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ORIGINAL ARTICLE

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Higher detection rate of hepatitis G and C virus RNA in liver tissue than in serum of deceased injection drug users

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Abstract To examine the prevalence of hepatitis G virus (HGV) and hepatitis C virus (HCV) infections in deceased injection drug users and for comparison of the detection rates of HGV and HCV RNA in liver tissue with detection rates in postmortem serum samples, RT-PCR was performed in 50 drug abuse-related fatalities. HGV RNA was detectable in liver tissue samples from 17/50 suddenly deceased drug abusers (34%). In 16 of these 17 positive cases, serum samples were also available but HGV RNA was detected in only 10. From 29/50 anti-HCV positive individuals, HCV RNA was detected in 23/50 liver tissue samples (46%), but HCV RNA was detectable in only 6/22 of the corresponding serum samples. In 12 anti-HCV positive cases (10 being also positive for HCV RNA in the liver), the examinations revealed a coinfection with HGV by detection of HGV RNA in the liver tissue samples. A significant association between the detection of HCV RNA in the liver and the occurrence of antibodies against the HCV NS4 protein, but not against HCV core antigen or NS3 protein was observed. The probability of anti-HCV and HCV RNA positivity increased with the age of the individuals. No HGV or HCV infection was detected in a control group of 50 persons who died suddenly by violent impact. The prevalence of active HCV and HGV infections in injection drug users detected by RT-PCR in liver tissue is in good accordance with data obtained from sera from living injection drug users. In contrast, the detection rate in postmortem serum samples was clearly lower. Possible reasons for this observation are discussed and the use of liver tissue for postmortem detection of hepatitis virus RNA is recommended.

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Introduction

Since the identification of hepatitis G virus as a further parenteral transmissible hepatitis agent, intravenous drug abusers are considered to be at high-risk for this infection [1]. However, as yet it is unclear whether hepatitis G virus (HGV) might be responsible for cases of so-called non A-E hepatitis [2]. In 8–13% of patients with chronic non A-E hepatitis HGV genome sequences were detectable [1]. Reports concerning the clinical relevance of HGV describe – if at all – only very mild clinical courses [3]. Hausmann et al. [4] discussed one case of postoperative liver failure due to enflurane exposure and high dose paracetamol treatment, where a hepatitis G infection might have influenced the fulminant clinical course.

In the meantime, several studies have been published regarding the prevalence of this new hepatitis virus, both in general and in selected populations [1, 3, 5–7]. In all of these studies the occurrence of viral RNA was determined in serum samples. Recently Laskus et al. [8] have determined the positive strand RNA of HGV and HCV in explanted livers and serum samples of 10 patients coinfected whith HCV and HGV who received a liver transplant. The authors detected genomic HGV RNA in all serum samples but only in 6/10 of the livers and suggested that this organ might not be the primary replication site of this virus.

Up to now the HGV infection rate of deceased injection drug users has not been investigated. The aim of this study therefore was to determine the prevalences of HGV- and HCV-RNA in the main target organ of hepatotropic viruses. The results obtained were compared with the detection rates of viral RNA in corresponding serum samples. Furthermore, we correlated the infection rates with serological data (for HCV) and epidemiological data, such as age and sex, as it has been described for hepatitis B and C virus [9–12].

Material and methods

The study group consisted of 50 drug abuse-related fatalities (42 males, 8 females; mean age 29 ± 12 years) with known long term abuse of intravenous drugs. In 48 cases the cause of death was an overdose of psychotropic drugs, in 2 cases severe pneumonia in combination with high concentrations of cannabinoids. The autopsy was carried out within 2 days after death in all cases. The liver tissue samples were frozen and the serum samples were centrifuged immediately after autopsy. In all cases serological investigations (HCV antibodies) and RT-PCR (HCV, HGV) in liver tissue were carried out. In all positive cases (apart from one case, where no more serum was available) RT-PCR was additionally performed in serum. In 16 negative cases these investigations were not carried out for lack of serum. Chemical-toxicological analyses were performed in 45 cases. Morphine and codeine were detected in 82% and the relative concentrations of both substances were characteristic for heroin intake. In 8 of these 37 cases no other drugs were found. The remaining 29 cases, however, showed an additional consumption of mainly benzodiacepines (59%) and cannabinoides (41%), but also of cocaine, amphetamines, barbiturates and methadone. In 5 out of 8 fatalities without evidence of current opiate intake, chemical-toxicological analyses revealed an intake of methadone combined with benzodiacepines. In one case cocaine and in two cases, as mentioned above, cannabinoides alone were detected.

The control group consisted of 50 persons (41 males, 9 females; mean age 30 ± 15 years) without injection drug abuse. All persons died suddenly by violent impact.

