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Intrahepatic long-term persistence of parvovirus B19 and its role in chronic viral hepatitis

Chun Wang, Albert Heim, Verena Schlaphoff, Pothakamuri Venkata Suneetha, Kerstin Stegmann, Hong Jiang, Martin Krueger, Paraskevi Fytili, Thomas F. Schulz, Markus Cornberg, et al.

► **To cite this version:**

Chun Wang, Albert Heim, Verena Schlaphoff, Pothakamuri Venkata Suneetha, Kerstin Stegmann, et al.. Intrahepatic long-term persistence of parvovirus B19 and its role in chronic viral hepatitis. *Journal of Medical Virology*, 2009, 81 (12), pp.2079. 10.1002/jmv.21638 . hal-00531828

HAL Id: hal-00531828

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Intrahepatic long-term persistence of parvovirus B19 and its role in chronic viral hepatitis

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|-------------------------------|---|
| Journal: | <i>Journal of Medical Virology</i> |
| Manuscript ID: | JMV-09-1347.R1 |
| Wiley - Manuscript type: | Research Article |
| Date Submitted by the Author: | 23-Jul-2009 |
| Complete List of Authors: | <p>Wang, Chun; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Heim, Albert; Hannover Medical School, Department of Virology Schlaphoff, Verena; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Suneetha, Pothakamuri; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Stegmann, Kerstin; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Jiang, Hong; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Krueger, Martin; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Fytli, Paraskevi; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Schulz, Thomas; Hannover Medical School, Department of Virology Cornberg, Markus; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Kandolf, Reinhard; University of Tuebingen, Department of Molecular Pathology Manns, Michael; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology Bock, Thomas; University of Tuebingen, Department of Molecular Pathology Wedemeyer, Heiner; Hannover Medical School, Department of Gastroenterology, Hepatology and Endocrinology</p> |
| Keywords: | Parvovirus B19, Chronic Hepatitis C, Intrahepatic persistence, Cellular immunity |
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For Peer Review



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23. Jul. 2009

Dear Prof. Zuckerman,

Thank you very much for considering a revised version of our manuscript on B19V-persistence in the liver of European patients. We thank the reviewers for the valuable comments which helped to improve the manuscript. All issues raised were addressed in the revised version. The manuscript was also re-edited by a native English speaker. We hope that this version is now acceptable for publication in the Journal of Medical Virology.

Kindest regards

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Point-to-point response:

Reviewer 1

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4 1. Can the authors explain why there is a difference in B19V DNA in the serum of Vietnamese vs
5 German patients given the presence of B19V DNA in the livers of German patients? Was B19V DNA
6 also present in the livers of the Vietnamese patients?
7

8 We thank the reviewer for this interesting question. Of note, B19V DNA detection in biopsy
9 specimens is usually associated with persistence in the absence of circulating B19V DNA in sera or
10 plasma (Norja, Simmonds et al., J Virol 2008). It also has to be considered that B19V genotype 1 is
11 almost exclusively found in Western Europe while genotype 2 is frequent in Asia. This may also
12 account for some differences. The previous study by Toan et al. (J Hepatol 2006) did not investigate
13 B19V DNA in livers, however, other reports from Asia detected viral DNA in liver tissues from
14 patients with fulminant hepatitis and biliary atresia (Abe K et al., Int J Med Sci 2007;4:105-9). We
15 clarified this point in the discussion.
16

17
18 2. Did the breadth and strength of the CD4+ T cell responses to B19V correspond to the severity of
19 liver disease?

20 T cell responses were tested in patients only in patients with mild liver disease (F score from 0-3,
21 activity score from 2-7) and thus this interesting question cannot be answered.
22

23
24 3. Why was the CD8+ T cell response not assessed? This may not be essential for publication but
25 might have been of interest.

26 The two MHC-class-II restricted peptides were selected based on previous studies (Kasproicz V, et
27 al. J Virol 2006;80:11209-17). As it was not our primary aim to perform a sophisticated and comprehensive
28 analysis of B19V-specific T cell responses, we focused on published epitopes first. Moreover, few class-I
29 B19V epitopes have been identified and there are restricted to a limited number of HLAs.
30

31 4. Why was the frequency of HCV-specific CD4+ T cell response against NS3, NS4 and core proteins
32 so low?

33 The frequency of HCV-specific CD4+ T cell response was similar to most previous studies on patients
34 with chronic HCV before therapy (e.g. Wedemeyer et al., JI 2002; Aberle et al., JID 2007).
35

36 5. Did the authors assess patients who cleared HCV spontaneously and who were B19V Ab positive
37 for B19V and HCV- specific CD4+ T cell responses? Could the B19V have contributed to an
38 increased clearance of HCV?

39 The author is raising an interesting point. However, no patients were included in this study who have
40 cleared HCV spontaneously. Thus and unfortunately, the question if B19V could have contributed to
41 an increased clearance of HCV remains speculative. To answer this question a much larger patient
42 cohort would need to be investigated.
43

44
45 6. Is it possible to look at intrahepatic B19V specific CD4+ T cell responses? Could this explain why
46 the virus is present in the liver but not in the serum?

47 Again, the reviewer is raising a very interesting question. These experiments are currently ongoing in
48 our laboratory. However, at this stage data are inconclusive and thus we did not want to include them
49 in this manuscript. A respective sentence has been added to the discussion.
50

51 7. Figures 2 and 3 present very similar data. The information contained in figure 3 is probably
52 sufficient and figure 2 could be deleted.

53 Figure 2 shows that B19V DNA was detected more frequently in explanted livers than in routine
54 biopsy samples while figure 3 presents the virus copy number in different cohorts. We think that this
55 information is not identical and thus would like to keep both figures. However, if the editor feels that
56 one of the two figures should be omitted we can delete one figure
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Reviewer 2

1.) Some of the numbers in the abstract need to be explained more clearly

The numbers in the abstract have been clarified.

2.) The method of patient selection needs to be clarified - were these sequential patients or were there specific criteria for selection

The reviewer is addressing an important issue. The 91 patients with chronic hepatitis C were randomly recruited from our outpatient clinic. Before antiviral therapy, baseline sera were collected. Also the 16 patients studied for T cell responses were randomly selected, however, with concomitant liver carcinoma, autoimmune liver disease or other viral hepatitis were excluded.

3.) Morbus Wilson should be Wilson's Disease.

Has been changed.

