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Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities

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The serologic reactivity and epidemiology associated with different hepatitis C virus (HCV) variants were investigated in a cohort of 113 anti-HCV-positive donors. In Scotland, HCV type 1 accounted for one-half of all infections; 40 percent of subjects were infected with HCV type 3, and the remainder were infected with type 2. Reactivity with the NS-4-encoded antigens in the first-generation anti-c100 assay was absent in 68 percent of donors infected with types 2 and 3, as compared with 10 percent for those infected with type 1. Even when combined with surrogate marker testing, first-generation tests would have failed to detect 12 percent of HCV-infected blood donors. The age distribution, incidence of past infection with hepatitis B virus, and reported risk factors were similar in donors infected with types 1 and 3 (mean ages were 31.9 and 29.9; 18 and 17.6% were positive for antibody to hepatitis B core antigen; and 47 and 48% had past intravenous drug abuse). However, the distributions of alanine aminotransferase levels were significantly different in those infected with type 3 (abnormally raised in 83%) and those infected with type 1 (55% abnormal alanine aminotransferase; $p < 0.05$) or type 2 (60%; $p < 0.01$) and those who were nonviremic (8%; $p < 0.0001$). These data suggest that HCV type 1 is the most common HCV infection in blood donors and that infection with HCV type 3 may be associated with more severe liver disease, because of more recent infection or because of a greater inherent pathogenicity of type 3 variants. TRANSFUSION 1993;33:7–13.

Abbreviations: ALT = alanine aminotransferase; anti-HBc = antibody to hepatitis B core antigen; EIA = enzyme immunoassay; HCV = hepatitis C virus; LIA = line immunoassay; NCR = noncoding region; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; RIBA = recombinant immunoblot assay.

THE IDENTIFICATION OF hepatitis C virus (HCV)[1,2] and the development and widespread use of screening assays for HCV antibody[3] are proving to be among the most significant factors in the prevention of posttransfusion hepatitis. However, concerns about the effectiveness of the original tests for anti-HCV followed the reporting of data showing continued transmission of HCV by screened donations.[4,5] The failure of screening may be due to infectious, seronegative samples that are collected in the "window" period between exposure and seroconversion in the donor. In addition, the humoral response to HCV antigens may be abnormally restricted or reduced in immunocompromised individuals.

Another possible cause of false-negative results is the existence of HCV variants that elicit antibodies that fail to react with currently available antigens. Extreme sequence variants of HCV,[6-10] variously described as K2[6] or type III,[11] have been reported, that have only 65 percent overall sequence homology with the prototype virus (HCV-1)[2] and other variants from Japanese patients.[12,13] Recently, we discovered another major type of HCV that was equally distinct from the prototype virus.[14,15] Phylogenetic analysis of sequences in the relatively well conserved 5′ non-coding region (5′NCR) and in the core, NS-3 and NS-5 coding regions led to our classification of our own and other documented sequences into three major types—1, 2, and 3. Within the type 1 group, a subdivision is apparent on analysis of coding region sequences between the prototype sequences (HCV-1; type 1a) and those from Japanese patients (type 1b). An

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equivalent subdivision is also apparent between type 2 sequences, corresponding to the K2a (type 2a) and K2b (type 2b) groups in the NS-5 region.[6]

In this study, we used restriction fragment length polymorphism (RFLP) analysis of viral sequences amplified in the 5'NCR to analyze the distribution of the three main types of HCV in Scottish blood donors. The extent to which sequence variability affects the recognition of HCV antigens has been assessed by analysis of serologic reactivity between individual peptides used in commercially available confirmatory assays for anti-HCV with sera from individuals infected with different HCV types. Finally, the recall of HCV-infected individuals has permitted a comparison of risk factors for infection, age distribution, and extent of liver function abnormality associated with each type.

