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Multiple infections with different HCV genotypes: prevalence and clinical impact

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Abstract

Background: In a HCV genotype 3a-infected patient, viremia with a different genotype (1b) was detected after 16 weeks of ineffective therapy. Serological typing revealed that this genotype had already been present prior to therapy. *Objectives:* To investigate the epidemiology of multiple HCV infections and the therapeutical consequences for patients superinfected with a new HCV strain. *Methods:* Sera of 600 patients were screened for infection with multiple genotypes by using sequencing and a serological assay in parallel. *Results:* Infection with two different HCV types was detected in 13 patients. The prevailing strain was genotyped by sequencing. From two of these patients additional sera were available which had been drawn up to 24 and 28 months prior to the current sample, respectively. Those early samples showed viremia with a HCV subtype that could not be detected by PCR afterwards. Only antibodies to the initial strain were detectable in the later samples. *Conclusion:* In patients serially infected by different HCV strains, one strain will prevail as the viremic virus. Under antiviral therapy, the displaced strain may become viremic again and may influence the outcome of therapy. Detection of inferior strains by serological assays before antiviral therapy may be important for choosing the adequate regimen.

Keywords: Infection; Hepatitis C virus; Antiviral therapy

1. Introduction

The genotype of hepatitis C virus (HCV) has been described as an independent predictor of success of antiviral therapy (Zeuzem et al., 2000). Therefore, different therapy regimens have been proposed depending on the infecting HCV genotype (Zeuzem et al., 2000). Before onset of therapy the actual viremic HCV strain is usually determined by a PCR-based method like nucleotide sequencing. However, it is well known from transplant patients that superinfection with a new HCV strain leads to suppression of one virus under the detection limit of PCR while the other

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one prevails as the viremic strain (Widell et al., 1995).

Repeated exposure to HCV is also common in high-risk groups like inravenous drug users (IVDU) or patients on maintenance hemodialysis (Alter et al., 1999). This study was initiated because only limited data are available about the epidemiology and about therapeutical consequences of superinfection with a new HCV strain.

2. Materials and methods

2.1. Serum samples

Sera were drawn from 600 unselected HCV PCR-positive individuals which were sent to our laboratory between July and December 2001. HCV viremia was proven by PCR as previously described (Schröter et al., 2001a). None of the patients was treated with α-interferon at the time of investigation. All of them gave informed consent to participate in this study.

2.2. Nucleotide sequencing of a part of the 5′ non-coding region:

Sequencing of a part of the 5′ non-coding region was performed as previously described (Schröter et al., 2001b). Briefly, RNA was extracted using Qiagen Viral RNA kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. For cDNA synthesis, the isolated RNA was resuspended in DEPC-treated H_2O and 50 pmol primer zap (5′-CTCGCAAGCACCCTATCAGGC-3′; 312–291 numbering of nucleotide sequences as previously described, Choo et al., 1991) as well as 200 U MMLV-reverse transcriptase (Life Technologies, Gaithersburg, USA) were added. After reverse transcription, amplification of the HCV cDNA was performed in a buffer containing 10 mM Tris–HCl (pH 8,3), 50 mM KCl, 2 mM $MgCl_2$, 160 μM (each) deoxynucleoside triphosphate, 30 pmol of each sense or antisense primer and 2 U Pfu thermostabile DNA polymerase (Stratagene, La Jolla, USA). For the first round of the nested PCR primers fap (5′-GCAGAAAGCGTCTAGCCATGG-3′; 68–88) and zap were used. In a second amplification step the primers fip (5′-TCTAGCCATGGCGTTAGTA-3′; 80–97) and zip (5′-CAGTACCACAAGGCCTTTC-3′; 291–270) were used.

