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Significance of the Immune Response to a Major, Conformational B-Cell Epitope on the Hepatitis C Virus NS3 Region Defined by a Human Monoclonal Antibody

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The nonstructural protein NS3 of hepatitis C virus (HCV) possesses two enzymatic domains which are thought to be essential for the virus life cycle: an N-terminal serine-type proteinase, responsible for the processing of nonstructural polypeptides, and a C-terminal nucleoside triphosphatase/helicase, presumably involved in the unwinding of the viral genome. The human antibody response to NS3 usually appears early in the course of HCV infection and is predominantly directed against the carboxyl-terminal portion; however, its fine specificity and clinical significance are largely unknown. We have generated a human monoclonal antibody (hMAb), designated CM3.B6, from a cloned B-cell line obtained from the peripheral blood of a patient with chronic HCV infection, which selectively recognized the purified NS3 protein expressed in bacteria or in eukaryotic cells transfected with full-length or NS3 cDNA. Fine-specificity studies revealed that CM3.B6 recognized a 92-amino-acid sequence (clone 8, amino acids 1363 to 1454) selected from an NS3 DNase fragment library but failed to bind to 12-mer peptides synthesized from the same region, suggesting recognition of a conformational B-cell epitope. Experiments using deletion mutants of clone 8 and competitive inhibition studies using a panel of NS3 peptide-specific murine MAbs indicated that limited N-terminal and C-terminal deletions resulted in a significant reduction of hMAb binding to clone 8, thus identifying a minimal antibody binding domain within clone 8. Competition experiments showed that binding of CM3.B6 to the NS3 protein was efficiently inhibited by 39 of 44 (89%) sera from HCV-infected patients, suggesting that the hMAb recognized an immunodominant epitope within the NS3 region. More importantly, recognition of the sequence defined by CM3.B6 appeared to accurately discriminate between viremic and nonviremic anti-HCV positive sera, suggesting potentially relevant clinical applications in the diagnosis and treatment of HCV infection.

Hepatitis C virus (HCV) is a heterogeneous, single-stranded RNA virus which frequently causes persistent infection leading to chronic liver disease and primary liver cancer (1, 8, 17, 20). The viral genome, approximately 9,400 nucleotides in length, encodes for a polyprotein of approximately 3,010 amino acids (aa) which undergoes extensive posttranslational processing (17). On the basis of limited nucleotide and amino acid sequence homologies, as well as similarities in hydrophobicity profiles (22), HCV appears to be related to the family *Flaviviridae*, which also includes the animal pestivirus and flavivirus genera (33); however, the assignment of definite function to putative polypeptide domains is still under investigation. The genetic organization of HCV is similar to those of other members of the *Flaviviridae*. The 5′ untranslated region is conserved and contains *cis*-acting regulatory elements (35). The structural region is located upstream from the nonstructural region and comprises a basic N-terminal nucleocapsid protein and two envelope glycoproteins, E1 (gp33–35) and E2/NS1 (gp70–72), which are thought to be processed by host cell signal peptidases (15).

The nonstructural region includes four proteins with poorly defined functions. NS2 and NS4 (a+b) are highly hydrophobic and probably membrane anchored. The NS5 subregion probably consists of two proteins, one of which, NS5b, contains the GDD consensus motif characteristic of several viral RNA-dependent RNA polymerases, suggesting that it is involved in viral replication (18, 25). The NS3 polypeptide (~70 kDa) comprises an N-terminal one-third, which contains the highly conserved catalytic triad typical of serine-type proteinases (3, 4, 7, 34), that is responsible for the cleavage of nonstructural proteins from the polyprotein precursor (2, 12, 29, 30). Moreover, the C-terminal portion contains polynucleotide-stimulated nucleoside triphosphatase and helicase domains that are presumably involved in the unwinding of the RNA genome (7, 9). As in other members of the *Flaviviridae*, these functions are usually not separated physically by a proteolytic cleavage (7, 34).

Antibodies to c33c, a recombinant protein fragment covering the carboxyl-terminal two-thirds of the NS3 region, usually appear significantly earlier than antibodies to c100-3 (NS4) and have been clearly shown to be useful diagnostic markers of acute HCV infection (26, 32). Unpublished data suggest that, unlike most HCV polypeptides, the fine specificity of the antibody response to the NS3 region cannot be defined with short synthetic peptides, suggesting that B-cell responses to this region are probably directed at conformation-dependent epitopes (31).

The fundamental importance of the NS3 protein in the virus life cycle suggests that identification of B-cell epitopes in this context is highly desirable. However, the wide number of specificities in polyclonal sera precludes accurate identification of immunodominant antibody-binding sites on the HCV

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polyprotein. To this end, we have generated a human B-cell cloned line derived from a patient with chronic HCV infection that secretes an immunoglobulin G (IgG) monoclonal antibody (MAb) specific for the NS3 protein. We show that this human MAb (hMAb) binds to a major, conformational epitope located at the carboxyl-terminal end of the NS3 region that is recognized by approximately 90% of sera from HCV-infected patients. More importantly, recognition of such an epitope was found to be closely related to viremia.

