Antibodies Directed to Envelope Proteins of Hepatitis C Virus
Outside of Hypervariable Region 1

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The relatively high variability of the hepatitis C virus (HCV) envelope proteins E1 and E2 suggests that parts of these proteins other than the hypervariable region 1 (HVR1) might be involved in the induction of virus neutralizing antibodies. To test this hypothesis, two HCV proteins, pE1 and pE2Δ, were generated by in vitro translation. They represent amino acids 174–437 of E1 and 411–688 of E2, respectively, of isolate HCV-AD78; the protein pE2Δ contained no HVR1. As a control, protein pG.HVR1, which represents amino acids 384–410 of HVR1 of isolate HCV-AD78, was expressed separately. These three proteins were used in an immunoprecipitation assay to detect the presence of antiviral antibodies in sera of patients infected with the same isolate of HCV (HCV-AD78). Sera were obtained 4–8 months postinfection from patients who later resolved an acute infection or developed chronic liver disease. A high prevalence of antibodies (up to 85.7%) against pE1 and pE2Δ could be detected in both groups of patients, suggesting that these forms of the HCV envelope proteins contain B-cell epitopes. The antibody responses against proteins pE1 and pE2Δ did not differ significantly between patients with resolving or chronic infection, whereas antibodies against protein pG.HVR1 were associated with resolution of infection. Rabbit antiseras raised against pE1 and pE2Δ were tested for their ability to neutralize the binding of HCV to susceptible cells in tissue cultures. The results suggested that although a few B-cell epitopes outside of HVR1 can induce virus neutralizing antibodies, these antibodies are probably not associated with the resolution of infection.

INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of parenterally transmitted hepatitis (Choo et al., 1989). It is associated with the majority of cases of posttransfusion hepatitis and community-acquired hepatitis worldwide (Alter et al., 1992, 1991). Chronic HCV infection is often followed by severe liver diseases, including liver cirrhosis and hepatocellular carcinoma (Alter, 1994; Saito et al., 1990; Miyamura et al., 1990). The high rate of chronic outcome of HCV infection is possibly due to an immune escape of the virus (Weiner et al., 1995, 1991). Clearance of virus most probably involves isolate-specific immune response because the genome of HCV possesses significant genetic heterogeneity (Bukh et al., 1995) and exists as a quasispecies population (Martell et al., 1992).

Immunization of chimpanzees with heterodimers of the two HCV envelope proteins E1 and E2 led to protection from challenge with a homologous isolate of HCV (Choo et al., 1994). Such protection seemed to correlate with the titer of neutralizing antibodies (Rosa et al., 1996). On the other hand, reinfection of chimpanzees and patients with other isolates have been observed, suggesting isolate-specific neutralization (Farci et al., 1992). Neutralizing antibodies were detected in sera of patients with chronic hepatitis C (Shimizu et al., 1994; Zibert et al., 1995; Rosa et al., 1996). Envelope-specific antibodies directed against a small region, located at the N-terminus of E2 and termed hypervariable region 1 (HVR1), have been postulated to be capable of neutralizing HCV (Zibert et al., 1995; Weiner et al., 1991). However, antibodies directed to epitopes of envelope proteins, other than HVR1, may also be important for neutralization (Rosa et al., 1996; Zibert et al., 1995).

Antibodies specific to envelope proteins E1 or E2 have been detected with prevalences ranging from 9 to 97% (Lanford et al., 1993; Yan et al., 1994; Hada et al., 1992; Harada et al., 1995; Lesniewski et al., 1995). These antibodies seem to persist in chronically infected patients (Chien et al., 1993). However, these studies used the same antigen for antibody detection in sera of patients infected by different isolates and may have missed isolate-specific immune responses. In addition, the time points of infection were mostly unknown in the patients studied. The prevalence of envelope-specific antibodies during the early phase of infection may differ in patients...
who have acute self-limiting or develop chronic infections. Such investigations with isolate specific proteins have been recently performed for HVR1 (Allander et al., 1997; Zibert et al., 1997b).