Sequencing was performed using ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Weiterstadt, Germany). The sequencing reaction was performed as recommended by the manufacturer (PE Applied Biosystems, Weiterstadt, Germany) using primer fip. Genotypes were determined by comparison with published reference sequences according to the classification proposed by Simmonds et al. (1994). EMBL accession numbers of the reference sequences are as follows: Subtype 1a HPCPLYPR acc. no. M62321; subtype 1b HCVJK1G acc. no. X61596; subtype 2a HPCPOLP acc. no. D00944; subtype 2b HPCJ8G acc. no. D10988; subtype 3a HPCK3A acc. no. D28917; subtype 4a HCV4APOLY acc. no. Y11604.

2.3. Serological determination of HCV genotypes by NS-4 IBA

The immunoblot assay (NS-4 IBA) for serotyping was previously described (Schröter et al., 1999). The HCV subtypes detectable by this assay are 1a, 1b, 2a, 2b, 3a, and 4a. Development of the NS-4 IBA was performed as previously described (Schröter et al., 1999). Briefly, serum samples were diluted 1:100 in TBST (20 mM Tris–HCl (pH 7,4), 150 mM NaCl, 0.05% Tween 20) and exposed to blot strips for 1 h on a rotation shaker at room temperature. Afterwards the strips were washed three times with 4 ml of TBST for 5 min each and then exposed for 30 min at room temperature on a rotation shaker to alkaline phosphatase-labeled goat anti-human-IgG antibodies (Sigma, Deisenhofen, Germany) diluted 1:7500 in TBST. Again the strips were washed three times with 4 ml TBST for 5 min each and finally once with 4 ml of TBS (TBST without Tween 20). Strips were developed with a substrate solution (Western Blue, Promega, Heidelberg, Germany) containing BCIP and Nitro Blue Tetrazolium.

To detect those antibodies which are directed against the subtype-specific epitopes of the respective recombinant proteins, serum samples needed

to be preabsorbed with a solution containing a surplus of 1a-recombinant protein and 2a-recombinant protein. All sera were tested in the NS-4 IBA before and after preabsorption, and the patterns of reactivity were compared. After preabsorption HCV subtypes could definitely be determined by the remaining reactivity against a single subtype-specific recombinant protein.

3. Results

A cross-sectional study was performed comprising 600 unselected HCV PCR-positive serum samples. HCV genotyping was determined in parallel by the antibody assay and by nucleotide sequencing. Discordant results in both assays occurred in 13 samples (2.2%) (Table 1).

Twelve of those (92.3%) were initially infected by a subtype of genotype 1 as detected by NS-4 IBA. Nine were determined as subtype 1a, three were subtype 1b, and in only one sample a 3a strain was detected initially. However, the subtypes of the actual prevailing strains as determined by PCR were different to the initial ones. In six patients the subtype 2b was detected, and the subtype 3a was also observed in six patients. One patient was actually infected by a subtype 4a strain (Table 1).

Demographical data revealed that all these samples were drawn from individuals practising IVDA (13/172; 7.6%), which is the main risk factor for HCV transmission and applies to about 70% of the newly acquired infections in our patient population. Due to the repeated exposition to HCV during IVDA, it must be assumed that sequential infection with different HCV strains occurs frequently in this group.

Table 1
HCV subtype determined by

No. of patients	Antibody assay	Sequencing
5	1a	2b
3	1a	3a
3	1b	3a
1	1a	4a
1	3a	2b

Thirteen of 600 examined samples were serologically assigned to a different HCV type than by sequencing. Among the entire study population, 172 patients had IVDA as the main risk factor for HCV acquisition, and all samples with different results belonged to this group (13/172; 7.6%).

From two of the 13 patients with discordant results in the antibody and the sequencing assay additional sera were available which had been drawn up to 24 and 28 months prior to the current sample, respectively. Sequence analysis of these samples revealed that both patients had been initially infected by HCV genotype 1a isolates. However, meanwhile the subtype had changed from 1a to 2b and from 1a to 3a, respectively. Using the antibody assay, antibodies exclusively against subtype 1a but not against 2b or 3a were detectable in all available samples of both patients.