MATERIALS AND METHODS

Generation of hMAb specific for the NS3 protein. Peripheral blood mononuclear cells were obtained from one patient with chronic HCV infection and high-titer circulating antibodies to a recombinant β-galactosidase (β-Gal) c33c fusion protein, expressed in *Escherichia coli*, encompassing residues 1192 to 1457 of the HCV deduced amino acid sequence. PBMC (4×10^6/ml) were cultured for 6 days at 37°C in 5% CO_2 in 25-cm^2 flasks in RPMI 1640 medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 4 mM l-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids supplemented with 10% heat-inactivated AB+ human serum, and 10% human endothelial cell culture supernatant in the presence of different concentrations (25, 125, and 625 ng/ml) of β-Gal c33c. Human cloned B-cell lines were obtained by immortalization of enriched B cells with Epstein-Barr virus (EBV) and repeated cloning cycles as extensively described previously (5, 6). Supernatants from EBV-transformed B-cell cloned lines showing specificity for c33c antigen (Ag) were further tested with the following reagents.

(i) Narrow-spectrum HCV enzyme-linked immunosorbent assay (ELISA) antibody test (Ortho Diagnostic Systems, Raritan, N.J.). The assay utilizes c100-3 as a solid-phase Ag.

(ii) A commercial broad-spectrum ELISA (Ortho HCV ELISA system, II generation; Ortho Diagnostic Systems). The following HCV recombinant polypeptides are incorporated in the assay: c22-3 (core) and c200, which includes the NS3 and NS4 regions.

(iii) Broad-spectrum recombinant immunoblot assay (RIBA II; Ortho Diagnostic Systems). In this assay, four recombinant proteins fused to superoxide dismutase are adsorbed on nitrocellulose strips: c100-3, a 363-aa polypeptide fused to human superoxide dismutase and expressed in yeast cells encompassing the C terminus of NS3 through NS4; 5-1-1, a 42-aa fragment comprised within the NS4 region; c33c, derived from the NS3 region; and c22-3, the putative nucleocapsid protein. Superoxide dismutase is also present on strips as a control.

(iv) Recombinant nucleocapsid (core) and NS5 proteins expressed in *E. coli*. Amino acid coordinates, according to the published HCV prototype sequence, were 1 to 177 for the core protein and 1948 to 2501 for NS5.

Only B-cell cloned lines generated from parental line CM3.B6 satisfied the requirements for monoclonality (5, 6, 23) and were therefore chosen for detailed characterization.

Fine specificity of NS3-specific hMAb. The fine specificity of hMAb CM3.B6 was initially analyzed by PEPSCAN as previously described (11). Dodecamers overlapping with 11 aa residues were synthesized in order to cover the sequence 1192 to 1457, which was previously utilized to select for anti-NS3-secreting B-cell lines. As an alternative approach, a recombinant epitope-mapping technique was used to define the fine specificity of hMAb, as described elsewhere (14, 21). Briefly, the sequence coding for a part of the NS3 region of HCV, amounting to 1,318 bp (nucleotides 3573 to 4890 of the HCV sequence), was amplified with specific primers by PCR from a cDNA clone constructed by reverse transcription-PCR from chimpanzee serum infected with the HCV prototype strain (16). PCR products were isolated from a Tris-borate-EDTA-polyacrylamide gel (8% polyacrylamide) by electroelution. Aliquots of the PCR-amplified material were digested under controlled conditions: 25°C, 10 to 60 min, in a final volume of 25 μl containing 1 mM $MnCl_2$, 20 mM Tris-HCl (pH 7.5), and 0.6 U of DNase (Worthington). The digestions were stopped by the addition of phenol-chloroform-isoamyl alcohol and DNA was extracted. DNase digestions were controlled by nick translation. Fragments with a length of approximately 50 to 200 bp were isolated from 8% acrylamide gels by diffusion (27). Parts of these fractions were tailed with oligo(dG) according to the manufacturer's recommendations (GIBCO/BRL, Gaithersburg, Md.). PCR was performed on the tailed product by using poly(C) as a primer attached to a terminal *Eco*RI site. After *Eco*RI digestion and phenol extraction, the products were cloned in λgt11 arms and transfected into *E. coli* as detailed by the manufacturer (Promega, Madison, Wis.). PCR on libraries, using λgt11 primers, revealed smears with lengths concordant with the desired length of 50 to 200 bp for the inserted fragments. The libraries were screened on duplo filters by standard procedures with human serum known to contain anti-NS3 antibodies (strongly positive for c33c by RIBA II). Positive phage were rescreened with hMAb CM3.B6 at a dilution of 1:50, and the reaction was detected with alkaline phosphatase-conjugated goat anti-human IgG. Positive phage were further examined to positively identify their immunoreactivities, and their inserts were subsequently transferred to the vector pGEM7Zf(+) (Promega). The inserts in this vector were sequenced with a commercial kit (Pharmacia T7 sequencing kit) as suggested by the manufacturer.

