Serological Markers of Posttransfusion Hepatitis C Viral Infection

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Serological markers for hepatitis C virus (HCV) infection were measured in serial samples from 14 posttransfusion chronic non-A, non-B hepatitis patients by a semiquantitative dot blot immunoassay. The assay detected antibodies to HCV by use of recombinant proteins that represent putative HCV capsid (core), nonstructural protein 3 (NS3) (33c), and NS4 (c100) epitopes. Seroconversion to anti-HCV antibodies (anti-HCV) was detected in all patients. The average time to active antibody production detected by any of the recombinant proteins was 13.8 (range, 3.6 to 22.0) weeks posttransfusion or 4.6 (range, −4.5 to 13.4) weeks after the first biochemical marker of illness. Anti-HCV were detected earliest by the core antigen in most cases; however, the patterns of anti-HCV responses varied significantly among individuals. Overall, the addition of the core and NS3 antigens to the assay enabled the detection of the antibody response 4 to 5 weeks earlier than that of the c100 antigen. The sole antigen used in current screening tests in the United States. Passively transferred antibodies were detected by at least one antigen in early posttransfusion samples from 12 patients and decayed below detectable levels for all antigens in only 2 patients. Antibodies to all three gene products were evident in the last sample from all five patients monitored for >3 years from transfusion indicating the persistence of antibodies in patients with chronic illness. Our data show that the period following the onset of hepatitis during which anti-HCV are not detected by current screening assays can be greatly shortened by the detection of anti-HCV responses by a combination of core, NS3, and c100 antigens.

Greater than 90% of transfusion-associated hepatitis cases worldwide are attributed to non-A, non-B hepatitis. The predominant etiological agent of non-A, non-B hepatitis, hepatitis C virus (HCV), has been cloned, and an immunodominant region designated c100 and encoded by the putative nonstructural protein 4 (NS4) genomic region, has been expressed, purified (3), and incorporated into immunoassays that are useful for detecting antibodies to HCV (anti-HCV) in infected blood (3, 6, 9, 11, 13).

In a study of 20 well-documented cases of posttransfusion non-A, non-B hepatitis, the mean delay to the development of anti-HCV was 21.9 weeks after transfusion and 15 weeks after the onset of hepatitis (1). Seroconversion occurred 6 months after transfusion in 28% of the patients and at approximately 1 year in one patient. Similar results were reported by Esteban et al. (6) for posttransfusion non-A, non-B hepatitis patients and by Shimizu et al. (12) for chimpanzees that were experimentally infected with the virus. The delay in seroconversion to anti-HCV detected by recombinant c100 antigen may account, in part, for the relatively low prevalence of seroconversion (15 to 30%) in individuals diagnosed with acute non-A, non-B hepatitis. Thus, anti-HCV-negative cases may actually be caused by HCV infection that does not elicit an immune response detectable by current assays for antibodies to c100.

We report on the use of a semiautomated dot blot immunoassay for detecting anti-HCV responses in transfusion recipients; this assay is done with a panel of purified recombinant HCV proteins derived from putative NS4 (c100 expressed in two different host systems), NS3 (clone 33c antigen), and 5' structural (core antigen) regions. The data documented the presence of antibodies to at least one recombinant protein in >95% of the samples throughout the posttransfusion course of follow-up. Passively transferred antibodies were evident in most of the early posttransfusion samples. In addition, the seroconversion interval detected by the addition of NS3 and the core protein was shortened relative to that in currently used assays.

MATERIALS AND METHODS

Selection of samples for antibody testing. Serum samples were collected from posttransfusion non-A, non-B hepatitis patients enrolled in an ongoing prospective study at the National Institutes of Health since 1973. The enrollment criteria and detailed follow-up procedures were described in a previous publication (1). Fourteen patients who were shown by a commercially available screening test for antibodies to recombinant c100 antigen (Ortho Diagnostics Systems, Raritan, N.J.) to have seroconverted to anti-HCV were selected for detailed analysis of antibody responses by use of additional recombinant HCV antigens as described below. The date of alanine aminotransaminase (ALT) elevation was the first date on which ALT levels exceeded 2.5 times the upper limit of normal levels. All additional antibody testing was conducted with an anti-HCV enzyme immunoassay (EIA) (Abbott Laboratories, North Chicago, Ill.) in accordance with the manufacturer's instructions.