4. Discussion

The driving force for the initiation of this study was the case of a chronically hepatitis C virus (HCV)- infected patient who failed to respond as expected to antiviral therapy with pegylated interferon-alfa (peg-IFN-α) and Ribavirin. Prior to therapy HCV subtype 3a, which is known for its favourable therapy outcome (response rate $> 70\%$) (Zeuzem et al., 2000), was determined on two occasions by nucleotide sequencing of the viral 5′-untranslated region as described earlier (Schröter et al., 2001b). The patient had a career in intravenous drug addiction (IVDA) several years before, which was the most probable route of acquisition. Due to the HCV subtype dependent treatment recommendations an antiviral therapy regimen of 6 months was planned (Zeuzem et al., 2000). After 10 weeks of therapy, viral load had decreased from 3×10^6 to 1×10^4 IU/ml. In a sample drawn 6 weeks later viremia had almost reached the initial level again (1×10^6 IU/ml), suggesting non-response to therapy. Therefore, the genotype analysis was repeated on two occasions by nucleotide sequencing and now revealed subtype 1b at both times. A recent reinfection could be excluded because the patient had stopped IVDA and other risk factors could not be evaluated.

Besides nucleotide sequencing, HCV subtypes can also be characterized by serological testing (Schröter et al., 1999). Using this assay, antibodies against subtype 1b only were detected in all available sera before and during therapy, including those sera which contained a subtype 3a strain as detected by sequencing. The different subtyping results by sequencing (3a) and by serotyping (1b), together with the risk behaviour of the patient and the clinical course during therapy, suggest that the patient was initially infected with a subtype 1b strain, that a superinfection with a subtype 3a strain occurred later on and then became predominant, and that during therapy the 3a strain was replaced by the 1b strain which reappeared as the viremic strain. Using PCR-based methods, only the actual prevailing strain can usually be detected in multiply infected patients (Widell et al., 1995). The detection of antibodies in such samples might correspond to double infection or to previous exposure to a genotype different to the one detected by PCR (Proust et al., 2000). The observation of different results in the two assays for genotype determination implies that the antibody assay detected a HCV strain which had been replaced by another subtype which established viremia as shown by PCR-based methods.

It is unclear why no serotype-specific antibodies against the new predominant strains were detectable. An immunological escape phenomenon, as previously described for the immune response to the V3-loop of HIV (Schreiber et al., 1994) could be a possible explanation. Recent data also suggest that past infection with HCV may attenuate the immune response to reinfecting virus, which could also explain the lack of serological reactivity against the strains acquired later (Mehta et al., 2002). In fact, the simultaneous detection of antibodies directed against different subtypes in the serotyping assay is a rare event (Schröter et al., 1999).

Predominance of 2b, 3a or 4a subtypes over the subtypes 1a and 1b was observed in 12 of 13 patients (92.3%) (Table 1). These results allow the conclusion that multiple infection with different HCV subtypes had occurred with displacement of the initial strains. This corresponds well to the observation that a dynamic change of HCV subtypes has occurred in Northern Germany during the last 2 years (Schröter et al., 2002). Meanwhile, the subtypes of genotype 2, subtype 3a, and 4a are the most prevalent strains in newly acquired HCV infections.

Besides the epidemiological significance, our results may influence antiviral therapy strategies. Patients infected with genotype 1 and 4 isolates have a less favourable response to pegylated-IFN/Ribavirin therapy than those infected with genotypes 2 or 3 (Zeuzem et al., 2000). There are different implications regarding the duration and the dose of the currently used drugs. The decision to choose a certain regimen depends on the HCV subtype which is detectable by PCR-based methods. Our results provide evidence that minor strains which respond less favourably might influence the outcome of therapy. This should be taken into account if patients do not respond as expected to the applied therapy regimen. In those cases, the actual viremic genotype has to be determined by PCR-based methods. However, serotyping assays would offer the possibility to detect minor strains by demonstration of respective antibodies prior to therapy initiation.

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