β-Gal fusion proteins were purified by affinity chromatography according to standard procedures (Protosorb lacZ column; Promega). ELISA plates were then coated with purified Ags and allowed to react with sera from patients with HCV infection as previously described (5). Alternatively, nitrocellulose strips containing β-Gal fusion proteins and β-Gal controls were prepared by application to a 12% acrylamide gel (30 min at 200 V) and then by blotting onto nitrocellulose (2 h at 100 V). Immunoblotting was performed by overnight incubation at room temperature of samples appropriately diluted in PBS containing 5% nonfat dry milk on a rocking platform. After washing with Tris-buffered saline plus 0.05% Tween 20, alkaline phosphatase-conjugated goat anti-human IgG was applied to the strips for 2 h under shaking. Following another washing step, 5-bromo-4-chloro-3-indolyl phosphate–*p*-nitroblue tetrazolium chloride was added as a substrate.

Competition experiments between hMAb CM3.B6 and human sera. hMAb CM3.B6 was purified on a protein A-Sepharose column and labeled with horseradish peroxidase (HRP) by using standard technology (24) at a HRP/IgG molar ratio of 4. Microtiter plates were coated with affinity-purified β-Gal–c33c at a concentration of 1 μg of 0.05 M bicarbonate buffer (pH 9.6) per ml at 4°C overnight. After incubation plates were aspirated and saturated with 0.1 M Tris buffer (pH 7.4) containing 0.2% bovine serum albumin (BSA). Human sera, diluted 1:10 in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/Tween), were added to each well and allowed to bind for 5 min. HRP-labeled hMAb CM3.B6 was then added, and the antibody mixture was incubated for 1 h at 37°C. After extensive washing with PBS/Tween, TMB (3,3',5,5' tetramethylbenzidine) was added and the color was allowed to develop for 30 min. The dilution of HRP-hMAb was adjusted

to give an A_{492} reading of 1.0 without the addition of human serum. Competition was then calculated as follows:

$$\% \text{ competition} = 1 - \frac{C - B}{A - B} \times 100$$

Where $A = A_{492}$ value obtained without competing human serum (0% competition), $B = A_{492}$ value in the presence of 100-fold molar excess of unlabeled hMAb (100% competition), and $C = A_{492}$ value obtained in the presence of competing human serum.

Seventeen sera from HCV-seronegative healthy subjects served as controls. Competition was considered significant if it was greater than 30%. This value was obtained by adding 5 standard deviations to the mean value of negative controls.

Production of murine MAb to c33c protein. BALB/c mice were immunized with recombinant c33c, and murine hybridomas specific for β-Gal–c33c were produced by conventional techniques (19). Fine specificity of murine MAb was analyzed by PEPSCAN (11), using consecutive 12-mers covering the entire c33c region. Murine MAbs were utilized in competition experiments with hMAb anti-NS3 as detailed above.

Immunofluorescence detection of NS3 protein in transfected cells. The vaccinia virus T7 transient-expression system was utilized for the transfection assay as previously described (30). Briefly, HeLa cell monolayers were seeded on glass coverslips and infected with vaccinia virus vTF7-3 at a multiplicity of infection of 5. After adsorption for 30 min at 37°C, 3 ml of Dulbecco modified Eagle medium supplemented with 10% fetal calf serum was added. Cells were incubated for an additional 30 min at 37°C. Twenty micrograms of plasmid DNA, pCD(38-9.4) encompassing nucleotides 1 to 9416 (full-length HCV construct), or pCITE(NS3) encompassing nucleotides 3351 to 5171 (NS3 region) of the HCV genome (30) was precipitated in calcium phosphate as previously described (27) and added directly to each plate. At 6 h posttransfection, cells were fixed with methanol for 4 min at −20°C, rehydrated in PBS, and incubated with culture supernatant from cloned line CM3.B6 diluted 1:20 in PBS containing 5% BSA. Cells were then labeled with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin, mounted in Moviol containing 1 mg of paraphenylenediamine per ml, and photographed by epifluorescence with a Leica Diaplan microscope with a 100× planar objective. Nontransfected or mock-transfected HeLa cells served as a control substrate.

Western (immunoblot) analysis of hMAb anti-NS3. Fifty nanograms of purified NS3 protein (aa 1187 to 1496), corresponding to the immunoreactive helicase domain, or 10 μg of total lysates of bacteria transformed with plasmid pT7-7, which contains the bacteriophage T7 RNA polymerase promoter (28), was separated by SDS–10% PAGE, and after transfer to nitrocellulose, strips were immersed in 1% skim milk in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) (blocking buffer) for 20 min at room temperature. The appropriate primary antibody diluted in blocking buffer was applied to the nitrocellulose strips and incubated for 1 h at room temperature. Dilutions were 1:50 for CM3.B6 supernatant, 1:2,000 for rabbit polyclonal anti-NS3 (30), and 1:200 for a control anti-HCV negative human serum. After three 10-min washings in blocking buffer, anti-human or anti-rabbit IgG conjugated with alkaline phosphatase was applied at a 1:2,000 dilution in blocking buffer. After 1 h of incubation at room temperature, membranes were washed thrice for 10 min and developed with 100 mM Tris-HCl buffer (pH 9.5), containing 100 mM NaCl, 5 mM $MgCl_2$, 0.33 mg of *p*-nitroblue tetrazolium–chloride per ml and 0.165 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml.

