An Epitope Chimeric Antigen for the Hepatitis C Virus Serological Screening Test

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The epitope chimeric antigen, CepCM, composed of the 9 selected major epitope regions (two in NS3, two each in the NS4 of two genotypes, two each in the core of two genotype, and one in the core in hepatitis C virus (HCV) polypeptide), was expressed as a fusion protein of the trpE peptide in *E. coli*. An ELISA test using this antigen produced the same judgements with most of the panel sera as a second generation HCV screening kit. Though discrepancies were found in twelve samples (5% of the samples), further analysis revealed that eleven samples were indeterminate sera as judged by an immunoblot test. The reactivity found in several seroconversion series sera suggested that CepCM has superior reactivity to HCV infected sera than some second generation kits. These data indicated that an epitope chimeric antigen with a man-made sequence will be a excellent tool for a diagnostic test kit.

Key words epitope chimeric antigen; hepatitis C virus; man-made antigen; serological screening

Hepatitis C virus (HCV) was first identified as cDNA clones encoding non-structural proteins.¹⁾ The development of a first generation anti-HCV testing kit using a recombinant antigen, c100-3, revealed that 70% of transmitted non-A, non-B (NANB) hepatitis had been caused by HCV infection.^{2,3)} The serological HCV test kit has drastically prevented the post-transfusion transmission of HCV.

To increase the sensitivity and specificity of detection for HCV infection, improvement of test kits has been performed. The so-called second generation testing kit, with improved specificity and sensitivity, proved that the anti-HCV antibody was detected in over 90% of patients.^{4,5)} A so-called third generation testing kits, recently introduced, has been reported to have improved detection of the anti-HCV antibody over the second generation kits.^{6,7)}

These improvements to anti-HCV testing kits have been accompanied by an increased number of recombinant antigens: the second generation kit uses c33c (NS3) antigen and c22-3 (core) antigen in addition to c100-3 (first generation),⁵⁾ and NS5 antigen was introduced into the third generation kit.⁸⁾ However improved sensitivity was due not only to the addition of NS5 antigen; many investigators have reported that the improvement of the third generation kit relied mainly on improved sensitivity in the NS3 antigen which had been used in a previous version.^{7,8)}

The increased number of recombinant antigens poses several problems: the increased number of antigens would increase the cost of the kit, resulting in an expensive testing kit; also, a false positive would be magnified, because the expanded amino acid sequence would increase the possibility of the appearance of cross reacting regions.

On the other hand, sequence analysis of the HCV genome revealed that there are at least six genotypes,⁹⁾ and epitope analysis of the HCV polypeptide revealed that there exist epitope regions which react in a genotype-specific manner.¹⁰⁻¹³⁾ Several of these genotype specific

epitopes were found in the NS4 region¹⁴⁾; most second generation kits use the NS4 region of genotype 1. Most human sera infected with genotype 2 HCV, which accounts for about 30% of the HCV infected people in Japan,¹⁵⁾ reacted weakly or not at all with 5-1-1 epitopes.¹⁶⁾ These data suggested that a test kit for HCV should not react in a genotype specific manner.

To resolve these problems, we constructed an epitope chimeric antigen that has a man-made sequence compromised of the necessary epitopes selected from the HCV polypeptide. It also contains epitopes of the two major genotypes found in Japan which do not react in a genotype specific manner.

In this report, we described the construction and performance of this epitope chimeric antigen, named CepCM, regarding anti-HCV detection.

MATERIALS AND METHODS

Sera, Monoclonal Antibodies, and Recombinant Antigens An in-house panel comprised 248 sera from blood donors. In-house indeterminate panels were composed of purchased serum samples. The HCV seroconversion panel, comprising a series of sera from a patient infected with HCV, were purchased from Serologicals (U.S.A.) and Boston Biomedical Incorporation (U.S.A.). We used 49 serum samples as a healthy control.

