
Rapid Communication

The Sensitive Detection and Quantitation of Antibody to HCV by Using a Microsphere-Based Immunoassay and Flow Cytometry

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Antibody to HCV core and NS3 was quantified by using a microsphere immunoassay and flow cytometry. Antibody to core and NS3 was elevated in the 85 seropositive blood donors tested. The amount of either antibody varied over two logs although greater variation was seen with the antibody to NS3 than was seen with antibody to core. In three documented acute HCV cases, the microsphere assay detected antibody prior to antibody detection using the reference methods. Twenty donor samples were indeterminate by the reference methods: 45% of these were indeterminate with the microsphere assay while

25% were negative and 30% were positive. As compared to enzyme immunoassay the microsphere assay showed a 5-fold increase in sensitivity. The microsphere assay demonstrated increased sensitivity for the quantification of specific antibody to HCV core and NS3 and was useful in resolving a significant proportion of indeterminate samples. Cytometry 29:106–112, 1997. © 1997 Wiley-Liss, Inc.

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Hepatitis C Virus (HCV) appears to be the causative agent for >90% of post-transfusion cases of hepatitis not attributable to Hepatitis A or Hepatitis B Virus (2). Prior to the implementation of screening for the antibody to HCV, the risk of acquiring HCV from a blood transfusion was estimated to be 1 in 526 units transfused (5). Owing to the elimination of antibody positive donors by screening, the current risk is estimated to be 1 in 103,000 units transfused (14). To date HCV has not been cultured in vitro and proteins representing portions of HCV, thought to be important in the development of the humoral immune response, are produced by using recombinant DNA methods. Proteins from two of the regions of the HCV genome, which have been shown to be the most sensitive and specific for the detection of antibody to HCV, are the viral core and the non-structural region 3, NS3 (2,4,13,17). However, the antibody response to HCV is variable with different levels of antibodies produced to the different antigens. Occasionally antibody is produced to only a single antigen with a protracted time needed for seroconversion in some individuals (7,11,15,20). The role that antibody may play in the resolution of HCV infection and

possible immunity to HCV is unknown (2,6). Currently screening for antibody to HCV is accomplished by using an enzyme immunoassay (EIA) with reactive samples tested with an immunoblot assay. There remain a significant proportion of blood donors and patients with indeterminate serology to HCV based upon the interpretation of the EIA and immunoblot testing (3,11,16,18,20).

The flow cytometric microsphere immunoassay (MIA) has been described previously for the detection of a variety of analytes (10). The assay has the benefits of the simultaneous detection of multiple antibodies and of having high analytic sensitivity. The simultaneous detection is dependent upon using different sized microspheres, each coated with a specific HCV protein, with the detection of fluorescence from each sized microsphere without interference from the other sized microsphere(s).

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This study was undertaken to evaluate the feasibility of the detection of antibody to HCV by using the microsphere method and to study the range of antibody levels among different blood donors to both the HCV core and NS3 proteins. In addition, the analytic sensitivity of the method was evaluated and compared to EIA.

MATERIALS AND METHODS

Enzyme Immunoassay

Enzyme Immunoassay (EIA) 2.0 was used to screen all samples (Abbott HCV EIA 2.0, Abbott Laboratories, Abbott Park, IL). The EIA uses proteins from the core, NS3, and NS4 regions of the HCV genome. The assay was performed following the manufacturer's instructions. Reactive samples were re-tested in duplicate and those positive for HCV antibody by EIA were tested with the immunoblot assay.

Strip Immunoblot Assay

An immunoblot assay was performed on all samples that tested positive by HCV EIA 2.0. The RIBA HCV 2.0 strip immunoblot assay (SIA) (Ortho Diagnostic Systems, Raritan, NJ) was performed following the manufacturer's instructions. The SIA uses proteins from the core, NS3, and NS4 regions of the HCV genome as well as a band of human superoxide dismutase (SOD) and two bands of human IgG. The reactivity pattern was read and samples with bands of 1+ or greater to at least two HCV bands from different regions of the genome were considered positive by the SIA. Samples with reactivity to a single band were considered to be indeterminate.