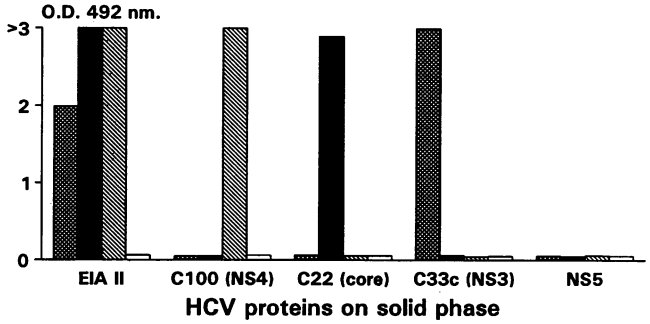

FIG. 1. Specificity of hMAb CM3.B6 (▩) determined by ELISA. A commercial broad-spectrum assay incorporating recombinant proteins derived from core, NS3, and NS4 regions of HCV was also utilized for comparison. Recombinant protein coordinates are reported in Materials and Methods. 01.D6 (□) is a control supernatant from an irrelevant human EBV-transformed B-cell line; D10.E6 (▨) is a hMAb specific for a linear epitope located within the NS4 protein (6); B12.F8 (■) is a hMAb specific for a conformational, continuous epitope on the nucleocapsid protein (5).

RESULTS

A total of 480 wells were seeded after in vitro stimulation of peripheral blood mononuclear cells with c33c and infection of enriched B lymphocytes with EBV; however, only 6 microcultures gave a positive signal by ELISA. Two cell lines, designated 2F5 and CM3, with an A_{492} of >2 were selected for subculture at densities of 12 to 25 cells per well. Following the first cloning cycle, only CM3 yielded a single positive well (CM3.B6) which, after another subcloning step, generated a number of cloned lines, all secreting NS3-specific antibody. Analysis of human B-cell line supernatants revealed that all cloned lines derived from parental line CM3.B6 secreted IgG1(κ) only. NS3-specific cloned lines were shown to be remarkably stable, secreting specific antibody for at least 18 months in continuous culture.

Specificity of hMAb CM3.B6. Supernatant from cloned line CM3.B6 was analyzed for specificity by ELISA and commercial immunoblot (RIBA II). As illustrated in Fig. 1, the hMAb exclusively recognized the recombinant c33c protein (also included as part of c200 in the commercial ELISA), whereas no reactions with c100-3 (NS4), c22-3 (core), and NS5 could be documented. These findings were further confirmed and extended by RIBA II, which demonstrated selective binding of CM3.B6 to the c33c polypeptide band (data not shown).

To determine whether CM3.B6 recognized the authentic NS3 protein expressed in eukaryotic cells, HeLa cells transfected with HCV cDNA clone pCD(38-9.4) were used as a substrate in an indirect immunofluorescence assay. This plasmid expresses the entire HCV genome and has been previously described (30). Immunofluorescence staining of pCD(38-9.4)-transfected HeLa cells with hMAb CM3.B6 revealed prominent cytoplasmic deposits (Fig. 2A). Staining was observed in 10 to 20% of the cells, consistent with the efficiency of transfection routinely observed, and was not detected in control nontransfected and mock-transfected cells (Fig. 2B). A similar overall staining pattern was observed when the hMAb was allowed to react with cells transfected with plasmid pCITE(NS3), which expresses the HCV NS3 protein (30). Moreover, a rabbit polyclonal antiserum raised against aa residues 1187 to 1496 of the HCV polyprotein gave a similar fluorescence pattern (data not shown).

Western blot analysis of a truncated form of NS3 protein,

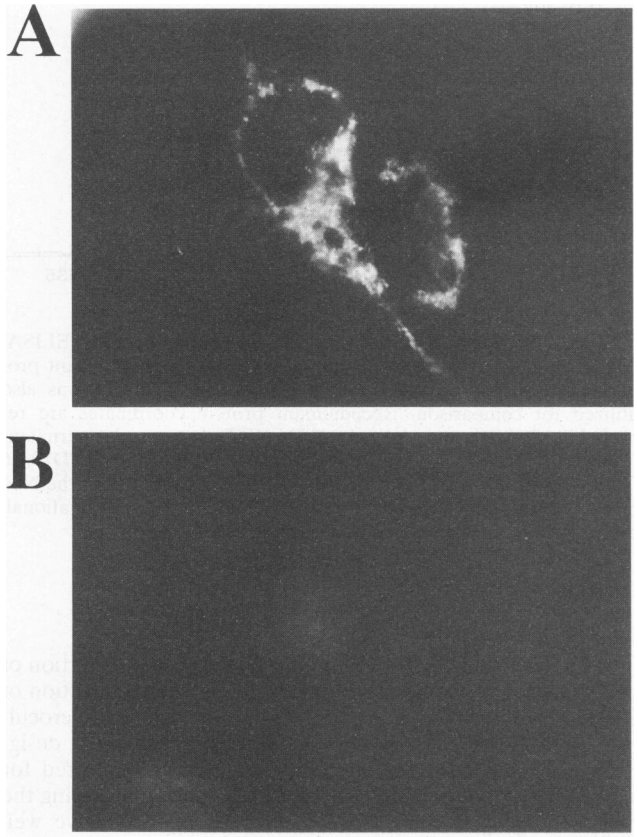

FIG. 2. Indirect immunofluorescence staining with hMAb CM3.B6 of HeLa cells transfected with pCD(38-9.4) (A) or nontransfected (B). Notice the exclusive cytoplasmic distribution of the fluorescence.