Materials and Methods

Samples

Approximately 147,000 volunteer blood donor samples collected in Scotland and Northern Ireland were screened between September 1, 1991, and January 15, 1992, for antibody to HCV using a second-generation HCV enzyme immunoassay (EIA, Abbott GmBH, Wiesbaden-Delkenheim, Germany) or a second-generation HCV enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Raritan, NJ). Repeatably reactive samples were tested by second-generation recombinant immunoblot assay (RIBA, Chiron Corporation, Emeryville, CA) for antibody to 5-1-1, c100-3, c33c, and c22-3 antigens and by line immunoassay (LIA, Innogenetics; Antwerp, Belgium) for antibody to NS-4, NS-5, and four different core oligopeptides. We carried out all antibody tests and interpreted them strictly in accordance with the manufacturers' instructions.

Donations that were positive (significant reactivity with two or more HCV antigens [1+ to 4+]) or indeterminate (reactivity with one antigen only) in the RIBA were tested for viral RNA by polymerase chain reaction (PCR). We tested donor samples for antibody to hepatitis B core antigen (anti-HBc) by radioimmunoassay (Abbott). Donors yielding RIBA-confirmed antibody-positive and/or PCR-positive donations were recalled for medical investigation and counseling in Glasgow and Edinburgh (Scotland). A single alanine aminotransferase (ALT) determination was made in samples from the 90 recalled donors and from 100 randomly selected HCV-negative donors. The upper limit of the normal range for the testing laboratory was 55 units per L. HCV sequences from hemophiliacs infected by factor VIII concentrates[16] were used for sequence comparisons.

RNA PCR

Amplification of HCV RNA used primers 269/939 and 211/940 to amplify the 5'NCR[15] and 205/208 and 206/207 to amplify part of the NS-3 region.[17] We directly sequenced amplified DNA as described previously.[18]

HCV typing

Predicted cleavage patterns produced by all common restriction enzymes of 59 blood donor and hemophiliac sequences, obtained in this and our previous study,[12] and 19 sequences reported elsewhere[2,7,8,12,18,19-21] were computed with standard sequence-analysis software.[22] We labeled amplified DNA by supplementing the PCR buffer with 2 μCi of [^{35}S]-dATP (Amersham International, Amersham, UK) and reducing the concentration of unlabeled nucleotide triphosphates to 8.25 μM (8.25 μmol/L).[23] One μL of the product was digested with 1 unit of *ScrFI* or 1 unit each of *RsaI* and *HaeIII* (all: Boehringer Mannheim UK, Lewes, UK) in 50 μL of supplied restriction buffer for 3 hours at 37°C.

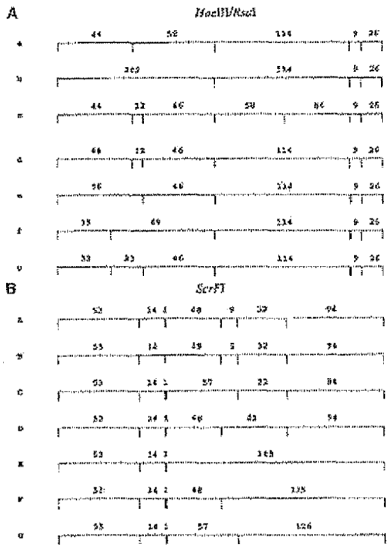

FIG. 1. Possible cleavage patterns for A) *HaeIII/RsaI* (patterns a-g) and B) *ScrFI* (patterns A-O) in the 5'NCR of published sequences of HCV (n = 19) and those obtained in this and our previous study[16] (n = 59).

The cleaved product was heated to 65°C for 5 minutes and electrophoresed on a 12-percent polyacrylamide gel in 1x TBE (134 mM Tris, 68 mM boric acid, 2.5 mM EDTA, pH 8.0) at 50 V for 18 hours or 160 V for 6 hours. After fixation in two changes of 5-percent acetic acid and 5-percent methanol (30 min each), the gel was dried and exposed to x-ray film for 3 days prior to development. We sized DNA fragments by comparison with migration distances of standard-size DNA markers (pBR322/*HaeIII* digest; Boehringer).