The monoclonal antibody (mAb) to the HCV core polypeptide, named 5E3, was described previously.¹⁷⁾ mAb to the HCV NS3 polypeptide, 1H10, was generated from mice immunized with purified recombinant NS3 polypeptide. (c7)¹⁸⁾

Human IgG fraction (hIgG) was purified from a patient's serum by protein A affinity chromatography (Pharmacia). hIgG was then labeled with biotin using a biotinylation reagent according to the manufacturer's recommended procedure (Amersham).

Recombinant antigens, c7, c11 and C14-1, C14-2 were purified as described previously.^{14,18)}

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Fig. 1. (A) Schematic Structure of the HCV Genome and Locations of Epitope Peptides. (B) The Structures of the Functional Domains of CepCM

(A) The top figure represents the organization of HCV genomic RNA (genotype 1b), and the junction of each structural and non-structural protein is indicated as a corresponding amino acid number. Boxes shown under the HCV genomic structure represent epitope peptides. The corresponding amino acid number of epitope peptides is indicated above the boxes.

(B) Each DNA fragment encoding epitope peptide was ligated at the restriction sites shown in the figure, then assembled into functional domains named NS3EP, Gr1EP, Gr2EP and CoreEP. The functional domains were then ligated into CepCM using the restriction sites indicated in the figure (middle). The corresponding amino acid numbers are shown at the bottom of the figure.

Construction of an Epitope Chimeric Antigen (CepCM)

All primers listed in Table 1 were synthesized using an automated DNA synthesizer (Applied Biosystems: 394A). Taq DNA polymerase (AmpliTaq) was purchased from Perkin-Elmer, Japan. Restriction enzymes and modification enzymes for DNA manipulation were purchased from New England Biolabs (U.S.A.), Takara Shuzo, and Toyobo (Japan). An *Escherichia coli* (*E. coli*) strain, XL1-Blue, was purchased from Stratagene (U.S.A.).

HCV cDNA clones were isolated from HCV infected human sera by RT-PCR (reverse transcription PCR) using specific primers for HCV (unpublished results). The genotypes of isolated cDNAs were identified by comparison with published DNA sequences. The nomenclature of genotypes of HCV was in accordance with the proposed one.¹⁹⁾ The fragments encoding NS3EP, NS4 regions in Gr1EP, the NS4 region in Gr2EP and Core-EP were generated by PCR using the primers listed in Table 1 using HCV cDNA clones as the target DNA. Each amplified fragment was cloned in pGEM-T (Promega, U.S.A.) to transform XL1-Blue cells and the nucleotide sequence was determined by an automate-sequencer (Applied Biosystems: 373A). The DNA oligomers for the genotype specific core regions in Gr1EP and Gr2EP were assembled into dsDNA by Taq DNA polymerase, and were cloned into the vector, followed by the sequence determination described above. The restriction fragments

from the cloned segments were ligated into other vectors to construct a block such as Gr1EP, as shown in Fig. 1-B. Except for Gr1EP, each combined block has a unique restriction enzyme site at the termini shown in Fig. 1-B.

Gr1EP was first constructed for expression alone; therefore, it has an EcoRI and a BamHI site at the termini (Table 1). The gene block was converted to contain the restriction sites shown in Fig. 1-B by PCR.

Purification of CepCM Antigen The assembled gene was inserted into an *E. coli* expression vector, pAT-trp,¹⁸⁾ and expressed as a fusion-protein with trpE in XL1-Blue cells. The expressed antigen was extracted from insoluble materials of sonicated cells by a buffer containing 6M urea. The antigen was purified by HiLoad Sp Sepharose FF ion-exchanged chromatography, and by HiLoad Superdex 200 pg (Pharmacia Japan) gel-permeation chromatography.

The SDS poly-acrylamide gel electrophoresis (SDS-PAGE) was performed as described previously using the precast-gradient (10–20%) gel (Daichi Pure-Chemical, Japan). Proteins were stained with Coomassie brilliant blue R250 (CBB).