Antibody detection

HCV antibodies were determined with the Abbott HCV EIA-kit (2nd generation, Abbott, Wiesbaden, Germany) by using peripheral blood samples retrieved from the femoral vein. All positive test results were verified using the recombinant dot-immunoassay MATRIX HCV (Abbott), which semiquantitatively detects specific antibodies against core, NS3 and NS4 proteins of HCV. All tests were carried out according to the manufacturer's instructions.

Extraction of RNA and cDNA synthesis

RT-PCR for HCV and HGV was carried out using frozen liver tissue samples or serum, respectively. Total RNA was extracted from approximately 50 mg of tissue or 150 µl serum, using the single step guanidinium thiocyanate method [13] and resuspended in a final volume of 50 µl DEPC-water. From the RNA solution 4 µl was subjected to reverse transcription in the presence of random hexanucleotides (Gibco, Eggenstein, Germany). Before transcription, the RNA was heated to 65 °C for 5 min. First strand synthesis of cDNA was carried out in a total volume of 10 µl containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 200 µM of each deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 100 pmol random hexanucleotides, 20 U of RNasin (Promega, Madison, Wisconsin) and 200 U M-MLV reverse transcriptase (Gibco BRL, Eggenstein, Germany). The reaction mixture was incubated for 60 min at 37 °C and the reaction was terminated by heating for 5 min at 95 °C, followed by a centrifugation for 1 min at $10{,}000 \times g$.

PCR and primers

Amplification of HCV cDNA was done by using one primer pair for a first PCR (HCV1 5′-GAA CTA CTG TCT TCA CGC-3′ and HCV2 5′-ACT ACT CGG CTA GCA GTC-3′, 210 bp) and an additional inner primer pair for a second, nested PCR (HCV3 5′-AAA GCG TCT AGC CAT GGC-3′ and HCV4 5′-TGA TCC AAG AAA GGA CCC-3′, 135 bp). Aliquots of 5 µl of the RT-reaction were transferred to 45 µl of reaction mixture containing 50 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 25 pmol of each primer, 0.01% (w/v) gelatine, 0.2% Tween 20, and 2.5 U of thermostable Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn., or MBI Vilnius, Lithuania). After an initial step of denaturation for 2 min at 94 °C, 15 cycles of amplification (first PCR) were carried out at 94 °C for 45 s, 53 °C for 50 s and 72 °C for 50 s. After this first amplification, 2 µl of the sample were transferred to a mixture containing the primers for the nested PCR and 30 amplification cycles were performed. After cycling, 10 µl of the amplified fragments were resolved on a 2% NuSieve-1% agarose gel and stained with ethidium bromide.

Similarly, for the detection of HGV two primer pairs were used for amplification. The first amplification was performed using the primers HGV1 5′-AAG ACA CTT TCC TCC CCT TCG GCT G-3′ and HGV2 5′-GAA CCG CAC ATT GAT GTC ATC ATG G-3′ (GenBank acc. U44402). For the second amplification the internal primer pair HGV3 5′-CTC TTT GTG GTA GTA GCC GAG AGA T-3′ and HGV4 5′-CGA ATG AGT CAG AGG ACG GGG TAT-3′ was used as published by Linnen et al. [1], resulting in a 156 bp fragment. Conditions for amplification and detection were the same as described for HCV, except for an annealing temperature of 55 °C instead of 53 °C.

Results

HGV RNA was detected in liver tissue of 17/50 individuals (34%). Additionally the corresponding serum samples were investigated. However, only 10 out of 16 available corresponding serum samples were also positive for HGV RNA, although viral genome was present in liver tissue of the same persons (Fig. 1). The serum of one individual who was positive for HCV RNA and HGV RNA in the liver tissue was not available for examination.

HCV RNA was detected in liver tissue of 23/50 cases (46%). However HCV RNA was detectable in only 6 out of 22 corresponding serum samples, while anti-HCV antibodies were found in 29/50 cases (58%). Thus, 6 individuals had acquired HCV-infection as shown by detection of HCV antibodies without HCV RNA now being detectable in serum or in liver tissue. Of the anti-HCV positive individuals 12 showed a coinfection with HGV and 10 were also positive for HCV RNA in liver tissue. Within the

Fig. 1 Flow chart of markers of HCV and HGV infections in 50 drug abuse-related fatalities (absolute numbers). The asterisks indicate one individual case being positive for HCV RNA and HGV RNA in liver tissue but without serum available for PCR

Table 1 Correlation between the semiquantitative determination of antibodies against the NS4, NS3 and core HCV polypeptides in the confirmation test and PCR positivity (only anti HCV positive cases). The figures represent mean values (with standard deviations) of the indicated HCV RNA positive and HCV RNA negative individuals. No serum was available from one HCV RNA positive case

	Antibody determination		
	NS4	NS3	Core
HCV RNA positive (liver) n = 22	11.70 ± 6.6	14.89 ± 7.1	18.54 ± 4.8
HCV RNA negative (liver) n = 6	2.73 ± 3.4	12.13 ± 6.6	16.97 ± 4.9
Wilcoxon's test	$p < 0.01$	–	–

control group no infections with HCV or HGV were detected.

