone marrow donors (anti-HCV negative and without evidence of liver disease) were used as controls to represent HLA distribution in the uninfected population.

All patients gave informed consent before entering the study. The protocol and all the procedures of the study were conducted in conformity with the ethical guidelines of the Declaration of Helsinki.

**Anti-HCV antibody testing**

Anti-HCV antibodies were measured by Microparticle Enzyme Immunoassay (MEIA) HCV version 3 (AXSYM System, Abbott Diagnostics, Wiesbaden, Germany). Anti-HCV positivity was confirmed by second generation RIBA (Deciscan, Diagnostic Sanofi Pasteur, Marnes la Coquette, France).

**HCV RNA determination**

Levels of serum HCV RNA were determined by using Amplicor HCV Monitor Kit version 3 (Roche Diagnostic Systems, Branchburg, NJ, USA; detection limit: 600 UI/ml).

When viral load was lower than 600 UI/ml, HCV RNA was evaluated by a qualitative PCR with a detection limit of 100 copies/ml. HCV RNA was isolated from serum by Qiamp viral RNA kit (Qiagen, Hilden, Germany). Five μl of purified RNA was used to generate cDNA in a 20 μl reaction mixture containing 10 U of RNAse inhibitor (Promega Inc, Madison, WI, USA), 50 ng random examer, and 200 μM (each) deoxynucleoside triphosphate. After incubation at 70°C for 10 minutes, reverse transcription was carried out at 37°C for three hours with 50 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD, USA). cDNA was amplified with primers for 5' UTR by nested PCR. Primer sequences were as follows: B1 (outer sense) 5' AAG TAC TGT CTT CAC GCA GAA 3'; A1 (outer reverse) 5' GAT GCA CGG TCT ACG AGA CCT C 3'; B2 (inner sense) 5' ATG GCG TTA GTC GTC 3'; A2 (inner reverse) 5' GCG ACC CAA CAC TAC TCG GCT 3'. PCR mixture contained 1.5 mM MgCl\textsubscript{2}, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM (each) deoxynucleoside triphosphate, 0.5 μM primers, and 1.75U of Taq Gold Polymerase (Roche Molecular Systems Inc, Branchburg, NJ, USA) in a total volume of 50 μl. One tenth of the product from the first round of amplification was used for the second PCR round. Cycling parameters consisted of 35 cycles of 1 minute 95°C (plus 15 minutes in the first cycle); 30 seconds at 45°C and 1 minute at 72°C for the first round and 30 cycles at 95°C (plus 15 minutes in the first cycle); 30 seconds at 50°C and 1 minute at 72°C for the second round.

HCV genotype in PCR positive samples was assessed using a reverse hybridisation line probe assay (Inno-LIPA HCV Kit Innogenetics, Gent, Belgium).

**HLA typing**

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMC) according to salting out method.\textsuperscript{44} HLA typing of DRB1 and DQB1 class II alleles was performed by molecular tests in low resolution. HLA class II DRB1*11, DRB1*07, DQB1*03, and DQB1*05 alleles were typed also in high resolution. Low resolution molecular typing of HLA class II alleles was performed by reverse hybridisation with sequence specific oligonucleotide probes following amplification of the second exon of the DRB1 and DQB1 genes (PPCR-SSO) with biotinylated primers (Inno Lipa, Innogenetics, Ghent, Belgium).\textsuperscript{45} High resolution molecular typing of HLA-DRB1 and HLA-DQB1 alleles was performed by PCR with sequence specific primers (PCR-SSP)\textsuperscript{46} using a commercial kit (Dynal Biotech Ltd, Bromborough, UK) according to the manufacturer's instruction.

