The Journal of Infectious Diseases

MAJOR ARTICLE

Repertoire and Neutralizing Activity of Antibodies Against Hepatitis C Virus E2 Peptide in Patients With Spontaneous Resolution of Hepatitis C

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Neutralizing antibodies can prevent hepatitis C virus (HCV) infection, one of the leading causes of cirrhosis and liver cancer. Here, we characterized the immunoglobulin repertoire of memory B-cell antibodies against a linear epitope in the central front layer of the HCV envelope (E2; amino acids 483–499) in patients who were infected in a single-source outbreak. A reverse transcription polymerase chain reaction–based immunoglobulin gene cloning and recombinant expression approach was used to express monoclonal antibodies from HCV E2 peptide–binding immunoglobulin G–positive memory B cells. We identified highly mutated antibodies with a neutralizing effect in vitro against different genotype isolates sharing similar gene features. Our data confirm the importance of V_H1–69 use for neutralizing activity. The data offer a promising basis for vaccine research and the use of anti-E2 antibodies as a means of passive immunization.

Keywords. HCV; HCV envelope (E2) protein; anti-D cohort; memory B cells; neutralizing antibodies.

With a rising incidence, chronic infection with hepatitis C virus (HCV) is a major public health problem. Approximately 3 to 4 million people worldwide are newly infected each year [1]. In the majority of cases, HCV infection results in chronic hepatitis, which may lead to liver cirrhosis and subsequent hepatocellular carcinoma. Spontaneous clearance of HCV, resulting in resolution of the infection without long-term sequelae, occurs only in a minority of acutely infected individuals [2].

Exceptional improvements in HCV therapy have been achieved in the last years with the development of highly effective direct-acting antivirals (DAAs). However, these therapies are expensive, resulting in highly restricted access in developing nations, where the disease burden is greatest [3]. Thus, because of the often asymptomatic course of the disease, the elimination of HCV will only be possible through the development of a prophylactic vaccine that prevents transmission [4]. A challenge for the development of vaccines lies in the genetic diversity of HCV, which makes it difficult to identify protective epitopes that are conserved in the majority of viral genotypes and subtypes [5]. To develop successful vaccination strategies, it is necessary to decode protective immune responses in vivo in patients with spontaneous resolution [6].

While HCV-specific T cells are of importance in the immune-mediated elimination of HCV [7], early generation of neutralizing antibodies is believed to play an extraordinary role in a successful immune response [8]. Further, humoral memory is maintained over a long period (eg, after vaccination), even in the absence of an antigen [9]. Memory B cells contribute to antibody production in vivo [10] and provide the option to analyze differences in antibody responses in these patients' peripheral blood. Several studies using animal models have shown that polyclonal anti-HCV immunoglobulins, as well as monoclonal antibodies, can prevent infection with HCV in vivo [11, 12].

A number of studies have suggested that antibodies against HCV envelope glycoproteins E1 and E2 are involved in controlling the viral load during acute infection [13–15]. These proteins are thought to form a heterodimer on the surface of the virus that interacts with several host factors, such as CD81 [16]. Binding to CD81 on host cells is needed for HCV entry into hepatocytes [17]. Within the HCV E2 region, amino acids 483-499 encompass a linear epitope [18, 19] forming the β2 loop, the outer layer of the central β-sandwich with little sequence variability [20]. The β-sandwich is part of the known antigenic domain C and comprises conformational epitopes mediating

Received 28 November 2018; editorial decision 15 May 2019; accepted 3 June 2019; published online June 4, 2019.

Presented in part: 52nd Annual Meeting of the European Association for the Study of the Liver (EASL), Amsterdam, the Netherlands, April 2017; 51st Annual Meeting of the EASL, Barcelona, Spain, April 2016; 49th Annual Meeting of the EASL, London, United Kingdom, April 2014.

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The Journal of Infectious Diseases® 2019;220:1209–18

DOI: 10.1093/infdis/jiz274

broad neutralization, owing to exposure on the virus surface that allows access for antibody binding [21].

Several neutralizing HCV antibodies that were induced by natural infection have been characterized and target different epitopes of the HCV envelope proteins [22–24]. However, these immunoglobulins vary in their neutralizing breadth and in the HCV strains that they were able to inhibit. The detailed repertoire analysis of anti-HCV memory B-cell responses is hampered by the fact that, in patients with spontaneous clearance, the infecting genotype and clinical data from the acute phase and long-term follow-up after infection are rarely available. For that reason, we studied a special cohort of patients infected with HCV in a single-source outbreak. In 1978 and 1979, 2867 women in East Germany were accidentally infected via contaminated rhesus prophylaxis (anti-D cohort) [25]. It was shown that the anti-D globulin batches contained 3 closely related but still distinct variants of the same HCV AD78 strain [26]. Of the patients regularly examined in follow-up, 93% developed acute hepatitis C. After 25 years, 46% of the women were able to clear the infection spontaneously [25]. Differences in immunological host factors are thought to be the major reason for the varying clinical outcomes.

Here, we characterize the naturally occurring immunoglobulin G (IgG) memory B-cell antibody repertoire against a linear epitope (amino acids 483–499) of the E2 β-sandwich region of HCV (hereafter, the "HCV E2 peptide") at the molecular and functional levels and identified 2 monoclonal antibodies with broad neutralizing activity against HCV. Our study provides insights in the molecular mechanisms underlying antibody-mediated viral clearance and informs antibody-based vaccine design.