4.) The number of T cell assays is too small for any definitive conclusions. The authors should state much DMSO was used in the T cell assays

We agree that T cell data are not conclusive yet and changed the discussion accordingly.

The DMSO was 0.5% which did not inhibit spontaneous proliferation of T cell according to our recent systematic study investigating the effect of peptide pools on effector functions of T cells. (Suneetha et al., J Immunol Methods 2009).

5). On P13 lines 254-6: The conclusion "Our findings clearly..." should be modified in view of the small sample size

We have made changes as suggested.

Tables

Table 1. Characteristics between B19V IgG antibody positive and negative patients with chronic hepatitis C

| | B19V IgG pos (n=67) | B19V IgG neg (n=24) | p value |
|--|------------------------|------------------------|---------|
| Age(years, mean±SD, range:19-73) | 48.4±9.8 | 43.9±8.7 | 0.26 |
| Gender | Male: 38 Female: 29 | Male: 12 Female: 12 | 0.50 |
| F score(mean±SD, range:0-6) | 2.6±2.1 | 2.2±1.5 | 0.34 |
| Percentage of live cirrhosis ^a (%) | 20.9 | 8.3 | 0.90 |
| Activity score(mean±SD, range:0-18) | 5.2±2.0 | 5.2±2.2 | 1.00 |
| ALT ULN(times) | 3.1±2.3 | 3.2±2.6 | 0.92 |
| AST ULN(times) | 2.4±1.8 | 2.3±1.5 | 0.71 |
| gGT ULN(times) | 2.1±2.2 | 2.2±2.8 | 0.85 |
| Total bilirubin(umol/l) | 12.2±7.9 | 12.7±5.6 | 0.74 |
| Albumin(g/l) | 43.7±3.4 | 44.9±3.1 | 0.20 |
| Prothrombin time (Quick value %) | 98.4±22.5 | 95.7±8.6 | 0.43 |
| Platelets(Tsd/ul) | 186.5±55.6 | 187.3±93.4 | 0.97 |
| Presence of B19V viremia | 1 | 0 | 0.55 |
| Presence of anti-B19V IgM | 1 | 0 | 0.55 |

Legend: a, F score ≥ 5 .

There were no significant differences of clinic characteristics in these two groups.

Table 2. Overview of CD4⁺ T cells responses determined by CFSE assay of PBMCs in the presence or absence of antigen in B19V IgG antibody positive individuals.

| | VP1/2 4.7 | VP1/2 7.2 | HCV NS3 | HCV NS4 | Core |
|---|--------------------|--------------------|------------------|--------------------|------|
| B19V IgG antibody positive healthy individuals | | | | | |
| HI 1 | - | - | NT | NT | NT |
| HI 2 | - | - | NT | NT | NT |
| HI 3 | - | - | NT | NT | NT |
| HI 4 | - | - | NT | NT | NT |
| HI 5 | - | - | NT | NT | NT |
| HI 6 | - | - | NT | NT | NT |
| HI 7 | - | + (SI=2.3;ΔPF=9) | NT | NT | NT |
| HI 8 | - | - | NT | NT | NT |
| HI 9 | - | - | NT | NT | NT |
| HI 10 | - | - | NT | NT | NT |
| HI 11 | - | - | NT | NT | NT |
| HI 12 | - | - | NT | NT | NT |
| HI 13 | - | - | NT | NT | NT |
| HI 14 | - | + (SI=2.4;ΔPF=6.3) | NT | NT | NT |
| HI 15 | - | - | NT | NT | NT |
| HI 16 | - | - | NT | NT | NT |
| HI 17 | - | + (SI=2;ΔPF=2.3) | NT | NT | NT |
| HI 18 | - | - | NT | NT | NT |
| HI 19 | - | - | NT | NT | NT |
| B19V IgG antibody positive patients with chronic hepatitis C | | | | | |
| HCV 1 | - | - | - | - | - |
| HCV 2 | - | - | - | - | - |
| HCV 3 | - | - | + (SI=3.6;ΔPF=1) | + (SI=5;ΔPF=1.5) | - |
| HCV 4 | - | - | - | - | - |
| HCV 5 | - | - | - | - | - |
| HCV 6 | + (SI=2.2;ΔPF=3.6) | + (SI=3.7;ΔPF=7.8) | - | + (SI=6.8;ΔPF=16) | - |
| HCV 7 | + (SI=2.6;ΔPF=1) | - | - | + (SI=6;ΔPF=3) | - |
| HCV 8 | - | - | - | - | - |
| HCV 9 | - | + (SI=2.3;ΔPF=1.6) | + (SI=2.7;ΔPF=2) | + (SI=2.3;ΔPF=1.6) | - |
| HCV 10 | - | - | - | - | - |
| HCV 11 | - | - | - | + (SI=2.6;ΔPF=1.5) | - |
| HCV 12 | - | - | - | - | - |
| HCV 13 | - | - | - | - | - |

A positive response was considered if the Δ PF was at least 1.0% and the stimulation index (SI) was >2.0. NT, not tested.