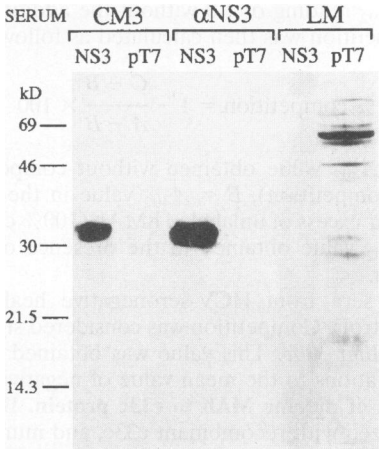

FIG. 3. Western blot analysis of hMAb CM3.B6. Lanes indicated with NS3 were loaded with truncated NS3 fusion protein (aa 1187 to 1496), whereas lanes indicated with pT7 were loaded with total lysates of bacteria transfected with plasmid pT7-7 (28). CM3 indicates strips incubated with hMAb CM3.B6; αNS3 indicates strips incubated with polyclonal rabbit anti-NS3 antiserum; LM indicates strips incubated with an anti-HCV negative control human serum. The relative positions of the molecular mass markers are also indicated. The position of the recombinant NS3 protein band corresponds to the predicted molecular mass of the helicase domain (30).

representing the helicase domain, revealed that hMAb CM3.B6 recognized a distinct protein band of the predicted molecular mass (30). Similar results were obtained with a polyclonal rabbit anti-NS3, but not with a control anti-HCV negative human serum. Control bacterial extracts were also negative (Fig. 3).

Fine specificity of hMAb CM3.B6. Initial efforts to identify the epitope defined by hMAb CM3.B6 by PEPSCAN analysis by the solid-phase pin technology (11) were unsuccessful, suggesting that the hMAb recognized a conformational epitope (data not shown). In order to overcome these problems, randomly expressed DNase fragments from a HCV cDNA library encompassing the complete NS3 region were analyzed for binding to hMAb CM3.B6. The best reactive recombinants were selected, and their nucleotide sequences were determined. The NS3-specific hMAb selected clone 8, a 92-aa fragment encompassing residues 1363 to 1454 of the HCV genome, as the best reactive recombinant. Other recombinant fragments were also selected in that subregion, although the signal was significantly weaker than that observed with clone 8. The considerable length of the fragment selected by hMAb CM3.B6 was indicative of recognition of a conformation-dependent epitope in agreement with the negative results obtained by PEPSCAN.

In an attempt to focus on the minimal sequence recognized by hMAb CM3.B6, we performed deletion experiments on clone 8. Deletion mutants were constructed, expressed as recombinant fusion proteins, and analyzed for reactivity with hMAb CM3.B6 (Fig. 4). Deletions of amino-terminal and carboxyl-terminal residues resulted in a significant reduction of reactivity with CM3.B6. The subregion covered by aa 1378 to 1443 appeared to contain the minimal sequence required for binding to the hMAb in this system, and flanking sequences greatly enhanced recognition. These findings indicated that CM3.B6 recognized a conformational, and probably discontinuous, epitope on the NS3 region defined by long peptides encompassing the region covered by clone 8 (aa 1363 to 1454). Additional evidence for the importance of sequences flanking the putative hMAb binding domain came from competitive inhibition studies with a panel of three murine MAbs specific for different sequential epitopes on the c33c protein. The coordinates of such epitopes were defined by PEPSCAN analysis with pin-bound 12-mer peptides covering the entire c33c sequence and were as follows: mAb 5I, 1203 to 1214; mAB 6L, 1214 to 1225; mAb 5D, 1444 to 1455. Figure 5 shows that only mAb 5D, which selectively binds to the C-terminal portion of clone 8, exerted significant inhibition of CM3.B6 binding to c33c Ag on the solid phase, suggesting that the sequence 1444 to 1455 probably contains critical residues for Ag-antibody interaction which are conceivably located outside the minimal antibody binding site for CM3.B6. Alternatively, steric hindrance may explain inhibition of CM3.B6 binding to c33c by murine MAb 5D.

Competitive inhibition studies with polyclonal sera from HCV-infected patients. We performed competitive inhibition studies with a panel of 44 sera from patients with HCV infection containing anti-c33c antibodies as determined in a recombinant c33c ELISA. Of these, 39 (89%) significantly competed with CM3.B6 for binding to recombinant c33c Ag, indicating that the epitope defined by this hMAb is immunodominant with respect to the NS3 region (Fig. 6). In contrast, control c33c antibody negative sera from HCV-infected patients failed to compete for binding to c33c (not shown).