Dot blot immunoassay. Antibodies to purified, recombinant HCV gene products were assayed with a semiautomated dot blot immunoassay (Abbott MATRIX; Abbott Laboratories), which includes an array of antigens spotted on nitrocellulose. This technology has been described in detail (5). The test panels consisted of the c100 antigen (purchased from Chiron Corp., Emeryville, Calif.), which is
expressed in yeast cells as a protein fused with human superoxide dismutase, and polypeptides corresponding to putative HCV NS4 (c100), NS3 (33c), and 5' structural (core) sequences (4, 8), which are expressed in *Escherichia coli* as fusions with CMP-KDO synthetase (2) and were supplied by the Molecular Biology Laboratory and the Rare Reagent Development Laboratory of the Abbott Diagnostics Division Hepatitis/AIDS Sector. The NS3 and NS4 polypeptides encompass unique highly immunoreactive HCV sequences, the antibody responses for which correlate well with viremia, as detected by the polymerase chain reaction (4a).

Each array also contained procedural controls (anti-human immunoglobulin G [IgG] and human IgG) to verify sample and reagent additions, as well as a negative control (casein) to adjust for nonspecific antibody binding to the nitrocellulose surface.

The prepared solid phase was incubated in a reaction cell with the test sample (diluted 1:100) for 1 h at 35°C and then sequentially for 30 min each at 35°C with biotin-labeled goat anti-human IgG (heavy and light chains), alkaline phosphatase-labeled rabbit antibody to biotin, and bromochloroindolyl phosphate. Upon completion of the incubation with the chromogen, the nitrocellulose surface was dried and the reflectance (660 nm) at defined locations within the array was determined to measure the extent of the individual reactions. Assay results are presented as the net reflectance density value (S/CO) for the test sample divided by a preassigned cutoff value (S/CO). Assay cutoff values were tested with various anti-HCV-positive and -negative specimens; the specificity of the test was also verified with a 50-member anti-HCV-negative specimen panel, for which the mean S/CO was at least 6 standard deviations from the cutoff value for each antigen. S/CO ratios of greater than 1.0 were considered to indicate reactivity with the specified HCV antigen.

**Evaluation of the dot blot immunoassay with serial dilutions of an anti-HCV-positive donor sample.** In Fig. 1, the performance of the dot blot immunoassay is compared with that of the licensed EIA for serial dilutions of an anti-HCV-positive donor sample which was obtained from the Sacramento Medical Center Blood Bank (10). The observed endpoint antibody titers were 1:32 and 1:64 for the c100 antigens from *E. coli* and yeast cells, respectively, and 1:128 for the NS3 and core antigens in the dot blot immunoassay. These data illustrate the comparable sensitivities of the dot blot immunoassay and the licensed EIA for antibodies to c100. Also, triplicate determinations in the dot blot immunoassay had a coefficient of variation of ≤15% for each of the reactive antigens. The overall coefficient of variation was also ≤15% (n = 30) for the reactivity of each of the two procedural controls present within the reaction cell. The presented results are representative of dilutions from 15 anti-HCV-positive donors.

**RESULTS**

**Characteristics of patient samples and frequency of anti-HCV seroconversion.** Fourteen patients with chronic transfusion-associated non-A, non-B hepatitis were assayed for serological markers of HCV infection. Eight recipients were monitored for 24 to 28 weeks, one was monitored for 50.7 weeks, and five were monitored for 3.1 to 10.0 years after surgery.

Presurgery samples were available for 12 patients. Ten of the presurgery samples were not reactive with any of the recombinant antigens, 1 was reactive with all antigens in the dot blot immunoassay (as well as the licensed EIA) and indicated prior HCV exposure, and 1 showed low levels of reactivity (S/CO, <2) only with the recombinant core and c100 (yeast) antigens; the last pattern did not provide convincing evidence of anti-HCV (Table 1). Donor samples implicated as a source of HCV were not available for testing by the methods described in the present study, although previously reported data demonstrated that approximately 85% of the recipients who developed non-A, non-B hepatitis were transfused with at least one anti-HCV (c100)-positive unit (1).
Seroconversion to anti-HCV was detected in all 14 patients by recombinant c100 and NS3 antigens and in 13 patients by the recombinant core antigen. All 14 were previously shown to seroconvert to anti-HCV by a commercially available screening test for antibodies to c100 (1). Passive transfer of antibodies from donors was also clearly evident in all but two patients (Table 1) and often was a dominant feature of the serology patterns. The population of patients showed various patterns of antibody responses to HCV infection detected by the recombinant c100, NS3, and core antigens (examples are shown in Fig. 2 to 4).

**Passive transfer of anti-HCV to core, NS3, and c100 antigens.** For 8 of the 14 patients, anti-HCV were detected by both c100 antigens in the dot blot immunoassay in the first postsurgery sample. Six of these patients had levels of reactivity greater than five times the assay cutoff. In all eight patients in whom anti-HCV were detected by c100 in the initial posttransfusion sample, reactivity levels declined to below the assay cutoff in subsequent samples. Similar patterns were detected by the licensed anti-HCV test for recombinant c100 protein. Passive transmission of anti-HCV from infected donor to recipient was previously detected by a radioimmunoassay with c100 antigen (1, 6).

