JOURNAL OF CLINICAL MICROBIOLOGY, May 1999, p. 1393–1397 Vol. 37, No. 5
0095-1137/99/$04.00+0

Use of a Novel Hepatitis C Virus (HCV) Major-Epitope Chimeric Polypeptide for Diagnosis of HCV Infection

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Received 18 August 1998/Returned for modification 10 December 1998/Accepted 19 January 1999

The genome of hepatitis C virus (HCV) consists of seven functional regions: the core, E1, E2/NS1, NS2, NS3, NS4, and NS5 regions. The U.S. Food and Drug Administration-licensed 2.0G immunoassay for the detection of anti-HCV uses proteins from the core, NS3, and NS4 regions (McHutchinson et al., Hepatology 15:19–25, 1992). The 3.0G enzyme-linked immunosorbent assay includes the protein from the NS5 region (Uyttendaele et al., Vox Sang. 66:122–129, 1994). The necessity of detecting antibodies to viral envelope proteins (E1 and E2) and to different genotype samples has been demonstrated previously (Chien et al., Lancet 342:933, 1993; Lok et al., Hepatology 18:497–502, 1993). In this study we have attempted to improve the sensitivity of the anti-HCV assay by developing a single multiple-epitope fusion antigen (MEFA; MEFA-6) which incorporates all of the major immunodominant epitopes from the seven functional regions of the HCV genome. A nucleic acid sequence consisting of proteins from the viral core, E1, E2, NS3, NS4, and NS5 regions and different subtype-specific regions of the NS4 region was constructed, cloned, and expressed in yeast. The epitopes present on this antigen can be detected by epitope-specific monoclonal and polyclonal antibodies. In a competition assay, the MEFA-6 protein competed with 83 to 96% of genotype-specific antibodies from HCV genotype-specific peptides. This recombinant antigen was subsequently used to design an anti-HCV chemiluminescent immunoassay. We designed our assay using a monoclonal anti-human immunoglobulin G antibody bound to the solid phase. Because MEFA-6 is fused with human superoxide dismutase (h-SOD), we used an anti-human superoxide dismutase, dimethyl acridinium ester-labeled monoclonal antibody for detection. Our results indicate that MEFA-6 exposes all of the major immunogenic epitopes. Its excellent sensitivity and specificity for the detection of clinical seroconversion are demonstrated by this assay.

The genome of the hepatitis C virus (HCV) consists of seven functional regions: the core, E1, E2/NS1, NS2, NS3, NS4, and NS5 regions. Since the discovery of HCV (7), significant progress in the development of serologic tests for the detection of antibodies to HCV has been made. The earliest tests, developed for blood screening, were enzyme immunoassays (EIAs) that detect antibody to a cloned HCV NS4 protein (C100) (1, 12). Second- and third-generation EIAs, which can detect a broader range of antibodies to HCV, include various combinations of recombinant antigens (14, 17).

Despite the proven utility of these assays for blood screening and for the diagnosis of HCV infection in symptomatic patients, important challenges to the improvement of assay performance remain. Examples of such challenges include reducing the window of seronegativity, improving the detection of HCV in samples from immunosuppressed patient, and increasing assay sensitivity in order to detect antibodies to the different HCV genotype-specific epitopes.

The commercial EIAs that have been developed to date have used synthetic peptides or recombinant chimeric polyproteins as antigens (14, 17). EIAs that use synthetic peptides as antigens are designed such that only a few synthetic peptides containing the major putative HCV epitopes are present (6). These types of EIAs usually lack the epitopes necessary for detection of the HCV antibodies present in certain samples positive for HCV. This has been demonstrated by the poor sensitivities of these assays for the detection of HCV in diluted sera and for the detection of seroconversion (3). EIAs that use chimeric polyproteins as antigens, on the other hand, show greater sensitivity than those that use the shorter synthetic peptides derived from the same HCV genomic regions (3). The more inclusive chimeric polyproteins, however, include many non-epitope sequences which add unnecessary length and which make it difficult to insert epitopes from different strains into their backbones without potentially altering the protein structure and consequently interfering with epitope recognition.