To determine whether additional B-cell sites were present on the NS3 protein, we examined in more detail the five sera from the above panel that did not compete with CM3.B6, using

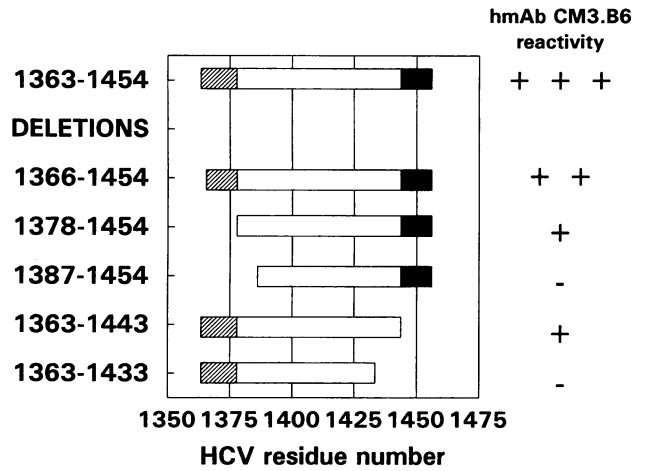

FIG. 4. Deletion analysis of recombinant CM3.B6 epitope. Amino- and carboxyl-terminal deletion mutants of clone 8 (aa 1363 to 1454) were generated by PCR with specific oligonucleotides which hybridized exactly at the indicated coordinates and contained *Eco*RI restriction sites at their ends. Truncated clone 8 fragments were put in frame with the gene encoding β-Gal by cloning amplimers digested with *Eco*RI into λgt11 arms. Upon lytic growth and induction by 1 mM isopropyl-β-d-thiogalactopyranoside, β-Gal clone 8 fusion proteins were produced, blotted onto nitrocellulose strips as described in Materials and Methods, and tested for their reactivities with hMAb CM3.B6. Open blocks indicate the putative minimal antibody binding site; hatched areas represent flanking sequences that enhance CM3.B6 recognition of recombinant Ag. The intensity of the signal on blot strips was semiquantitatively evaluated on a −(negative) to +++ (strongly positive) scale by comparing the signal observed with clone 8 as Ag (+++) with that of the various deletion mutants utilized in the experiment.

a validated PCR assay (10) and RIBA II. Three of these five sera were positive in both assays; interestingly, however, the remaining two sera did not contain detectable levels of viral RNA and were indeterminate by RIBA II because of the

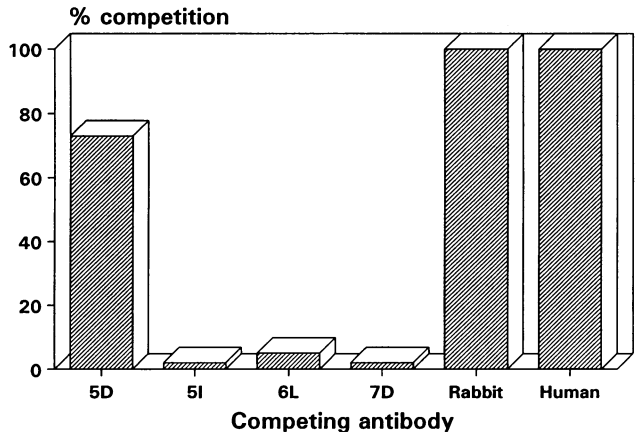

FIG. 5. Competitive inhibition of CM3.B6 binding to recombinant c33c (representing the C-terminal two-thirds of the NS3 protein) by four murine MAbs with different fine specificities. Coordinates of the linear epitopes defined by murine MAb and experimental conditions are reported in Materials and Methods. Competing MAbs were utilized at a 100-fold molar excess. Rabbit and human polyclonal antibodies were also included as positive control inhibitors. 7D is a control murine MAb which recognizes the HCV nucleocapsid protein.

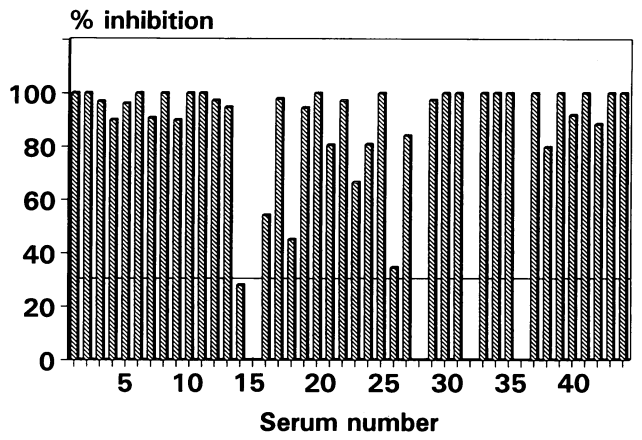

FIG. 6. Competitive inhibition of hMAb CM3.B6 binding to recombinant NS3 Ag by 44 sera from patients exposed to HCV. An ELISA was developed for the detection of human antibodies specific for NS3, using purified recombinant β-Gal–c33c as an antigen. CM3.B6 was labeled with HRP as previously described (24). Percent inhibition was determined as described in Materials and Methods. A cutoff value of 30% was obtained by adding 5 standard deviations to the mean percent inhibition obtained with sera from 17 healthy HCV-seronegative controls.