SDS-PAGE and Western Blotting SDS-PAGE was performed using a precast-gradient (15–25%) gel. Proteins were then transferred to Immobilon-PVDF membranes (Millipore) using a semi-dry blotter. The blots were treated with TTBS [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20] containing 4% Block Ace

Table 1. The Regions of Epitopes in CepCM and Primer Sequences for Cloning

Domain	Region (genotype)	Primer name sequence	
NS3EP	1238—1313	NS3EP1F	CCAGAATTCACTAAAGTGCCGGCTGCA
	HCV1b	NS3EP1R	CTCTCCGGACCCTATGATGTCATAAGCGCC
	1363—1460	NS3EP2F	GGGTCCGGAGAGGAGGTGGCCCTGTCT
	HCV1b	NS3EP2R	GAAGCTTACCAGTCCAGATCCCTGGGTGACGCATCTGTTACA
Gr1EP	1712—1750	Gr1EP1F	GCGAATTGCGCTCACACCTCCCTTACATC
	HCV1b	Gr1EP1R	GGGTACCTACCACGGGAGCAGCAGCTC
	66—80	CoreGR1-F	AGGTACCCCTAAAGCTCGTTCGTCGGAAGGTCGTGCT
	HCV1b	CoreGR1-R	TCCCGGGTTGAGCCCAAGCACGACCTTCCGGACG
	1670—1705	Gr1EP2F	ACCCGGGAGTGTGGTCATTGTGGGTAGG
	HCV1b	Gr1EP2R	GAGGATCCTTATCAATCGAACTCCCGGTAGAGGACTTC
		Gr1EP-F	GTAAGCTTCGCTCACACCTCCCTTACATC
		Gr1EP-R	GCCTCGAGAAAGAACTCCCGGTAGAGGAC
Gr2EP	1716—1750	Gr2EP1F	GACTCGAGGCCTCTAGAGCGGCTCTCATT
	HCV2a	Gr2EP1R	GCTATCGATTTTTATGTCTTGGGCTG
	66—80	CoreGr2S	CGGTCGACGGTACCCTGATTATTCCGAAAGATCGTCGCAGCACCGGTA
	HCV2a	CoreGr2AS	GCTATCGATGAGGAAGCCCGGTTTACCCAGCTTTTACCGGTGCTGCG
	1690—1713	Gr2EP2F	AAAATCGATAGCTTGCATATCAACCAG
	HCV2a	Gr2EP2R	GACGAGCTCCATCTCATCAAAAGCCTC
Core	1—43	Core-F	ATGGAGCTCGCCATGGGCATGGGCACGAAT
	HCV1b	Core-R	TATGGATCCTTATCTGGGGCCCCTGCGCGGCAA

(Yuki-Jirushi, Japan) for blocking. For the binding competition assay, 1 μ g of biotin hIgG was incubated with 50 μ g of the purified recombinant antigen (C14-1 or c7) in a serum dilution buffer (TTBS containing 1% Block Ace).^{14,18} The blots were then incubated for 2 h in serum dilution buffer containing purified antibodies, biotin hIgG, or biotin hIgG which had been incubated previously with the antigen at a concentration of 1 μ g/ml. After washing twice with TTBS, the blots were incubated in a serum dilution buffer containing either goat antibodies to mouse immunoglobulin conjugated with alkaline phosphatase (Bio-Rad, U.S.A.) for detecting mAb, or streptavidin conjugated with alkaline phosphatase for detecting biotin hIgG (Boehringer Mannheim, Germany). Enzymatic activity on the blot was detected using Western Blue reagent (Promega, U.S.A.).

Enzyme Immunological Assay The micro-well plates (Maxi-sorb, Nunc) were coated with CepCM antigen at 2.5 μ g/ml in phosphate buffer (pH 7.5) containing 8 M urea. The antigen-coated wells were blocked with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.5% casein. The human IgG in eleven-fold diluted sera, which was bound to the antigen, was detected as described previously¹⁸ using an automated micro-well plate reader (ELISA Auto: International Regent Corporation, Japan). Samples were assayed in duplicate.