In this study we have designed a novel multiple-epitope fusion antigen (MEFA). Our objective was to design a chimeric antigen that would contain only the essential immunodominant epitopes from the HCV structural and genotype-specific regions necessary for optimal sensitivity and specificity. We describe the construction of this chimeric HCV polypeptide, termed "MEFA-6," which incorporates the major epitope domains of the core, E1, E2, NS3, NS4, and NS5 regions. We compare the immunoreactivity of MEFA-6 with those of individual recombinant polyproteins and peptides derived from several regions of the HCV genome and evaluate the exposure of HCV epitopes within the MEFA-6 polypeptide. Furthermore, we describe the use of MEFA-6 in the design of a chemiluminescent immunoassay (CLIA; MEFA-6 CLIA) for the detection of antibodies to HCV.

Our results demonstrate that the MEFA-6 CLIA is capable of detecting HCV at a two- to fourfold greater dilutional sensitivity than a previously developed EIA which uses a chimeric fusion polyprotein made up of the NS3-NS4-core (C25) region (4). The MEFA-6 CLIA exhibits a sensitivity and specificity equivalence to those of the licensed Ortho 3.0G enzyme-linked

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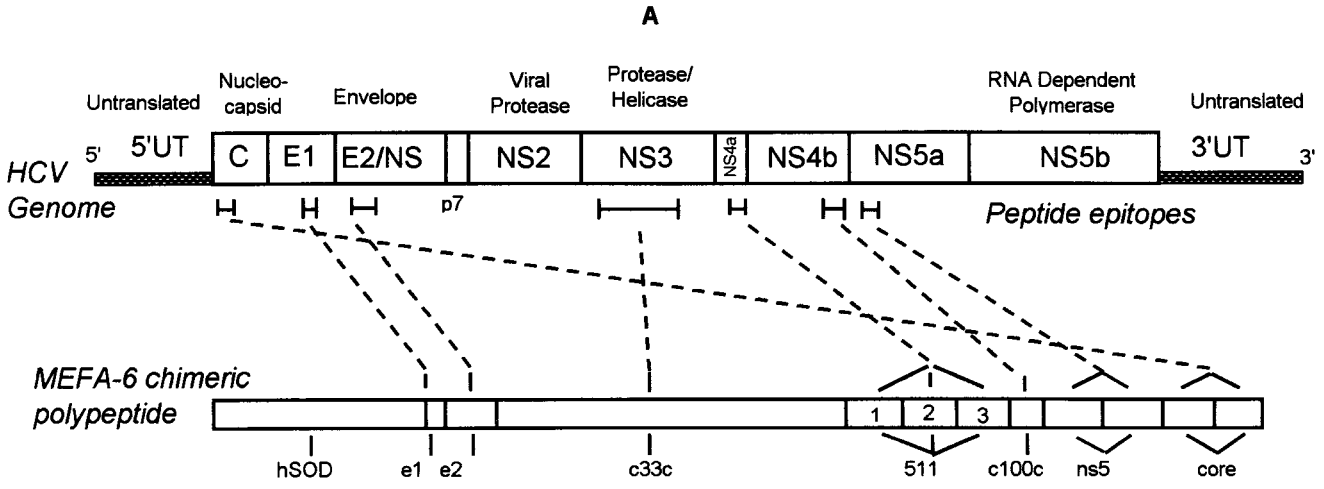

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MEFA-6 aa sequence	Epitope	HCV aa sequence	HCV genotype
1-154	hSOD	N/A	N/A
159-176	el	303-320	1a
179-217	e2	405-444	1a
218-484	c33c	1192-1457	1a
487-533	5-1-1	1689-1735	3
536-582	5-1-1	1689-1735	2b
585-631	5-1-1	1689-1735	1a
634-673	c100c	1901-1940	1a
676-711	NS5	2278-2313	1a
714-749	NS5	2278-2313	1a
750-793	core	10-53	1a
796-839	core	10-53	1a

FIG. 1. (A) Genetic organization of HCV and relative location of peptide antigens in MEFA-6 polypeptide. (B) MEFA-6 epitope sequence, HCV genotype, and corresponding HCV amino acid (aa) sequence.

immunosorbent assay (ELISA). As demonstrated by our analysis of MEFA-6, MEFAs should prove to be valuable in the development of improved HCV serodiagnosis and screening assays.