METHODS

Patient Cohort and Patient Samples

Peripheral blood samples were obtained after approval of the study protocol by the local ethics committee of the University of Leipzig (ethics committee project no. 133-14-14042014) and receipt of written consent from patients of the anti-D cohort and healthy non–HCV-infected donors.

Isolation of HCV E2 Peptide–Binding Memory B Cells

Peripheral blood mononuclear cells (PBMCs) were isolated and purified by density gradient centrifugation using lymphocyte separation medium (Histopaque 1077, Sigma-Aldrich). Cell pellets were suspended in heat-inactivated fetal calf serum (FCS; Biochrom) containing 10% dimethyl sulfoxide (Carl Roth) and stored in liquid nitrogen. After thawing and washing, purified PBMCs were stained with antibodies to human molecules (ie, anti-CD19 [dilution 1:100], anti-CD27 [1:10], and anti-IgG [1:10]; BD Pharmingen) and with N-terminal–labeled HCV E2 peptide (RPYCWHYAPQPCGIVPA) [18, 19]). $CD19^+CD27^+IgG^+HCV\ E2^+$ B cells were analyzed and sorted as single cells by fluorescence-activated cell sorting (FACS) into 96-well plates (Eppendorf) containing 4 µL/well of ice-cold 0.5× phosphate-buffered saline, 10 mM DTT, and 8 U RNAsin (Promega). Plates were stored at −80°C.

Immunoglobulin Gene Amplification

Paired immunoglobulin heavy chain (*IgH*) and corresponding immunoglobulin light chain (*IgL*) genes were amplified and analyzed as previously described [27]. All polymerase chain reaction (PCR) products were sequenced and analyzed for V(D)J gene segment use, CDR3 features, and number of somatic hypermutations (SHMs) by alignment and comparison to germ line sequences, using IgBLAST (available at: http://www.ncbi.nlm.nih.gov/igblast/) and the IMGT database (available at: http://www.imgt.org).

Recombinant Antibody Expression

Human embryonic kidney 293T cells (catalog no. CRL-11268; ATCC) were cultured under standard conditions in Dulbecco's modified Eagle's medium supplemented with a high level of glucose, GlutaMax-I, and sodium pyruvate (Invitrogen); with 10% heat-inactivated FCS; and with 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Polyethylenimine (Sigma-Aldrich)–mediated transient transfection was performed as previously described [27]. Supernatants were analyzed by an enzyme-linked immunosorbent assay (ELISA) for recombinant antibody production, using the human IgG total Ready-Set-Go kit (e-Bioscience). Antibodies were purified with protein G beads (GE Healthcare) according to the manufacturer's instructions.

HCV E2 Peptide ELISA

Antibodies were tested by ELISA for their binding to the HCV E2 peptide. Streptavidin-coated ELISA plates with a binding capacity of 10 pmol biotin/well (ThermoFisher Scientific) were incubated with the peptide (10 µg/mL) diluted in assay buffer (25 mM Tris, 150 mM NaCl [pH 7.2], 0.1% bovine serum albumin, and 0.1% Tween) for 2 hours at room temperature. Antibody concentrations after purification were adjusted to 10 µg/mL. Bound antibodies were detected with horseradish peroxidase (HRP)–coupled goat anti-human IgG (Jackson ImmunoResearch Laboratories) and HRP chromogenic substrate (Siemens Behring Dade).

Polyreactivity ELISA

Antibodies were tested by ELISA for polyreactivity [27]. ELISA plates (Siemens Behring Dade) were coated with antigens (salmon sperm double-stranded DNA, human recombinant insulin, or lipopolysaccharide from *Escherichia coli* serotype 055:B5; all from Sigma-Aldrich) at a concentration of 10 µg/mL in sodium phosphate buffer (pH 7.4) overnight. Antibody concentrations were adjusted to 1 µg/mL. Recombinant human monoclonal antibodies mGO53 (no polyreactivity) and eiJB40

(low-level polyreactivity) [28], as well as human serum (high-level polyreactivity), were used as controls for polyreactivity on each plate. Bound antibodies were detected with HRP-coupled goat anti-human IgG and HRP chromogenic substrate. Antibodies were considered reactive if reactivity was confirmed in at least 2 independent experiments, and antibodies were considered polyreactive if they bound at least 2 different antigens.

HCV Pseudoparticle (HCVpp) Neutralization Assays

HCVpp of different genotypes (genotype 1a, H77; genotype 1b, HCV-J; genotype 2a, JFH1; genotype 2b, J8; genotype 3, S53; genotype 4, UKN4.21.15; genotype 5, UKN5.14.4; and genotype 6, UKN6.5.340) were produced as previously described [29]. Neutralization assays were performed with HCVpp-containing supernatants. The supernatants were preincubated with the expressed antibodies for 1 hour at 37°C before inoculation of Huh7.5.1 cells seeded the day before (10^4 cells/well). Infected cells were incubated for 72 hours at 37°C and then lysed with GloLysis buffer (Promega) according to the manufacturer's instructions. Luciferase activity was measured with the Mithras LB 940 Multimode Microplate Reader (Berthold Technologies).