presence of antibodies reacting exclusively with the c33c protein band. Further analysis of the remainder of the 44-member panel by RIBA II showed that those sera were from bona fide HCV-infected patients, since they contained antibodies reactive with multiple HCV antigens. This observation suggested that the epitope recognized by anti-NS3 antibodies present in sera containing only anti-c33c might differ from the epitope recognized by the majority of HCV-infected patients. To address this issue, we employed a panel of 58 sera which were positive only for anti-c33c by RIBA II (36). Seven of those sera were obtained from patients with HCV infection and were HCV RNA positive by PCR; 51 sera were from blood donors, and only 1 was PCR positive. Sera from this panel were tested for their abilities to inhibit binding of hMAb CM3.B6 to c33c antigen on the solid phase. Eight of 8 sera from viremic anti-HCV carriers effectively competed for binding, whereas only 1 of 50 sera from nonviremic anti-HCV carriers showed significant competition. We therefore suggest that anti-NS3 antibodies present in nonviremic sera react with a B-cell epitope(s) distinct from that defined by our hMAb. Additional evidence in support of this contention came from experiments with a synthetic peptide called 377, which encompassed the CM3.B6 epitope as defined by clone 8 (aa 1363 to 1454). This peptide was allowed to compete with recombinant c33c for binding to anti-NS3 antibodies present in human sera. Figure 7A shows that, in viremic patients, binding of circulating polyclonal NS3 antibodies to recombinant c33c was very efficiently inhibited by peptide 377, indicating that, during viral replication, B-cell responses to NS3 are almost invariably directed to the major immunodominant epitope described above. In contrast, nonviremic sera often contain antibodies directed against B-cell sites different from those recognized by CM3.B6. Indeed, binding of anti-c33c positive sera from nonviremic patients to recombinant c33c was largely unaffected by peptide 377 (Fig. 7B). These findings are in complete agreement with those obtained in a direct binding assay with peptide 377 as an antigen, which almost perfectly discriminated PCR-positive from PCR-negative sera containing anti-c33c antibody only (13). We conclude that hMAb CM3.B6

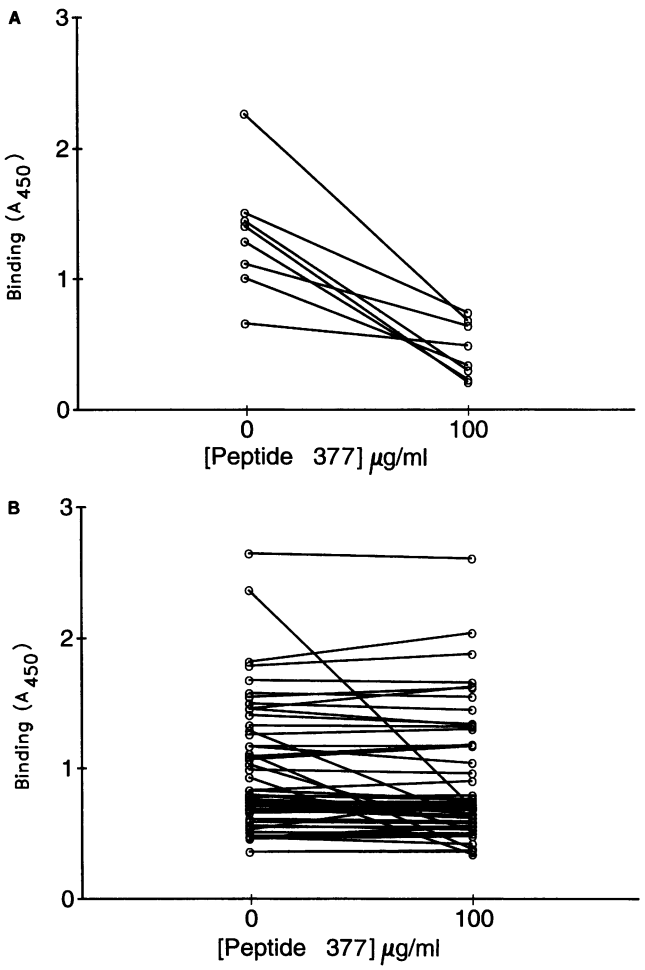

FIG. 7. Binding of anti-c33c positive human sera to recombinant NS3 Ag is inhibited by a single synthetic peptide, 377, encompassing the epitope defined by hMAb CM3.B6. Binding of sera to β-Gal–c33c on the solid phase was measured in the absence and in the presence of 100 μg of synthetic peptide. Recognition of β-Gal–c33c by viremic sera (A) was efficiently inhibited by the competitor peptide, whereas only 5 of 51 sera from nonviremic individuals competed for binding to β-Gal–c33c (B).

identifies a unique B-cell epitope which is predominantly recognized by sera from viremic anti-HCV carriers.

