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Distinguishing Acute from Chronic Hepatitis C Virus (HCV) Infection Based on Antibody Reactivities to Specific HCV Structural and Nonstructural Proteins[▽]

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Currently available serological assays for detection of antibodies to hepatitis C virus (HCV) cannot reliably discriminate acute from chronic HCV infection. We developed a multiplexed, flow-cytometric microsphere immunoassay to measure anti-HCV-IgG reactivities to the core, NS3, NS4, and NS5 HCV recombinant proteins and applied it to 99 serum samples from 24 anti-HCV seroconverters and 141 anti-HCV-IgG and HCV RNA-positive plasma specimens from chronically infected people. Differences in the geometric means or means of signal/cutoff ratios between the two sample sets were statistically significant for all the antigens tested. A multivariate logistic regression model correctly classified the samples in two groups, with a cross-validation accuracy of 90.8% for the acute group and 97.2% for the chronic group. The immunoassay described has the potential to distinguish acute from chronic HCV infection.

The diagnosis of acute hepatitis C virus (HCV) infection is based on the detection in serum or plasma of HCV RNA, anti-HCV IgG, and elevation of alanine aminotransferase levels (5). However, none of these markers alone or in combination can be used to identify acute infection, since they may also be detectable during the chronic phase of infection. Further, distinguishing acute from chronic infection on the basis of clinical history, epidemiological risk factors, and symptoms can be difficult because for most patients acute infection is asymptomatic (12, 3). Several approaches, which include detection of anti-HCV IgM (4, 2), measurement of the anti-HCV IgG avidity index (9), and observation of serial changes in viral load (10), have been proposed as indicators of acute HCV infection. The usefulness of anti-HCV IgM as a marker of acute infection remains controversial (4, 2). The recently published approach of using viral load fluctuations to identify acute HCV infection requires serial testing of samples (10). We report here the development of a high-throughput microsphere immunoassay, which simultaneously detects anti-HCV IgG responses to multiple structural and nonstructural HCV recombinant proteins, and its application to serum and plasma samples collected from people in the acute and chronic phases of HCV infection. The assay has the potential to discriminate between the acute and chronic phases by testing of single specimens.

MATERIALS AND METHODS

Study serum specimens. This study was performed using unlinked anonymous serum or plasma specimens and specimens obtained commercially from blood donor anti-HCV seroconversion panels. Ninety-nine plasma samples were obtained from 24 donor panels; the number of samples per panel varied between 1

and 11. Ten panels were acquired from Zeptometrix (Buffalo, NY) (batch numbers 6211, 6212, 6213, 6214, 6215, 9041, 9047, 9054, 9055, and 9058), 5 from NABI (Boca Raton, FL) (batch numbers 10, 20, 30, 40, and 60), 4 from Serologicals (Clarkston, GA) (batch numbers 4812B, 4813, 4814, and 4814B), 3 panels (batch numbers 908, 920, and 921) from BBI (West Bridgewater, MA), and 2 from Profile Diagnostic (Sherman Oaks, CA) (batch numbers RP006 and RP040). The samples were taken within 62 days after the last anti-HCV-IgG-negative result: 23 samples between 1 and 10 days, 25 samples between 11 and 20 days, 17 samples between 21 and 30 days, 17 samples between 31 and 40 days, 12 samples between 41 and 50 days, 3 samples between 51 and 60 days, and 2 samples between 61 and 62 days. These samples are here called the "acute" group. Of the 24 batches, the HCV genotype could be determined for 11; batches 6212, 6213, 6214, 6215, 9041, 9047, 9058, and 920 belonged to genotype 1, and batches 9054, 9055, and 921 belonged to genotype 3. Genotyping could not be determined for the remaining batches due to insufficient samples and/or low HCV RNA titers.

The chronic hepatitis C patients (the "chronic" group) consisted of anti-HCV-IgG-positive plasma samples from 141 blood donors: 64 samples were from BBI, and 77 were from the American Red Cross (9). All samples were confirmed to contain anti-HCV IgG by the Ortho recombinant immunoblot assay (RIBA) and HCV RNA by reverse transcriptase PCR (9). The HCV genotype was determined for 96 samples; 73 samples (76%) belonged to genotype 1, 16 samples (17%) to genotype 2, and 7 samples (7%) to genotype 3. In addition, a control group of 30 human serum samples negative for all markers of infection for hepatitis A, B, and C viruses (BBI, West Bridgewater, MA) was included in the study.