Anti-HCV were detected by the recombinant core poly-peptide in 13 of the initial posttransfusion samples; S/CO ratios were >100 in 6 of these samples. Anti-HCV core reactivity declined in subsequent samples from 12 patients but remained above the cutoff in 9 of these patients. Antibody reactivity to the core antigen increased from the earliest reactive sample on in patient 6 (for whom the presurgery sample was reactive with all antigens; Table 1), suggesting that the antibodies to the core antigen that were present in this patient sample were due to a prior HCV infection as opposed to passive antibody transfer. In contrast, patient 9, who lacked detectable antibodies to all antigens in the initial samples, produced detectable antibodies to the core antigen at 4 weeks after transfusion (Fig. 3).

Passively transferred antibodies were detected by the NS3 antigen in 11 patients; S/CO ratios ranged from 3.2 to >100.
posttransfusion and 8 to 9 weeks after the onset of hepatitis (Table 3). However, anti-HCV were detected by NS3 and/or core proteins an average of 3.3 weeks and as many as 17.6 weeks before detection by the c100 protein. The mean window from the onset of hepatitis until the detection of anti-HCV by any of the recombinant antigens was 4.6 (range, –4.5 to 13.9) weeks. Antibodies detected by all recombinant HCV proteins persisted until the final sample that was obtained from all five of the long-term patients.

**Relationship among anti-HCV markers.** Correlation coefficients were calculated from a pairwise linear regression analysis of the reactivities of the HCV markers in the nine patients monitored for <12 months from transfusion. The highest quantitative correlation was evident between reactivity with the superoxide dismutase-c100 fusion protein expressed in yeast cells and the truncated c100 antigen expressed in *E. coli* (correlation coefficient, 0.988; *P* < 0.05). Antibodies to NS3 also demonstrated a weak correlation with antibodies to each of the c100 antigens (correlation coefficients, 0.091 to 0.102; *P* < 0.05), but antibodies to neither of the two putative nonstructural antigens demonstrated a significant quantitative correlation with antibodies to the core antigen (correlation coefficient, <0.01; *P* > 0.300). Samples from the long-term patients demonstrated a higher correlation among all recombinant antigens, presumably reflecting the preponderance of reactive samples in this group.

**DISCUSSION**

Whereas previous studies established HCV as the primary cause of transfusion-associated hepatitis, anti-HCV detected by the c100 antigen are likely to be absent during acute infection. Our data show that the addition of recombinant HCV core and NS3 polypeptides to anti-HCV assays decreases the likelihood that transfusion-associated acute HCV infection will not be detected by anti-HCV. Passive transfer of anti-HCV was primarily responsible for anti-HCV positivity at 0 to 14 weeks posttransfusion and was detected as high levels of reactivity (e.g., 100 times the assay cutoff) with core and/or NS3 antigens; passive anti-HCV detected by c100 were modest by comparison, as noted in previous studies of posttransfusion patients (1, 6, 9, 11, 13). The decline in the levels of reactivity of the passive antibodies was consistent with first-order decay of IgG (16). Also, the initial high levels of reactivity seen in recipients are consistent with our experience that anti-HCV titers often exceed 1,000 in donor samples (unpublished data) and that antibody titers detected by NS3 and core antigens commonly are higher than antibody titers detected by c100 (e.g., Fig. 1). In only 2 of the 12 cases in which passive antibodies were evident did the antibody titers decay below detectable levels for all antigens prior to the active response.

Detection of active production of anti-HCV was clearly more sensitive when core and NS3 antigens were incorporated into the assay (Tables 2 and 3). The interval to seroconversion was reduced from approximately 18 weeks posttransfusion (9 weeks after the onset of hepatitis) when c100 alone was used to detect anti-HCV to 14 weeks posttransfusion (4.6 weeks after onset) when an increase in reactivity to any of the three antigens was taken as evidence for anti-HCV seroconversion. By the latter criterion, seroconversion occurred concomitantly with or before the first ALT elevation in six cases. The earliest antibody response to HCV was detected most frequently by the core antigen (earliest marker or coincident with the earliest marker in 10

### TABLE 2. Intervals between transfusion and anti-HCV seroconversion

<table>
<thead>
<tr>
<th>Patient</th>
<th>Onset of hepatitis (weeks)</th>
<th>Wk posttransfusion at which anti-HCV was detected&lt;sup&gt;a&lt;/sup&gt; by EIA</th>
<th>Wk posttransfusion at which the following was detected by dot blot immunoassay&lt;sup&gt;b&lt;/sup&gt;:</th>
<th>Yeast cells</th>
<th>E. coli cells</th>
<th>NS3</th>
<th>Core</th>
<th>Any</th>
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<tr>
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<td>14</td>
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<td>18.1</td>
<td>18.1</td>
<td>18.1</td>
<td>24.6</td>
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</table>

<sup>a</sup> Seroconversion was defined as the point at which reactivity with a specific antigen increased over that in the preceding bleed.