**Interferon (IFN-γ) enzyme linked immunospot (ELISpot)**

Synthetic peptides (20 amino acids in length and overlapping by 10 residues) were derived from the sequence of HCV BK strain.\textsuperscript{47} All peptides were synthesised with free N-terminal amine and free C-terminal carboxylic acid, using standard Fmoc solid phase methods and were purified by preparative high pressure liquid chromatography. The peptides were reconstituted in 100% DMSO at 20 mg/ml and used in the assay at 5 μg/ml. To facilitate the analysis, the 216 peptides were combined in seven pools covering Core, NS3 protease (NS3p), NS3 helicase (NS3h), NS4, NS5A, and NS5B (split in two pools NS5B-I and NS5B-II). Polyvinyl difluoride membrane plates (96 well; Millipore MAIP5 4510) were coated with 10 μg/ml of antihuman IFN-γ monoclonal antibody (MABTech clone # 1-D1K) in sterile phosphate buffered saline (PBS). Cryopreserved PBMC were thawed, recovered overnight at 37°C, 5% CO\textsubscript{2} and added to the wells at two different cell concentrations (200 000 and 100 000 cells/well) in 100 μl of R10 medium (RPMI medium 1640 (GibcoBRL, Ghatersburg, MD, USA), 10% fetal bovine serum (HyClone, Logan, UT, USA), supplemented with 10 μM HEPES buffer, 2 μM L-glutamine, 50 U/ml of penicillin, and 50 μg/ml of streptomycin (all GibcoBRL).
Five μg/ml peptide solutions were added, while concanavalin A and DMSO served as positive and negative controls, respectively. After incubation for 18–20 hours at 37°C in 5% CO₂, plates were washed with PBS/0.05% Tween 20, and 100 μl/well of 1 μg/ml biotinylated mouse anti-human IFN-γ mAb (MABTech clone 7-B6-1) in 0.5% BSA/PBS was added per well and incubated for three hours at RT. The assay was developed by incubation with alkaline phosphatase conjugated antibiotin mAb (Vector Laboratories, Burlingame, CA, USA) diluted to 1:750 in 0.5% BSA/PBS followed by incubation with BCIP/NBT substrate solution (Pierce, Rockford, IL, USA). Plates were washed with deionised water and dried before automated counting by the ELISpot reader system (ELR03 AID, Elispot Scientific, Strassberg, Germany).

To differentiate between antigen specific and background production of IFN-γ, a cut off value was defined based on fold increase over background and overall spot count. To this end, PBMC from 20 HCV seronegative subjects were stimulated using individual HCV peptide pools and compared with cells incubated in the absence of peptides (“mock”). Values measured in the presence of peptides were divided by the mock value to determine fold increase over background. In this analysis, the background ranged between 0 and approximately 55 spots per million cells. Therefore, spot counts within the range of 0–55 spots/million cells (SFC/10⁶) may not be clearly antigen specific. The mock ratios were similar regardless of antigens and we estimated the maximum mock ratio as the sample average plus twice the sample standard deviation.

Based on these analyses, we have defined threefold over mock plus at least 55 specific spots/million cells as a stringent cut off for the definition of a significant positive response.

Statistical analysis
The Mann-Whitney test was used for continuous variables to assess differences between distributions. The χ² test and Fisher’s exact test were used for comparisons of proportions. A p value of <0.05 was considered significant. A multiple regression model was constructed to explore the independent association of the clinical-epidemiological variables with the outcome of disease. The adjusting variables were selected because of their association (p<0.10) in the previous analyses.

Comparison of HLA alleles frequencies in different groups (resolving, chronically evolving, and bone marrow donors) was carried out using the Fisher’s exact test. The observed probability (p) was corrected (pc) according to Bonferroni.

The Bio Medical Data Processing statistical package (University of California, Los Angeles, CA, USA) was used for all calculations above cited. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with Fleiss’ quadratic confidence intervals (CI) were calculated by Epi-info statistical package.

RESULTS
Patients’ characteristics
A total of 34 subjects with acute hepatitis C (seven women, 27 men; median age 30 years, range 20–61 years) were included in the study (table 1). The most frequent at risk exposures reported within six months before disease onset were: intravenous drug use (19 subjects), surgical or medical invasive procedures (nine), accidental needle injuries (two), and sexual intercourse with a HCV positive individual (one). No recognised risk factor could be identified for three subjects. HCV genotype was determined in 31 patients, showing a clear prevalence of genotypes 3a and 1b (12 subjects each), followed by genotype 1a (three), 2a/2c (two), and 1a/1b or 2b (one). All but one subject (97%) were symptomatic at the onset of the disease and 23 of them (68%) had jaundice.

Median peak ALT and bilirubin levels at the onset of the disease were 1058 U/L (range 20–3276 U/L) and 5.78 mg/dL (range 0.43–31.80), respectively. Median HCV RNA titre at the entry into the study was 29,950 UI/mL (range 590–2,38 × 10⁸). All samples with viral load lower than 600 UI/mL resulted HCV RNA negative when tested by a qualitative PCR.