Cell Culture–Derived HCV (HCVcc) Neutralization Assays

HCVcc virions of genotype 2a (Luc-Jc1) were produced as previously described [30]. Neutralization assays were performed with HCVcc-containing supernatants, using a protocol analogous to that for the HCVpp assays described above.

Statistical Analysis

Statistical calculations were performed using GraphPad Prism. The nonpaired 2-tailed Student *t* test was used to obtain *P* values for the evaluation of *IgH*-CDR3 length and mutation numbers. *P* values for immunoglobulin gene repertoire analysis were calculated by the Fisher exact test. *P* values of ≤ .05 were considered statistically significant.

RESULTS

Successful Identification of HCV E2 Peptide–Binding Memory B Cells >30 Years After Infection

Peripheral blood specimens from 15 patients from the anti-D cohort, of whom 7 experienced spontaneous recovery (the SR group) from HCV infection and 8 had chronic HCV infection (the CHC group), and blood specimens from 12 healthy volunteers without HCV infection (Table 1) were analyzed for HCV binding memory B cells, using the labeled HCV E2 peptide. We found circulating anti-HCV memory B cells >30 years after viral clearance, emphasizing that humoral memory is maintained even in the absence of antigen. Figure 1 shows representative FACS blots from patients of each clinical group that were performed to isolate $CD19^+CD27^+IgG^+$HCV E2 $peptide^+$ cells on a single-cell level. The SR group showed a significantly higher number of HCV E2 peptide–reactive memory B cells than the healthy volunteer group ($P = .0339$). Moreover, these cells were significantly more frequent in the CHC group as compared to the SR group ($P = .0078$; Figure 2A).

Immunoglobulin Gene Repertoire of HCV E2 Peptide–Binding Memory B Cells

Using FACS, HCV E2 peptide–binding IgG^+ memory B cells from 4 patients in the SR group and 3 in the CHC group were sorted as single cells. Heavy chain (*IgH*) and corresponding light chain (*IgL*) gene transcripts were amplified by nested reverse transcription PCR for each cell. A total of 570 individual paired and complete *IgH* and *IgL* sequences were exploited to describe the HCV E2 peptide–binding IgG memory repertoire (Supplementary Tables 1–7).

Sequence analyses revealed that the distribution of HCV E2 peptide–binding memory B cells coding for a κ light chain (*Igκ*) or a λ light chain (*Igλ*) (κ use, 64% in the SR group vs 69% in the CHC group; Supplementary Figure 1*A*) were unbiased, compared with the healthy volunteer group [31]. Moreover, no significant differences in the use of immunoglobulin gene families were detected between the SR and CHC groups (Figure 2B). Gene segment use was also similar to findings for IgG antibodies derived from non-HCV E2 peptide–binding IgG memory B cells recovered from non–HCV-infected individuals [31]. In addition, important features in binding characteristics—namely, the length of *IgH* CDR3 regions (Figure 2C) and the number of positively charged amino acids in the *IgH* CDR3 regions of the HCV E2 peptide–binding antibodies—showed no significant differences in patients from the CHC or SR groups (Supplementary Figure 1*B*).

Notably, the analyzed anti-HCV E2 peptide antibodies from both patient groups were highly mutated (Figure 2D). However, gene transcripts from the SR group showed a higher mean number (±SD) of SHMs in *IgH* (7.8 ± 3.8 mutations/100 bp v. 6.7 ± 2.9 mutations/100 bp; $P = .0016$) than transcripts from

Table 1. Characteristics of the Patient Cohort

Group	Patients, No.	Female Sex, No.	Age, y, Mean ±SD	HCV Therapy (No.)	ALT Level, μkat/L, Mean ± SD
Patients with spontaneously resolved HCV infection	7	7	56 ± 7	Untreated	1.4 ± 1.8
Patients with chronic HCV infection	8	8	60 ± 5	Untreated (5), pegylated interferon alfa + ribavirin (2), interferon alfa 2a + ribavirin (1)	0.9 ± 0.3
Healthy volunteers	12	7	39 ± 13	…	…

Abbreviations: ALT, alanine aminotransferase; HCV, hepatitis C virus.