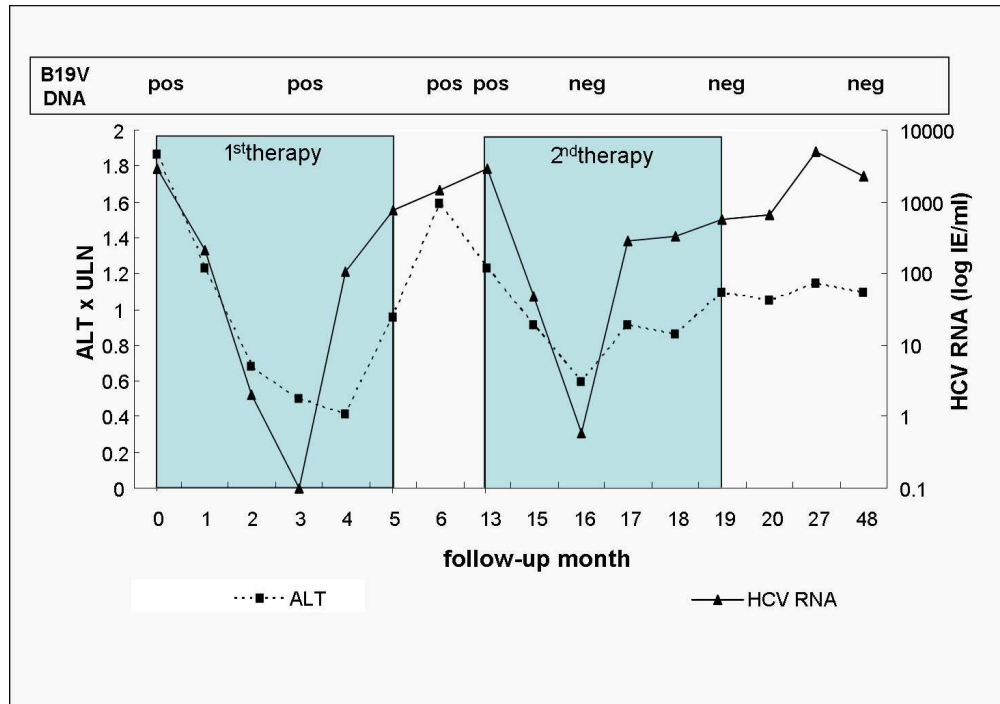


Fig. 1. Fluctuation of ALT, HCV-RNA and B19V viremia in the patient with chronic hepatitis C. The patient was followed for 48 months and treated twice with interferon alpha and ribavirin. B19V viremia lasted at least one year even during the first combination therapy. The first combination therapy (from follow-up month 0 to 5) consisted of interferon alpha-2b plus ribavirin for 22 weeks. The dose of interferon alpha-2b was 10 MU per day in the first two weeks followed by 3 MU per day for 8 weeks followed by 3 MU every 2nd day. The second combination therapy (from follow-up month 13 to 19) consisted of PEG-interferon alpha-2b (100 µg qw) plus ribavirin for 20 weeks.

248x174mm (150 x 150 DPI)

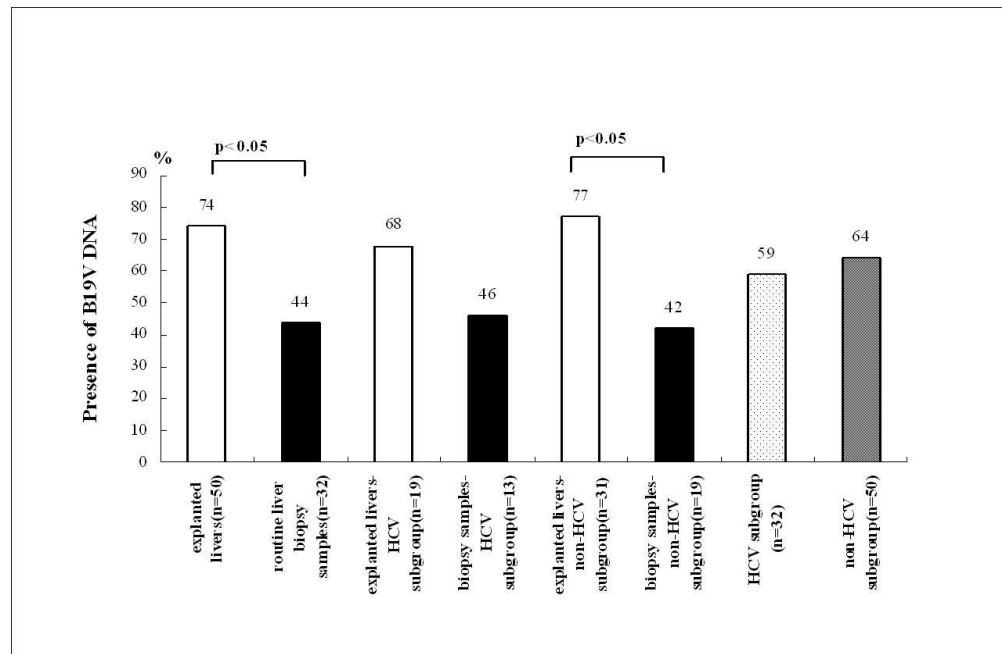


Fig. 2. Presence of B19V DNA in explanted livers and routine liver biopsy samples. B19V DNA was amplified more frequently in explanted end-stage liver tissues (74%) than in biopsy samples (44%) ($p < 0.05$). The same trend was found in non-HCV subgroups (non-HCV explanted liver tissues vs non-HCV liver biopsy samples, $p < 0.05$)

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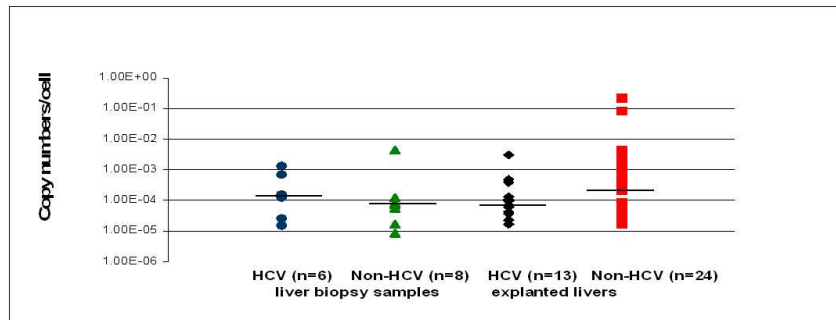


Fig. 3. B19V DNA copy numbers per cell in explanted livers and routine liver biopsy samples. The median of B19V DNA copy numbers/cell in routine liver biopsy samples was 8.29E-05 (in chronic hepatitis C subgroup was 1.38E-04, while in non-HCV liver disease subgroup was 7.18E-05). The median of B19V DNA copy numbers/cell in explanted livers was 9.63E-05 (in end-stage hepatitis C subgroup was 6.95E-05, while in non-HCV end-stage liver disease subgroup was 2.42E-04). There was no significant difference of virus load in these groups.