Results

Sequence variation in the 5'NCR

We compared 78 HCV sequences in the 5'NCR by standard phylogenetic methods[25] and assigned them to HCV types 1, 2, and 3 (data not shown). A total of seven distinct cleavage patterns were predicted for the restriction endonucleases *HaeIII* and *RsaI* in combination (Fig. 1A). All of the type 1 sequences obtained in this and our previous study,[16] as well as all pub-

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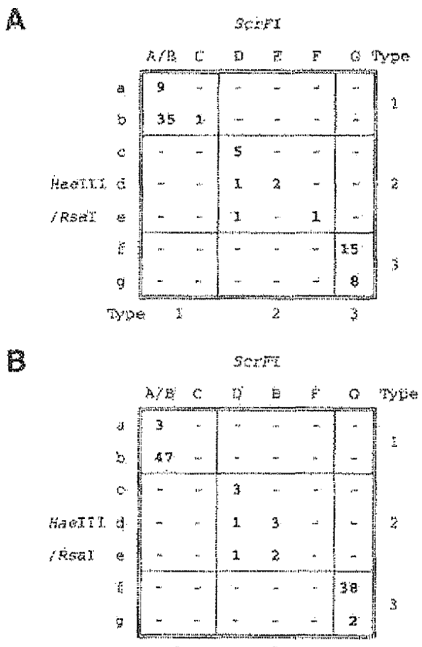

FIG. 2. A) Predicted combinations of RFLP patterns obtained from cleavage with *HaeIII/RsaI* and *ScrFI* associated with sequences of HCV types 1 to 3. Patterns A and B for *ScrFI* (Fig. 1) are not differentiated. B) Observed RFLP patterns of HCV variants amplified from 100 blood donor samples, showing inferred HCV type.

lished type 1 sequences, produced restriction patterns a and b; type 2 sequences produced patterns c, d, and e, and all type 3 sequences produced patterns f and g (Fig. 2A).

ScrFI independently differentiates all known type 1 sequences (pattern A, B) from type 2 (C, D, E, and F) and type 3 (G; Figs. 1B, 2A). The two adjacent *ScrFI* sites present in all but two of the sequence variants (pattern B) could not be simultaneously cleaved, so patterns A and B are, for practical purposes, equivalent and will subsequently be referred to as A/B.

HCV typing

Amplified and radiolabeled 5'NCR sequences from a total of 100 PCR-positive, RIBA-confirmed or -indeterminate subjects were typed by RFLP with both *ScrFI* and *HaeIII/RsaI*. Observed combinations of restriction patterns and thus the inferred HCV type for each sample are shown in Fig. 2B. No sample produced restriction patterns incompatible with the combinations of published sequences or our own sequences (Fig. 2A). Types 1 and 3 are the predominant variants in Scotland (Table 1); 50 percent of donors were infected with type 1 and 40 percent with type 3, while type 2 accounted for the remaining 10 percent.

Serologic reactivity

A total of 45 of the 50 samples from subjects infected with HCV type 1 were positive in the first-generation anti-c100 EIA (Abbott) and showed broad reactivity with all four antigens in the RIBA and with the NS-4, NS-5, and core peptides in the LIA (Table 1; Fig. 3). By contrast, fewer of those infected with HCV types 2 and 3 (16/50) were anti-c100 positive, and samples showed weak or absent reactivity with the NS-4-derived antigens in the RIBA and LIA. Type 3 sera also showed weaker reactivity with c33c in the RIBA. Sera from type 3 samples were, however, equally reactive to the NS-5 peptides in the LIA and to each of the four core peptides. Almost all sera from infected individuals (98/100) reacted with the c22-3 antigen with a score of 4+.

Sera from eight subjects found by RFLP to be infected with type 1 failed to react with some or all of nonstructural antigens. One possible explanation is that these individuals were infected with variants that had type 1 RFLP pattern in the 5'NCR but were divergent in sequence elsewhere in the genome. To investigate this, we amplified samples and sequenced them in the NS-3 (coding) region. Of the six samples that could successfully be amplified with the NS-3 primers, five corresponded closely to those of type 1a variants, while the last was more similar to type 1b (data not shown).

Clinical correlations with HCV type

Anti-HBc was detected in 19 percent of samples from HCV-infected subjects (100 PCR-positive subjects; 13 samples from individuals who were anti-HCV positive but PCR negative; Table 1). We found similar frequencies among subjects infected with different HCV types.