The serological data revealed a significant association between HCV RNA detection and the mean indices of antibodies against HCV NS4 protein as a marker of ongoing active infection (Wilcoxon's test, $p < 0.01$, Table 1). No association with PCR results was apparent for the core antigen and the NS3 protein.

Correlating the HCV/HGV infection rates with age, a significant increase with age was found for anti-HCV (Fisher's Exact Test, $p < 0.01$) and for HCV RNA (Fisher's Exact Test, $p < 0.01$), but not for HGV RNA. An association with sex was found neither for HGV nor for HCV infections, but it should be taken into account that only 8 female cases could be investigated. Since most of the individuals of the study group proved to be polytoxicomanic (78%), a correlation of HCV and HGV infections with specific drugs was not possible.

No correlation was found between the postmortem interval (10–48 h) and the evidence of virus RNA.

Discussion

The prevalences of 34% for HGV RNA and 46% for HCV RNA in tissue samples showing active infections are in good accordance with data obtained from sera of living drug abusers [1, 5, 14]. The detection rates in serum were clearly lower than in liver tissue, in particular for HCV. One explanation could be that HCV RNA in serum is more susceptible to degradation than RNA in liver tissue. But this could also be due to a higher amount of viral RNA in liver than in serum. Another hypothesis could be that viral replication is continued in liver cells for a certain time even after cessation of blood circulation. Our investigations confirm that blood taken at autopsy does not seem to be an ideal material for detection of viral RNA. These results suggest searching for viral RNA in autoptic cases in particular in the target organ and not only in serum.

In some cases no HCV RNA was detectable in liver tissue, although HCV antibodies were detected in serum. For this constellation two explanations are possible: either the detection of antibodies is attributed to a previous HCV infection with subsequent elimination of the virus, or a carrier state had been established but with an amount of HCV RNA in the liver tissue being too low for detection. It has been published that the presence of antibodies against NS4 protein of HCV might be a serological marker for an active infection [15]. Therefore, we correlated the presence of antibodies against different HCV peptides with the detection of HCV RNA. Indeed, we found a significant association between the occurrence of antibodies against NS4 protein and the detection of HCV RNA in liver tissue. This result could indicate that the negative HCV PCR results in antibody positive individuals might in fact be due to elimination of the virus during lifetime.

In contrast to Laskus et al. [8] we found a higher detection rate of both HCV RNA and HGV RNA in liver tissue than in the corresponding serum samples. However, the differences between the both study groups have to be considered, since Laskus et al. investigated patients who received liver transplants due to end-stage liver diseases, whereas no obvious liver disease could be observed or was reported in the individuals in our study. It is possible that in end-stage liver diseases HGV is not present in high amounts in liver, similarly to hepatitis B virus [16]. In agreement with our results Hausmann et al. [4] reported one case of postoperative liver failure, where the detection of HGV RNA was possible only in liver tissue and not in serum.

Correlation of cumulative incidences of infections with age obviously needs the determination of persistent markers detecting active infections as well as infections in which the virus has already been eliminated. This holds true for many antibody determinations. Viral RNA, however, is found only in active or persistent infections and an age dependence thus can only be expected if elimination of the virus is a rare event. Our data revealed a significant positive correlation between age and anti-HCV and, although to a lesser extent, HCV-RNA. This result underlines the known fact that HCV establishes chronic infections in most cases. In contrast, the age distribution of HGV infections, as determined by detection of viral RNA, was well-balanced. This could point to a higher rate of virus elimination for HGV. The proof of the latter assumption will be possible with tests for permanent infection markers for HGV in the future. New investigations point to the HGV surface proteins E1 and E2 and the nonstructural proteins NS3 and NS4 which seem to provoke a persistent immune reaction of the host. Antibodies against these proteins were recently detected in serum of injection drug users [17, 18]. The occurrence of antibodies after a viraemia (months up to several years) seemed to be followed by virus elimination [18].

Neither HGV infections nor HCV infections were seen within the control group. This observation is in good accordance with data obtained from healthy blood donors, where anti HCV was detected in 0–0.42% [9, 19] and HGV RNA in 0.5–3.2% [7, 20].

In conclusion, the detection of HGV and/or HCV genome in liver tissue revealed a much higher sensitivity

than the determination of viral RNA in serum samples of deceased drug abusers. Therefore, we recommend the use of target organ tissues for investigations on viral RNA after autopsies to offset the reduction in quality of postmortem blood samples.

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