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Detection of acute hepatitis C virus infection by ELISA using a synthetic peptide comprising a structural epitope

(early diagnosis/blood screening)

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ABSTRACT **An enzyme-linked immunosorbent assay (ELISA) was developed by using a synthetic polypeptide (SP) whose sequence was derived from the structural region of hepatitis C virus (HCV). Results of several coded panels of sera obtained from volunteer blood donors and patients with apparent non-A, non-B hepatitis and/or hepatitis B virus used in this ELISA were compared with those of a commercially available first-generation C-100 ELISA (using nonstructural HCV antigens), an experimental second-generation C-200/C-22 ELISA (using both structural and nonstructural HCV antigens), and recombinant immunoblot assays RIBA-I and RIBA-II. In the majority of cases, the results obtained with the HCV-SP ELISA correlated well with those obtained by RIBA-II and C-200/C-22 ELISA. In contrast, many samples that were repeatedly reactive in the C-100 ELISA results were nonreactive with RIBA and HCV-SP ELISA. In addition, HCV-SP detected HCV-specific antibody that appeared within a month of infection and coincided with the earliest increase in alanine aminotransferase. In summary, we have developed an ELISA based on a structural HCV synthetic polypeptide, HCV-SP, that has high specificity and sensitivity and is capable of detecting specific antibodies in the acute phase of HCV infection.**

There are several assays for the serodiagnosis of hepatitis C virus (HCV) infection. The "first-generation" enzyme-linked immunosorbent assay (ELISA) and the first recombinant immunoblot assay (RIBA-I) are dependent on detecting antibodies to the C100 fragment, a product of the third and fourth nonstructural genes of HCV (1–3). More recently, second-generation assays have been developed which, besides utilizing the C100 region, also detect antibodies to epitopes on the C22 region expressed by the capsid gene and the C33 region from the third nonstructural gene (4). While these second-generation assays appear to be more sensitive and specific than the first-generation assays, there still are a number of false positive and false negative test results, especially in volunteer blood donors and patients with acute HCV infection, respectively (5–8). In addition to these serological tests, the polymerase chain reaction (PCR) has been used to detect HCV RNA in serum samples (9–13). However, the RIBA-I, RIBA-II, and PCR tests are not practical substitutes for an ELISA as a routine assay in blood banks to screen millions of donor samples. Our goal has been to develop a sensitive and specific ELISA for the detection of HCV antibodies at an early phase of HCV infection. We achieved this objective by analyzing the sequence of the putative HCV polyprotein in order to determine the most probable immunogenic regions. Among several HCV polypeptides that were designed, HCV-SP was found to be the most specific and sensitive antigen. In addition, on conjugation of HCV-SP to a carrier protein and injection in rabbits, we obtained significantly high-titer antibodies (1:1,000,000) to HCV-SP (14). This antibody enabled us to detect HCV-specific antigens in semen from non-A, non-B (NANB) hepatitis patients (14). This indicates that the structural region of the putative HCV polyprotein encompassing the HCV-SP sequence is an immunodominant epitope. Several coded panels of sera have been tested with HCV-SP in an ELISA. Comparison of the results with those from C-100 ELISA, C-200/C-22 ELISA, RIBA-I, RIBA-II, and a neutralization test indicates that the HCV-SP ELISA may be more sensitive and specific than the other tests.

METHODS

Coded Panels of Sera from Patients and Healthy Blood Donors. Five coded panels were used in this study. Coded panels of 44 (panel 1) and 115 (panel 2) sera, representing samples from patients with clinical evidence of NANB (type C) viral hepatitis or volunteer blood donors with or without evidence of NANB (type C) viral hepatitis, and 1076 (panel 3) sera from healthy volunteer blood donors were first tested with a licensed HCV C-100 ELISA (Ortho Diagnostics). Subsequent testing involved a RIBA-I test (Chiron); a RIBA-II test (Chiron); a second-generation ELISA, HCV C-200/C-22 (Ortho Diagnostics); and/or a C-100 neutralization assay (Abbott).