A total of 90 HCV-infected blood donors (38 infected with HCV type 1, 10 with type 2, 29 with type 3, and 13 who were confirmed antibody positive but were PCR negative and therefore were not typed) attended a donor follow-up clinic. As the ages of the donors were not distributed normally, they were compared by the nonparametric Wilcoxon rank-sum test. This showed a significant difference between those infected with HCV types 1 and 3 (combined mean age, 30.8) and those infected with HCV type 2 (mean age, 37.9; $p<0.05$; Table 2; Fig. 4A). No significant difference was observed in the age distributions of those infected with type 1 or type 3 or those who were PCR negative (Fig. 4A).

ALT values from each of the four categories of HCV-infected individuals and a control group of 100 HCV antibody-negative donors are shown in Fig. 4B. The Wilcoxon rank-sum test was used to compare the distribution of values, and the χ^2 test was used for contingency analysis of numbers of samples over the upper limit of the normal range (55 units/L; Table 2). Both statistical methods indicated significant differences in ALT values in PCR-positive donors (i.e., types 1-3) and PCR-negative and HCV-uninfected donors (all p values <0.02). We observed no difference between the PCR-negative group and the controls. In both statistical tests, ALT levels were significantly higher ($p<0.05$, <0.025) in donors infected with HCV type 3 than in those infected with other types (type 3, 24/29 had abnormal ALT; type 1, 21/38; type 2, 6/13; Fig. 4B).

A total of 27 of the 90 donations had normal ALT values (<55 units/L) and were anti-HBc negative (totals of 12, 3, 4,

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Table 1. *Comparison of anti-c100, anti-HBc*, and history of intravenous drug abuse in subjects infected with different HCV† types*

HCV type	Frequency (%)	Anti-c100 (%)	Anti-HBc (%)	IVDA‡ (%)
1	50/100 (50)	45/50 (90)	9/50 (18)	18/38 (47)
2	10/100 (10)	3/10 (30)	3/10 (30)	2/10 (20)
3	40/100 (40)	13/40 (33)	7/40 (18)	14/29 (48)
Total				
PCR§-positive	100/113 (88)	61/100 (61)	19/100 (19)	34/77 (44)
PCR-negative	13/113 (12)	10/13 (77)	2/13 (15)	7/13 (54)
Total HCV-positive		71/113 (63)	21/113 (19)	41/90 (46)

* Antibody to hepatitis B core antigen.
† Hepatitis C virus.
‡ Number of individuals admitting to past intravenous drug abuse. Other risk factors (e.g., multiple blood transfusion) not analyzed.
§ Polymerase chain reaction.

and 8 for HCV types 1, 2, and 3 and PCR negatives, respectively). Among these samples, 11 were also negative on anti-c100 testing (2, 3, 4, and 2 for HCV types 1, 2, and 3, and PCR negatives, respectively).

Discussion

Distribution of HCV types

The data presented in this article confirm our previous conclusion[14] that all three major types of HCV are present in the British blood donor population. In Japan, type 1 appears more commonly than type 2 in blood donors and patients with non-A,non-B hepatitis, cirrhosis, and hepatocellular carcinoma.[6,8,24,25] When it has been possible to differentiate type 1a sequences from type 1b, it has been reported that the majority of Japanese and Taiwanese patients are infected with type 1b, with the exceptions being hemophiliacs treated with imported (from the USA) factor VIII.[6,8] However, while almost all blood donors in this and our previous study[15] appear to be infected with type 1a, it has recently been reported that type 1b is more common in Holland[26] and Belgium (Maertens G, verbal communication, December 1991); type 1b also accounts for a proportion of HCV infections in France.[27]

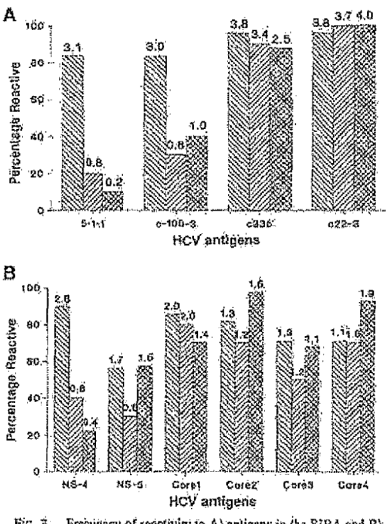

FIG. 3. Frequency of reactivity to A) antigens in the RIBA and B) peptides in LIA, *by 50 type 1* (▨), *10 type 2* (▨), *and 40 type 3* (▨) sera. Figures above histograms indicate mean score for each type with each antigen, from 0 (nonreactive) to 4+.