Recombinant HCV antigens. Eight recombinant HCV proteins purchased from RPC Diagnostic Systems Inc., Nizhniy Novgorod, Russia, were used as antigens. Five of the proteins were derived from the NS3 region: NS3#201 (amino acids [aa] 1192 to 1459) from a genotype 1b strain and NS3#207, NS3#208, NS3#210, and NS3#215 (aa 1356 to 1459) from genotype 1a, 1b, 2c, and 1c strains, respectively. The other 3 proteins originated within the HCV core region (aa positions 1 to 100) of a genotype 1b HCV strain: a mosaic protein containing immunodominant regions of the NS4 protein (aa 1691 to 1710, 1712 to 1733, and 1921 to 1940) derived from HCV genotype 1, 2, 3, and 5 strains (1) and an NS5 protein (aa 2061 to 2302).

Coupling methods. All purified recombinant HCV proteins were coupled to individual color-coded carboxylated xMAP microspheres (Luminex Corp, Austin, TX) according to the manufacturer's protocol (7). Briefly, 12.5 × 10⁶ microspheres (assigned 8 individual identification [ID] numbers) were activated for 20 min in 100 mM monobasic sodium phosphate, pH 6.2, with 50 mg/ml N-hydroxysulfosuccinimide and 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce, Rockford, IL). The recombinant proteins (200 µg each) were added to the resuspended microspheres, bringing the total volume

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TABLE 1. Coefficients of multivariate logistic regression model

Covariate	Coefficient	SE	P value
Constant	16.689	4.9521	0.0008
Core	−1.4036	0.4345	0.0012
NS3#201	0.2627	0.1756	0.1347
NS3#208	2.9682	0.9569	0.0019
NS3#215	−1.8973	0.6331	0.0027
NS3#210	−0.3956	0.1927	0.0401
NS4	−1.4768	0.4669	0.0016

TABLE 2. Comparison of anti-HCV IgG responses between acute and chronic groups

Antigen	Anti-HCV IgG response[a]			
	Acute group		Chronic group	
	Geometric mean	95% confidence interval	Geometric mean	95% confidence interval
Core	3.74[b]	2.52–4.95	13.107[b]	12.5–13.69
NS3#207	0.55	0.35–0.86	2.79	2.32–3.40
NS3#201	0.87	0.51–1.49	6.82	5.75–8.09
NS3#208	0.51	0.32–0.81	2.25	1.87–2.71
NS3#215	0.76	0.46–1.25	7.01	5.67–8.67
NS3#210	0.64	0.37–1.10	8.31	6.44–10.72
NS4	0.79	0.47–1.33	8.55	7.20–10.14
NS5	0.58	0.37–0.90	3.37	2.60–4.36

[a] A P value < 0.001 was found for each of the antigens tested when differences between the 2 groups were compared. For the acute group, $n = 24$; for the chronic group, $n = 141$.
[b] Mean (normal distribution).

to 500 μl with phosphate-buffered saline (PBS), pH 7.4, and vortexed. After incubation for 2 h by rotation at room temperature, the coupled microspheres were washed twice with PBS-TBN (PBS, pH 7.4, 0.1% [wt/vol] bovine serum albumin [BSA], and 0.05% sodium azide) and resuspended in 1 ml of PBS-TBN, after which the yield was determined by counting the microsphere suspensions using a hemacytometer. The microspheres were protected from light and stored in PBS-TBN at 4°C until use.

Multiplex HCV antibody assay. The anti-HCV-IgG multiplex assay was performed by mixing each one of the different antigen-coupled microspheres to a working concentration of 2,000 beads per ID number per well, as described above in the coupling methods. Serum or plasma samples were diluted 1:100 in PBN (PBS, pH 7.4, 0.5% [wt/vol] BSA, and 0.02% sodium azide) for measurement of antigen-specific IgG binding activity. Briefly, 25 μl per diluted sample was incubated with the microsphere mixture in a 96-well filter microtiter plate (Millipore, Billerica, MA) in the dark at room temperature by shaking for 1 h. The plate was washed twice with wash buffer (0.05% Tween 20 in PBS). The microspheres were resuspended in 25 μl of PBN and 25 μl of a 1:200 dilution of 1.1 mg/ml of Biotin-SP-conjugated AffiniPure goat anti-human IgG (Jackson Immuno Research, West Grove, PA). After incubation in the dark at room temperature by shaking for 30 min, 25 μl of a 1:5 dilution of 1 mg/ml of streptavidin–R-phycoerythrin (SA-PE) (Molecular Probes, Eugene, OR) was added. The plate was incubated for 15 min in the dark at room temperature on a plate shaker and washed. The microspheres were then resuspended in 150 μl of PBN. Measurements were obtained using the Luminex 100 instrument (Qiagen, Valencia, CA) with the MasterPlex CT v 1.0 software program (Mirai-Bio, San Francisco, CA) installed. All tests were performed in duplicate, and the mean fluorescence intensity (MFI) values were averaged. The coefficient of variation (CV) was calculated from MFI values obtained by testing eight replicates of five sera in the same run for intra-assay variation or in 2 separate assays for interassay variation, and the values were averaged for each antigen. The intra-assay CVs varied between 2% and 12%, and the interassay CVs varied between 2% and 22%. The positive cutoff value was determined as the MFI value for 30 normal human serum samples plus 2 standard deviations; the signal-to-cutoff (S/CO) ratio was determined as the MFI divided by the cutoff.

