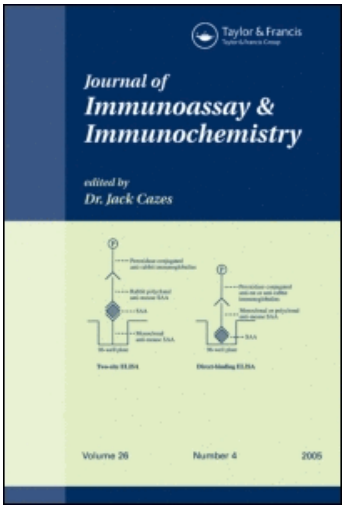

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A Novel Semi-Automated Paramagnetic Microparticle Based Enzyme Immunoassay for Hepatitis C Virus: Its Application to Serologic Testing

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A NOVEL SEMI-AUTOMATED PARAMAGNETIC MICROPARTICLE BASED ENZYME IMMUNOASSAY FOR HEPATITIS C VIRUS: ITS APPLICATION TO SEROLOGIC TESTING

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ABSTRACT

A new rapid serologic enzyme immunoassay for antibodies to hepatitis C virus (HCV) is described. The assay combines synthetic peptide and recombinant antigens representing putative structural and non structural HCV gene products with paramagnetic microparticle assay (MP assay) technology. Assay readout is based upon an enzymatically generated fluorescent product which is quantified with a novel semi-automated washer/reader instrument system. Assay sensitivity and specificity was determined to be greater than the first generation HCV C-100 EIA using a non-A, non-B hepatitis disease panel, an HCV performance panel, an HCV seroconversion panel, dilutions of HCV reactive sera, and random volunteer blood donor specimens.

INTRODUCTION

Infection with hepatitis C virus (HCV) has been strongly linked with the blood borne disease of non-A, non-B hepatitis (NANBH) in humans and chimpanzees (1-4). A convincing serologic relationship between anti-HCV antibodies and infectivity has led to the implementation of testing blood donors for the presence of such antibodies (5,6). Current testing procedures are based on the detection of antibodies to a recombinant non-structural antigen (C-100) coated onto the solid phase utilized in standard enzyme immunoassay (EIA) formats (5,7). Although not generally available, second generation anti-HCV EIAs have been developed using multiple antigens representing putative structural and non-structural gene products (8-10). We report here the development of an anti-HCV EIA that utilizes both synthetic peptide and recombinant antigens in a novel paramagnetic microparticle fluorescent immunoassay (MP assay). Performance data demonstrate that this assay is significantly more sensitive and specific than the first generation anti-C-100 EIA.

MATERIALS AND METHODS

Reagents and Specimens.

Unless stated otherwise all reagents were obtained from Sigma Chemical Company (Saint Louis, MO) and

Baxter Scientific Products (McGaw Park, IL). A coded NANBH performance panel was provided by Dr. Harvey Alter (National Institutes of Health, Bethesda MD) and was tested under code. Random volunteer donor EDTA plasma specimens were obtained from the Milwaukee Blood Bank (Wisconsin) and were tested within 7 days of collection. A HCV performance panel was obtained from Boston Biomedica, Inc. (Boston, MA). Seroconversion specimens were obtained from a Japanese female, 23 years of age. The first serum sample tested was obtained from the patient 30 days after onset of disease.

Reference Test Procedures.

Anti-HCV C-100 reactivity was determined by EIA (Ortho Diagnostic Systems, Inc., Raritan, NJ). Ortho HCV recombinant immunoblot assay (RIBA2) testing was performed and interpreted according to the package insert. Reactivity with 2 or more of the 4 antigens was required for the specimen to be termed reactive, whereas, reactivity to a single antigen was considered "indeterminate."

HCV Antigens.

The HCV MP assay utilized synthetic peptides and recombinant antigens representing immunodominant sequences of HCV (5,11,12). Synthetic peptides

corresponding to immunoreactive domains of the putative HCV capsid (aa1-aa38) and NS4 (aa1693-aa1734, representing part of C-100) were synthesized on Milligen-Biosearch model 9050 and 9600 peptide synthesizers (Burlington, MA), respectively, using a 9-fluorenylmethoxycarbonyl (FMOC) amino protection scheme. In order to avoid proline mediated diketopiperazine formation during peptide synthesis (since the carboxy terminal amino acid at position #38 was proline), we incorporated leucine as an additional amino acid at the carboxy end of this peptide (13). Crude peptides were purified by semi-preparative HPLC using a Waters micro Bondapak C-18 column (7.8 x 300 mm; 125^{O}A, 10um). Peptides demonstrated greater than 90% purity. Unless otherwise stated, the terms capsid and NS4 refer to the specific synthetic peptides described above. The amino acid positions of these peptides correspond to the amino acid sequence for HCV described by Choo et al (14).