Microsphere Immunoassay

Microspheres of 3.1, 5.1, and 7.6 μm in diameter (Bangs Laboratories, Fishers, IN) were used. An aliquot of each microsphere population was washed twice in distilled water and once in 0.1 M Na_2CO_3 , pH 9.6 buffer. The microspheres were adjusted to 10^8 microspheres/mL in 0.1 M Na_2CO_3 buffer. The microspheres were coated with optimum concentrations of their respective proteins for 3 h at 37°C followed by 18 h at 2–8°C. The 3.1 μm microspheres were coated with human IgG (Armour Pharmaceutical Co., Kankakee, IL) at 4 $\mu\text{g}/10^7$ microspheres. The 5.1 μm microspheres were coated with the HCV core protein at 10 $\mu\text{g}/10^7$ microspheres. The 7.6 μm microspheres were coated with the HCV NS3 protein at 22 $\mu\text{g}/10^7$ microspheres. After washing, the excess binding sites on the microspheres were blocked by incubating the microspheres in 0.5% bovine serum albumin in phosphate-buffered saline, pH 7.2 (PBS-BSA) for 24 h at 2–8°C. After separate coating and blocking of remaining binding sites, the microspheres were mixed to a final concentration of $10^6/\text{mL}$ of PBS-BSA (1.0×10^5 of the 3.1 μm and 4.5×10^5 of each of the 5.1 and 7.6 μm microspheres).

Serum or plasma samples were diluted to 1:40, 1:160, 1:640, 1:2,560, and 1:10,240 in sample diluent (PBS + 10% calf serum + 0.1% Tween 20); 100 μL of each dilution was added to 100 μL of the microsphere mixture in a microplate and incubated for 3 h at 37°C. The microspheres were washed twice and 100 μL of phycoerythrin (PE)

conjugated goat anti-human IgG (1:100 final dilution; DAKO, Carpinteria, CA) was added to each well and incubated for 1 h at 37°C. Fifty microliters of PBS-BSA was added to each well and the microspheres were aspirated directly from the 96-well microplate by using the Microtiter Tray Sample Injector System (Cytek Development, Fremont, CA). The microspheres were analyzed by using flow cytometry (FACScan, Becton Dickinson, San Jose, CA) and the mean of the log PE fluorescence from each microsphere population was calculated. The PE vs. forward scatter (FSC) plot was displayed for each sample dilution. The log serum dilution was plotted vs. the mean fluorescence for the core and the NS3 microspheres. The reciprocal of the serum dilution predicted to produce a fluorescence intensity in channel 450 for core and 1,000 for NS3 on a 4 decade log scale was calculated and used as the antibody titer for each sample. The flow cytometer was set up daily by using caliBRITE Beads (Becton Dickinson).

Serum and Plasma Samples

One hundred thirty-six blood donors were HCV antibody positive by EIA and one hundred twenty had sufficient volume for all testing. One hundred donors that were HCV antibody negative were selected randomly and used to determine a cut-off for positivity. Donor samples were identified by number from 1 through 136 for the positive samples and 200–300 for the negative samples. All samples were tested without knowledge of the EIA or SIA results.

Three commercially available serum panels from documented cases of HCV seroconversion (PHV901 with 11 members, PHV902 with 7 members, and PHV903 with 8 members; Boston Biomedica, Inc., Boston, MA) were tested with the microsphere immunoassay (MIA). The panels are provided with EIA, SIA, and alanine amino transferase (ALT) results.

Alanine Amino Transferase Assay

ALT was measured by using a standard kinetic assay based upon NADH consumption (Boehringer Mannheim/Hitachi 747 analyzer, Indianapolis, IN). Results >75 U/L were considered to be elevated.