We investigated the early antibody response (months 4–8 p.i.) in a group of patients infected by the same isolate of HCV (HCV-AD78). This single source outbreak was caused by a contaminated immunoglobulin (Dittmann et al., 1991). Patients had either self-limiting infections or developed chronic hepatitis C infections. Two different sets of experiments were performed in order to characterize the antibody response against regions of the HCV envelope which are located outside of HVR1. We first investigated the prevalence of antibodies directed against three proteins of HCV-AD78; pE1, pG.HVR1, and pE2Δ by an immunoprecipitation assay. Second, the significance of antibodies directed against epitopes outside of HVR1 was characterized using rabbit sera which were raised against regions of E1 and E2. These sera were tested for their ability to neutralize binding of HCV-AD78 to human fibroblasts (in vitro neutralization of binding).

RESULTS

Antibodies directed against E1, HVR1, or E2Δ in patients with acute self-limiting and chronic infections caused by HCV-AD78

The distribution of a quasispecies of HCV-AD78 which is present in the contaminated immunoglobulin (IgG) was demonstrated by cDNA cloning and sequencing of HVR1 using different aliquots of the inoculum and RT–PCR primers (Zibert et al., 1995; Hohne et al., 1994). These analyses revealed only limited sequence heterogeneity, suggesting a HCV contamination of the IgG preparation by one donor only. In order to characterize the envelope-specific antibody response in patients who had self-limiting or developed chronic infections after administration of the contaminated IgG, an immunoprecipitation assay with three in vitro translated envelope-specific proteins, termed pE1, pG.HVR1, and pE2Δ, was performed (Fig. 1A). The proteins corresponded to the main variant of isolate HCV-AD78. The patient sera analyzed were obtained at early time points after infection with HCV-AD78. Protein pE1 encodes aa 174–337 and lacks the putative transmembrane region. Protein pG.HVR1 encodes the amino acids of HVR1 (aa 384–410) which also include the two amino terminal insertions present in isolate HCV-AD78 (Zibert et al., 1997a; Hohne et al., 1994). For immunoprecipitation analysis, this 29-amino-acid region was fused to GST. Protein pG.HVR1 encodes the amino acids of HVR1 (aa 384–410) which also include the two amino terminal insertions present in isolate HCV-AD78 (Zibert et al., 1997a; Hohne et al., 1994). For immunoprecipitation analysis, this 29-amino-acid region was fused to GST. Protein pE2Δ encodes the remainder of E2 (aa 411–688) and lacks HVR1 and the putative transmembrane region (Fig. 1A). The three proteins were mixed after in vitro translation at equal amounts of radioactivity. GST protein was included as a negative control. After SDS–PAGE (Fig. 2A) the protein bands showed the expected molecular weights of 20 kDa for pE1 (lane 1), 27 kDa for GST (lane 2), 30 kDa for pG.HVR1 (lane 3), and 31 kDa for pE2Δ (lane 4).

Three examples of immunoprecipitations are given in Fig. 2B. Sera of patient 1 (lane 1) and patient 2 (lane 2) immunoprecipitated all three proteins, while the serum of patient 3 (lane 3) precipitated only pE1 and pG.HVR1. Different relative intensities of the individual protein bands were observed (data not shown), indicating that titers of antibodies directed to these proteins differed for each serum. Protein pG.HVR1 was more efficiently precipitated than pE1 and pE2Δ with most of the patient sera (exemplified in Fig. 2B).

Sera of 37 patients which were obtained between
months 4 and 8 p.i. were analyzed by immunoprecipitation. Sera of 21 patients who had acute self-limiting infections and 16 sera of patients who later developed chronic infections were compared. Only 1 of 37 patients did not contain antibodies to either of the three proteins (Table 1). Protein GST alone was not precipitated. Antibodies against HVR1 had the highest prevalence (95.2%) in sera of patients with acute self-limiting infection of HCV-AD78. The prevalence of antibodies directed against pE1 and pE2Δ was also high. Eighteen of 21 (85.7%) patients with acute self-limiting infection had antibodies against pE1 as well as against pE2Δ. In patients with chronic infections the prevalence of antibodies directed against pE1 and pE2Δ was lower, but not with statistical significance. In this group, 13 of 16 patients (81.2%) had antibodies against both proteins. Only 2 patients with acute self-limiting infection or chronic infection were negative for antibodies against both proteins, and 2 other patients of each group were negative for antibodies to either of the two proteins. In contrast to antibodies against pE1 and pE2Δ, the prevalence of antibodies against pG.HVR1 differed significantly (P < 0.005) between the two patient groups. Twenty of 21 (95%) patients with acute self-limiting infections had such antibodies, while only 5 of 16 (31.2%) patients with chronic infections were anti-HVR1 positive (Table 1). Both patients who did not contain antibodies against pE1 and pE2Δ were positive for anti-HVR1. Fifteen anti-HCV-negative sera did not precipitate any of the three proteins.