A clone of NS3 c33c was isolated from NANBH serum by use of reverse-transcription PCR (15). The c33c DNA was subcloned and expressed as a recombinant antigen using the pET-5a vector expression system in E. coli BL 21 (DE3) (16). After extraction of antigen from a cell pellet with lysozyme, sonication and Triton X-100, it was purified using a Mono Q 5/5 FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ). Fractions were

analyzed for absorbance at 280nm and immunoreactivity by dot blotting (17). Fractions containing c33c were pooled and purity was assessed to be approximately 90% using SDS PAGE with Coomassie Brilliant Blue R250 and silver staining of the gel (18,19).

Paramagnetic Microparticles

Paramagnetic microparticles (MP) were obtained from Baxter Diagnostics, Inc., Pandex (Mundelein IL). They consisted of a fluorescent polystyrene paramagnetic core and a polystyrene surface or a polystyrene surface modified with carboxyl groups. All particle preparations were characterized by a narrow size distribution (average diameter was 4.5 um).

Antigen Coating Onto Paramagnetic Microparticles.

Peptide (capsid and NS4) and recombinant (c33c) antigens were coated separately onto microparticles (2.5% w:v) by gently mixing the antigen with MP overnight at room temperature. The antigen coating conditions were: 400ug c33c/ml of acetate buffer (100mM, pH 5.0) or 125ug capsid peptide/ml of CAPS buffer (100mM, pH 11.0). MP were then washed extensively with PBS using centrifugation (5000 x g, 5 min). NS4 peptide was coated onto MP as reported elsewhere (20).

Antigen coated MP were either stored separately or mixed together to provide a working MP concentration of 0.025% (w:v) in phosphate buffer saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.4).

MP Assay Washer and Analyzer.

All reactions were carried out in 96-well, U-bottom microtitre-type assay plates that were designed specifically for MP assays. These were processed with two prototype automated modules controlled by an IBM Model 70 computer. One of the modules washed the particles and dispensed substrate, while the other made fluorescence readings. Each module was controlled by a custom Z-80 microprocessor computer board which drove stepper motors on the various mechanisms, regulated temperature and sensed inputs. The temperature of the modules was maintained at 42 +/- 0.5 deg C.

MP Assay Procedure.

Serum or plasma samples were tested using either individual antigens or combinations of antigens coated onto MP. Specimens were diluted 1:100 (dilution buffer: 15% newborn calf sera, 500 mM NaCl, 100 mM Tris-HCl, 0.3% Nonidet P-40; pH 7.4), and 50 ul was placed into each well of a black plastic microtitre plate. Twenty ul of MP (0.025% w:v) were added to

each well and allowed to incubate at 42°C for 30 minutes. Particles in each well were then washed 5 times with PBS containing 0.05% Tween-20 using a magnetic field to keep the particles at the bottom of each well during the washing steps. Fifty ul of goat anti-human Ig (H+L) beta-galactosidase conjugate (American Qualex, La Mirada, CA) diluted 1:800 in conjugate diluent (8% newborn calf sera, 50 mM Tris-HCl, 200 mM NaCl and 1 mM $MgCl_2$; pH 7.4) was then added to each well and incubated at 42°C for 15 minutes. The plate was washed as described above. Fifty ul of substrate (4-methyl umbelliferyl beta-galactoside, MUG: 0.5 mM MUG, 20 mM Tricine, 0.05% Tween-20; pH 8.5) was added to each well and product fluorescence was measured (365 nm excitation and 450 nm emission) at timed intervals (2 min and 14 min). Fluorescence values were converted into nM coumarin values using dilutions of coumarin as a standard curve. A kinetic value for substrate turnover (the amount of coumarin in nM generated over a 12 minute period) was determined. The dynamic range of kinetic values was 0 to 5,000 nM coumarin. A preliminary algorithm for cutoff calculation was established by testing random volunteer blood donor samples as well as panels of serial serum samples from patients who were monitored during seroconversion. The algorithm (nM coumarin of positive

calibrator x 0.75) resulted in a cutoff of approximately 200 nM coumarin.

Fluorescence in each well was additionally determined at 525 nm excitation and 580 nm emission. These values were converted to nM rhodamine and were a measure fluorescence contributed by the fluorescent dye located in the core of each MP.

RESULTS

Determination of Assay Parameters.

Multiple parameters were systematically tested during the development of the MP assay, resulting in the optimized assay conditions described in the Methods section. During development, each antigen was found to have different optimal solid phase coating conditions, consequently, the use of different peptides and recombinant antigens would mandate re-evaluation of the antigen coating procedures. The pET-5a expression system used for c33c antigen yielded highest positive signal and lowest negative signal. Performance of the pET 5a expressed c33c antigen was superior to that produced in other expression systems evaluated, namely, bacteriophage lambda gt11 (21), pGEX (22) and trpLe (23).