HCV Core and NS3 EIA

To separate wells of 96-well microplates, 100 μL of a 5 μg of HCV core/mL or 2 μg of HCV NS3/mL in Tris, pH 8.0 buffer was added and incubated for 24 h at 2–8°C. The protein solutions were removed and the excess binding sites were blocked with PBS-BSA for 24 h at 2–8°C; 100 μL of each sample dilution was incubated in the well for 0.5 h at 37°C, washed, and 100 μL of peroxidase conjugated goat anti-human IgG (Protos Laboratories, South San Francisco, CA) was added to each well and incubated for 0.5 h at 37°C. The wells were washed as before and 200 μL of the substrate 2,4-tetramethylbenzidine was added and incubated for 10 min at ambient temperature. Fifty microliters of 2 N H_2SO_4 were added to each well to stop the reaction and the absorbance was read at 540 nm. The

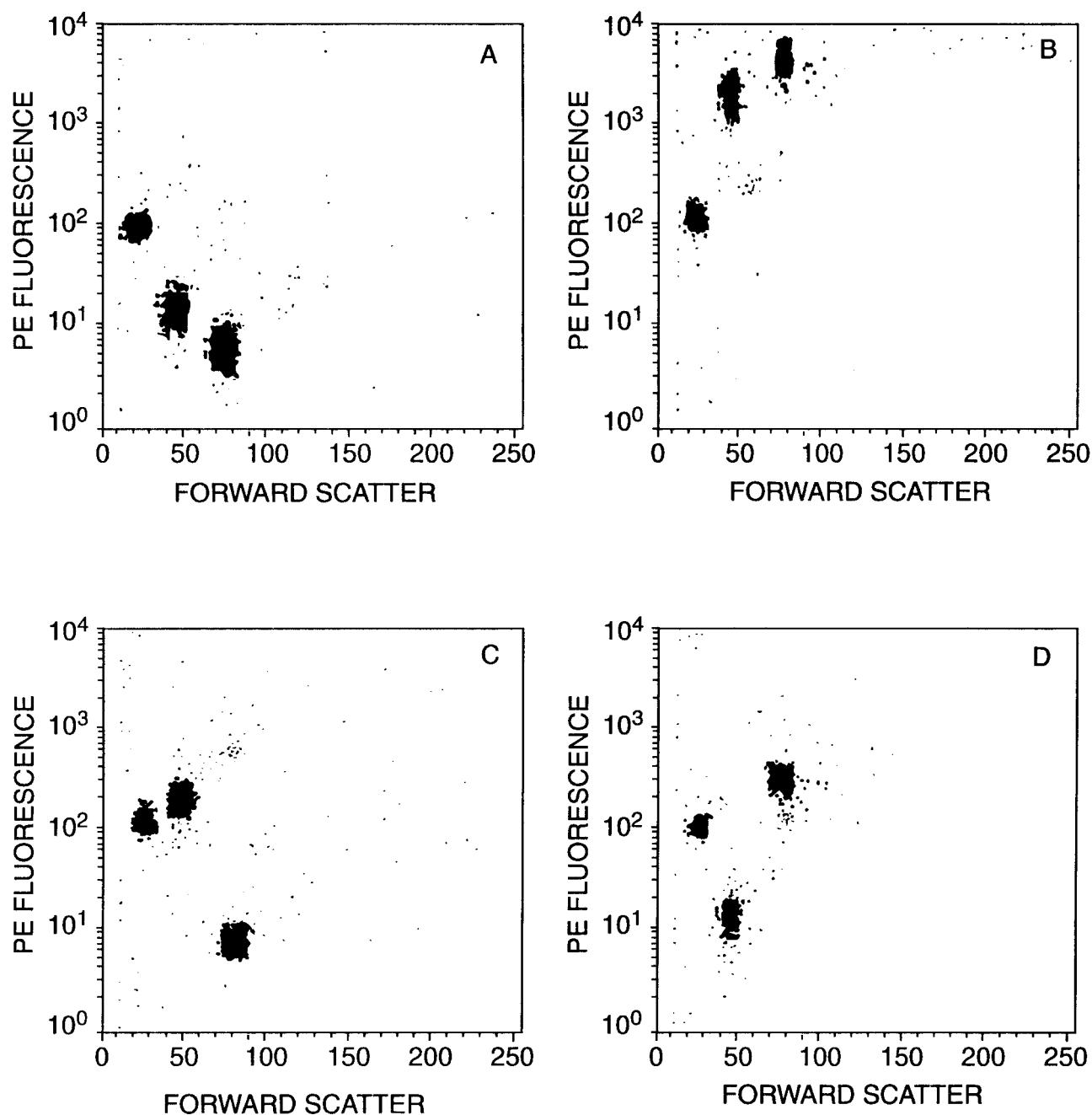


FIG. 1. Dot plots of PE fluorescence (ordinate) versus FSC (abscissa) for four different donor samples. **A:** A donor negative for antibody to HCV. **B:** A donor positive for antibody to both HCV core and HCV NS3. **C:** A donor positive for antibody to HCV core but negative for antibody to HCV NS3. **D:** A donor negative for antibody to HCV core but positive for antibody to HCV NS3.

cut-off for positivity was 3 S.D. above the mean absorbance from the 100 negative samples.

RESULTS

Figure 1 shows the FSC vs. PE fluorescence pattern of the three mixed microsphere populations from a number of samples with different antibody reactivity to the HCV proteins. The 3.1 μ m microspheres were positive with all

samples indicating that the PE-goat anti-human IgG reagent was added to the reaction well. The 3.1 μ m human IgG microspheres generally produced a fluorescence signal at channel 100 (mean fluorescence channel of 101 with a S.D. of 11, $n = 14$). The intra-assay coefficient of variation (CV) for the 3.1 μ m IgG microsphere mean fluorescence was 14% with an inter-assay CV of 22%. For the two HCV-coated microspheres the fluorescence signal of the