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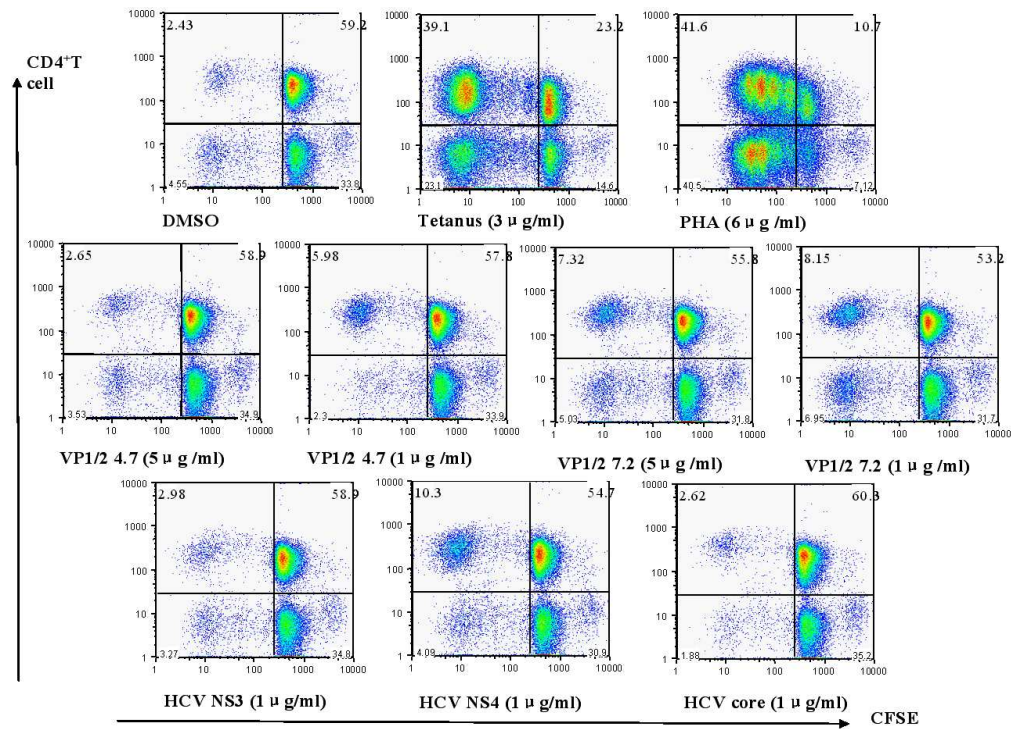


Fig. 4. Proliferation of CD4+ T cells in patients with chronic hepatitis C and healthy individuals with serologically recovered B19V infection.

Dot plots show the flow cytometric analysis of the proportion of CD4+ T cells proliferating in response to stimulation of PBMCs with no stimulus (DMSO), phytohemagglutinin (PHA), tetanus (TT), two synthetic B19V peptides (VP1/2 4.7, VP1/2 7.2) in two different concentrations (1 μg/ml and 5 μg/ml) or recombinant HCV NS3, NS4 and core protein. Values in the upper left quadrants of dot plots represent the proportion of CFSElow CD4+ T cells (daughter cells), which have proliferated during the 7-day culture. Values in the upper right quadrants of dot plots are CFSEhigh CD4+ T cells (parent cells).

4A shows the positive responses of CD4+ T cells in a B19V IgG antibody positive patient with chronic hepatitis C after stimulation of PBMCs with VP1/2 4.7, VP1/2 7.2 and HCV NS4.

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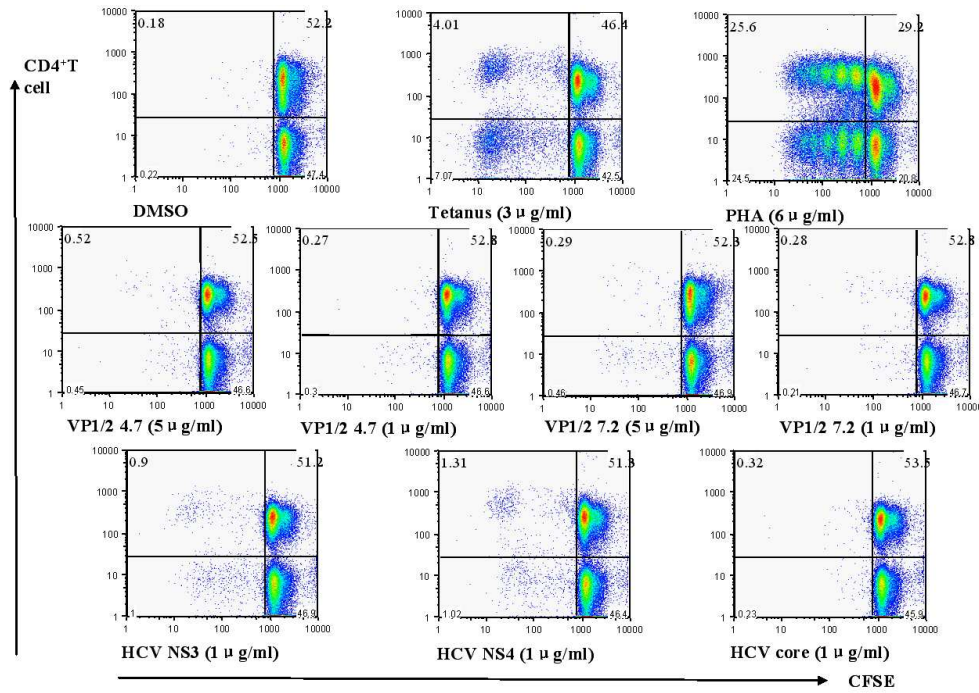


Fig. 4B shows the negative responses of CD4+ T cells in a B19V IgG antibody positive patient with chronic hepatitis C after stimulation of PBMCs with B19V peptides, while positive with HCV antigen (NS3 and NS4).
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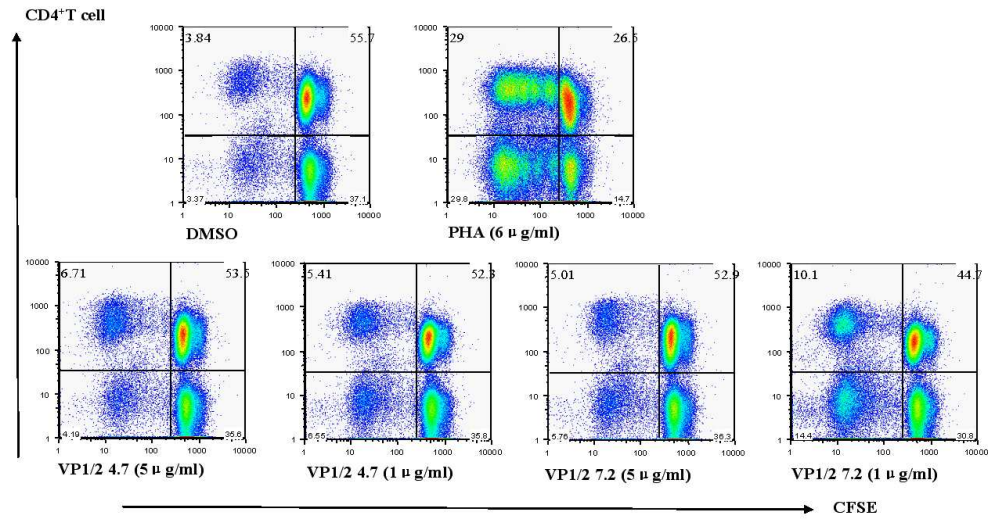