MATERIALS AND METHODS

Samples. Hepatitis C seroconversion panels were purchased from Boston Biomedica Inc. (West Bridgewater, Mass.). Samples for dilutional sensitivity testing and with chronic HCV infection were acquired from Uniglobe Research Corporation (Reseda, Calif.). The HCV pedigree patient samples were obtained from M. Tong (Huntington Memorial Hospital, Pasadena, Calif.). The samples from hemophiliacs, intravenous drug abusers (IVDAs), kidney dialysis patients, and patients with chronic HCV infections and the different genotype samples were provided by Harvey Alter of the National Institute of Health (Bethesda, Md.) and Johnson Y. N. Lau from the University of Florida Shands Clinic (Gainesville, Fla.). Randomly selected volunteer blood donor samples were obtained from G. Tegtimeier (Community Blood Center of Greater Kansas City, Kansas City, Mo.).

Expression and purification of MEFA-6 chimeric polypeptide. Yeast strains AB122, JSC310, and AD2 (Chiron, Emeryville, Calif.) were transformed with the pS.MEFA-6 yeast expression plasmid which contained the ADH2-GAPDH promoter (9), followed by the coding sequence for human superoxide dismutase (h-SOD) fused to the N terminus of the MEFA-coding sequence, followed by the alpha-factor terminator. This plasmid was cloned into a shuttle vector that included pBR322 sequences for the ampicillin resistance gene and the ColE1 origin of replication, 2μm sequences, and the yeast *leu2-d* and *URA3* genes as selectable markers. The h-SOD fusion protein was used to increase the level of expression of MEFA-6 (10, 11). A lithium acetate protocol, described in a previous publication, was used for the transformation (10). The Ura^- transformants were streaked for single colonies and patched on Leu^- 8% glucose plates to increase the plasmid copy number. The Leu^- starter cultures were grown for 24 h at 30°C and were then diluted 1:20 in yeast extract-Bacto Peptone glucose medium. The cells were subsequently grown for 48 h at 30°C and harvested. To test for expression of recombinant MEFA-6, an aliquot of the cells was boiled in the sodium dodecyl sulfate (SDS)-gel electrophoresis sample buffer of Laemmli containing 50 mM dithiothreitol. The protein components of the cell mixture were then separated by gel electrophoresis on a Tris-glycine polyacrylamide gel.

Cells expressing MEFA-6 were harvested by centrifugation. The cells were then suspended in lysis buffer (50 mM Tris, 0.15 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [pH 8.0]) and lysed in a Dyno-Mill (Basel, Switzerland) apparatus with glass beads. The lysate was centrifuged at low speed for 15 min, and the pellet containing the insoluble protein fraction was washed with increasing concentrations of urea in lysis buffer. The MEFA-6 protein was then extracted from the cell pellet with 0.1 N NaCl–4 M urea in lysis buffer, and the cell debris was removed by low-speed centrifugation. The supernatant was adjusted to pH 8.0, and the precipitate was removed by centrifugation. SDS and dithiothreitol were added to the supernatant, and the mixture was then boiled for 3 min at pH 8.0. The proteins in this mixture were fractionated by gel filtration on a Pharmacia Sephacryl S-400 column in phosphate-buffered saline containing 0.1% SDS and 1 mM EDTA adjusted to pH 7.4. Column fractions containing MEFA-6 were collected, pooled, and concentrated on an Amicon YM-30 membrane. Protein purity was determined by SDS-polyacrylamide gel electrophoresis and Western blot analysis (with anti-HCV monoclonal antibodies). The final purity of MEFA-6 was greater than 95%.

Monoclonal and polyclonal antibody binding assays. Monoclonal and polyclonal antibodies raised against HCV-specific recombinant core, E1, E2, NS3, NS4, and NS5 antigens were used to evaluate the antigenicity and epitope exposure of the chimeric antigen, MEFA-6. Purified MEFA-6 was diluted to an