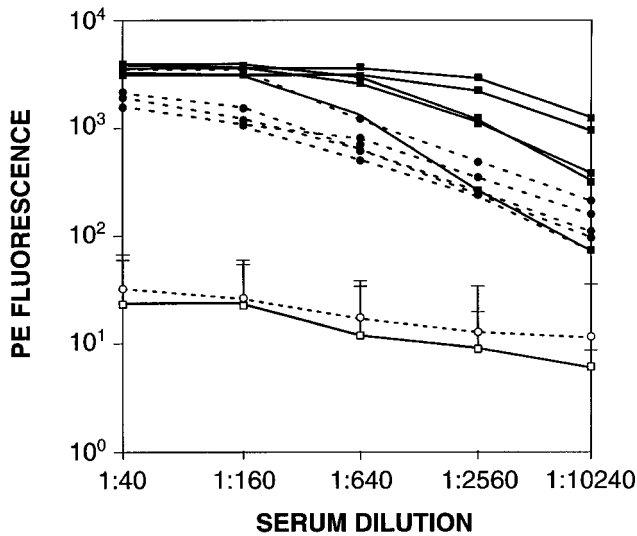


FIG. 2. The plot of fluorescence signal from each microsphere population at each serum dilution tested. Five random donor samples are displayed. The upper dashed lines connected to the solid circles are HCV core antibody from the MIA. The upper solid lines connected with the solid squares are HCV NS3 antibody from the MIA. The lower dashed line is the mean of the HCV core antibody and the lower solid line is the mean of the HCV NS3 antibody level from the negative donors. The vertical lines represent the 3SD range from the negative donors. The 3SD cutoff for core is 59, 55, 32, 28, and 33 for each dilution, respectively. The 3SD cutoff for NS3 is 53, 42, 23, 16, and 10 for each dilution, respectively.

individual microspheres is displayed against the FSC signal for a typical negative serum sample and three positive samples with different antibody reactivities to core and NS3.

Greater than 90% of each microsphere population was singlets and there was no change in the percentage of aggregates in the presence of antibody positive serum. In repeated analyses, <5% of the microspheres in the 5.1 or 7.6 μm region gate was due to aggregates of the smaller microspheres (data not shown).

Figure 2 shows the dilution studies from five antibody positive samples, as well as the mean + 3 S.D. of the negative samples. At low serum dilutions with high antibody levels, a prozone-like effect was seen as evidenced by a reduced fluorescence signal at lower dilutions. However all antibody positive samples were well above the mean + 3 S.D. cutoff calculated from the negative samples at each dilution. The dilution curves shown in Figure 2 represent the range of curves seen with the positive samples. The location of the linear portion of the dilution curve was dependent upon the concentration of antibody to HCV in the sample.