Clinical outcome
Clinical outcome was determined after a follow up of at least six months (median 13; range 6–29). Ten out of 34 subjects (29%) normalised serum ALT levels and displayed undetectable HCV RNA within the first six months after the onset of the disease, with half of them achieving normalisation of both viriological and biochemical parameters within the first four weeks from the onset. In these subjects, ALT remained normal and HCV RNA undetectable until the end of the follow up (median period 12 months; range 6–29 months) (fig 1). Thus, they were considered to have a self-limiting acute infection.

Twenty four subjects who showed increased serum ALT levels and/or detectable serum HCV RNA for more than six months after disease onset were considered to have developed a persistent infection. Their median follow up period was 13 months (range 6–26 months). Eighteen of these patients (75%) had detectable HCV RNA and persistently high ALT values throughout the entire follow up period, while four patients tended to normalise serum ALT values, but had persistently detectable HCV RNA in their serum. Two patients tested negative for HCV RNA at six and 12 months after disease onset, respectively, but could not be further evaluated to assess an eventual delayed resolution of their infection.

All subjects with self-limiting infection showed at least a negative HCV RNA test within three months of follow up, while eight and six of them had a normal ALT determination or a combination of negative HCV RNA and normal ALT, respectively (fig 1). When such an analysis was carried out among patients with chronic evolution, only five of them displayed a negative HCV RNA test within three months from the onset of the infection, whereas seven and three patients had a normal ALT determination or tested negative for both viremia and increased ALT levels, respectively. The differences between subjects with self-limiting infection and those with chronic evolution in the detection frequency of at least one HCV RNA negative test or a normal ALT determination or both within the third month from the disease onset were statistically significant (p<0.01, p<0.05, and p<0.01, respectively). A similar figure was obtained by analysing the same parameters already within the first month from the onset of the symptoms (9/10 v 4/24 for HCV RNA, p<0.01; 6/10 v 4/24 for ALT, p<0.05; 6/10 v 2/24 for both parameters at the same time, p<0.01). Focusing on the detection of HCV RNA within the first month from the disease onset, 90% (95% CI 54.1 to 99.5) of the resolving patients had at least a negative test (sensitivity), whereas 83.3% (95% CI 61.8 to 94.5) of the patient with chronic evolution had not at least a negative HCV RNA determination (specificity). The post-test probability of recovery (or PPV) following a positive result (that is, at least a negative HCV RNA test) and the post-test probability of chronic evolution (or NPV) following a negative result (all determinations positive for HCV RNA) of such a test were 69.2% (95% CI 38.9 to 89.6) and 95.2% (95% CI 74.1 to 99.8), respectively.

With regard to the other clinical-epidemiological features (table 1), females experienced a self-limiting course of disease in a significantly higher proportion than males (5/7 v 5/27; p<0.01) and patients with a resolving hepatitis had
higher median peak bilirubin level at the onset of disease (7.66 mg/dL vs 3.39 mg/dL; \( p < 0.05 \)). After multivariate analysis including age, sex, ALT, and bilirubin levels at the onset, the only independent factor associated to a self-limiting course of acute hepatitis C was female sex (OR = 19.4; 95% CI 1.65 to 227).

Multispecific T cell response during the early phase of acute HCV infection is associated to disease resolution

To define the role of adaptive T cell immunity in determining the course of disease, we measured the breadth and magnitude of anti-HCV CD4\(^+\) and CD8\(^+\) T cell responses ex vivo in PBMC of 30 acutely infected subjects by the quantitative and functional IFN-\(\gamma\) ELIspot assay. As resolution of HCV infection occurs in most cases within the first three months from the onset of the infection,\(^8\)\(^11\)\(^13\)\(^-\)\(^15\) this analysis was performed for each patient on PBMC collected at the time of the enrolment (T = 0) and after one month (T = 1). Furthermore, to avoid bias towards preselected epitopes and HLA alleles, we used as a source of antigen a panel of 216 peptides covering about 72% of the HCV polyprotein and spanning the Core and NS3-NS5B region of a 1b HCV isolate.

The results of this analysis showed that HCV infection induced CMI in 18 out of 30 acutely infected patients. This high frequency of T cell response is twice as much the rate of disease resolution we observed in the same cohort (60% vs 27%), implying that T cell response per se is not sufficient to achieve spontaneous clearance of the virus.