optimal coating concentration in phosphate-buffered saline (pH 7.4) and was coated on Immulon I (Dynatech, Chantilly, Va.) microtiter plates. Monoclonal antibodies to the core, NS3 (C33C), NS4 (C100c and 5-1-1), and NS5 regions and rabbit polyclonal antisera to the E1 and E2 regions were prepared by standard techniques. The antibodies were then diluted 200-fold in sample diluent, added to the plate, and incubated for 1 h at 37°C. The plate was then washed with wash buffer. Affinity-purified goat anti-mouse $F(ab')_2$ or goat anti-rabbit immunoglobulin G (IgG; heavy and light chain) conjugated to horseradish peroxidase was then added to the appropriate wells. The plates were incubated for 1 h at 37°C and washed with wash buffer. A developing buffer containing hydrogen peroxide and the substrate *o*-phenylenediamine dihydrochloride was then added to the wells, and optical density (OD) readings at 492 and 620 nm were determined with a plate reader. The cutoff value for each antigen (the core, E1, E2, NS3, NS4, and NS5 regions and SOD) was defined as three times the mean for three negative control serum samples included in each assay. If any sample reacted with the SOD control, then that sample was considered to be nonreactive or indeterminate.

Junction sequence peptide assays. Peptides comprising 7 to 9 amino acids derived from the sequences existing at each junction within the MEFA-6 cassette were synthesized and biotinylated. Each sequence was compared with the sequences in Genbank by scanning for all existing pathogen-related epitopes in order to rule out any matches. Peptides were coated on strepavidin-coated plates and were subsequently tested against more than 10,000 random, HCV-negative samples.

Inhibition assays. To test whether the genotype-specific epitopes present on MEFA-6 are capable of detecting HCV type-specific antibodies in HCV-reactive human sera, we performed a peptide inhibition assay. Inhibition assays were performed by the multiantigen ELISA protocol described previously (4). This assay measures the degree to which MEFA-6 in solution was capable of competing with serum HCV type-specific antibodies from the genotype-specific peptides coated on the solid support. Prior to the assay, sample dilution breakpoints were determined. The dilution breakpoint was defined as the greatest dilution at which binding was still detectable. For optimal detection, patient samples were diluted twofold less than the dilution breakpoint. Samples from HCV-infected patients were serially diluted and tested for a reaction to each recombinant antigen: core (C22c), NS3 (C33C), and NS4 (C100c) regions immobilized on Immulon I microtiter plates. The genotypes of the HCV isolates present in these samples were determined with an Innogenetics INNO-LIPA HCV II kit. The ability of MEFA-6 to bind to antibodies to HCV genotypes 1, 2, and 3 from patient samples was demonstrated by measuring the percent inhibition of antibody binding to the NS4 5-1-1 region (amino acids 1689 to 1735) in the presence of MEFA-6.

MEFA-6 immunoassay procedure. The immunoassays were performed as follows. A total of 10 μl of test sample was added to a polystyrene tube (75 by 12 mm; Sarstedt, Newton, N.C.), followed by the addition of 100 μl of sample diluent, 100 μl of the solid-phase reagent, which had an optimal concentration of mouse monoclonal anti-human IgG covalently linked to paramagnetic particles (PMPs; Chiron Diagnostics, Walpole, Mass.), 50 μl of recombinant MEFA-6, and 100 μl of a chemiluminescent tracer (mouse monoclonal anti-h-SOD conjugated to dimethyacridinium ester). The tubes were then vortexed and incubated for 18 min in a 37°C water bath. The tubes were subsequently placed on a magnetic rack for 3 min to allow the PMPs to separate from the solution. The unbound fractions were decanted, and the aggregated PMPs were washed with 1 ml of wash buffer. This decant-wash cycle was repeated two additional times. After the final decanting, the tubes were loaded in an illuminometer (Magic Lite Analyzer II; Chiron Diagnostics). Each tube was then treated with a 300-μl aliquot of 0.5% hydrogen peroxide followed by a 300-μl aliquot of 0.25 N NaOH for light signal detection (measured in relative light units). The cutoff value was defined as three times the mean for the negative control.

Commercial immunoassay procedure. The total HCV antibody titer was determined with commercial anti-HCV ELISA kits (Abbott 2.0 ELISA and Ortho 3.0 ELISA). Positive anti-HCV reactivity was confirmed with the Chiron 3.0G RIBA (recombinant immunoblot assay) kit. All experiments were performed according to the manufacturer's specifications.

Nucleotide sequence accession number. The MEFA-6 sequence has been deposited in GenBank under the accession number BankIt 253337 (AF127762).