In order to standardize instrument to instrument optical variations, fluorescence values were converted

TABLE 1.

Performance of the HCV MP Assay with a NANBH panel.

ID	MP Assay nM Coumarin[a]	RIBA2	Diagnosis
A	**>5000**	**Positive**	NANBH-Chronic
B	28	Negative	Normal Control
C	**>5000**	**Positive**	Implicated Donor
D	23	Negative	Control
E	**>5000**	**Positive**	NANBH-Chronic
F	**>3130**	**Positive**	NANBH-Acute
G	31	Negative	Control
H	27	Negative	Control
I	**>5000**	**Positive**	Implicated Donor
J	**>5000**	**Positive**	Implicated Donor
K	42	Negative	Normal Control
L	46	Negative	Alcoholic
M	111	Negative	Normal Control

[a] nM coumarin values indicative of positive reactivity are in bold type

to nM coumarin. Coumarin was diluted in MUG substrate buffer and 50 ul of each dilution was added to a well in singlet. A linear response between fluorescence and nM coumarin (5 to 5000 nM) was obtained (data not shown), thus validating the use of such an approach.

Sensitivity of MP Assay.

Performance of the MP assay using a coded NANBH panel (Alter panel) is displayed in Table 1. After decoding the panel, we found all NANBH disease associated sera specimens to be MP assay reactive (indicated by bold type), whereas, normal and alcoholic hepatitis

controls were non-reactive. Complete agreement was obtained with the MP assay and RIBA2 results.

A comparison of MP assay and first generation C-100 EIA sensitivity assessed by Boston Biomedica's Mixed Titre HCV Performance Panel is presented in Table 2. All samples that were C-100 EIA reactive and confirmed with RIBA2 were MP assay reactive as well. In addition, the strength of signal intensity, as measured by signal/cutoff (S/C) ratio, was much greater for the MP assay; 21% of the C-100 reactive specimens yielded a S/C between 1.0 and 2.0 for the C-100 EIA and a S/C >20.1 for the MP assay. One specimen (#19) was C-100 EIA reactive yet MP assay nonreactive. Since this specimen yielded RIBA2 negative results, it was considered a C-100 EIA false positive reaction.

Finally, assay sensitivity was evaluated during the course of HCV seroconversion in a NANBH patient. HCV seroreactivity was observed after 38 and 67 days of disease onset with the MP assay and C-100 EIA, respectively (Figure 1). The earlier seroconversion noted with the MP assay was due to detection of antibodies to the c33c antigen. Antibodies to NS4 and capsid became detectable at a later stage of disease.

Specificity of MP Assay.

MP assay specificity was established by a variety of criteria. First, normal and alcoholic controls of

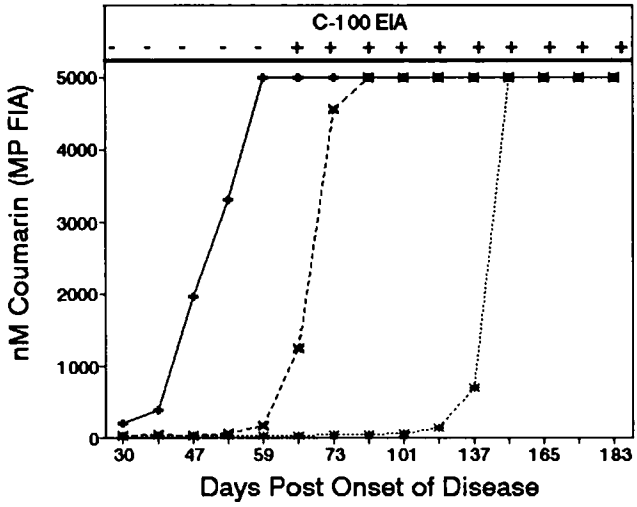

Figure 1. Reactivity of a NANBH patient to specific antigens with the MP Assay during the course of HCV seroconversion. Recombinant c33c, solid line; NS4 peptide, dashed line; capsid peptide, dotted line.

the Alter panel were non-reactive (Table 1). Second, the C-100 EIA false positive specimen (# 19, S/C 1.5 and RIBA non-reactive) of the Boston Biomedica, Inc. mixed titre HCV performance panel was non-reactive (S/C 0.2) with the MP assay (Table 2). Third, reactivity with 225 random volunteer blood donor plasma specimens was low in that no specimens yielded nM coumarin values greater than the provisional cutoff value of 200 nM coumarin and only 4 specimens were within 50% of the cutoff value (data not presented). The mean of this

TABLE 2

Evaluation of HCV MP Assay Using Boston Biomedica's Mixed Titre HCV Performance Panel