Panel 4 consisted of 94 serum samples obtained under code from the Transfusion Transmitted Virus Study (TTVS) Repository at the National Heart, Lung, and Blood Institute (NHLBI), Bethesda, MD (15). These samples were selected from among those collected from blood-transfusion recipients at 2-week intervals for 3 months, 3-week intervals for the next 3 months, and then at 10 months. A serum sample was also obtained, in most cases, on day 7 after the first transfusion (recipients) or after surgery (controls) (15). After reporting our results on this coded panel, we learned that this panel consisted of 18 transfusion recipients and 10 control (hospitalized, but not transfused) subjects. The transfusion recipients had been previously diagnosed as cases of acute or chronic hepatitis B and/or NANB hepatitis. Panel 5 consisted of 79 additional serum samples also obtained under code from the TTVS Repository at NHLBI. These serum samples were provided as a result of successful analysis of the previous panel; they included 5 serum samples from a control and 74 serum samples from six transfusion recipients

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Abbreviations: ALT, alanine aminotransferase; HCV, hepatitis C virus; NANB, non-A, non-B; NHLBI, National Heart, Lung, and Blood Institute; TTVS, Transfusion Transmitted Virus Study; RIBA, recombinant immunoblot assay.

who had been previously diagnosed as cases of NANB hepatitis and confirmed to be of type C by three tests: C-100 ELISA, RIBA-II, and HCV-SP ELISA (see Table 4).

HCV-SP Synthesis. The polypeptide HCV-SP has the following sequence: NH_2-Cys-Lys-Pro-Gln-Arg-Lys-Thr-Lys-Arg-Asn-Thr-Asn-Arg-Arg-Pro-Gln-Tyr-COOH. It was synthesized by a solid phase strategy on an Applied Biosystems model 430A peptide synthesizer according to the manufacturer's instructions. The polypeptide was subsequently purified to >95% by reversed-phase HPLC. The authenticity of HCV-SP was determined by amino acid analysis with an Applied Biosystems 420A automated hydrolyzer and by molecular weight determination with a Bio-Ion mass analyzer.

HCV-SP ELISA. HCV-SP polypeptide was used in an ELISA to detect antibodies to HCV in serum samples. Stock solutions (1 mg/ml) of HCV-SP were prepared and further diluted 1:100 in phosphate-buffered saline (137 mM NaCl/10 mM phosphate, pH 7.2) prior to coating 96-well Microtiter plates (Dynatech Immunolon, U-shaped wells); 100 ng of the polypeptide was adsorbed to each well. Patient or normal donor serum (20 μl) was diluted with 80 μl of PBS/0.1% Tween 20 and applied to each well. The plate was covered and incubated overnight on a shaker platform at room temperature. Samples were removed and the wells were washed three times with 100 μl of PBS/Tween. A 1:1 mixture of rabbit anti-human IgG and rabbit anti-human IgM conjugated to horseradish peroxidase was diluted 1:200 in PBS/Tween. One hundred microliters of the diluted rabbit anti-human IgG/IgM was added to each well. The plate was covered and incubated for 3–6 hr on a shaker platform at room temperature. Rabbit anti-human IgG/IgM was removed and the plate was washed three times with 100 μl of PBS/Tween. One hundred microliters of substrate solution was added to each well. Substrate solution was prepared by dissolving four 1,2-phenylenediamine dihydrochloride tablets (2 mg per tablet) in 12 ml of 0.1 M citric acid/phosphate, pH 5.0, and then adding 5 μl of 30% H_2O_2. Plates were covered and incubated for about 5 min at room temperature. Fifty microliters of 1 M H_2SO_4 was added to each well. Absorbance of yellow end-product was read by a microwell plate reader at 450 nm/492 nm. The cutoff value was obtained by multiplying the mean absorbance value of ten individual negative controls by a factor of 1.5.

To shorten the duration of the HCV-SP ELISA, modifications included a 1-hr sample incubation at 37°C, 1-hr conjugate incubation at 37°C with goat anti-human immunoglobulin (1:3500 dilution), and a 15-min substrate incubation at room temperature. The 1076 healthy blood donor samples were assayed with the modified procedure.