Assays using a commercially available anti-HCV test kit, EIA2.0 (Dinabott, Japan) and Imucheck HCV "Kokusai" (International Reagent Corporation, Japan) were performed according to the manufacturer's instruction. The results were expressed as the ratio of absorbance value of the sample with the absorbance value of the cut-off proposed by the manufacturers. Sera with a ratio of over 1.0 were considered as positive in the test kits. For the immunoblot assay [RIBA-2 (Ortho Diagnostic, Japan)], the response to the antigens was judged by comparing intensity to the internal control on the same blot.

RESULTS

We selected 9 epitope regions from the HCV polypeptide according to results reported previously (cited later) and our epitope analysis (unpublished data), as described in the following sections.

The reports of the evaluation of second and third-generation confirmation test kits, such as the RIBA (recombinant immunoblot assay)-2 and RIBA-3 test kits, revealed that there are HCV RNA positive serum in indeterminate samples with a single positive core, NS3 or NS4 antigen.^{6,7,20} These results showed that these epitope regions are important for the detection of HCV.

The improved third generation ELISA and RIBA kits were reported to contain NS5 peptide antigen and also serum with a single-positive to NS5 antigen exist. However, these serum were rare in donor and patient serum and negative to the HCV RNA test.^{7, 21} Therefore, we decided to omit the NS5 region in our construct. The correctly folded and glycosylated E2/NS1 antigen, which would be a component of the envelope of HCV, is a highly antigenetic polypeptide; more than 90% of chronic hepatitis C patients have antibodies to this antigen.^{21,23} But we have no evidence of HCV RNA with a single-positive to E2/NS1 antigen, and recombinants polypeptide expressed in *E. coli* did not show strong reactivity. So, we did not use this region in our construct, either.

We further selected an epitope peptide in these selected regions. An epitope peptide corresponding to amino acids 1238 to 1313 of HCV1b polypeptide has a strong linear epitope, and an epitope peptide of 1363—1460 of HCV1b has a strong conformational epitope.^{24,25} In the c-HC (chronic hepatitis C) patients' sera which reacted to an NS3 antigen (c7 antigen),¹⁸ 60% and 100% of them were positive to these epitope peptides, respectively (data not shown).

Since genotype-specific reactivity was reported in NS4 antigenetic peptides,¹¹⁻¹³ we selected an epitope peptide

from HCV1b and HCV2a, which are shown to be major genotypes found in Japan.¹⁵⁾ The epitope peptides shown in Fig. 1 were selected in accordance with the previous data.¹¹⁾

We selected the major antigenic peptide of 1 to 43²⁶⁻²⁸⁾ of HCV 1b, and an antigenic region-reacting genotype specific manner¹⁰⁾ was selected from the core region (66 to 80).

The cDNA fragment encoding each epitope fragment was generated from HCV 1b, 2a or 2b cDNA clones by a polymerase chain reaction to provide a restriction site on each side (Table 1, Fig. 1). The DNA fragment for the genotype-specific peptide of the core region was constructed using chemically synthetic DNA oligomers (Table 1).

The most important point of designing an epitope chimeric antigen is not to offer a new epitope introducing non-specific binding. In order to avoid generating such an epitope, we inserted a hydrophobic amino acid such as leucine, isoleucine and valine or poly-glycine at the junction of each epitope peptide. The antigenicity of the junctions was estimated on the basis of the antigenic index calculated from their amino-acid sequences.

The epitope peptides were assembled into functional units; that is, the epitopes of NS3 were located at the amino-terminal half of the chimeric antigen, and each genotype-specific epitope was assembled into a segment, Gr1EP or Gr2EP. Because the core peptide was known to be a target site of some *E. coli* metal-protease (our unpublished observation), it was positioned at the c-termini.