During the time period covered by this study, 11,830 blood donors were tested with the Abbott HCV EIA 2.0. Of these, 136 samples (1.15%) were repeatedly reactive for HCV antibody by the Abbott HCV EIA 2.0. Of the 136 samples, 16 were either unavailable or had insufficient volume for complete testing and were not used. The remaining 120 specimens were tested with the SIA: 85 (70.8%) were positive, 20 (16.7%) were indeterminate, and 15 (12.5%) were negative. Of the 20 samples that were

Table 1
Distribution of Specific Antibody to HCV Core and NS3 Proteins*

	Core	NS3
Minimum	26	10
10th percentile	134	170
25th percentile	333	890
Median	1,639	4,200
75th percentile	4,620	7,000
90th percentile	6,798	13,800
Maximum	8,580	40,000

*Values are the reciprocal of the serum dilution that produced a fluorescence intensity in channel 450 for core and 1000 for NS3.

indeterminate by the SIA, 16 (80%) had only antibody to core detected, 3 (15%) had only antibody to NS3 detected, and 1 (5%) had only antibody to NS4 detected.

The relative titer of antibody to core and NS3 by the MIA was expressed as the reciprocal of the serum dilution required to produce a fluorescence signal in channel 450 for core and channel 1,000 for NS3. From the 85 EIA and SIA positive samples the relative antibody titers to core and NS3 by MIA are listed in Table 1. There was considerable range in the level of antibody to core and NS3 by the MIA. The range of antibody to NS3 was greater than the range of antibody to core as shown in Table 1. This is supported by the SIA results, which showed that 83 of 85 positive donors (98%) were scored as 4+ for core antibody, whereas for NS3 antibody, 62 (73%) were 4+, with 13 (16%) scoring as 3+, 4 (5%) scoring as 2+, and 5 (6%) scoring as 1+. The scoring of band intensity in the SIA can mask significant difference in antibody levels. For example, in the 62 donors with a 4+ NS3 antibody band in the SIA, the median NS3 antibody level by the MIA was 4,900 with 0.10 and 0.90 percentile values of 503 and 16,000, respectively. However the median NS3 antibody level by the MIA did decrease with lower band scoring in the SIA as follows: median of 4,900 for 4+, 1,160 for 3+, 155 for 2+, and 55 for 1+.

Results from the samples that were indeterminate by the SIA and the seroconversion samples are listed in Table 2. Of the 16 samples that had antibody only to core by the SIA, seven (44%) had antibody only to core by the MIA, five (31%) did not have antibody to core nor to NS3 by the MIA, three (19%) had antibody to both core and NS3 by the MIA, and one (6%) did not have antibody to core but had a low level of antibody to NS3 by the MIA. The three samples with antibody only to NS3 by the SIA had antibody to NS3 as well as antibody to core by the MIA. One sample with antibody only to NS4 by the SIA lacked antibody to core and had a low level of antibody to NS3 by the MIA. The seroconversion series had early samples in which levels of antibody to NS3 and/or core was detectable by the MIA prior to being detectable by the EIA or SIA.

Of the 120 HCV antibody positive donors, 21 (17.5%) had an elevated ALT, with a mean ALT value of 112 units. In a group of seronegative blood donors, with an elevated ALT, the mean ALT value was 109 units. Only two of the