Characterization of rabbit sera directed to epitopes outside of HVR1

Antibodies directed to HVR1 were suggested to be capable of in vitro neutralization of HCV (Farci et al., 1996). The significance of antibodies directed against the part of the HCV envelope that does not contain the HVR1 with respect to virus neutralization is not known. In order to investigate whether such antibodies are important for neutralization of HCV, we raised antisera to E1 and E2 (lacking HVR1) in rabbits and characterized these sera by their ability to neutralize the binding of HCV to cells (Zibert et al., 1995; Shimizu et al., 1994). E1- and E2-specific proteins were expressed in E. coli (Fig. 3). Protein pGST.E1 (Fig. 1) encodes almost complete E1 in fusion to GST (Fig. 3, lane 1). However, the complete E2 could not be expressed as was reported before (Mita et al., 1992) and was represented by two protein fragments termed pGST.E2a and pGST.E2b (Fig. 1; Fig. 3, lanes 2 and 3). These proteins were used for immunization of rabbits after purification of major protein bands showing the expected molecular weights of approximately 48 kDa each.

### Table 1

<table>
<thead>
<tr>
<th>Patients</th>
<th>No. of sera examined</th>
<th>Month p.i. (mean)</th>
<th>pE1 (aa 174±337)</th>
<th>pG.HVR1 (aa 384±410)</th>
<th>pE2Δ (aa 411±688)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute self-limiting infection</td>
<td>21</td>
<td>4±7 (5.4)</td>
<td>18 (85.7%)</td>
<td>20 (95.2%)</td>
<td>18 (85.7%)</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>16</td>
<td>4±9 (5.9)</td>
<td>13 (81.2%)</td>
<td>5 (31.2%)</td>
<td>13 (81.2%)</td>
</tr>
<tr>
<td>Anti-HCV negative</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

FIG. 2. In vitro translation and immunoprecipitation of HCV envelope-specific proteins. (A) Autoradiograph after SDS–PAGE of in vitro translated pE1 (lane 1), GST (lane 2, control), pG.HVR1 (lane 3), and pE2Δ (lane 4). (B) Immunoprecipitation of in vitro translated proteins. All four proteins were mixed at almost equal amounts of radioactivity and immunoprecipitated by three patient sera (lanes 1±3). The film was exposed for 7 days. Lane M, molecular weight markers (kDa).

FIG. 3. SDS–PAGE of partially purified HCV envelope proteins. Coomassie stain after SDS–PAGE. Partially purified E. coli lysates (approximately 5 μg of protein/lane) were applied to the gel. Lane 1, pGST.E1; lane 2, pGST.E2a; and lane 3 pGST.E2b. Lane M, molecular weight markers (kDa).
Five rabbits were immunized and the specificity of the resulting sera (R1–R5) was demonstrated by immunoprecipitation (Fig. 4) and by ELISA (Fig. 5). As expected, in vitro translated pE1 was precipitated by rabbit serum R1, and pE2Δ by sera R2–R5, respectively. Rabbit serum R3 seemed to precipitate these proteins rather inefficiently. A threefold amount of serum had to be used to yield similar amounts of radioactivity. The HCV E1-specific (R1) and the four HCV E2-specific rabbit sera (R2–R5) were characterized by ELISA using overlapping peptides (20-mers, overlapping by 10 amino acids) covering aa 171±340 or aa 411±690 of HCV-AD78, respectively. Except serum R3, all rabbit sera recognized one or more peptides (Fig. 5). Serum R1 reacted to peptides representing aa 231±250 and 281±230. Serum R2 reacted to five peptides, which represent aa 441±460, aa 511±530, and aa 561±599. Sera R4 and R5, which were raised against the same protein (pGST.E2b), recognized two different sets of peptides (aa 481±510 and aa 641±680, respectively).