RESULTS

The major epitopes of the HCV type 1 (HCV-1) polyprotein were determined with 3,000 octapeptides in a pin solid-phase pepscan study (5). The major HCV-1 epitope regions (the core, E1, E2, NS3, NS4, and NS5 regions) and genotype 1-, 2-, and 3-specific epitopes of the NS4 region were chosen for peptide synthesis. A multipeptide ELISA containing each of these epitope-specific peptides was developed to evaluate samples from paid blood donors, patients with posttransfusion hepatitis C, and IVDAs. The most reactive and prominant epitopes (i.e., those that were able to detect antibodies over the greatest period throughout the course of infection) were chosen in the design of MEFA-6. More than one copy of immunodominant epitopes as well as epitope sequences from strains with different genotypes were included in the design of MEFA-6 in order to enhance its overall sensitivity and its ability to detect genotype-specific antibody.

TABLE 1. Prevalence of antibodies to HCV recombinant antigens and peptide epitopes in U.S. patient groups at high risk of HCV infection

HCV antigen (amino acid positions)	% reactive samples in the following groups:		
	Paid blood donors[a]	Patients with posttransfusion** HCV infection[b]	IVDAs[c]
Core			
Recombinant polyprotein c22 (2–120)	18	92	100
Peptide epitope (10–53)	18	92	100
E1			
Recombinant polyprotein e1 (192–380)	12	58	44
Peptide epitope (303–320)	9	53	44
E2			
Recombinant polyprotein e2 (404–662)	9	55	33
Peptide epitope (405–444)	8	53	31
NS4			
Recombinant polyprotein C100c (1569–1930)	17	71	69
Epitope (1901–1940)	13	58	66
NS5			
Recombinant polyprotein NS5 (2054–2995)	17	74	82
Peptide epitope (2278–2313)	10	53	69
MEFA-6 chimeric polypeptide	20	95	100

[a] Samples collected in 1989 from paid blood donors (prior to the earliest HCV antibody screening assays) ($n = 58$).
[b] Samples from patients with confirmed HCV infection posttransfusion ($n = 38$).
[c] Samples from IVDAs with confirmed HCV infection ($n = 39$).

Figure 1A illustrates the genetic organization of HCV and the relative locations of the major peptide antigens arranged in the MEFA-6 polypeptide. The MEFA-6 epitope sequence, the HCV genotype, and the corresponding HCV amino acid sequence are specified in Fig. 1B. The genotype 1a sequence was deduced from HCV-1 (8), the genotype 2b sequence was deduced from clone A (16), and the genotype 3 sequence was deduced from T0040 (15).

The prevalence of antibodies to HCV recombinant antigens, peptides, and MEFA-6 chimeric polypeptides in U.S. patient groups at high risk of infection (comprising paid donors, patients with posttransfusion HCV infection, and IVDAs) was evaluated by ELISA (Table 1). MEFA-6 demonstrates equivalent or greater reactivity with these groups of samples than the recombinant antigens and peptides alone.

The epitopes of MEFA-6 were characterized with HCV-specific monoclonal and polyclonal antibodies (Table 2). Each of the epitope-specific antibodies tested reacted with MEFA-6, indicating that all of the major epitopes present on MEFA-6 are exposed and accessible for detection.

In order to compare the combined epitope reactivities of MEFA-6 to those of the individual recombinant antigens, core (C22c), NS3 (C33c), and NS4 (C100c), we set up an inhibition

TABLE 2. ELISA analysis of MEFA-6 for exposed HCV epitopes

Antibody specificity	HCV sequence recognized by antibody	MEFA-6 reactivity (S/CO[a])
Monoclonal antibodies		
Anti-core (c22c)	aa[b] 10–50	6.71
Anti-NS3 (c33c)	Linear epitope of c33c	6.93
Anti-NS4 (C100c)	aa 1901–1940	8.56
Anti-NS4 (5-1-1, type 1)	aa 1689–1735	6.68
Anti-NS5	aa 2278–2313	6.43
Polyclonal antibodies		
Anti-E1	aa 192–380	9.72
Anti-E2	aa 404–662	3.91

[a] S/CO, signal OD/cutoff OD ratio (cutoff OD = 0.45).
[b] aa, amino acids.

assay using the individual antigens coated on ELISA plates. MEFA-6 was able to inhibit 71 to 97% of the immunoreactivity of these individual recombinant antigens when they were tested with reactive samples from three patients with chronic HCV infection (Table 3).