Table 2
Results in Blood Donors With Indeterminate Serology by SIA*

Donor sample ID	EIA Abs.	EIA S/CO	SIA NS4 (5-1-1)	SIA NS4 (c100)	SIA NS3 (c33)	SIA Core (c22)	SIA N, I or P	MIA Core Ab	MIA NS3 Ab	ALT
16	0.574	1.8	—	—	—	1	I	33	<	13
51	0.863	2.3	—	—	—	1	I	<	<	82
74	0.482	1.4	—	—	—	1	I	<	<	49
114	0.494	1.2	—	—	—	1	I	33	50	11
122	0.496	1.2	—	—	—	1	I	<	5	20
96	0.872	2.4	—	—	—	2	I	22	<	45
115	0.899	2.2	—	—	—	2	I	<	<	6
12	1.315	3.3	—	—	—	3	I	18	<	7
27	0.631	1.8	—	—	—	3	I	143	<	10
32	1.607	4.6	—	—	—	3	I	<	<	14
18	1.943	4.8	—	—	—	4	I	59	14	52
23	1.898	5.7	—	—	—	4	I	<	<	15
44	>2.200	>5.5	—	—	—	4	I	73	8	183
53	1.476	4.2	—	—	—	4	I	22	<	10
66	2.134	5.3	—	—	—	4	I	396	<	60
72	1.276	3.2	—	—	—	4	I	209	<	8
95	0.520	1.4	—	—	2	—	I	22	10	25
100	0.640	1.5	—	—	2	—	I	18	84	11
45	1.636	4.2	—	—	4	—	I	44	240	6
21	0.543	1.3	—	1	—	—	I	<	12	18
PHV901-01	N/A	0.2	—	—	—	—	N	<	14	35
PHV901-02	N/A	0.2	—	—	—	—	N	<	14	44
PHV901-03	N/A	1.6	+/-	+/-	1	—	I	<	250	158
PHV901-04	N/A	1.7	+/-	+/-	1	—	I	<	225	122
PHV901-05	N/A	1.7	+/-	+/-	1	—	I	<	215	86
PHV901-06	N/A	1.6	+/-	+/-	1	—	I	<	220	89
PHV901-07	N/A	3.8	3	1	3	—	P	<	265	68
PHV901-08	N/A	3.6	3	1	2	—	P	<	210	51
PHV901-09	N/A	>5.0	4	3	3	—	P	<	350	59
PHV901-10	N/A	>5.0	4	4	2	—	P	<	275	91
PHV901-11	N/A	>5.0	4	4	4	—	P	<	380	44
PHV902-01	N/A	0.2	—	—	—	—	N	<	18	143
PHV902-02	N/A	0.2	—	—	—	—	N	<	6	128
PHV902-03	N/A	0.4	—	—	+/-	—	N	<	19	429
PHV902-04	N/A	0.8	—	—	+/-	—	N	<	53	>450
PHV902-05	N/A	3.9	—	—	3	+/-	I	44	770	>450
PHV902-06	N/A	5.0	—	—	4	+/-	I	40	1500	>450
PHV902-07	N/A	>5.3	+/-	—	4	1	P	55	3300	331
PHV903-01	N/A	0.2	—	—	—	—	N	<	22	148
PHV903-02	N/A	0.4	—	—	—	+/-	N	15	22	>450
PHV903-03	N/A	0.5	—	—	—	+/-	N	18	18	>450
PHV903-04	N/A	0.8	—	—	+/-	1	I	33	55	>450
PHV903-05	N/A	0.7	—	—	+/-	1	I	16	66	>450
PHV903-06	N/A	1.7	—	—	2	2	P	13	150	>450
PHV903-07	N/A	1.9	—	—	2	2	P	12	170	>450
PHV903-08	N/A	2.3	—	—	2	2	P	10	225	>450

*EIA Abs, absorbance in the Abbott HCV 2.0 EIA; EIA S/CO, signal/cutoff with 1 or greater being reactive; —, no band seen by SIA; +/-, weak band by SIA; 1, 2, 3, or 4, band intensity by SIA; N, I, or P, negative, indeterminate or positive by SIA; <, less than the 3SD cutoff for fluorescence by the MIA; ALT values over 75 were considered elevated; N/A, not available.

samples with indeterminate results in the SIA had elevated ALT levels.

Samples from 12 random antibody positive donors were serially diluted in 4-fold increments starting at 1:40 until the fluorescence in the MIA was at or below the cutoff. Each dilution was tested by the MIA, the HCV core, and the HCV NS3 EIA. The average end point titer for antibody to core by the MIA was 65,483, for antibody to core by the EIA was 13,617, for antibody to NS3 by the MIA was 66,558, and for antibody to NS3 by the EIA was 12,567. This increase in detection of diluted samples represents a

5-fold increase in analytic sensitivity of the MIA as compared to the EIA (data not shown).