Fig. 4C shows the positive responses of CD4⁺ T cells in a B19V IgG antibody positive healthy control after stimulation of PBMCs with VP1/2 7.2
338x187mm (96 x 96 DPI)

Intrahepatic long-term persistence of parvovirus B19 and its role in chronic viral hepatitis

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Running title: Parvovirus B19 infection in hepatitis C

** CTB and HW contributed equally to this work and thus shared last authorship*

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Abstract

Parvovirus B19 (B19V) has been detected in the liver of Asian patients infected with HBV and may contribute to acute and chronic liver disease. This study aimed to investigate the impact of B19V infection in European patients with viral hepatitis. B19V DNA was detected in 1/91 and 0/50 serum samples from patients with chronic hepatitis C and B, respectively. In contrast, B19V DNA was amplified frequently from explanted end-stage liver tissues (37/50, 74%) and from routine biopsy samples (14/32, 44%) ($p < 0.05$). However, there was no significant difference in B19V copy number per cell between these two groups. B19V-specific CD4+ T cell responses to two dominant MHC-class-restricted epitopes were detected in a similar frequency in healthy anti-B19V-positive individuals (3/19; 16%) and patients with chronic hepatitis C (3/13; 23%). These results indicate that B19V can persist in the liver. However, there is no evidence that B19V is a “hepatitis virus” worsening liver disease in European patients with chronic hepatitis C.

Keywords : Parvovirus B19; Chronic Hepatitis C; Intrahepatic persistence; Cellular immunity

1 Introduction

Human parvovirus B19 (B19V) is a species of the genus *Erythrovirus*, family *Parvoviridae*, which has been discovered by Cossart et al. [1975] during the measurement of hepatitis B virus surface antigen in 1974. B19V is the causative agent for *erythema infectiosum*, also termed fifth disease or “slapped-cheek” disease. In the past decade, a growing number of studies have been published aiming to investigate a possible association between B19V and liver diseases especially in the etiology of fulminant liver failure and the persistence of B19V in the liver [Abe et al., 2007; Drago et al., 1999; Eis-Hubinger et al., 2001; He et al., 2003; Hillingso et al., 1998; Karetnyi et al., 1999; So et al., 2007; Sokal et al., 1998; Wong et al., 2003; Yoto et al., 1996]. Recently, B19V-infection has been linked with severe liver disease in Vietnamese patients with chronic hepatitis B [Toan et al., 2006]. However, only few studies have investigated B19V in European patients and available results are controversial. Thus the first aim was to investigate if B19V DNA can be detected in the liver.

The hepatitis C virus (HCV) is the type species of the genus *Hepacivirus*, family *Flaviviridae*. HCV is a major causative agent of liver cirrhosis and hepatocellular carcinoma. Viral co-infection (e.g. HBV, HIV) is one major factor which influences the natural history of HCV infection and contributes to the development of liver cirrhosis [Manns et al., 2006]. The prevalence of B19V co-infection in European patients with chronic hepatitis C in different stages of liver damage is unknown. Based on the recent finding of B19V being involved possibly in the disease progression of Asian patients infected with hepatitis B, the second aim was to investigate whether B19V infection is a co-factor for the disease progression in chronic hepatitis C.

Immune responses against HCV are crucial to control viral replication, elimination, as well as the outcome of chronic infection [Rehermann and Nascimbeni, 2005]. Both innate

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4 26 and adaptive immune responses against a virus can be altered significantly by past or
5
6 27 ongoing exposure to third pathogens [Welsh et al., 2006]. For hepatitis C it has been
7
8 28 suggested that T cell cross-reactivity between HCV and the influenza A virus [Wedemeyer
9
10 29 et al., 2001] may cause subfulminant courses of acute HCV infection [Urbani et al., 2005].
11
12 30 However, T cell responses in coinfections with other pathogens than HIV have rarely been
13
14 31 investigated in patients with chronic hepatitis C. Thus, the third aim was to study the
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16 32 frequency and strength of B19V-specific and HCV-specific T cell responses in individuals
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18 33 with persistent HCV infection.
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34 **Patients and methods**

35 **Patients infected with hepatitis C: sera and liver biopsy samples**

36 Stored baseline sera were studied from randomly selected 75 patients with chronic
37 hepatitis C infection who have been treated with interferon alpha and ribavirin combination
38 therapy. Another 16 sera and paired peripheral blood mononuclear cells (PBMCs) were
39 collected from patients with chronic hepatitis C infection without antiviral therapy for
40 evaluating cellular immune responses. For screening B19V DNA, 49 paraffin treated liver
41 biopsy samples out of these patients were also recruited. These 91 patients had a mean age
42 of 46 years ranging from 19-73 years (male: 49; female: 42). Characteristics are summarized
43 in Table 1. The presence of anti-HCV antibody (ELISA) and HCV RNA \geq 600 IU/ml for
44 more than 6 months were required for the diagnosis of chronic HCV infection. Patients with
45 concomitant hepatocellular carcinoma, autoimmune liver disease or other viral hepatitis
46 were excluded.

47 **Patients infected with hepatitis B**

48 Serum samples were taken from 50 patients with chronic hepatitis B infection selected at
49 random (mean age: 47 years, range: 15-72; male: 33, female: 17). The presence of hepatitis
50 B surface antigen (HBsAg) for more than 6 months was required for the diagnosis of
51 chronic HBV infection.

52 All patients were treated in the outpatient clinic of the Department of Gastroenterology,
53 Hepatology and Endocrinology of Hannover Medical School, Germany.