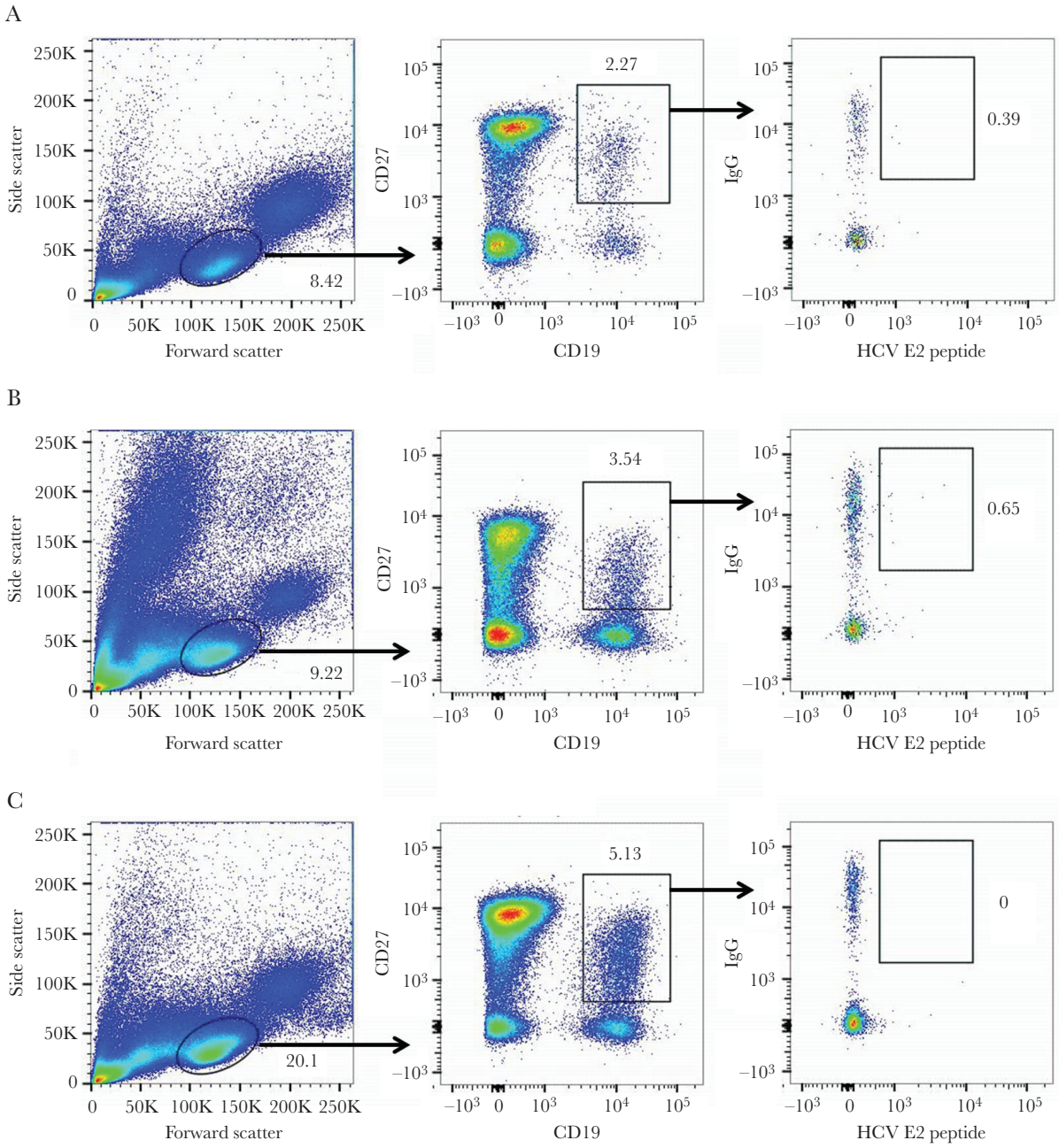

Figure 1. Fluorescence-activated cell-sorting (FACS)–based isolation strategy of single hepatitis C virus (HCV) E2 peptide (amino acids 483–499)–binding immunoglobulin G (IgG)–expressing memory B cells. Peripheral blood mononuclear cells from spontaneous resolvers from the anti-D cohort (*A*), chronically infected patients from the anti-D cohort (*B*), and healthy non–HCV-infected blood donors (*C*) were surface labeled with fluorochrome-coupled anti-CD19, anti-CD27, anti-IgG, and HCV E2 peptide (amino acids 483–499). Representative sorting gates for FACS single-cell isolation of $CD27^+CD38^+CD19^+$HCV E2 $peptide^+$ B cells are shown. Numbers indicate the percentage of the gated cells in relation to all cells in the corresponding blot.

the CHC group. A similar tendency of higher numbers of mutations in transcripts from the SR group as compared to the CHC group was observed in *Igκ* chains.

Expanded Cell Clones Were Mainly Identified in the SR Group

Surprisingly, we identified clonally related antibodies with identical *IgH* V(D)J and *IgL* VJ gene rearrangements, as well as shared SHMs, in all individuals in the SR group and 1 of 3 patients in the CHC group, indicating the presence of vastly expanded clones in the HCV E2 peptide–binding memory B-cell compartment. Despite the limited number of analyzed immunoglobulins, up to 14% of antibodies isolated from individuals in the SR group (4 of 116 (3.5%) in patient 1 [SR-1], 12 of 86 (14.0%) in SR-2, 9 of 101 [8.9%] in SR-4, and 14 of 100 [14.0%] in SR-5) but only 1.2% of antibodies from individuals in the CHC group (2 of 79 in patient 3 [CHC-3], 0 of 53 in