Eight of the 30 HCV acutely infected subjects screened by IFN-\(\gamma\)-ELIspot assay had a self-limiting infection. All but one of these individuals developed an HCV specific CMI (fig 2A), whereas 12 of 22 patients who developed a persistent infection (fig 2B) displayed anti-HCV T cell responses, but the difference between the two groups was not statistically significant. All T cell responding subjects with self-limiting acute hepatitis C displayed a multispecific response: A43, A45, and A50 responded to two or three antigens, A24 to 4,

<table>
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<tr>
<th>Parameter</th>
<th>Number of patients</th>
<th>Self-limiting</th>
<th>Chronic course</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>34</td>
<td>10 (29%)</td>
<td>24 (71%)</td>
</tr>
<tr>
<td>M/F</td>
<td>27/7</td>
<td>5/5*( \dagger )</td>
<td>22*( \dagger )</td>
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<tr>
<td>Age (years); median (range)</td>
<td>30 (20–61)</td>
<td>40, 5 (20–61)</td>
<td>29 (20–56)</td>
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<td>Risk factor</td>
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<td>IVDU</td>
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<td>4</td>
<td>15</td>
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<tr>
<td>Invasive procedure</td>
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<td>4</td>
<td>5</td>
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<td>1</td>
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<td>HCV+ sexual partner</td>
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<td>0</td>
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</tr>
<tr>
<td>No data</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ALT (U/L); median (range)</td>
<td>1058 (20–3276)</td>
<td>1201.5 (20–1829)</td>
<td>861.5 (5.17–3276)</td>
</tr>
<tr>
<td>AST (U/L); median (range)</td>
<td>519 (29–1962)</td>
<td>618.5 (29–1962)</td>
<td>496.5 (56–1751)</td>
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<td>Peak total bilirubin (mg/dL); median (range)</td>
<td>5.78 (0.43–31.8)</td>
<td>7.6 (0.9–31.8)</td>
<td>3.4 (0.4–14.0)</td>
</tr>
<tr>
<td>RNA titer (UI/mL); median (range)</td>
<td>29950 (590–1.94 ( \times ) 10^6)</td>
<td>32250 (590–2.38 ( \times ) 10^6)</td>
<td>29950 (590–1.94 ( \times ) 10^6)</td>
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<tr>
<td>Genotype</td>
<td>1a</td>
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<tr>
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<tr>
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<td>2</td>
<td>1</td>
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<tr>
<td>Symptoms at the onset (n. pts)</td>
<td>33 (97%)</td>
<td>10 (100%)</td>
<td>23 (96%)</td>
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<tr>
<td>Jaundice at the onset (n. pts)</td>
<td>23 (68%)</td>
<td>9 (90%)</td>
<td>14 (58%)</td>
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<tr>
<td>Follow up (month); median (range)</td>
<td>13 (6–29)</td>
<td>12 (6–29)</td>
<td>13 (6–26)</td>
</tr>
</tbody>
</table>

*\( p < 0.01 \) self-limiting vs chronic course (univariate analysis).
†\( p < 0.05 \) self-limiting vs chronic course (univariate analysis).
‡OR = 19.4 (CI 1.65 to 227).

Figure 1  Follow up of HCV RNA and ALT level in patients with self-limiting HCV acute infection. (A) Viral load. Different symbols represent data points for each individual subject. Viral loads are indicated on the vertical axis. The shaded area represents the range of values below the threshold of sensitivity of the employed HCV RNA determination method. The time scale is shown on the horizontal axis. (B) ALT levels. Different symbols represent data points for each individual subject. ALT values are indicated on the vertical axis. The shaded area represents the normal range of values. The time scale is shown on the horizontal axis.
The difference in the breadth of the T cell response between the two groups was statistically significant (p<0.01). On the contrary, no significant statistical difference was found between individuals with a self-limiting and those with chronic evolution, as regards the overall median strength of CMI response evaluated by adding up the reactivities against individual antigens (784 SFC/10⁶ PBMC, range 412–6872 v 448 SFC/10⁶, range 240–1510; p = 0.063).