HCV type 2 was originally found in Japanese and Taiwanese patients,[6,8,9,24,25] its presence in Italy[28] and the UK hints at a much more widespread distribution than anticipated by the original reports. Sequences of the type 3 group have been found in the UK[14,15] and in blood donors from India, Finland (McOmish F. and Simmonds P., unpublished observations, 1992), and Brazil (Maertens G, verbal communication, February 1992). Two of the donors in this study appear to have been infected with HCV type 3 outside of the UK (Belgium, Italy). We have also detected type 3 sequences in patients with non-A,non-B posttransfusion hepatitis from numerous countries (Sweden, Italy, USA; unpublished data). Together, these data indicate that all three types have a worldwide distribution, with varied frequencies in particular countries.

Serologic crossreactivity

The region of the HCV genome encoding to c100-3 and 5-1-1 is known to be extremely variable between HCV types 1 and 2[2,7] and within types.[9,10,12,13] Thus, it is not surprising that serologic crossreactivity between type 1a antigens and antibody elicited by infection with type 2 and 3 is limited. In this study, sera from only 21 of the 51 patients infected with type 2 and 3 (4/11 and 17/40, respectively) reacted with NS-4-encoded antigens

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Table 2. *Comparison of the distributions of ALT* values in donors infected with HCV† types 1, 2, and 3 and in PCR‡-negative and HCV-uninfected donors using χ^2 § (upper right quadrant) and Wilcoxon Rank-Sum ¶ (lower left quadrant)*

	HCV type				
	1	2	3	PCR-negative	HCV-uninfected
1		0.002 (NS)	5.63 (p<0.025)	8.94 (p<0.005)	42.46 (p<0.005)
2	222 (NS)		3.39 (NS)	6.57 (p<0.025)	24.22 (p<0.005)
3	1135 (p<0.05)	136 (p<0.01)		20.99 (p<0.005)	74.9 (p<0.005)
PCR-negative	160 (p<0.001)	105 (p<0.02)	126 (p<0.001)		0.057 (NS)
HCV-uninfected	4010 (p<0.001)	865 (p<0.02)	3155 (p<0.001)	610 (NS)	

* Alanine aminotransferase.
† Hepatitis C virus.
‡ Polymerase chain reaction.
§ χ^2 values for each pair-wise comparison of numbers of ALT values greater than 55 units/L (probability of obtaining the observed numbers by chance in parentheses).
¶ Rank Sums of ALT values, W, for each pair-wise comparison.
‖ p values > 0.05 considered nonsignificant (NS).

(Fig. 3; Table 2). Reactivity with c100-3 was lower in Japanese patients infected with type 2 in one study[16] but similar to that in type 1-infected patients in another.[25] It is possible that such "crossreactivity" is the result of multiple infection, as has been described for hemophiliacs.[24] It could be hypothesized that antibody to type 1 antigens persists longer than the type 1 viremia upon reinfection with type 2 or 3. Multiple exposure is possible in intravenous drug abusers, as that practice is the predominant risk factor for infection identified in blood donors in this study (45%) and others.[29]

Reactivity to c33c in the RIBA (encoded by NS-3) and the NS-5 peptides (LIA) with sera from type 2- and 3-infected donors was more frequent than reactivity with the NS-4 antigens (Fig. 3A), and almost all sera from donors infected with any of the three types reacted with c22-3, the most highly conserved protein. Similarly, the frequency and strength of reactivity with each of the core peptides in the LIA differed little in regard to HCV type (Fig. 3B). In interpreting these results, it must be borne in mind that the sera were preselected by the original second-generation screening test. Thus, there is no information on the proportion of type 2- and 3 infections that elicit such a restricted serologic response as to be undetectable by current blood donor screening.