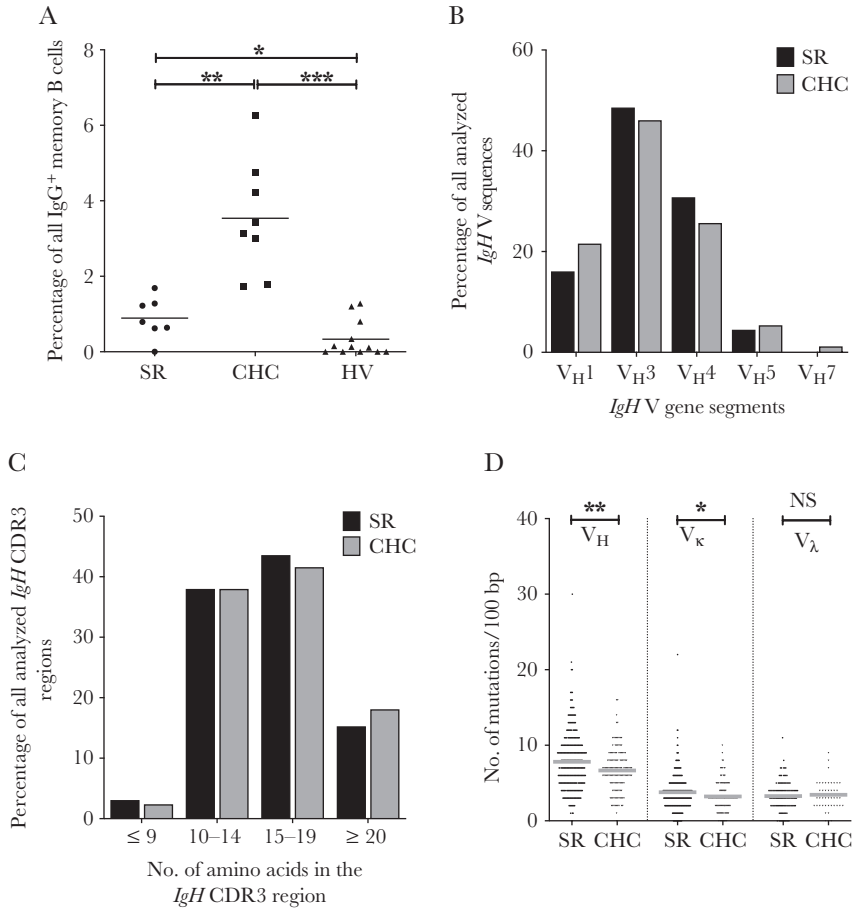

Figure 2. Immunoglobulin gene features of hepatitis C virus (HCV) E2 peptide (amino acids 483–499)–binding immunoglobulin G (IgG)–expressing memory B-cell antibodies isolated from patients with chronic HCV infection (the CHC group) and patients with spontaneous resolving infection (the SR group). *A*, Frequency of HCV E2 peptide (amino acids 483–499)–binding IgG^+ memory B cells from the SR group, the CHC group, and healthy volunteers (the HV group) among all IgG^+ memory B cells. Means are indicated by horizontal lines. *B*, Transcripts from the SR group (black) and the CHC group (gray) were analyzed for the *IgH* V gene family repertoire. The graph shows the V_H gene family distribution. *C*, Transcripts from the SR group (black) and the CHC group (gray) were analyzed for positively charged amino acids in the *IgH* CDR3 regions. The bar graph shows frequencies of *IgH* CDR3 regions with ≤9 amino acids, 10–14 amino acids, 15–19 amino acids, or ≥20 amino acids. *D*, Number of somatic hypermutations per 100 bp in the *IgH* V genes. Means are indicated by horizontal lines. *P* values were calculated to compare data from both patient groups. NS, not significant. $*P < .05$, $**P < .01$, and $***P < .001$, by the Student *t* test.

CHC-6, and 0 of 35 in CHC-8) belonged to expanded B-cell clones. We conclude that B-cell memory against HCV E2 peptide is dominated by a limited number of expanded cell clones.

A Fraction of Expressed Antibodies Showed Anti-HCV Neutralizing Activity

After the gene transcript analyses, a randomly chosen set of antibodies from all 4 patients in the SR group were recombinantly expressed in vitro. Using a peptide-specific ELISA, we found that the majority (86%) of the antibodies expressed were able to bind the HCV E2 peptide used as bait for FACS analysis (Supplementary Figure 2).

To determine whether the anti-HCV E2 peptide–binding antibodies bound the bait peptide due to polyreactive or promiscuous binding properties, we measured binding to a panel of defined structurally distinct antigens by ELISA [31]. More than half of the HCV E2 peptide–binding antibodies expressed by patients in the SR group were polyreactive (61.0%; Figure 3A). To investigate the neutralizing properties of the anti-HCV E2 peptide antibodies, an HCVpp assay was used. Antibodies were screened against the HCVpp isolates H77 (from genotype 1a), HCV-J (from genotype 1b), JFH1 (from genotype 2a), and J8 (from genotype 2b), using final antibody concentrations of 5 μg/mL, and HCVpp entry was evaluated relative to control cells, which were infected with the appropriate HVCpp subtype (Figure 4). The screening experiment revealed that 53 tested antibodies (63%) were able to inhibit HCVpp entry of at least 1 tested isolate (40%–90% inhibition relative to the uninhibited controls). Of these antibodies, 32 (38%) were able to neutralize one of the HCV isolates tested, 15 (18%) inhibited entry of 2 isolates, 4 (5%) inhibited entry of 3 isolates, and 2 (2%; M-240 and M-407) inhibited entry against all 4 tested isolates.