<sup>b</sup> The onset of hepatitis was defined as the first date on which ALT levels exceeded 2.5 times the upper limit of normal.

For all 11 patients, reactivity declined in subsequent samples and decreased to S/CO ratios of <1.0 in 7 patients.

**Intervals to active anti-HCV responses.** The intervals from the date of transfusion to anti-HCV seroconversion detected by each recombinant antigen are summarized in Table 2. Active antibody production was defined as the point at which reactivity with a specific antigen increased over that in the preceding bleed. Thus, for transcription recipients, high levels of passively acquired antibodies may mask the initial rise in the anti-HCV titer. Anti-HCV were detected by the core polypeptide as the earliest marker (n = 6) or coincident with the earliest marker (n = 4) in most cases. Detection by NS3 as the earliest marker was evident in one case and coincident detection as the earliest marker was evident in five cases; detection by c100 as the earliest marker and coincident detection as the earliest marker were evident in two and three cases, respectively.

The mean intervals to seroconversion detected by the individual antigens were similar—approximately 18 weeks

### TABLE 3. Mean intervals to the appearance of antibodies to different recombinant HCV proteins

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mean interval (range) (wk) between seroconversion and posttransfusion</th>
<th>Onset of hepatitis</th>
</tr>
</thead>
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<tr>
<td>c100</td>
<td>18.3 (11.3–36.9)</td>
<td>9.1 (0–28.6)</td>
</tr>
<tr>
<td>Yeast cells</td>
<td>18.2 (11.3–36.9)</td>
<td>9.0 (0–28.6)</td>
</tr>
<tr>
<td>E. coli cells</td>
<td>17.2 (10.9–24.6)</td>
<td>7.9 (2.4–13.9)</td>
</tr>
<tr>
<td>NS3</td>
<td>17.2 (10.9–24.6)</td>
<td>7.9 (2.4–13.9)</td>
</tr>
<tr>
<td>Core</td>
<td>18.0 (3.6–50.7)</td>
<td>9.2 (4.5–42.4)</td>
</tr>
<tr>
<td>Any</td>
<td>13.8 (3.6–22.0)</td>
<td>4.6 (4.5–13.9)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Patient 6 was excluded because of evidence of HCV exposure prior to transfusion.
patients), although a shorter mean seroconversion interval was not detected for this antigen. A significant correlation was evident between the antibody responses detected by the NS3 and c100 antigens, as opposed to the responses observed when either of these putative HCV epitopes was compared with the core epitope. However, the pattern for patient 5 (Fig. 4) suggests that neither NS3 nor the core nor a combination of both obviates the need for c100 antigen in anti-HCV assays.

The finding of passive transfer of antibodies directed to HCV nonstructural and core epitopes illustrates that the presence of these antibodies does not prevent infection by the virus. It is not known whether HCV core polyepitopes circulate as an immune complex or are sequestered in viral particles. In hepatitis B carriers, core antigen contained within Dane particles and high serum titers of antibodies to this structural antigen are known to occur. Additional studies are required to determine whether antibodies directed to other HCV structural components, e.g., the envelope protein(s), confer protection against the virus. However, our present understanding of HCV serology points out that individuals who possess anti-HCV are potentially infectious (7, 14). This notion is supported by reports that >37% of donors positive for antibodies to c100 have detectable HCV RNA (15) and by a demonstration of transmission of infection to chimpanzees from antibody-positive serum (1). However, documentation that anti-HCV (c100) can persist for years after the loss of RNA and, presumably, the resolution of infection (12) demonstrates the limitation of the diagnostic value of the antibody assay.

The data reported in this study indicate that non-A, non-B hepatitis patient samples that are negative for anti-HCV in licensed tests may be positive for anti-HCV by NS3 and core antigens and thereby reveal HCV infection. The newer screening assays which are under development include these additional HCV epitopes and should substantially increase the sensitivity of assays for the detection of HCV exposure. Implementation of these assays should further reduce the risk of transfusion-associated hepatitis and will provide a clearer understanding of the epidemiology of community-acquired non-A, non-B hepatitis.

Different assay formats may further improve the sensitivity of anti-HCV detection. In this regard, HCV RNA has been detected by the polymerase chain reaction as early as 3 days postinfection of chimpanzees and well before the peak elevation of ALT (12). Also, we are investigating whether the detection of the IgM class of anti-HCV may differentiate acute infection from either passive antibody transmission or prior infection and recovery (12a).

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REFERENCES