To determine the importance of HCV envelope-specific antibodies directed to epitopes outside of HVR1, sera R1–R5 were tested for their ability to neutralize the binding of HCV-AD78 to human fibroblast cells (VH3) (Table 2). These cells have been shown to bind HCV and support replication of HCV (Zibert et al., 1995). The experiments were carried out at least three times with the same results and a HVR1-specific rabbit serum which has previously been shown to neutralize binding of HCV-AD78 (Zibert et al., 1997a) was used as a positive control (data not shown). Serum R2 (raised against aa 411±613) was able to block viral attachment. In contrast, rabbit serum R3, which was raised against the same protein, but only inefficiently precipitated E2 (Fig. 4) and showed no antibodies against E2-specific peptides (Fig. 5), did not neutralize viral binding. Rabbit sera R1, R4, and R5 could not neutralize binding of HCV-AD78 even at high concentrations (1:5).

**DISCUSSION**

Humoral immune response to the HCV envelope is considered essential for elimination of the virus. However, the analysis of antiviral antibodies during the natural course of infection is hampered by the high variability of the envelope sequences found in different isolates. Therefore, our study included patients infected with the same isolate of HCV with known time points of infection and characterized by a long-term follow-up of infection. Analysis of such a well-characterized patient group should allow us to determine some characteristics of anti-E1 and anti-E2 response in acute self-limiting or chronic infections. It is likely that the sera studied here, obtained after 4±8 months p.i., contained antibodies which are mainly directed to the amino acid sequence of the virus present in the contaminated anti-D IgG (HCV-AD78), although the amino acid sequence of the HCV envelope was shown to rapidly accumulate mutations within a few months of HCV infection (Kato et al., 1994; Higashi et al., 1993; Kurosaki et al., 1994; Taniguchi et al., 1993; van Doom et al., 1995; Kojima et al., 1994). Isolate-specific antibodies which may have been missed in other studies of the HCV anti-envelope response (Saracco et al., 1994; Lesniewski et al., 1995; Yuki et al., 1996; Chien et al., 1993) are likely to be detected by analysis of our patient group.

Different forms of the envelope proteins E1 and E2 have been used for antibody detection in sera of HCV-
infected patients. The rate of antibody detection was generally lower when denatured proteins were used (Yan et al., 1994; Ray et al., 1994) compared to putative native, glycosylated proteins (Chien et al., 1993; Harada et al., 1995; Ray et al., 1994; Lesniewski et al., 1995), suggesting that a substantial portion of the patient antibodies are directed to nonlinear B-cell epitopes of the HCV envelope. Using proteins pE1 and pE2 directed to nonlinear B-cell epitopes of the HCV envelope, that a substantial portion of the patient antibodies are directed to nonlinear B-cell epitopes of the HCV envelope (Chien et al., 1995; Ray et al., 1994), which are nonglycosylated, partially truncated forms of the HCV envelope generated by in vitro translation, specific antibodies were detected with a rate of up to 85.7%, which is almost as high as the rates observed with potentially native envelope proteins (Chien et al., 1993; Yuki et al., 1996). This suggests that proteins pE1 and pE2Δ contain major B-cell epitopes which are recognized by antibodies of the majority of HCV-infected patients during the early phase of infection (months 4+8 p.i.). However, the rate of antibody detection observed here could have been slightly higher when native envelope proteins of HCV, e.g., by using heterodimers of E1 and E2 expressed by recombinant vaccinia viruses, had been used. This latter form of the HCV envelope was suggested to be the functional unit of the virion (Dubuisson et al., 1994) and induced an immune response in chimpanzees which could prevent infection with HCV (Choo et al., 1994). Although our own studies involving HCV envelope proteins expressed by recombinant vaccinia virus led to similar high rates of antibody detection (data not shown) compared to those of other studies (Chien et al., 1993; Yuki et al., 1996), such proteins have little value for investigations of the specific antibody response outside of HVR1. The native proteins which also include HVR1 could distort the determination of the specific antibody response to other regions of the envelope since HVR1 includes immunodominant B-cell epitopes (Scarselli et al., 1995; Zibert et al., 1997a). On the other hand, deletion of HVR1 which is located adjacent to hydrophobic sequences involved in the translocation of the proteins into the ER would possibly abrogate the correct processing of the HCV envelope and also modify the folding of E2.