Antibodies that showed the most promising neutralization potential in the screening experiment were selected for dose-response analyses (serial dilutions, 5–0.005 μg/mL) against an

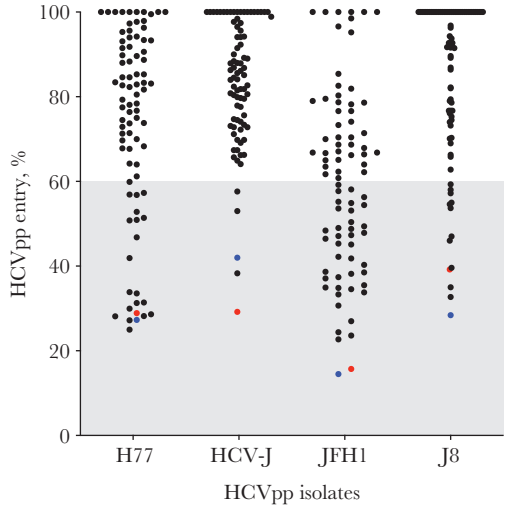

Figure 3. Neutralization activity in detected by the hepatitis C virus (HCV) pseudoparticle (HCVpp) assay. Screening experiment of the antibodies, using common HCVpp isolates (H77, genotype 1a; HCV-J, genotype 1b; JFH1, genotype 2a; and J8, genotype 2B) with an antibody concentration of 5 µg/mL. HCVpp were incubated with the antibodies and added to Huh7.5.1 cells. HCVpp entry was analyzed by luciferase reporter gene expression and normalized to controls. Antibodies that were able to keep HCVpp entry <60% (gray shading) in comparison to the controls were considered to have neutralizing properties. The blue (M-240) and red (M-407) dots highlight 2 antibodies that exhibited broadly entry-inhibiting activity within the tested HCVpp isolates.

expanded panel of HCVpp bearing envelope glycoproteins from the most common HCV genotypes (ie, 1a, 1b, 2a, 2b, 3a, 4, 5, and 6). M-240 and M-407, which showed the broadest activity in the screening experiment, inhibited the entry of all tested HCVpp isolates, with a half-maximal inhibitory concentration of 0.8–4.7µg/mL (Figure 4A). These results were confirmed using an infectious cell culture–based HCVcc system (genotype 2a isolate Luc-Jc1). M-240 and M-407 dose-dependently inhibited HCVcc infection (Figure 4B).

Interestingly, comparison of antibody sequences with the ability to neutralize HCV revealed that gene segment use appeared to influence the inhibitory activity in the HCVpp assay. Use of the V_H1–69 gene family was correlated with the inhibitory ability of the antibodies, whereas the V_H1–46, V_H3–15, and V_H3–30 gene families were correlated with no or weak inhibitory strength.

M-240 and M-407 Share the Same V_H Gene Segment

Antibodies with broad neutralizing activity (ie, M-240 and M-407) were encoded by the same V_H1–69*13 gene segment but were not clonally related because they did not express the same Vκ gene segment and had unique CDR3 regions. Both antibodies displayed a high number of positively charged amino acids in the *IgH* CDR3 region (Table 2) and did not show promiscuous binding features. The CDR2 region included 2 (for M-240) and 4 (for M-407) hydrophobic residues.

DISCUSSION

Using a unique cohort of patients who were infected in a single-source outbreak, we identified IgG memory B cells binding to a linear section of the HCV E2 β-sandwich region. Although the role of antibodies in HCV clearance is still ambiguous, it was shown that the development of a neutralizing antibody response during the early phase of viral infection is associated with resolution of the disease [32]. Moreover, several broadly neutralizing immunoglobulins showed an ability to protect against HCV infection in vivo, indicating that antibodies contribute to viral clearance [12, 33, 34]. Therefore, the neutralizing antibodies of patients with spontaneous resolution identified in this study >30 years after primary infection may have contributed to viral clearance.

Here we intended to characterize the repertoire of antibodies directed against the HCV E2 antigen of patients from the SR and CHC groups. Dominant IgV_H gene use has been identified in antibodies directed against several pathogens [35]. However, with regard to antibodies against the HCV E2 peptide, we found no significant differences in gene family use or CDR3 features between the SR and CHC groups or between our patients and previously published data from healthy volunteers. Missing evidence of biased gene segment use might be due to variations between individuals and the small cohort size in our study.

Most of the analyzed sequences showed high frequencies of SHMs, indicating that anti-HCV E2 peptide memory B cells are strongly selected after germinal center cells. These findings are in line with those from previous studies describing extensive SHMs in B cells from patients with CHC [36, 37]. Notably, in our study, the SR group displayed higher numbers of mutations, indicating stronger antigenic selection than in chronically infected individuals. Chronic exposure to a genetically fast-mutating virus may lead to short affinity maturation times of antibodies, less efficient binding to the virus, and recruitment of naive cells [38].

Compared with deep-sequencing approaches, we analyzed only a small fraction of immunoglobulin sequences. Regarding the estimated variety of the entire B-cell memory compartment it was surprising to find a high amount of clonally related sequences in our study, especially in the SR group (3.5%–14% in different sample sizes of 86–116 sequences per individual in the SR group). Modeling of deep-sequencing data estimated the size of the circulating B-cell heavy chain repertoire to be around 11 million different clonotypes per individual [39]. Although our data do not allow modeling of clonal expansion, we can conclude that humoral response to the HCV E2 peptide we used is realized by a limited number of clonotypes. Although other techniques (ie, deep sequencing) may allow more-robust coverage of the gene repertoire than the single-cell reverse transcription PCR assay used in this study, single-cell sorting and deep-sequencing approaches nevertheless have recently demonstrated congruent results for genetic characterization of memory B cells [40].