The assembled epitope chimeric antigen, named CepCM, was expressed in *E. coli* as a fusion protein with trpE peptide.¹⁸⁾ It had a 421 amino acid length with a molecular mass of 45.2 kDa. The chimeric antigen was recovered from the inclusion body by extraction with a buffer containing 6 M urea, then purified to homogeneity by gel-permeation chromatography after ion-exchange chromatography (Fig. 2).

ELISA was performed with a micro-well plate coated with the purified CepCM antigen. The cut-off value was set at A492/A690 value (the value of the absorbance at 492 nm was subtracted by that at 690 nm) of 0.300 according to the distribution of A492/A690 values of 49 healthy donor sera. The reactivity of CepCM was examined with commercially available panel sera and in-house panels (248 specimens). The results are summarized in Table 2 with comparison to the results determined by a second generation screening kit (Imucheck HCV "Kokusai") that uses two recombinant HCV antigens (c7 and c11)¹⁸⁾ for detecting anti-HCV antibodies. In 95.1% of the sera, two ELISA systems gave the same judgement results.

The twelve specimens whose results were discrepant between the two ELISA systems (CepCM and Imucheck) were further analyzed with other systems, and the results obtained are presented in Table 3. All seven samples with CepCM positive/Imucheck negative results were indeterminate with the RIBA-2 test. Two specimens (no. 468 and 10) were RIBA-2 negative. However, three specimens (no. 640, 356, and 79) were RIBA-2 indeterminate

Fig. 2. Purification of CepCM Antigen

The upper figure shows the HiLoad Sp Sepharose ion-exchange chromatography of the extract by a buffer containing 6 M urea. The extracts were applied to a HiLoad Sp Sepharose FF column (2.6 cm × 10 cm) that was previously equilibrated with buffer A [50 mM Bicine NaOH (pH 8.5), 6 M urea, 20 mM EDTA, 5 mM EDTA], and eluted by the gradient of NaCl in buffer A at a flow rate of 5 ml/min. Fractions were collected every 2 min. Shadowed peak fractions were combined, and proteins were concentrated by ultra-filtration using a Centricon cell (Amicon). The lower figure shows the HiLoad Superdex 200pg column (2.6 cm × 60 cm) chromatography of concentrated fractions. The flow rate was 2 ml/min, and fractions were collected (2 min/fractions). The total cell extract (lane 2), soluble materials (lane 3), urea extract of insoluble materials of sonicated cells (lane 4), and purified CepCM (lane 5) were subjected to SDS-PAGE and stained with CBB (inlet). In lane 1, a molecular weight marker containing 94.5, 67, 45, 30, 20.1, and 14.4 kD marker proteins, was applied.

Table 2. Sensitivity of CepCM Compared with a Second Generation Kit (Imucheck HCV "Kokusai")

		CepCM ELISA test	
		Positive result	Negative result
Imucheck	Passive result	162	5
	Negative result	7	74

Total number of samples is 248.

or positive.

Table 4 shows the results of CepCM antigen ELISA in specimens with single positive results using a RIBA-2 test kit. CepCM antigen gave positive results to all the c33c positive sera. Two c22-3 positive sera were also positive in CepCM ELISA. These data indicated that major epitopes in the core and NS3 antigens were functional on the chimeric antigen.

The functions of epitopes in CepCM were also analyzed by Western blotting using mAbs or human serum IgG (Fig. 3). Both mAbs, 5E3 and 1H10, whose epitopes were identified in the regions corresponding to amino acid position (a.a.) 21—40 of the HCV core polypeptide¹⁷⁾ and a.a. 1230—1250 of the HCV NS3 region (Kashiwa-

kuma, unpublished result), respectively, bound CepCM. A hIgG of a patient clearly bound CepCM, c7 and C14-1 antigen (Fig. 3). The addition of C14-1 antigen to hIgG inhibited binding to C14-1, but not to c7 or the CepCM antigen, suggesting that some NS3 epitopes in CepCM

antigen were functional. Also, binding competition by the addition of c7 antigen indicated that some NS4 epitopes were functional for the binding of human anti-HCV antibodies.