The lack of serologic reactivity on the part of the eight donors infected with HCV type 1 to some or all of the nonstructural antigens could be explained by proximity to seroconversion, although only three of the six individuals recalled showed abnormal ALT values. Sequential studies have shown that antibody to c22-3 or c33c may appear before antibody to c100-3[30-32] and that this restricted profile may persist for over a year after primary infection, particularly in elderly or immunocompromised individuals. We therefore postulate that some individuals infected with types 2 or 3, who have a delayed or absent response to the core protein of HCV, may not be identified by present serologic screening assays. To detect such "seronegative," HCV-infected donors, the sensitivity of current second-generation assays could be improved by incorporation of the homologues of the c100-3 and other proteins from HCV types 2 and 3 as antigens in screening and confirmatory assays.

Approximately one-third of HCV-infected donors were negative for anti-HBc and had ALT values in the normal range, which is consistent with previous studies.[33-35] Even if surrogate marker testing were combined with anti-

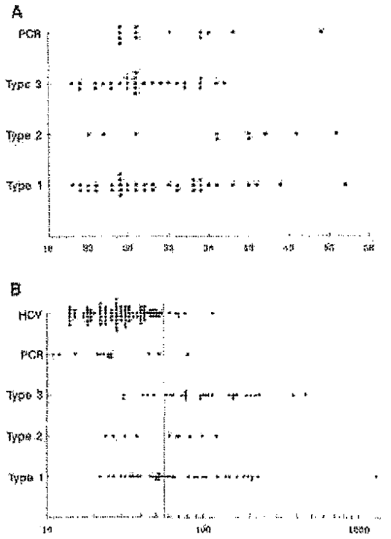

FIG. 4. A) Age distribution of recalled HCV-infected donors and B) ALT values; maximum normal value of 55 indicated. ALT values for 100 anti-HCV-negative controls included for comparison.

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c100 (first-generation) screening, 11 of the 90 HCV-infected donors would have been missed. Whereas most donors infected with HCV type 1 would be excluded on the basis of anti-c100 reactivity, exclusion of those infected with type 3 would have been, in almost all cases, due to raised ALT values.

Course of disease associated with different HCV types

All of the recalled donors were asymptomatic, although approximately 60 percent had ALT values above the upper limit of the normal range. A significant finding was a clear difference in the distribution of ALT values in those who were PCR positive and those who were PCR negative ($p<0.0001$). Indeed, the ALT values of the latter group were comparable to those of the HCV-negative control donors.

It has been suggested that HCV type 2 may cause more severe disease and be less susceptible to interferon therapy than type 1.[28] In our study, the distribution of ALT values of type 2-infected donors was similar to that of type 1-infected donors. However, their age distribution was not typical of blood donors and tended to be higher than that of donors infected with type 1 and type 3 variants, which suggests that infection had occurred longer ago and had had more time to resolve.

In contrast, donors infected with HCV types 1 and 3 were of similar age and had a similar incidence of past hepatitis B virus infection and reported risk factors for infection (in both cases, approximately 50% admitted past intravenous drug abuse). There was no association between HCV type and the geographical region within which the donor lived in Scotland or Northern Ireland, nor was there any association with specific areas, such as public housing, within a city. All but three of the donors were born in Scotland or Northern Ireland, and the majority are likely to have acquired infection from there. Thus, it is unlikely that simple epidemiologic differences can account for the marked difference in the distribution of ALT values in the two groups (type 3: 83% abnormal ALT, compared with type 1: 55% abnormal; $p<0.05$). These differences raise the possibility that infection with type 3 is associated with greater liver damage and possibly a more severe course of disease than infection with type 1 and indicate a potential clinical role for HCV typing. However, despite the epidemiologic investigations, it remains possible that the higher ALT values in type 3-infected donors are the result of more recent infection and are perhaps associated with the infection of current drug abusers and their contacts in certain areas of Scotland. In addition, the higher ALT values do not necessarily reflect greater long-term damage to the liver or the likelihood of complications such as cirrhosis and hepatocellular carcinoma. The full significance of the differences in ALT values can therefore be determined only by further observation of the donors, to study the course of infection in more detail and to carry out liver biopsies to investigate directly the extent of liver disease.

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