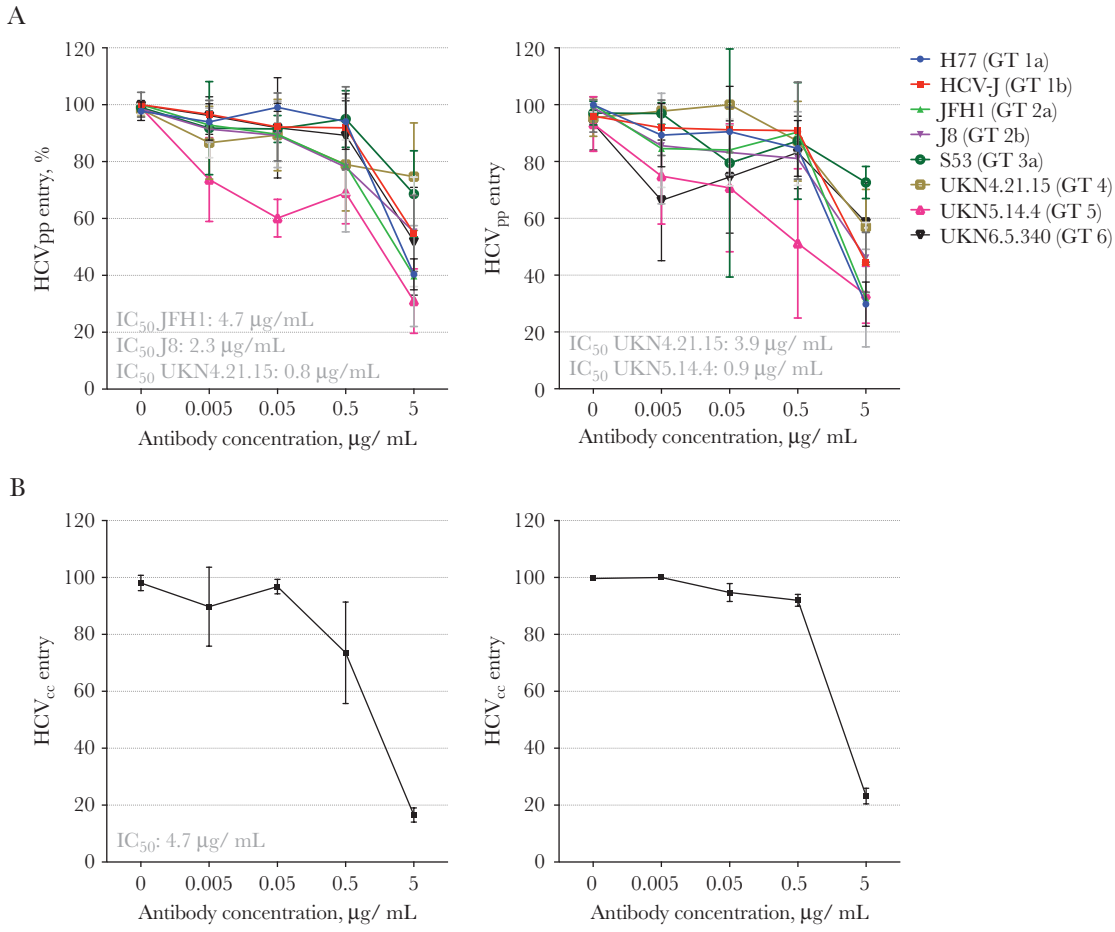

Figure 4. Neutralization activity of antibodies M-240 and M-407. *A*, Common hepatitis C virus (HCV) pseudoparticle (HCVpp) isolates (genotypes [GTs] 1a [H77], 1b [HCV-J], 2a [JFH1], 2b [J8], 3a [S53], 4 [UKN4.21.15], 5 [UKN5.14.4], and 6 [UKN6.5.340]) were incubated with serial dilutions (5–0.005 µg/mL) of antibodies M-240 (left) and M-407 (right) and added to Huh7.5.1 cells. HCVpp entry was analyzed by luciferase reporter gene expression and normalized to controls. Mean values (±SDs) are indicated. Half-maximal inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism and are indicated when available. *B*, Confirmation of the neutralizing activity of M-240 (left) and M-407 (right), using the cell culture–derived HCV (HCVcc) system (Luc-Jc1, genotype 2a). Luc-Jc1 was incubated with serial dilutions (5–0.005 µg/mL) of antibodies M-240 and M-407 and added to Huh7.5.1 cells. HCVcc entry was analyzed by luciferase reporter gene expression and normalized to controls. Mean values (±SDs) are indicated. IC_{50} values were calculated using GraphPad Prism and are indicated when available.