54 **Liver tissue samples**

55 Explanted liver tissues were obtained at the time of liver transplantation from 50 patients
56 with end-stage liver damage undergoing orthotopic liver transplantation for various reasons
57 from 1993 to 2000 at the Hannover Medical School (mean age: 47 years, range: 8-69). Of
58 these, 19 had HCV related end-stage liver disease, while 31 had non-HCV liver cirrhosis

(e.g. primary biliary cirrhosis, primary sclerosing cholangitis, Wilson's disease and Polycystic liver disease). Another 32 liver biopsy samples were obtained from patients with chronic liver disease (mean age: 46 years, range: 33-75). Of these, 13 had chronic hepatitis C. All samples were stored at -20°C until used.

63 **Healthy individuals**

64 Sera and paired PBMCs were collected from 30 healthy volunteers (mean age: 37.5 years, 65 range: 25-65; male: 16, female: 14). Among them, 19 were B19V-IgG antibody positive 66 while 11 were negative.

67 **Methods**

68 **Serological tests**

69 Anti-B19V IgM and anti-B19V IgG antibodies were tested using the Parvovirus B19 70 IgM/IgG Enzyme Immunoassay (Biotrin, Dublin, Ireland) according to the manufacture's 71 instruction. Anti-HCV antibodies were tested using ARCHITECT Anti-HCV assay (Abbott, 72 Abbott Park, IL, USA).

73 **Extraction of nucleic acids from liver tissue**

74 DNAs were extracted from 20-30g frozen explanted liver tissue using the QIAamp DNA 75 mini kit (Qiagen, Hilden, Germany) according to the manufacture's instruction.

76 DNAs from paraffin treated liver samples was extracted as described previously 77 [Lehmann et al., 2007].

78 **Polymerase chain reaction (PCR)**

79 Quantitative real-time PCR (qPCR) was performed using the B19V artus LC-PCR kit 80 (Qiagen, Hilden, Germany) according to the manufacture's instruction. A qPCR of genomic 81 C-reactive protein (CRP) DNA was performed with same samples in order to determine the 82 amount of human CRP DNA representing the actual amount of amplifiable cellular DNA in

each sample [Gan et al., 2002; Wandinger et al., 2000]. B19V copy numbers per cell were calculated from the amplification of B19V divided by the amount of human CRP DNA.

Samples were defined as a “serologically recovered” cohort by the presence of anti-B19V IgG with the absence of IgM and DNA.

Isolation of PBMCs and carboxy fluorescein succinimidyl ester (CFSE)-based T cell proliferation assay

PBMCs were separated from heparinized blood samples by gradient centrifugation on Ficoll-Paque and stored in liquid nitrogen until used.

CFSE-based T cell proliferation assay was performed as described previously [Wedemeyer et al., 2002]. Briefly, frozen PBMCs were thawed and suspended at 10^7 /ml in PBS plus 0.2% BSA and incubated at 37°C for 7 min with 2.5 μ M CFSE (Sigma, St. Louis, MO, USA). An equal volume of FCS was added thereafter and cells were incubated on ice for 5 min. to stop reaction, followed by 3 times washing. Then, labelled cells were resuspended in medium (RPMI 1640 supplemented with 10% inactive AB serum and penicillin), plated at 0.3×10^6 cells per 200 μ l per well in round-bottom 96-well microtiter plates and cultured with DMSO alone (background), synthetic peptides VP1/2 7.2 (LASEESAFYVLEHSSFQLLG) and VP1/2 4.7 (FLIPYDPEHHYKVFSPAASS) [Kasprovicz et al., 2006] (ProImmune, Oxford, UK) at a final concentration of 1 μ g/ml and 5 μ g/ml, tetanus toxoid (TT, 3 μ g/ml) and PHA (6 μ g/ml) as positive controls at 37°C with 5% CO₂ for 7 days. Each condition was duplicated. DMSO concentration was 0.5% which did not inhibit spontaneous proliferation of T cells according to the recent systematic study investigating the effect of peptide pools on effector functions of T cells [Suneetha et al., 2009].

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4 106 PBMCs from patients with chronic HCV infection were stimulated also with recombinant
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6 107 genotype 1a-derived HCV core, NS3 and NS4 protein (Microgen, Munich, Germany) at a
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9 108 final concentration of 1µg/ml.

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11 109 Flow cytometric analysis: Flow cytometric data (100,000 nongated events) were acquired
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13 110 on a BD FACS-Calibur 4-color flow cytometer using BD Cellquest software (both from BD
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15 111 Biosciences, San Jose, CA, USA). For analysis, BD FlowJo 6.1.1 (Treestar, Ashland, OR,
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17 112 USA) was used to gate on CD4⁺CD3⁺ T cell populations. The background of CD4⁺ T cells
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19 113 proliferative frequency (%) was calculated as the number of CFSE^{low} CD4⁺ T cells / (numbers
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21 114 of CFSE^{low} CD4⁺ T cells + numbers of CFSE^{high} CD4⁺ T cells) *100 in the absence of antigen.
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23 115 The ΔPF was calculated by subtracting the mean background proliferation from the
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25 116 proliferating fraction in response to specific antigen. The SI was calculated by dividing the
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27 117 antigen-induced PF by the background PF. Both a ΔPF of 1.0% or more and an SI of 2.0 or
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29 118 more are considered as a positive response, as previously defined [Crawford et al., 2004;
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31 119 Semmo et al., 2007] .
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37 120 The study was approved by the Institutional Review Board of the Hannover Medical
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39 121 School, Germany.

40 41 42 122 **Statistical analysis**

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44 123 Statistical analysis was performed using t-test and χ^2 test with SPSS 10.0 software.
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124 **Results**

125 **Prevalence of B19V antibodies and B19V DNA in serum samples from patients with** 126 **chronic hepatitis C and B infection**

127 Anti-B19V IgG antibodies were detected in 67/91 of patients with chronic hepatitis C
128 (74%) and in 19/30 healthy individuals (63%). Only one HCV sample was positive for
129 B19V IgM antibody. In contrast to previous studies on Asian patients infected with HBV
130 and HCV [Hsu et al., 2005; Toan et al., 2006], all but one HCV sera were negative for
131 B19V DNA by qPCR. Importantly, B19V DNA was also undetectable in all 50 sera
132 collected from HBsAg positive patients. Thus, B19V viremia is an extremely rare finding in
133 German patients with viral hepatitis B and C.

134 **Clinical characteristics in relation to B19V serology**

135 Clinical characteristics including age, sex, liver enzymes, albumin and bilirubin levels,
136 prothrombin time and platelets, as well as histological staging and inflammation scores did
137 not differ between B19V IgG antibody positive and negative subgroups as shown in Table 1.
138 Thus, past B19V infection had no apparent effect on the course of HCV infection in this
139 cross-sectional study.