The association of antibodies against HVR1 with resolving from HCV infection was reported previously (Allander et al., 1997; Zibert et al., 1997b). However, the antibody response against B-cell epitopes outside of HVR1 was not addressed in these investigations (Zibert et al., 1997b) or was not assessed with isolate-specific proteins that exclude HVR1 (Allander et al., 1997). In the current study, we demonstrated the marked antibody response against the isolate specific envelope proteins lacking HVR1 in patients who either resolved the infection or developed chronic liver disease (Table 1). It should be noted, however, that although we did not detect differences of the antibody response between these patient groups using proteins pE1 and pE2Δ, our immunoprecipitation assay might not measure antibodies appropriately (e.g., neutralizing antibodies) directed to single epitopes. In addition, subtle differences of antibody titers against pE1 and pE2Δ could not be determined, although our previous investigation of HVR1 suggests that such differences might not be significant for the course of HCV infection (Zibert et al., 1997b). Nevertheless, our study using an isolate-specific test system shows that antibodies to epitopes of the envelope proteins lacking HVR1 appear during the early phase of HCV infection but can be associated with neither the resolution nor the development of a chronic infection.

The existence of HCV isolate-specific neutralizing antibodies was shown by experimental infection of chimpanzees (Farci et al., 1996,1994) and suggested by tissue culture experiments (Zibert et al., 1997a; Rosa et al., 1996; Shimizu et al., 1994). For other viruses, e.g., HIV, it is known that some of the neutralizing antibodies are di-

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### Table 2

**Characterization of Envelope-Specific Rabbit Sera**

<table>
<thead>
<tr>
<th>Rabbit serum</th>
<th>Antigen for immunization</th>
<th>Immunoprecipitation</th>
<th>Reactivity to peptides of E2 (ELISA)</th>
<th>Neutralization of binding (HCV-AD78)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>pGST.E1 (aa 174–337)</td>
<td>+</td>
<td>aa 231±250; 281±320</td>
<td>D</td>
</tr>
<tr>
<td>Pre R1a</td>
<td>D</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>pGST.E2a (aa 411–613)</td>
<td>+</td>
<td>aa 441±460; 511±530; 561±600c</td>
<td>+</td>
</tr>
<tr>
<td>Pre R2</td>
<td>D</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>pGST.E2a (aa 411–613)</td>
<td>(+)d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre R3</td>
<td>D</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R4</td>
<td>pGST.E2b (aa 481–688)</td>
<td>+</td>
<td>aa 481±510</td>
<td></td>
</tr>
<tr>
<td>Pre R4</td>
<td>D</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R5</td>
<td>pGST.E2b (aa 481–688)</td>
<td>+</td>
<td>aa 641±680</td>
<td></td>
</tr>
<tr>
<td>Pre R5</td>
<td>D</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Rabbit serum taken prior immunization (pre serum).

b Not determined.

c Amino acids of reactive peptides.

d R3 had to be used at high concentration (1:15), compared to all other sera (1:100).

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rected against epitopes located outside the extremely variable regions (Sattentau and Moore, 1995). For HCV such evidence was also obtained by several authors (Zibert et al., 1995; Rosa et al., 1996; Choo et al., 1994). We characterized antibodies of five rabbit sera directed to epitopes other than HVR1 by their ability to neutralize binding of HCV to cells (Table 2). One rabbit serum (R2; immunized with aa 411–613) contained such antibodies. HCV-specific antibody titers in the rabbit sera were rather low. The absence of antibodies which block viral binding in sera R1 and R3 or R5 might be due to inefficient presentation of important epitopes in the SDS-denatured antigens (Fig. 3). On the other hand, our results suggest that at least one epitope which might be important for induction of neutralizing antibodies resides outside of HVR1. Whether this epitope is located in one of the three regions identified by ELISA using serum R2 (Fig. 5) has to be established by monospecific antisera. Serum R3 obtained after immunization with the same protein as R2 did not neutralize binding of HCV-AD78, probably due to the absence of antibodies directed to these epitopes or due to the lower titer of HCV-specific antibodies (Fig. 4). Further studies are needed to investigate whether the presence of antibodies directed to these epitopes can be correlated with resolving from disease.