Statistical analysis. Since multiple samples were available from a majority of donors in the acute group, the mean S/CO values were calculated per antigen, per donor. Comparison of IgG anti-HCV responses to each of the 8 HCV antigens between acute and chronic groups was performed after the normality assumption was verified. Since all antigens, except the core antigen, fit a lognormal distribution, the geometric mean or mean (normal distribution) and variances were calculated for each group. The F test was performed to test the equality of the two group variances. If the F test generated a significant outcome, the t test assuming unequal variances was used. Otherwise, the t test assuming equal variances was used. A P value of <0.001 was considered to be statistically significant. Analyses were performed using the SAS software program (SAS Institute, Cary, NC).

To facilitate the classification of samples as belonging to acute or chronic HCV infection, a multivariate logistic regression model was built in the MATLAB software program (6). A multivariate model using the reactivities to all 8 antigens as covariates had a deviance of 24.95. The P value of the reactivity to the NS5 antigen was 0.65. Consequently, this covariate was removed from the model, yielding a deviance of 25.39. The P value of the likelihood ratio test for the two models was $\Pr[\chi^2 (1) > 0.44] > 0.1$, confirming that the NS5 antigen is not significant for the model. Similarly, the covariate corresponding to the response to the antigen NS3#207 was removed, yielding a deviance of 25.44. The coefficients of the resulting model are given in Table 1.

To study the predictive ability of the model, a leave-one-out cross-validation was performed. A panel from the acute group and one sample from the chronic group were removed in turn, following which a multivariate logistic regression model using the six covariates was built from the remaining data. The model was then used to predict the membership of the panel or sample set aside in the acute or chronic group. In the case of a panel with more than one sample, the accuracy was calculated as the percentage of correctly classified samples out of all samples in the panel. The calculated accuracies were averaged by group.

RESULTS

Some panels used in the study showed incomplete seroconversion profiles, presenting anti-HCV IgG reactivity exclusively to one protein over time; six (40, 6215, 9054, 9055, RP006, and 4812B) of them seroconverted to the core protein exclusively, two (908 and 6214) to the NS4 protein only, and one (6212) panel to the NS3#201 protein only. Additionally, we observed that the core was the most immunoreactive protein, reacting with 78.8% (78 out of 99) of the acute samples and 99.3% (140 out of 141) of the chronic samples.

More than one sample was available for most of the donors in the acute group; in this situation, the S/CO mean per antigen per donor was calculated. The differences in the mean, geometric mean, and variances per protein per group were significant for all proteins tested (Table 2) or when each sample was considered independently (Fig. 1). However, in some cases, we observed that the S/CO ratios for acute and chronic samples overlapped (Fig. 1).

A multivariate logistic regression model based on the immune response to the 8 HCV antigens was developed to facilitate the classification of samples. The HCV antigens NS3#207 and NS5 were later removed because they did not improve the classification of samples. The multivariate analysis correctly classified 100% of acute samples and 97.9% of the chronic samples. To further evaluate the goodness of fit of the model, a receiver operating characteristic (ROC) curve was plotted. The area under the ROC was 0.9972, indicating that a clear separation exists between the acute samples collected within 62 days after seroconversion to anti-HCV IgG and the chronic samples. The predictive ability of the model to classify unknown future samples from one of these two groups was measured by external validation. The leave-one-out accuracy was 90.8% for acute samples and 97.2% for chronic samples.
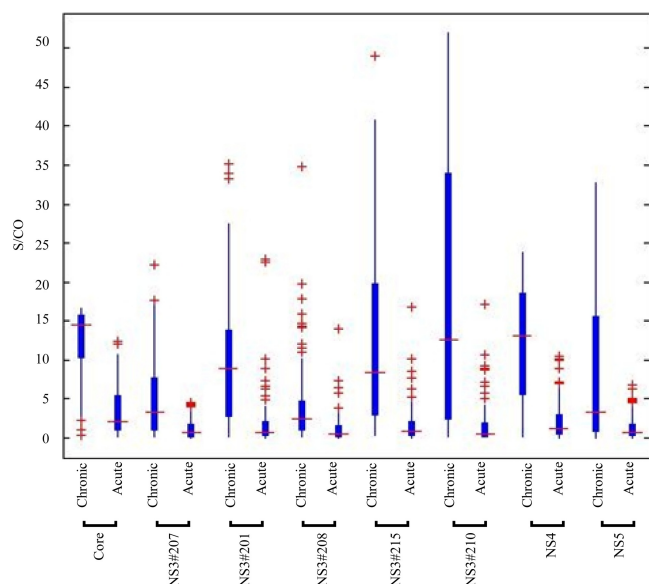
FIG. 1. Distribution of S/CO values per antigen in acute and chronic groups. For each box, the central horizontal bar is the median, the edges are the 25th and 75th percentiles, and the vertical bars extend to the most extreme data points. Outliers are plotted individually as red crosses.