When we evaluated the ability of a multispecific CMI response (≥3 antigens) to predict a favourable outcome in these 30 acutely infected patients, sensitivity and specificity were 62.3% (95% CI 25.9 to 89.8) and 90.9% (95% CI 69.4 to 98.4), respectively. The post-test probability of recovery following a positive response and the post-test probability of chronic evolution following a negative response were 71.4% (95% CI 30.3 to 94.9) and 87.0% (95% CI 65.3 to 96.6), respectively. By combining this test with the detection of at least a negative HCV RNA test within one month from disease onset, sensitivity, specificity, post-test probability of recovery following a positive result and post-test probability of chronic evolution following a negative result of the two combined test were 50.0%, (95% CI 17.4 to 82.6) 100% (95% CI 81.5 to 100), 100% (95% CI 93.6 to 100), and 84.6% (95% CI 64.3 to 95.0), respectively, demanding that both tests gave a positive response (for HCV RNA it means at least a negative determination in the first month) to consider the test result as positive. Demanding that either or both tests gave a positive response to consider the test result as positive sensitivity, specificity, post-test probability of recovery following a positive result, and post-test probability of chronic evolution following a negative result of the two combined test were 100% (95% CI 17.4 to 82.6), 77.3% (95% CI 54.2 to 91.3), 61.5% (95% CI 32.3 to 84.9), and 100% (95% CI 77.1 to 100), respectively.

To further extend these findings, we measured HCV specific CMI in a cohort of 56 chronically infected patients by IFN-γ ELISPOT assay. Patients with chronic hepatitis C were older than acutely infected patients (median 59 years, range 31–75 years), but no statistically significant difference was found between them with regard to sex and genotype distribution. In this cross sectional study, only 10 patients (18%) were found to be positive by ELISPOT assay (fig 4). The magnitude of the overall responses in patients with chronic hepatitis C was similar to that observed in acutely infected subjects presenting a chronic evolution and clearly lower than that observed in acutely infected subjects showing a self-limiting course (range 201–1464 SFC/10⁶ PBMC, with a median value of 346; fig 4). Most importantly, CMI was directed against a single antigen in nine of the 10 chronic patients with a positive T cell response, whereas in the
remaining patient it targeted two peptide pools (fig 3). The difference in the frequency of IFN-γ positive responses observed between these patients and subjects with self-limiting acute hepatitis C was statistically significant (p<0.01).

Overall, these data indicate that a strong T cell response targeting multiple HCV antigens in the early phase of infection is a distinctive feature of individuals who clear the virus.

**HLA class II typing, HCV-specific CMI, and outcome of acute hepatitis C**

The frequency of DRB1 (table 2) and HLA DQB1 (table 3) alleles was determined in both resolving and chronically evolving acutely HCV infected subjects and compared to that of a control population of 40 healthy bone marrow donors. No statistically significant difference was observed in the distribution of these alleles among these groups of subjects after Bonferroni correction. Interestingly, allele DRB1*1101 (that was more frequent in resolving patients than in those with chronic evolution and less frequent in patients with chronic evolution than in controls; tables 2 and 3) was detected in two female subjects (A26 and A46) who showed the strongest and broadest T cell response as measured by IFN-γ ELIspot assay (fig 2A). Both individuals showed a rapid resolution of their infection with HCV clearance and ALT normalisation occurring within the first (A26) and second (A46) month from disease onset.

**DISCUSSION**

A number of recent studies have shown that spontaneous elimination of HCV most often occurs in the first few months of infection, and that once chronic infection is established spontaneous viral clearance is rare. Thus, the initial months after infection seem to be of crucial importance for the subsequent course of disease. However, a prospective study on a statistically significant number of acutely infected subjects, which measured clinical, virological, and, most importantly, cell mediated immunity without bias for the patient haplotype was still missing.