The genotype-specific epitopes present in MEFA-6 were tested against the genotype-specific peptides of the NS4 (5-1-1) region by an inhibition assay. MEFA-6 was added to samples of genotypes 1a, 1b, 2a, 2b, and 3. This mixture was subsequently added to the genotype-specific peptides coated on ELISA plates. The results are given as percent inhibition (Table 4). The MEFA-6 antigen inhibited 83 to 96% of the binding of the genotype-specific antibodies to the genotype-specific peptides. As demonstrated in Table 4, we observed cross inhibition between samples containing genotype 1a and those containing genotype 1b.

We then compared the sensitivity and specificity of the assay with MEFA-6 with those of an assay with HCV chimeric polyprotein C25. C25 is a fusion protein consisting of the NS4 (C100), NS3 (C33c), and core (C22) regions. This recombinant antigen contains a greater number of HCV epitopes than the commercial 2.0G ELISA kits. To make an unbiased comparison, both antigens were highly purified (>95% pure by SDS-polyacrylamide gel electrophoresis analysis) by the same purification scheme. Because both antigens contain SOD fusion proteins, a universal conjugate reagent for detection (monoclonal anti-h-SOD labeled with dimethyl acridinium ester) was designed as our chemiluminescent tracer. We also used a universal solid phase consisting of paramagnetic particles coated with murine monoclonal anti-human IgG (Fc). Assay sensitivity was evaluated with dilutional sensitivity and seroconversion panels of samples from patients of known HCV infection status. The results demonstrated that MEFA-6 was able to detect HCV in these panels with a two- to fourfold greater dilutional sensitivity than the C25 antigen was (data not shown).

TABLE 3. Inhibition of HCV antibodies to HCV antigens by MEFA-6 chimeric polypeptide as determined by ELISA

Patient no.	% Inhibition of the following HCV antigens[a]:		
	rC22	rC33C	rC100
LL57366	89.9	97.2	93.2
LL57454	84.5	95.9	70.9
FF25946	89.9	92.8	96.1

[a] Percent inhibition = [(OD without MEFA-6 − OD with MEFA-6) ÷ OD without MEFA-6] × 100.

TABLE 4. Inhibition of HCV genotype-specific antibodies by MEFA-6 chimeric polypeptide as determined by ELISA

HCV serotype	% Inhibition of HCV by the following type-specific 5-1-1 peptides[a]:				
	1a	1b	2a	2b	3
1	84.9	0	0	0	0
1b	54.6	83.1	33.0	0	0
2	0	0	95.9	88.0	0
3	37.4	32.0	16.1	16.2	88.3

[a] Percent inhibition = [(OD without MEFA-6 − OD with MEFA-6) ÷ OD without MEFA-6] × 100.

Next we compared the sensitivity of the MEFA-6 CLIA with that of the commercially licensed 3.0G Ortho ELISA for the detection of seroconversion. At day 7, the earliest time of seroconversion detected by the Ortho assay, both assays showed equivalent sensitivities (Table 5).

The specificity of the MEFA-6 CLIA was compared with that of the Ortho HCV 3.0G ELISA using 65 HCV seropositive and 201 seronegative prescreened samples (prescreened by the Ortho 3.0G ELISA). No discrepancies were observed between the two assays (data not shown).

The MEFA-6 CLIA was tested for clinical sensitivity by using 303 anti-HCV reactive samples, and its results correlated 100% with those of the Ortho 3.0G ELISA for all reactive and randomly negative samples (data not shown).

DISCUSSION

The overall goal in the development of an immunoassay is to achieve the greatest possible sensitivity and specificity. To a large extent, these two variables are dependent upon both the accurate characterization of the most immunodominant epitopes and the design of the recombinant antigen. Current assays use relatively few recombinant polyproteins or synthetic peptides (6, 17). In these assays, sensitivity is dependent upon major epitopes bound to the solid support, which are capable of capturing virus-specific antibodies in the test sample. Due to the limited capacity of the solid phase to bind to peptides or proteins, an inherent limitation is always imposed on epitope availability, thereby limiting sensitivity. This limitation often results in poor dilutional sensitivity for the detection of viral antibody in diluted samples, poor sensitivity for the detection of seroconversion, and false-negative results (3).