DISCUSSION

The NS3 region of HCV contains enzymatic domains that are likely to play a pivotal role in the virus life cycle. The N-terminal serine-type proteinase is involved in the processing of nonstructural proteins from the polyprotein precursor (2, 12, 29, 30), while the C-terminal portion contains nucleoside triphosphatase-RNA helicase activities (29). Expression of these proteins most probably occurs during active viral replication, and this hypothesis is supported by prospective clinical studies which demonstrated that antibodies to NS3 appear early in the course of HCV infection, usually before or concomitantly with seroconversion to anticore (32). This observation and the finding that anti-NS3 is often detected during persistent HCV infection suggest, however, that the antibody is unlikely to exert significant inhibitory activity on HCV replication in vivo.

In this study, we report for the first time the isolation and fine characterization of a B-cell cloned line generated from a patient with chronic HCV infection which secretes a MAb specific for a major conformational epitope located at the C-terminal one-third of the NS3 protein. Moreover, the hMAb was shown to recognize the authentic NS3 protein expressed in transfected eukaryotic cells or purified from transformed bacteria by immunofluorescence and Western blot, respectively. The immunodominance of the carboxyl-terminal region of NS3 was clearly demonstrated by direct binding and competition experiments in which the overwhelming majority of sera from bona fide HCV-infected subjects were shown to recognize a β-Gal fusion protein or a synthetic peptide containing the sequence defined by hMAb CM3.B6. These findings have been recently confirmed with a panel of 105 sera from consecutive patients with chronic HCV infection (13).

Evidence for the presence of a conformation-dependent antibody binding site(s) within the NS3 C-terminal domain came from preliminary experiments in which we were unable to show recognition of short synthetic peptides by anti-NS3 positive human sera. This observation is in contrast with findings in the murine system, in which three major sequential B-cell epitopes were identified, by different MAbs, within the c33c portion of NS3 with 12-mer peptides arranged in PEPSCAN format. Here we have shown that hMAb CM3.B6 selectively bound to a 92-aa sequence comprised in c33c (clone 8), strongly suggesting the presence of at least one conformational epitope within the NS3 region. Moreover, competition experiments indicated that the majority of human sera very efficiently inhibited binding of our hMAb to recombinant c33c, indicating the existence of a major, immunodominant B-cell site within the NS3 protein. The fine specificity of CM3.B6 could be restricted to a minimal immunoreactive sequence of 66 aa residues, with N- and C-terminal flanking sequences greatly enhancing immunoreactivity. In addition, binding of CM3.B6 to recombinant c33c could be inhibited by a murine MAb, 5D, which recognizes the C-terminal flanking sequence within clone 8, suggesting that flanking residues, located outside the putative minimal immunoreactive sequence, are important for antibody recognition.

The most important aspect of the present study was the finding of a close relationship between HCV viremia and antibody recognition of the amino acid sequence defined by hMAb CM3.B6. In contrast, sera from nonviremic anti-HCV carriers appeared to recognize a different B-cell epitope(s). Evidence in support of this contention came from experiments showing (i) complete inhibition of CM3.B6 binding to c33c by viremic (PCR positive) sera, but not by the overwhelming majority of nonviremic (PCR-negative) sera; (ii) inhibition of binding of serum anti-NS3 antibodies to c33c by a synthetic peptide encompassing the major immunoreactive sequence in viremic patients, but not in the vast majority of nonviremic patients; and (iii) recognition of peptide 377 by viremic patients only, in a direct binding assay (13). On the other hand, failure to detect viral RNA in some of anti-HCV positive sera may also be explained on the basis of extremely low, fluctuating levels of circulating HCV RNA, below the detection limits of our sensitive PCR assay. This phenomenon may account for the apparent discrepancies observed in competition experiments using five PCR-negative sera which were significantly inhibited from binding to c33c by peptide 377 (Fig. 7B). Indeed, preliminary evidence indicates that such sera react with additional HCV Ags, including the putative viral replicase NS5 which was not incorporated in the RIBA II generation

assay, suggesting that those patients have a low titer of circulating virus (13). The clinical relevance of the findings presented in this study is immediately apparent. Indeed, incorporation of the amino acid sequence defined by hMAb CM3.B6 in diagnostic assays may be an extremely valuable tool to discriminate between viremic and nonviremic anti-HCV carriers, therefore representing a first line or supplemental test to more sophisticated assays that qualitatively or quantitatively measure HCV RNA. Obviously these preliminary observations should be validated with a larger panel of sera from several patient categories. Furthermore, since expression of the NS3 protein, which contains essential enzymes for HCV life cycle, would occur in hepatocytes that actively sustain viral replication, it would be interesting to develop a targeted delivery system whereby the anti-NS3 hMAb is coupled to a toxin or a nucleoside analog, in order to explore potential antiviral effects in vitro and, eventually, in vivo.

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