On the basis of the clinical criteria adopted to define the outcome of acute HCV infection, 29% (10/34) of the patients in our study cohort experienced a self-limiting infection. This figure is consistent with previous reports on patients with symptomatic acute HCV infection, for which significant rates of resolution were measured. Among the 10 subjects with self-limiting infection, seven achieved virus clearance within one month and eight within three months after the onset. Similarly, normalisation of ALT values occurred within one or three months in six and eight subjects, respectively. In addition, we have found that detection of at least one negative HCV RNA test, with or without a normal ALT value, within one or three months from the onset of symptoms, was significantly associated with resolution of acute HCV infection. These findings suggest that repeated evaluation of HCV RNA status until the third month from the onset of disease can serve as a good predictor of the outcome of the infection and can be used as useful criterion to decide the beginning of antiviral treatment. Previous studies have shown that HCV specific CMI can be detected in individuals who resolved infection, but very rarely in chronic patients, supporting the view that the cellular arm of anti-HCV immunity plays a major role in control of viral infection. Thus, the majority of the existing information was gathered employing a limited number of epitopes, mostly restricted by the HLA A2.1 haplotype, an approach that clearly misses many responses. The cohort of acutely infected patients described in this study provided for a unique opportunity to evaluate the influence of adaptive T cell immunity in the outcome of HCV infection without these biases. A first question we wished to answer was whether HCV induces a T cell response in most of the infected individuals or if the virus can overcome T cell surveillance by providing only weak immunological stimuli. By ex vivo IFN-γ ELIspot assay using a complete set of overlapping peptides covering most of the HCV polyprotein, we found a significantly higher frequency of responses in acutely infected individuals than in a group of control patients with a long history of persistent infection (60% vs 18%). Furthermore, among those individuals of the latter group where chronic infection persisted in the face of measurable T cell response, fewer antigens were targeted,

### Table 2

<table>
<thead>
<tr>
<th>Allele</th>
<th>SLI v PI controls</th>
<th>PI v controls</th>
<th>SLI-PI v controls</th>
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<tbody>
<tr>
<td>DRB1</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 24)</td>
<td>(n = 40)</td>
</tr>
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<td>3 (7.5%)</td>
</tr>
<tr>
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<td>3 (30%)</td>
<td>3 (12.5%)</td>
<td>3 (7.5%)</td>
</tr>
<tr>
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<td>2 (20%)</td>
<td>4 (16.7%)</td>
<td>8 (20%)</td>
</tr>
<tr>
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<td>10 (41.7%)</td>
<td>7 (17.5%)</td>
</tr>
<tr>
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<td>13 (54.2%)</td>
<td>25 (62.5%)</td>
</tr>
<tr>
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<td>4 (16.7%)</td>
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<td>2 (20%)</td>
<td>3 (12.5%)</td>
<td>1 (2.5%)</td>
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<td>15</td>
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<td>4 (16.7%)</td>
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<tr>
<td>16</td>
<td>2 (20%)</td>
<td>2 (8.3%)</td>
<td>5 (12.5)</td>
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NS, not significant.
and chronically infected subjects to address ex vivo T cell responses in large cohorts of acutely infected individuals also indicate that in patients with chronic evolution T cell immunity becomes undetectable after a few months from the onset of the infection. These data support previous observations indicating that HCV induces a lower number of epitopes than those observed during the early phase of infection. Preliminary data from our cohort of acutely infected individuals indicate that in patients with chronic evolution T cell immunity becomes undetectable after a few months from the onset of the infection. These data support previous observations indicating that HCV induces significant CMI during the early phase of infection, but that this response is not sustained. 

Because of the difficulty in using peptides matching the infecting genotypes in each patient, we have used a panel of peptides derived from a 1b HCV isolate corresponding to regions highly conserved among different strains. T cell response was measured in subjects bearing different HLA alleles and infected by different viral genotypes. All but one subjects infected by 3a genotype were scored positive in our assay, consistent with previous findings of cross genotype T cell reactivity for epitopes located within the NS3-NS5 region. Therefore, even if there is some underestimation of the strength and the frequency of CMI responses in the study cohort, we think our approach remains a viable option to address ex vivo T cell responses in large cohorts of acutely and chronically infected subjects.

The high frequency of T cell responses measured within the first month from disease onset suggested that a significant proportion of chronically evolving patients would also develop anti-HCV CMI. In fact, about half (12/22) of the acutely infected subjects who subsequently developed a chronic infection displayed a T cell response during the first four weeks from the onset of the symptoms. Although the difference in the frequency of T cell responses between resolving and chronically evolving subjects was evident, it did not reach statistical significance, possibly also because of the limited number of individuals analysed. These data imply that, if present, the immunological signature of a self-limiting course of disease is not simply the presence of specific T cells, but rather consists of some qualitative feature of this response, which is missing in patients progressing toward chronicity. Consistently with this hypothesis, a T cell response targeting multiple HCV antigens in the early phase of the infection emerged as a statistically significant distinctive feature of individuals who cleared the virus. This finding is in agreement with the notion that a response against multiple antigens may reduce the likelihood of escaping immune response through antigenic variation, as recently shown in the chimpanzee model.