DISCUSSION

A high-throughput Luminex multiplex immunoassay using 8 recombinant proteins from 5 HCV genotypes was developed. This assay detects specific IgG reactivities to HCV structural and nonstructural proteins. The core protein was the most immunoreactive in both groups. In the acute group, it reacted with nearly 80% of the first anti-HCV-positive samples in the seroconversion panels, and in the chronic group it reacted with almost all of the samples. Netski et al. (11) examined antibody responses to HCV structural (E1, E2, and core) and nonstructural (NS3, NS4, and NS5) proteins during acute infection in samples from 12 injection drug users before, during, and after HCV infection. They detected antibody responses to NS4 proteins in samples from 60% of patients, and between 60% and 70% showed a positive response to the NS3 and NS5 proteins, compared to only 40% with positive responses to the core antigen. Compared to the findings of Netski et al., the marked differences in anti-HCV IgG reactivity we found among the nonstructural and core proteins likely reflect the use of HCV antigens that are larger and represent a wider array of genotypes, as well as a larger study sample size.

The most significant difference between the acute and chronic groups was in the levels of anti-HCV IgG responses (Table 2 and Fig. 1). These differences are probably due to increasing antibody titers as the infection progresses, as we previously reported (9). This increase in IgG antibody titer during the progression of disease has also been observed for patients with HIV infection (8).

Our assay detected anti-HCV IgG in samples missed by the IgG avidity index assay previously described (9). In the avidity assay, 24 out of 99 samples from the acute group were negative. These samples belong to 7 seroconversion panels belonging to genotypes 1A and 3A or unknown genotypes, and the time to seroconversion varied between 1 and 46 days (9; also data not shown). This disparity between outcomes of the 2 assays could be due to the fact that the Luminex anti-HCV assay is based on reactivities to 3 other NS3 proteins and to the NS5 protein, which are additional to the proteins used in the IgG avidity test we previously described (which was a mixture of the core, NS3#207, NS3#201, and NS4 proteins) (9). Because the Luminex assay measures specific reactivities to each of these antigens, a sample can be detected as being positive earlier than with the avidity test, whose outcome is an aggregate of avidity to the 4-protein mix. Moreover, when we compared the Luminex IgG results with RIBA reactivities, we observed that among 63 acute samples that could be compared with RIBA, 12 samples were indeterminate and 9 were negative. These findings suggest that the Luminex assay described here can discriminate between samples in the acute phase of HCV infection and those in the chronic phase more accurately than the IgG avidity test or RIBA. With it being multiplexed and high throughput, antibody reactivities to several HCV antigens can be detected simultaneously in a single test run. Further, the assay requires only a small amount of serum or plasma. Last, the discrimination is based on a single sample rather than serially obtained samples. The differences in anti-HCV IgG responses to each of the 8 antigens is statistically significant (Table 2); however, none of them alone can be used to discriminate between the acute and chronic phases of infection with a high degree of accuracy due to some overlapping of S/CO ratios between the two groups. Therefore, we generated a multivariate logistic regression model built upon differences in S/CO ratios between acute and chronic groups through measurements of anti-HCV IgG reactivities. According to this model, only 6 antigens are required for the classification of a sample into the acute or chronic group (Table 1). The cross-validation accuracy of this model was 90.8% for the acute group and 97.2% for the chronic samples.

One limitation of this study is that we used HCV seroconversion panels instead of clinical samples. Furthermore, the number of samples between days 51 and 62 after seroconversion was small (5 out of 99 samples). The associations we found here should be confirmed in other clinical settings.

It has been reported that early identification of acute HCV infection followed by treatment with alpha interferon 2b and ribavirin can lead to eradication of the virus in up to 98% of cases, compared to 42% to 82% of cases if the treatment starts during the chronic phase of infection (13). The assay we describe here for detection of antibody levels for multiple HCV antigens may be of diagnostic value if a patient is in the acute phase of infection and therefore is able to benefit from an early course of antiviral therapy. Moreover, because only a single sample, rather than serially obtained samples, is needed for testing, the assay also can be applied in surveillance studies to identify trends in incident HCV infection.

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