140 **Interferon treatment of a patient with persistent B19V viremia**

141 The single B19V DNA positive serum sample tested also positive for both anti-B19V
142 IgM and IgG. Consecutive serum samples of the same patient were collected for up to 48
143 months. The B19V viremia lasted at least one year even when the patient received
144 combination therapy of daily injections with recombinant interferon alpha-2b plus ribavirin.
145 B19V viremia showed no obvious association with ALT levels or HCV viremia (Fig. 1).
146 However, the patient became B19V-negative during a second course of antiviral therapy
147 with pegylated interferon alpha-2b plus ribavirin.

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149 **B19V DNA in liver biopsy samples and explanted livers**

150 Because of the rare occurrence of B19V viremia, B19V DNA has been screened in paired
151 routine biopsy blocks from these patients. However, the amount of cells of these blocks was
152 very low, only 4 out of 49 were positive. Therefore additional liver tissues from biopsy
153 samples and explanted liver tissues were recruited for further study.

154 The presence of B19V DNA in the liver by qPCR is shown in figure 2. B19V DNA was
155 amplified from more than half of liver tissues which has been studied and detected more
156 frequently in explanted end-stage liver tissues (37/50, 74%) than in biopsy samples (14/32,
157 44%) ($p < 0.05$). This held also true for non-HCV subgroups (non-HCV explanted liver
158 tissues vs non-HCV liver biopsy samples, $p < 0.05$).

159 In biopsy samples, the presence of B19V DNA was similar in severe liver disease (F
160 score ≥ 5) as in moderate liver disease (F score < 5). In the same group, there was no
161 difference of grading (inflammatory activity) in B19V DNA positive samples versus
162 negative samples.

163 While the frequency of B19V DNA-positive tissues differed between explanted liver
164 tissues and routine biopsy samples, there was no significant difference of virus copy number
165 per cell between explanted liver tissues and routine biopsy samples, as well as HCV-positive
166 versus HCV-negative liver samples (Fig. 3).

168 **Proliferation of B19V-specific CD4⁺ T cells in patients with chronic hepatitis C and** 169 **healthy individuals with B19V recovered serologically**

170 A flow cytometry-CFSE assay was performed to evaluate the proliferation of
171 B19V-specific CD4⁺ T cell after stimulation of PBMCs with synthetic peptides. In B19V
172 serologically recovered healthy individuals, 3/19 (16%) showed a positive CD4⁺ T cell
173 responses to the peptide VP1/2 7.2. In anti-B19V IgG positive patients with chronic

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4 174 hepatitis C, 3/13 (23%) patients had a positive response to the peptide VP1/2 7.2 and/or the
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6 175 peptide 4.7. Representative examples of three individuals are shown in figure 4.
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9 176 HCV-specific CD4⁺ T cells against NS3, NS4 and core proteins were detectable in 2/13,
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11 177 5/13 and 0/13 patients with chronic hepatitis C, respectively. The summary of CD4⁺ T cell
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14 178 responses after stimulating with specific antigens is shown in Table 2.
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For Peer Review

179 Discussion

180 B19V is the causative agent of *erythema infectiosum* (fifth disease) [Anderson et al.,1983;
181 1985]. The presence of anti-B19V IgG antibody increases with age, 2-15% in young
182 children (1-5 years), 15-60% in school age children (6-19 years), 30- 60% in adults, and
183 more than 85% in the older population [Heegaard et al., 2002]. B19V is thought to be a “hit
184 and run” virus which can be eliminated completely. However, it was reported that B19V
185 may persist in the bone marrow [Cassinotti et al., 1997; Heegaard et al., 2002; Lundqvist et
186 al., 1999a; Lundqvist et al., 1999b], muscle [Chevrel et al., 2000], synovia [Nikkari et al.,
187 1995] and the skin [Vuorinen et al., 2002] in immunocompetent individuals with the
188 absence of viremia or symptomatic infection. Likewise, three groups of investigation
189 demonstrated the persistence of B19V DNA in the liver [Abe et al., 2007; Eis-Hubinger et
190 al., 2001; Wong et al., 2003]. Strong additional evidence is provided that B19V may indeed
191 persist in the liver as B19V DNA was detectable in more than half of the snap frozen liver
192 tissues investigated.

193 Whether B19V is a pathogenic agent of fulminant liver failure and non-A-E hepatitis or a
194 major risk factor to worsen liver function and accelerate disease progression is still
195 controversial. One study from China suggested that the co-infection of B19V and HBV did
196 not lead to more severe liver damage [He et al., 2003]. Nevertheless, two other studies on
197 B19V infection in patients with chronic viral hepatitis led to different conclusions. Hsu et al.
198 [2005] detected B19V DNA in serum samples from patients in Taiwan infected with HBV
199 (37%) and patients infected with HCV (24%) by nPCR. Furthermore, IgM antibodies were
200 detected in patients with HBV (35%) and HCV (16%). However, co-infection of B19V with
201 HBV or HCV did not increase the frequency of liver dysfunction in patients with chronic
202 hepatitis. In contrast, Toan et al. [2006] demonstrated that the persistent B19V infection was

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4 203 not only frequent in Vietnamese patients with HBV but also associated with severe hepatitis
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6 204 B-associated liver disease.

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9 205 In order to find out whether B19V acts indeed as a bystander or a participator in the
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11 206 progression of liver disease, the prevalence of B19V infection in patients with chronic
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13 207 hepatitis C and B as well as healthy individuals in Germany were assessed. The frequency
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15 208 of serologically recovered was not different in patients infected with HCV than in healthy
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17 209 individuals and coincided with the average level reported previously [Young and Brown,
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19 210 2004]. Past B19V infection had also no apparent impact on biochemical and histological
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21 211 parameters of liver disease in patients with HCV. These findings suggest that B19V does
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23 212 neither worsen liver disease nor accelerate disease progression in patients with chronic
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25 213 hepatitis C in central Europe, however, larger studies are needed to confirm these results.