The results of commercially available seroconversion panels, which were composed of a series of sera of HCV infected patients, are shown in Table 5. The PVH901 series became positive with anti-c33c antigen with the RIBA-2 kit. CepCM gave a positive result at the same point as the other 2nd generation kits. The PVH903 series were positive with the c22-3 antigen. CepCM gave positive results faster than the other kits. With the no. 4810 series, serum was converted to positive in CepCM ELISA at 20/11/92. Courouc reported the result with third generation testing kits, in the same series of the seroconversion panel, that only EIA3.0 (Ortho diagnostics) gave a positive result at 17/11/92, and other third generation kits gave positive results at 20/11/92 or later.⁶⁾

DISCUSSION

The ELISA system using an epitope chimeric antigen, CepCM, showed better performance in several specimens than those with a second-generation HCV screening test. In the investigation of discrepant results, positive results were concentrated in the c22-3 positive sera, indicating that the epitopes of the core antigen in CepCM would show sufficient function for screening.

Recently, the importance of the NS3 antigen in serological screening has been emphasized by many researchers.^{7,29)} The results in single positive sera and several seroconversion series indicated that the major epitope in NS3 would be functionally displayed on the CepCM antigen. Western blot experiments also suggested that several epitopes in the NS3 region and the epitopes in the NS4 region of genotype 1 functioned in our construct. However, we can not conclude whether the other epitopes in the chimeric antigen would be functional.

The discrepant result found in specimen no. 79, that the

Table 3. Discrepant Results between CepCM ELISA and Second Generation Kits

	CepCM	Imucheck	EIA2.0	RIBA-2			
	A492/A690	COI ^{a)}	COI	5-1-1	c100-3	c33c	c22-3
311	0.358	0.40	0.70	—	—	—	+/-
286	0.404	0.46	0.98	—	—	—	+/-
492	0.517	0.70	1.12	—	—	—	1
986	0.325	0.66	0.55	—	—	—	+/-
973	0.310	0.78	1.64	—	—	—	1
174	0.459	0.40	0.74	—	—	—	+/-
593	0.512	0.86	0.94	—	—	—	+/-
468	0.131	1.20	0.24	—	—	—	—
640	0.284	1.09	1.09	—	—	+/-	+/-
356	0.049	1.34	0.23	—	—	—	+/-
10	0.042	1.21	NT	—	—	—	—
79	0.169	2.20	NT	3	4	3	—

a) COI means cut-off-index value. Cut-off-index values were calculated according to manufactures' instructions (Materials and Methods). A492/A690: The value of absorbance at 492 nm that was subtracted by that at 690 nm. Positive judgements are indicated by an underline. NT means not tested.

Table 4. In-House RIBA-2 Single-Positive Panel

	Imucheck	CepCM	RIBA-2			
	COI	A492/A690	5-1-1	c100-3	c33c	c22-3
004	9.28	1.579	+/-	—	2	—
68	4.71	0.915	—	—	3	1
131	2.01	0.647	—	—	2	—
277	>6.64	1.026	+/-	—	4	—
280	1.84	0.319	—	—	2	—
135	3.62	2.591	+/-	+/-	+/-	2
285	>6.64	>3.000	—	—	+/-	4

Details are shown in Table 3.

Fig. 3. Western Blot Analysis of Recombinant HCV Antigens

The recombinant antigens, CepCM (lane 1), c11 (lane 2), c7 (lane 3), C14-1 (lane 4) and C14-2 (lane 5), were applied to polyacrylamide gels. The positions of prestained molecular weight markers were marked at the left side of the figure. Antibodies which reacted to antigens on each blot were indicated at the bottom of the figures.