ID	Signal/Cutoff[a]		RIBA2 Antigen			
	C-100 EIA	MP Assay	5-1-1	C-100	C33C	C22
1	**1.4**	**>20.1**	+/-	-	2+	4+
2	**5.6**	**>20.1**	1+	2+	4+	4+
3	**3.9**	**>20.1**	-	1+	-	4+
4	0.2	0.2	-	-	-	-
5	**2.5**	**>20.1**	2+	+/-	+/-	4+
6	**4.8**	**>20.1**	2+	1+	1+	4+
7	0.1	0.1	-	-	-	-
8	**1.2**	**>20.1**	-	+/-	3+	-
9	**1.2**	**>20.1**	1+	-	4+	4+
10	**2.7**	**>20.1**	+/-	+/-	2+	-
11	**2.1**	**>20.1**	2+	+/-	3+	3+
12	**4.7**	**>20.1**	2+	2+	2+	3+
13	**5.6**	**>20.1**	4+	4+	4+	4+
14	**4.3**	**>20.1**	2+	+/-	4+	4+
15	**5.6**	**>20.1**	2	+/-	4+	4+
16	**5.6**	**>20.1**	4+	4+	4+	4+
17	**5.6**	**>20.1**	4+	4+	4+	4+
18	**3.9**	**>20.1**	+/-	2+	3+	3+
19	**1.5**	0.2	-	-	-	-
20	**2.3**	**>20.1**	1+	1+	1+	+/-
21	**2.6**	**>20.1**	1+	+/-	3+	3+
22	**1.6**	**>20.1**	+/-	-	4+	4+
23	**5.6**	**>20.1**	4+	3+	4+	4+
24	**2.6**	**>20.1**	2+	4+	4+	4+
25	**5.6**	**>20.1**	4+	1+	4+	4+

[a] Values greater than 1.0 are positive reactive and are indicated in bold type.

donor population was 29.3 nM coumarin with a standard deviation of 17.4. These results determined a provisional assay cutoff of 10 standard deviations away from the mean of the population.

Precision of MP Assay.

Precision was determined by analyses of 5 samples (2 negatives, NEG1, NEG2; 2 low positives, LOW POS1, LOW POS2; and 1 moderately reactive positive, MOD POS1) tested in replicates of 16 on 4 separate plates (runs). The intra-plate (intra-assay) coefficient of variation (CV) ranged from 9-12% for the negative samples, 6-10% for the low positive samples and 10-12% for the moderately reactive sample. These calculations were determined from nM coumarin values generated with each run. Inter-plate precision was determined after the nM coumarin values were first converted to signal/cutoff values using the algorithm described in METHODS. The inter-plate CVs were 14% for the negative samples, 7-8% for the low positive samples and 13% for the moderately positive sample.

DISCUSSION

Evaluation of the MP assay with HCV performance panels, dilution panels and a seroconversion panel demonstrated that the MP assay was more sensitive than the currently used C-100 EIA. This increased sensitivity was due to the inclusion of multiple HCV structural and non-structural antigens. Specificity of the assay was established with a NANBH panel and random volunteer donor specimens. Although the blood donor

pool in the United States is estimated to have a 0.4% reactive rate for HCV (5), we did not find any reactive specimens among the 225 donors tested. The development of second generation HCV serologic assays comprising multiple structural and non-structural antigens has been reported (8-10). Since the MP assay contains the important immunodominant regions reported in these other assays, it is anticipated that the HCV MP assay will yield assay performance equal to if not better than second generation HCV assays.

The MP assay format is very amenable to incorporating multiple antigens onto the solid phase at specific ratios. This is easily achieved by mixing MP that were coated with individual antigens. Other EIA formats such as coated microtitre plates are restricted in that antigens must be coated simultaneously or sequentially onto the solid phase. Such an approach may result in different antigens competing for solid phase binding sites resulting in antigens with low binding characteristics being excluded from the solid phase. We have observed this phenomenon when the HCV antigens were coated simultaneously onto the MP. Additionally, different chemistries can be used to couple different antigens to the solid phase.

A high degree of precision was noted for the MP assay. The inter-plate precision for a low positive

near the decision-making point (ie: cutoff) was 7% and reflects many variables of the assay format. These variables include: dilutions, pipetting, washing, incubation time and temperature and optics of the instrument system.

The MP assay was designed to provide an EIA format where the kinetics of antigen-antibody reactivity would be rapid. During the incubation steps, MP settle slowly and allow for a higher frequency of intermolecular collisions than that obtained with a stationary solid phase format. The MP assay format allows for thorough washing, resulting in carryover of less than 10^{-7} with 6 wash steps. Additionally, the assay was designed for automation which will improve sample throughput and assay precision.

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