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30 214 The frequency of B19V viremia was much lower in this study than in several reports
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32 215 studying Asian cohorts of patients with liver disease (e.g. [Toan et al., 2006]). There is no
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34 216 evidence that a technical problem to amplify B19V DNA was responsible for the low
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36 217 frequency of B19V DNA in this study. First, the positive control worked always well and
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38 218 secondly, the technique applied was able to detect B19V DNA at high frequencies in liver
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40 219 tissue samples. Therefore, the most likely explanation for different results of different
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42 220 studies is that B19V viremia is more frequent in East-Asia than in Germany. Further studies
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44 221 on additional cohorts in Europe and other regions of the world need to clarify this issue.

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49 222 In sharp contrast to biopsy samples, B19V DNA was found in more than half of the
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51 223 explanted liver tissues studied. Interestingly B19V DNA was found more frequently in
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53 224 end-stage liver tissues than in routine biopsy samples. However, there was no significant
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55 225 difference in virus copy number per cell between these two groups. The presence of B19V
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57 226 DNA in explanted liver tissues with different types of liver disease has been reported
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59 227 previously ranging from 24%-66% [Abe et al., 2007; Eis-Hubinger et al., 2001; Langnas et

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4 228 al., 1995; Wong et al., 2003]. Eis-Huebinger et al [2001] also demonstrated that B19V DNA
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6 229 can be found frequently in livers of adults with severe liver damage. In this study, the
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9 230 frequency of B19V DNA in explanted liver was slightly higher than in previous studies.
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11 231 This is the first study reporting the persistence of B19V DNA in routine biopsy liver
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13 232 samples. However, whether the detection of B19V DNA is the cause or consequence of
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16 233 progressive liver disease is unknown. Only a prospective study comparing the outcome of
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18 234 liver disease in individuals with detectable and undetectable B19V DNA in liver biopsies
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21 235 would be able to answer this question.

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23 236 The only patient with detectable viremia for more than one year had no B19V-related
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25 237 symptoms. Prolonged B19V infection seems to be not rare as clearance of B19V viremia
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28 238 has been reported to be more slowly than previously considered, despite early resolution of
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30 239 symptoms [Lindblom et al., 2005]. Thus this case would be in line with this report.
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32 240 Importantly, the patient showed persistent viremia despite parallel treatment with high doses
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35 241 of interferon alpha-2b and ribavirin. Whether the second course of antiviral therapy with
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37 242 pegylated interferon alpha-2b and ribavirin has contributed to the clearance of B19V
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39 243 viremia remains speculative. In this respect it is worthy to note that treatment trials for
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42 244 B19V-myocarditis with interferon beta are ongoing [Kuhl, 2005].

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44 245 Persistence of B19V DNA detection is usually associated with B19V DNA detection in
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46 246 biopsy specimens in the absence of detectable B19V DNA in sera or plasma [Norja et al.,
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49 247 2008]. The finding of B19V DNA detection in the liver but not in sera is therefore in line
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51 248 with previous studies. It also has to be considered that B19V genotype 1 is almost
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53 249 exclusively found in Western Europe while genotype 2 is frequent in Asia. This may also
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56 250 account for some differences. The previous study by Toan et al. [2006] studying B19V in
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59 251 hepatitis B patients did not investigate B19V DNA in livers, however, other reports from
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252 Asia detected viral DNA in liver tissues from patients with fulminant hepatitis and biliary
253 atresia [Abe et al., 2007].

254 B19V specific CD4⁺ T cell responses have been first described in 1996 using
255 recombinant protein in IgG positive but IgM negative individuals [von Poblitzki et al.,
256 1996]. Thereafter, several studies [Franssila et al., 2001; Mitchell et al., 2001; von Poblitzki
257 et al., 1996] reported that in serologically recovered individuals VP1, VP2, VP1/2 and VP1u
258 were major targets of CD4⁺ T cells. In 2006, Kasprovicz et al [2006] characterized B19V
259 CD4⁺ T cell epitopes using overlapping peptides. In this study, two key immunodominant
260 peptides were employed to investigate T cell proliferation on a single-cell basis sensitive
261 flow cytometry-based CFSE-assay. Few positive responses were detected in patients with
262 chronic hepatitis C and healthy individuals who carried anti-B19V IgG antibody at the same
263 frequency and strength. Thus this study did not reveal an impairment of recall T cell
264 responses to another pathogen in patients with HCV. This finding is of importance as HCV
265 may alter the function of several immune cells including dendritic cells [Bain et al., 2001;
266 Rehermann and Nascimbeni, 2005]. The normal T cell response to B19V is also in line with
267 the clinical finding that in patients with HCV infection the frequency of B19V viremia was
268 not increased, thus the low level of persisting intrahepatic B19V is controlled by intact
269 immunological mechanisms. Future studies will also need to investigate if B19V-specific T
270 cell responses are associated with severity of liver disease. In addition, studying intrahepatic
271 B19V-specific T cell responses might of interest.

272 HCV-specific CD4⁺ T cell responses against recombinant HCV protein NS3, NS4 and
273 core were detectable in 2/13, 5/13 and 0/13 patients with chronic hepatitis C who infected
274 with serologically recovered B19V infection, respectively. This frequency of HCV-specific
275 CD4⁺ T cell response was similar to most previous studies on patients with chronic hepatitis
276 C before therapy [Wedemeyer et al., 2002; Aberle et al., 2007] but lower than in patients

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4 277 after spontaneous HCV clearance [Meyer et al., 2007]. Thus, memory to B19V had no
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6 278 apparent influence on T cell responses to HCV proteins in this study. However, future
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8 279 studies will have to investigate potential cross-reactivities between B19V and HCV in more
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10 280 detail and specifically also for CD8⁺ T cells.
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13 281 In summary, B19V DNA may persist in the liver at a low level for a long period after
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15 282 acute infection. However, even though B19V DNA can be detected intrahepatically, there is
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17 283 no evidence that B19V is a “hepatitis virus” worsening liver disease and accelerating
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19 284 disease progression of chronic hepatitis C in European patients. T cell responses to B19V
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21 285 are also not affected by HCV infection suggesting that persisting B19V can well be
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23 286 controlled by the host’s immune system.
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32 289 **Acknowledgements:** We are grateful to Ms. Heike Kaiser for her excellence assistance.
33
34 290 This work was supported by the Deutsche Forschungsgemeinschaft SFB-Transregio 19, TP
35 291 B5, by a grant from the German Network of Competence on Viral Hepatitis to HW (projects
36 292 10.2.2 and 10.2.4), the DFG Center grant SFB 738, project B2 and the IFB-Tx of Hannover
37 293 Medical School.
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