### MATERIALS AND METHODS

**Patient sera**

Sera of 37 patients, who received a HCV-contaminated anti-D immunoglobulin in 1978/1979 (isolate HCV-AD78) (Dittmann et al., 1991), were studied. Sera were obtained shortly after administration of the immunoglobulin (months 4±8 p.i.). Twenty-one sera were derived from patients who, as diagnosed retrospectively, resolved HCV infection and 16 sera were obtained from patients who developed chronic infections. Acute self-limiting infections were characterized by initial (months 2±7 p.i.) elevated ALT levels that dropped thereafter and remained normal in the follow-up period of up to 17 years p.i. Sera were HCV RT±PCR negative in follow-up. In contrast, chronically infected patients were consecutively RT±PCR positive and had elevated ALT levels throughout follow-up. Fifteen sera which were anti-HCV, anti-HBs, and anti-HIV negative served as negative controls.

**Cloning of E1 and E2 of HCV-AD78**

RNA preparation of HCV-AD78 and RT were performed as described previously (Zibert et al., 1995). Nested PCR was carried out using 5 μl of respective cDNA mixture, 0.3 U Taq DNA polymerase (Promega, U.S.A.), and 50 pmol of primers in a total volume of 50 μl. For reverse transcription of E1, RT-antisense (A) primer 5′-GTCAACGCGCAAGATAGCAT-3′ (nt position 1475, Kato et al., 1990) was used. The first round of PCR was with RT primer and the sense primer (S) 5′-TTCTGGAGGACGCGGTGA-CTA-3′ (788). The second round of PCR was performed with primers S 5′-TGGCTTCTCTCTATCTTCCC-3′ (843) and A 5′-GTAGCATCACAATCAAGACC-3′ (1458). For cloning, this cDNA was amplified with tagged primers S 5′-GTCTCCATGGTTCTCTATCTTT-3′ (Ncol) and A 5′-TTGTGGAGACTCGAGTAGCTGCATA-3′ (Xhol). After digestion with the corresponding enzymes, the cDNA was cloned into the Ncol and Xhol sites of vector pTM1 (Moss et al., 1990). The resulting plasmid, termed pTM1E15, encodes amino acids 174±337. Cloning of a 1.3-kb fragment encoding E2 was described previously (Zibert et al., 1995). This cDNA was cloned into vector pTM1 after amplification of E2 with tagged primers S 5′-CAGAGCCATGGCGCTTGGATTTGATGTAC-3′ (Ncol) and A 5′-GTGCCAGCTCTAGGTACCA-GCGGCGCAC-3′. The resulting cDNA was digested with Ncol and ligated (sticky/blunt) into the Ncol/Smal sites of vector pTM1. The resulting plasmid was termed pTM1B139 and encodes aa 371±78.

In vitro translation and immunoprecipitation

Plasmids pTM1E15, pTM18.19, and G.HVR1A (see below) served as templates for PCR of envelope-specific regions. Individual sense primers were preceded by a T7 promoter sequence followed by an ATG start codon and the HCV envelope-specific sequence. In case of GHVR1A, the primer contained the GST (glutathione S-transferase)-specific sequence. Five micrograms of the PCR products served as a template for in vitro transcription using T7 RNA polymerase (20 U) (Promega) in a volume of 30 μl. Transcription was carried out for 60 min at 37°C. One microliter of the resulting transcripts was translated in 30 μl of rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine (30 μCi) for 60 min at 30°C. Proteins were analyzed on 17% sodium dodecyl sulfate (SDS)–polyacrylamide gels. Gels were dried and exposed to Kodak-XOMAT film. For immunoprecipitation, reticulocyte lysates (2±μl, depending on the amount of radioactivity of the respective proteins) were diluted with 300 μl buffer 1 (2% Triton X-100, 50 mM Tris±HCl, pH 8.0, 5 mM EDTA). Either 1 μl of patient serum or 5±15 μl of rabbit sera was used for immunoprecipitation. Incubation time was 2 h at 4°C. Protein A-Sepharose 4B (Pharmacia, Sweden) (20 μg bed volume) was then added to the immune complexes and incubated for 30 min under vigorous shaking. The complexes bound to protein A-Sepharose were washed three times with buffer 1 and once with buffer 1 lacking Triton X-100. Retained immunocomplexes were analyzed by SDS±PAGE followed by autoradiography. Films were exposed for 7 days and the presence of individual bands was determined by visual inspection. Longer exposition times (3 weeks) did not yield different patterns of immunoprecipitated protein.
Expression and purification of recombinant E1 and E2