In an attempt to decrease these limitations in terms of sensitivity, we constructed an MEFA (MEFA-6). This antigen con-

TABLE 5. Comparison of MEFA-6 CLIA, Ortho 3.0G ELISA, Abbott 2.0G ELISA, and Chiron RIBA 3.0 for detection of anti-HCV antibodies in a pedigree seroconversion panel

Patient bleed day	S/CO[a]				Reactivity by Chiron RIBA 3.0
	MEFA-6 CLIA	C25 CLIA	Ortho 3.0G ELISA	Abbott 2.0G ELISA	
1	0.28	0.23	0.02	0.2	Nonreactive
2	0.94	0.26	0.02	0.2	Nonreactive
7	**1.17**[b]	0.24	**1.45**	0.4	Intermediate
9	1.27	0.93	2.74	0.8	Intermediate
14	3.54	**3.85**	4.11	**3.9**	Intermediate
16	6.38	5.25	4.11	5.0	Intermediate
20	10.9	8.31	4.11	5.3	**Reactive**

[a] S/CO, signal OD/cutoff OD ratio.
[b] Boldface indicates reactive bleed date.

tains two copies each of the major core and NS5 epitopes, a single copy of the major E1 and E2 linear epitopes, and a single copy of genotype 1-, 2-, and 3-specific epitopes derived from NS4 (5-1-1). Because the helicase region is very immunogenic, we included most of the NS3 region, which consists of the C-terminal viral protease and most of the viral helicase. The nucleotide sequence of the fusion protein h-SOD was fused at the N terminus of the MEFA-6 gene cassette (Fig. 1A).

Theoretically, by increasing the number of copies of the major epitopes present in a recombinant antigen and decreasing the number of nonessential amino acids, a higher percentage of exposed epitopes should become available. Furthermore, the presence of repeating epitope sequences should decrease the steric hindrance caused by binding of the antigen to the solid phase because the higher percentage of a given epitope present would mean that there is less chance of steric hindrance of that epitope. This hypothesis was supported by the comparison of MEFA-6 and C25 tested with dilutional sensitivity and seroconversion panels (Table 5).

Another possible advantage to the MEFA design lies in the antigen purification scheme. Most yeast and *Escherichia coli* intracellular proteins are very insoluble. Because the MEFA-6 protein primarily contains hydrophilic epitopes, it should be more soluble than conventional recombinant polypeptides and much easier to purify.

When deciding the organization of MEFAs in the gene cassette, it is necessary to ensure that the antigen arrangement does not create an artificial epitope at any of the individual antigen junctions. Each MEFA-6 junction was tested for the presence of artificial epitopes. ELISA plates were coated with synthetic peptides comprising each junction sequence, and more than 10,000 randomly negative samples were tested. No false-positive reactions were observed at these junction sequences (data not shown).

The use of a universal solid phase (anti-human IgG Fc) and a universal light reagent (dimethyl acridinium ester-labeled anti-SOD monoclonal antibody) greatly simplifies the manufacturing process. The combination of the high binding capacity of the PMPs with the high affinity of anti-human IgG (Fc) monoclonal antibody covalently conjugated to the solid-phase particles greatly enhanced our assay and allows the solid phase to be used in other assays.

One limitation of the MEFA-6 is its inability to bind to conformational antibodies at its E2 region, due to the denaturing conditions to which it is exposed during the purification protocol. Further improvements to MEFA-6 might involve the addition of a computer-generated E2 consensus sequence for genotypes 1 and 2 and the addition of genotype 4-, 5-, and 6-coding sequences from the NS4 region of the HCV genome.

ACKNOWLEDGMENTS

We are grateful to Steve Tirrell, Wanda Zeigler, and Nick Masiello for providing the PMP and dimethyl acridinium ester research reagents. We also thank Myron Tong, Harvey Alter, and Johnson Y. N. Lau for providing sera. Last, but not least, we thank Peter Anderson and Eric Dahlstrom for assistance in manuscript preparation and the drawing of diagrams for publication.

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