When applied in our cohort to predict a favourable course of infection, the detection of a multispecific T cell response (≥3 antigens) by IFNy ELISpot assay showed low sensitivity and post-test probability of recovery following a positive test. This test’s characteristics imply that we might consider having an unfavourable course—and eventually subject to early treatment—a non-negligible proportion of subjects who indeed will resolve the infection.

What is the mechanism for the lower spectrum (or total lack) of anti-HCV T cell responses in chronically evolving subjects? In some models of infection, prolonged exposure to high amounts of viral antigens could lower immune responses both in terms of number as well as function of antigen specific cells. In line with this hypothesis, we observed that CMI induced in chronically evolving patients was on average of lower intensity than that measured in subjects with self-limiting acute infection. Our observation of similar viral loads at the onset of the symptoms in subjects with self-limiting acute infection and those with chronic evolution does not rule out this hypothesis because hepatic measurements of viremia could not be performed for ethical reasons. Finally, other mechanisms like deficient induction of T helper cell response, incomplete T cell maturation, or liver induced immunotolerance may be responsible for the observed attenuation of the immune response in chronically evolving patients.

Recent studies have described important associations between some HLA alleles, particularly DRB1*1101 and/or DQB1*0301, and clearance of HCV. We found no statistically significant difference in the distribution of HLA DRB1 and DQB1 alleles between patients with resolving infection and those with chronic evolution probably because of the insufficient number of individuals analysed. It is worthy of note that the strongest and broadest HCV specific T cell response with a rapid clearance of the virus, was found in patients carrying haplotype DRB1*1101. Further prospective studies on larger cohorts of HCV acutely infected individuals are needed to exhaustively evaluate the role of specific HLA class II alleles in the outcome of the infection and to characterise the mechanisms leading to T cell failure during primary infection. Notwithstanding this, our results suggest that induction of a broad CMI already within one month from the onset of the symptoms, and negative detections of HCV RNA during this period are predictive of a favourable outcome of HCV acute infection. Indeed, when these tests were used in combination in the first month from disease onset, to predict a favourable outcome, demanding that either or both of them were positive, we were able to identify all patients with a self-limiting infection, even though about 20% of patients with a predicted favourable courses (false positive) finally showed a chronic evolution. Nonetheless, it is necessary to underline that these estimates were based on a small sample of patients, and so they need to be further confirmed by analysing prospectively a larger group of acutely infected individuals.

These findings could provide for a convenient alternative to the immediate treatment of all patients with acute hepatitis C as suggested by Jaeckel et al, who recently reported sustained

<table>
<thead>
<tr>
<th>Table 3</th>
<th>DQB1 alleles distribution in patients with self-limiting HCV infection (SLI), with persistent HCV infection (PI) and in controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td>SLI (n = 10)</td>
</tr>
<tr>
<td>DQB1</td>
<td></td>
</tr>
<tr>
<td>*02</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>*0301</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>*0302</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>*0303</td>
<td>2 (20%)</td>
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<tr>
<td>*05</td>
<td>6 (60%)</td>
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<td>*0501</td>
<td>2 (20%)</td>
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<tr>
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<tr>
<td>*06</td>
<td>1 (10%)</td>
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</tbody>
</table>

NS, not significant.
response of 95% with starting treatment of patients within 4–16 weeks after infection. The very high rate of response found by these authors could have been due to the adjuvant effect of anti-HCV CMI which is often attenuated (if not absent) in the chronic phase of infection. Thus, by implementing a T cell assay together with RNA detection tests within the first month from the onset of symptoms, one could select with high diagnostic sensitivity a group of patients with high probability to resolve the infection, preventing them having to undergo an early antiviral treatment. If such a combined test failed in predicting a favourable course (false positive results), treatment could still achieve high frequency of sustained virological response if started within the first six months from disease onset.\(^9\) 

ACKNOWLEDGEMENTS

This study was supported in part by the Viral Hepatitis Project, Istituto Superiore di Sanità (D. Leg.vo 30/12/1992 n. 502).

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Gut 2004 53: 1673-1681
doi: 10.1136/gut.2003.037788