E. coli strain XL1-blue carrying the respective plasmids was grown overnight in LB medium containing ampicillin (100 μg/ml). After dilution (1:50) with 2× YT medium (100 μg/ml ampicillin) cells were grown at 37°C to an OD600 of 1.3. Induction of expression was started by addition of IPTG (1 mM). After 4 h of incubation, cells were harvested by centrifugation. Lysis was performed according to Marston (1987) with a lysis buffer (1 mM EDTA, 100 mM NaCl, 50 mM Tris, pH 8) containing 1% (w/v) lysozym. Proteins pGST.E1, pGST.E2a, and pGST.E2b were expressed as inclusion bodies in E. coli and harvested by centrifugation (12,000 g).

Immunization of rabbits with recombinant E1 and E2

Immunization of rabbits was performed by standard procedures (Eurogentec, Seraing, Belgium). Briefly, partially purified bacterial lysates obtained from 500 ml of culture (approximately 3 mg of GST-specific fusion protein) were used for blotting onto nitrocellulose. HCV-specific protein bands were cut from the blot after staining with Ponceau S. A slice of the filter (approximately 100 μg of protein) was used for immunization. Boosts were performed at weeks 2, 4, and 8. One rabbit (R1) was immunized with pGST.E1, and two rabbits were immunized with pGST.E2a (R2 and R3) or pGST.E2b (R4 and R5). Animals were bled 2 weeks after the last boost.

Peptide ELISA

Sixteen or 27 overlapping peptides (20-mers) were used for ELISA to detect antibody reactivities to HCV E1 or E2, respectively. Peptides cover aa 171–340 or aa 411–690 of HCV-AD78. Peptides overlapped by 10 amino acids and carried a biotinylated spacer at their N-terminus (lysyl(e-biotinyl)-β-alanyl-e-aminohexanoyl-β-alanyl-) (Kraas et al., 1995). ELISA plates were coated with avidin (10 mg/ml) overnight at 4°C in carbonate buffer, pH 9.5. Plates were washed with 1% Tween in PBS. Peptides (1 μg/ml) were solved in buffer A (20% FCS, 1% NP-40 in PBS) and bound to microtiter wells for 2 h at 37°C. After washing, rabbit serum (1:200) was incubated in 0.5× buffer A for 2 h at 37°C. Another washing step was followed by incubation with peroxidase-labeled anti-rabbit antibody (1:4000; DAKO Diagnostika, FRG) diluted in 0.25× buffer A. After five additional washing steps, the ELISA was developed with ABTS (Sigma, FRG) according to standard procedures. Preimmunization sera were used as negative controls and produced OD405 values of 0.05±0.1 (mean 0.07). Sera were scored positive when OD405 values were above 0.25 (3× mean_neg + 15%) in at least two experiments.

HCV binding to cells (in vitro neutralization assay)

The binding assay used was described before (Zibert et al., 1995). Briefly, 5 μl of HCV-AD78 (2±5×10^5 PCR HCV genome equivalents/ml) was preincubated with 20 μl of rabbit serum for 2 h at 4°C in a total volume of 100 μl. This incubation mixture was adjusted to 500 μl with MEM medium and incubated with 2×10^6 human fibroblast cells (VH3) for binding of the virus. After 2 h of incubation at room temperature, cells were washed extensively with PBS (five times). Binding of HCV to cells was monitored by RT-PCR specific to the 5′ nontranslated region of HCV after RNA extraction of cells.

Statistical analysis

Statistical analysis for group comparison was performed by the χ² method. Values of P < 0.005 (two tailed) were considered a significant difference between groups.

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