To identify HCV binding memory B cells, we picked a small linear epitope as selection bait, which is known to show only little sequence variability [41]. The chosen linear peptide forms the outer layer of the β-sandwich and includes residues counting to the nonneutralizing (495) and neutralizing face (499) of HCV as parts of the overlapping antigenic regions 1 and 3 [21]. Although antigenic domains of HCV E2 peptide, such as amino acids 412–423, 426–443, and 529–535, have been identified as essential targets for binding of neutralizing antibodies [42], less is known about the contribution to neutralization of the β-sandwich area [21]. It is known that HCV E2 peptide shows high conformational flexibility, which could

Table 2. Sequence Characteristics of Broadly Neutralizing Antibodies M-240 and M-407

Antibody	V_H	J_H	*IgH* CDR3, Amino Acids	Positively Charged Amino Acids, No.[a]	Mutations, No./100 bp[b]
M-240	1-69*13	6*04	ARGRYHDSSDHYLEVPYYYGMDV	4	5
M-407	1-69*13	4*02	ATDILRDLPDS	3	12
	Vκ	Jκ	*Igκ* CDR3, Amino Acids		
M-240	1-9*01	3*01	QHLNTYLLFT	…	3
M-407	2-30*01	1*01	MQGTHWPWT	…	4

[a]Data are no. of positively charged amino acids within the *IgH* CDR3 region.

[b]Data are no. of mutations within the V region per 100 bp.

be detrimental for vaccine development [43], whereas the β-sandwich area itself represents a structural and less inflective area of the peptide. Located in direct proximity to the previously described CD81 binding area on the front layer, the linear structure comprising amino acids 483–499 represented a promising target for our approach. Although only a small and not yet well-characterized epitope was used for single-cell sorting, we were able to detect and monoclonally express antibodies from the SR group with neutralizing activity against several isolates of different genotypes in HCVpp and HCVcc tests.

We identified 2 antibodies, M-240 and M-407, with isolate-independent neutralization ability, both of which used the V_H1–69 gene segment. It was reported that HCV E2 antigen drives the expansion of B-cell clones encoding for antibodies using the IgV_H1–69 gene segment [42]. Our data support the potential importance of B cells expressing V_H1–69-encoded antibodies for HCV neutralization. V_H1–69-derived broadly neutralizing antibodies also play a role in other viral diseases, such as influenza virus [44] and human immunodeficiency virus type 1 infection [45], where they are thought to interact with conserved hydrophobic clusters in their antigens via hydrophobic residues at the tip of their CDRH2 loops, and have been proposed as primordial pattern-recognition receptors [46].

Both broadly neutralizing antibodies that we detected had different light chains, which corroborates data for other neutralizing antibodies against HCV whose binding activity is not compromised when its light chain is exchanged [20].

As expected, most antibodies were able to bind the bait antigen; however, our sorting strategy also favored memory cells producing promiscuous binding antibodies. Polyreactivity is a common serologic feature of certain viral infections in humans [47, 48] and may increase antibody affinity for a pathogen. Nevertheless, promiscuous binding properties do not play a role in the neutralizing properties of the broadly neutralizing antibodies M-240 and M-407. The HCVpp assay showed neutralizing activity of M-240 and M-407 against 8 different HCV isolates, however use of the HCVcc assay to examine entry inhibition was only performed for the Luc-Jc1 isolate, representing genotype 2a. Further studies are needed to confirm genotype-independent neutralizing activity and the clinical significance of the detected antibodies.

In conclusion, we described the molecular characteristics of the immunoglobulin repertoire against a central part of the β-sandwich of HCV E2 peptide in patients with SR or CHC. Expression of monoclonal antibodies from the SR group and reactivity testing identified 2 broadly neutralizing nonpolyreactive antibodies. We confirmed the importance of V_H1–69 for the broadly neutralizing activity of antibodies, suggesting that V_H1–69-encoding antibodies may be used for the development of passive vaccines. Detection of neutralizing antibodies directed against part of the β-sandwich of the HCV E2 protein underlines the importance of this structure as a potential target for protective antibody responses. Moreover, the presence of a long-term memory B-cell pool strengthens the argument for the development of an HCV vaccine. The results of the present study may contribute to an improved understanding of antibody responses against HCV, as well as other medically relevant viruses.

SUPPLEMENTARY DATA

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank the patients, relatives, and volunteers cooperating in this study; the East German HCV Study Group, particularly Prof Manfred Wiese; the DNA Technologies (Birgit Oelzner) and Fluorescence (Dr Andreas Lösche and Kathrin Jäger) core units of the Medical Faculty of the University Hospital Leipzig; Cornelia Kreschel, for experimental support; Dr Christian Busse, for helpful discussions; Benjamin Michling, for laboratory assistance; the FACS facility of the Max Planck Institute for Infection Biology, Berlin, for technical support; and Prof Jonathan Ball, for provision of HCVpp expression plasmids.

A. O. conducted the experiments; acquired, analyzed, and interpreted the data; and drafted the manuscript. S. B., H. W., and T. F. B. participated in the concept, design, and planning of the study. K. R. participated in interpreting the data and drafting the manuscript. C. C. C. and F. W. participated in acquiring of the data. T. B. participated in the conceptualization, design, and planning of the study. J. B. planned the conceptualization and design of the study, analyzed and interpreted the data, and drafted the manuscript. All authors critically reviewed iterations of the manuscript and approved the final draft for submission.

Financial support. This work was supported by MSD (grant to J. B.) and HepNet Deutsche Leberstiftung (support to J. B.).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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