DISCUSSION

The three selected microsphere populations were easily resolved by FSC. This allowed for the simultaneous and discrete detection of antibody to different analytes based upon the separate coating of the microsphere populations. The low amount of microsphere aggregates introduced little contamination from one sized population into an-

other and did not interfere with the determination of antibody level.

All donor samples with antibody to HCV by the EIA and the SIA had elevated levels of antibody to both core and NS3 by the MIA. There was clear separation of the antibody positive donors from the antibody negative donors. The antibody levels in the HCV antibody positive donors covered two logs. The range of antibody level to core was less than the range of antibody to NS3, which was supported by the band scoring results in the SIA.

We had sufficient sample volume in 120 of the 136 blood donors who were EIA positive. These 136 HCV antibody positive donors were from 11,830 donors screened, which is an antibody reactive rate of 1.15% in our blood donor population and is in agreement with other reports (14); 20 of the 120 blood donors were indeterminate by the SIA. Indeterminate HCV serology has been investigated in a number of studies and attempts at the resolution has employed testing for antibody to different HCV proteins, testing for antibody with different methods, using ALT to suggest hepatitis, or testing for HCV RNA (3,11,16,18–20). A high percentage of samples with isolated antibody to core in the SIA also had antibody to NS3 when tested with the new version SIA (RIBA 3.0; 13) and/or antibody to an envelope protein (8). However another study suggested that isolated antibody to core in the SIA represents a false positive reaction (16). In our 20 blood donors who were indeterminate in the SIA, the 16 with isolated antibody to core had mixed results in the MIA; however, one-half of these were resolved as either negative or positive by the MIA.

The most frequent indeterminate pattern seen is the isolated antibody to core (3,9,11,19). With the MIA the level of fluorescence in seronegative samples to core was higher than was the level of fluorescence to NS3. This suggests either increased non-specific binding of immunoglobulin to core or perhaps epitopes on the core protein to which antibodies are present in the absence of HCV infection. Isolated antibody to NS3 by the SIA is generally thought to represent true infection and other HCV antibodies can be found using more sensitive techniques (4,8,11,13). The three samples we tested with isolated antibody to NS3 by the SIA had antibody to NS3 as well as to core by the MIA. The detection of antibody to core or NS3 in these isolated SIA reactive samples as well as in the seroconversion samples suggests an increase in analytic sensitivity of the MIA. This is supported by the dilution studies of known antibody positive samples analyzed in the EIA and MIA in which the MIA produced a result over the cutoff at greater dilutions than seen with the EIA. The increase in sensitivity of the MIA may be due to the antigens used as well as due to the use of sensitive fluorescent conjugates with a high quality optical system in the flow cytometer.

Measurement of ALT has been used as a indicator of hepatitis; however ALT has been shown to be insensitive as well as non-specific (12). While the frequency of an elevated ALT was much higher in the seropositive donors

as compared to the seronegative donors, the ALT is still too insensitive and non-specific to be used as independent supplemental evidence for HCV infection in the blood donors with indeterminate serologic evidence. Owing to the concerns about lack of sensitivity and specificity, ALT has been discontinued as a mandated blood donor test (1).

Our dilutions studies show that the analytic sensitivity is greater with the MIA than with the EIA. The MIA showed significant differences in antibody levels in samples that scored the same in band intensity in the SIA. Both the increase in sensitivity and the greater precision to quantify antibody levels allowed us to compare antibody levels to core and NS3 among a series of infected blood donors. The quantification of antibody may prove useful in situations such as following antibody levels in newborns in whom maternal specific IgG to HCV is present, in hypogammaglobulinemic individuals treated with intravenous gamma globulin, and in immunocompromised patients, either due to HIV infection or due to immunosuppressive therapy in whom the antibody response may be significantly compromised (7,9,17). In these individuals, a sensitive and quantitative assay may be an important adjunct to other testing such as EIA screening and possibly HCV RNA detection by the PCR. There are instances in which the MIA, with increased sensitivity, the ability to quantitate specific antibody, and the ability to simultaneously test for multiple analytes may prove to be useful. Further studies are underway to evaluate the antibody response to HCV in a group of immunocompromised patients.

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