Table 5. Results of Seroconversion Series

Day/Month/ Year	Imucheck	CepCM	RIBA-2			
	COI	A492/A690	5-1-1	c100-3	c33c	c22-3
PHV901						
23/ 9/93	0.014	0.08	—	—	—	—
27/11/93	0.001	0.05	—	—	—	—
29/12/93	4.39	0.512	+/-	+/-	1	—
31/12/93	4.58	0.515	+/-	+/-	1	—
5/ 1/94	4.84	0.625	+/-	+/-	1	—
7/ 1/94	5.24	0.632	+/-	+/-	1	—
1/ 2/94	8.61	2.105	3	1	3	—
9/ 2/94	8.70	2.242	3	1	2	—
1/ 3/94	>9.35	>3.000	4	3	3	—
8/ 3/94	>9.35	>3.000	4	4	2	—
14/ 4/94	>9.35	>3.000	4	4	4	—
PHV903						
7/ 2/92	0.05	0.065	—	—	—	—
12/ 2/92	0.24	0.235	—	—	—	+/-
14/ 2/92	0.50	0.417	—	—	—	+/-
19/ 2/92	1.10	0.733	—	—	+/-	1
21/ 2/92	1.45	0.733	—	—	+/-	1
26/ 2/92	3.83	1.756	—	—	2	2
28/ 2/92	5.61	2.602	—	—	2	2
4/ 3/92	6.48	2.746	—	—	2	2
4810						
20/10/92	0.04	0.015	—	—	—	—
23/10/92	0.10	0.013	NT	—	—	—
27/10/92	0.12	0.007	NT	—	—	—
30/10/92	0.11	0.007	—	—	—	—
17/11/92	0.21	0.057	—	—	—	—
20/11/92	0.76	0.412	—	—	+/-	+/-
8/12/92	3.82	1.777	NT	—	—	—
17/12/92	3.36	1.645	NT	—	—	—
21/12/92	5.64	2.742	NT	—	—	—
28/12/92	4.76	>3.000	2	2	3	3
31/12/92	4.62	>3.000	NT	—	—	—
4/ 1/93	6.44	>3.000	NT	—	—	—
7/ 1/93	7.19	>3.000	4	4	3	3

Details are shown in Table 3.

CepCM ELISA test alone gave a negative result, illustrated that sensitivity in the NS3 or NS4 epitopes of CepCM should be improved for the screening test. The epitopes in the NS3 and NS4 region have been shown to react in genotype-specific manner.^{11,13,25} In our construct, we used the NS3 region of genotype 1b, and the NS4 regions of 1b and 2a. We therefore need to determine another epitope with more general reactivity.

Khudyakov recently reported an epitope chimeric antigen combining linear epitopes, which was selected by epitope mapping using a synthetic peptide, in hepatitis E virus.³⁰ The concept of their antigen and ours was nearly identical. Nevertheless, the point of difference is that the major epitope in the NS3 antigen used in CepCM is a conformational epitope, which needs an amino-acid length of about a hundred to display sufficient function.^{23,25} Therefore, CepCM will not be constructed according to the strategy of a mosaic protein composed of epitope oligo-peptides that was identified using synthetic oligopeptides as a PEPSCAN method,³¹ proposed by Khunyakov.

Although fusion proteins comprised of several antigens were also reported,^{32,33} our construct has several advantages over them. One is that CepCM is more compact than those reported; the second is that CepCM has more

antigenetic regions than the previous ones. The compactness will lead us to construct a novel chimeric antigen for the detection of multiple virus infection, because the chimeric antigen for the HEV and HB virus were also reported.³⁴

We are now trying to shorten CepCM using mimotope technology. Short oligo-peptides expressed as a fusion protein with a surface protein of filamentous bacteriophages have been able to efficiently mimic a conformational epitope with a large amino-acid length.³⁵ The mimotope for NS3 antigen has been isolated from a peptide library expressing random 9 mer peptides (our unpublished data). The combination of a mimotope with short linear epitope peptides will be a new strategy to reduce the molecular weight of an epitope chimeric antigen.

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