RESULTS

Serodiagnosis of HCV in Five Coded Panels by HCV-SP ELISA. The results of the first coded panel (44 sera) are shown in Table 1 and confirm that 8 sera reactive in the C-100 ELISA were reactive in all the other tests, including HCV-SP ELISA. Conversely, 14 sera nonreactive with C-100 were nonreactive with HCV-SP. A total of 18 sera reactive in the licensed C-100 ELISA were nonreactive in the HCV-SP ELISA and were found to be nonreactive or indeterminate by either RIBA-I or RIBA-II. This probably reflects the rate of false positive samples (40.9%) that was found in this coded panel with the C-100 ELISA. In addition, 2 serum samples from a patient who had clinical NANB hepatitis tested negative with the C-100 ELISA but were reactive with HCV-SP and subsequently determined to be reactive by both RIBA-I and RIBA-II. This coded panel indicated that the ELISA utilizing HCV-SP was in general agreement with both RIBA results but appeared to be more sensitive and specific than the C-100 ELISA.

Table 1. Comparative testing of coded panel 1 (44 serum samples)

No. of samples	C-100 ELISA	RIBA-I	RIBA-II	HCV neutralization	HCV-SP ELISA
10	10r	8r (2nt)	10r	8y (2nt)	10r
6	6r	6i	6nr	3y	6nr
12	12r	12nr	9nr, 3i	6y	12nr
14	14nr	nt	nt	nt	14nr
2*	2nr	2r	2r	nt	2r

r, Reactive; nr, nonreactive; nt, not tested; y, positive; i, indeterminate (only one band out of two was reactive with RIBA-I or one band out of four was reactive in RIBA-II).
*Clinically diagnosed NANBH patient, nonreactive with C-100 ELISA.

Table 2. Comparative testing of coded panel 2 (115 serum samples reactive in the C-100 ELISA)

No. of samples	HCV-SP ELISA	RIBA-II	C-200/C-22 ELISA
52	52r	49r, 3i	52r
40	40nr	40nr	40nr
18	18nr	11nr, 7i	18r
2	2nr*	lr, 1i	2nr
1	1r†	1nr	1nr
2	2nr‡	2r	2r

r, Reactive; nr, nonreactive; i, indeterminate (only one band out of four was reactive).
*RIBA-II results for these two samples were as follows: sample HCV055, indeterminate (reactive only with the C-100 band; nonreactive with the 5-1-1, C33c, and C22 bands); sample HCV203, reactive (reactive with the 5-1-1 and C-100 bands; nonreactive with the C33c and C22 bands).
†Sample HCV225; HCV-SP ELISA sample/cutoff ratio was 1.425.
‡These samples (HCV214 and HCV234) were reactive upon retesting with HCV-SP ELISA.

Panel 2 (115 sera reactive by the C-100 ELISA) was used to compare the ELISA utilizing HCV-SP with an experimental ELISA using C-200/C-22 (second-generation assay of Chiron/Ortho). Fifty-two sera (Table 2) that were reactive with C-200/C-22 and HCV-SP were verified to be reactive in RIBA-II, with the exception of 3 sera that were indeterminate (one single band was reactive on RIBA-II). Forty sera nonreactive in C-200/C-22 and HCV-SP ELISAs were also found to be nonreactive in RIBA-II. Of 2 sera (Table 2, 2nr*) nonreactive with C-200/C-22 and HCV-SP, 1 was reactive and 1 was indeterminate in RIBA-II. One serum sample (Table 2, 1r†) that was reactive with HCV-SP was nonreactive in both the C-200/C-22 ELISA and the RIBA-II test. On the other hand, two serum samples (Table 2, 2nr‡) that were reactive in both C-200/C-22 ELISA and RIBA-II tested negative with HCV-SP. Both of these samples were reactive upon retesting in the HCV-SP ELISA. Finally, 18 sera that were reactive with C-200/C-22 tested negative with HCV-SP; 11 were negative with RIBA-II and 7 were indeterminate. While the majority (11/18, 61%) were false positive by the C-200/C-22 ELISA, the 7 (39%) that were indeterminate were reactive with one nonstructural antigen (C-100) of RIBA II and therefore may be false positive by C-200/C-22 ELISA.

Panel 3 (1076 serum samples from healthy volunteer blood donors) was tested with HCV-SP (Table 3). Eleven samples

Table 3. Comparative testing of healthy blood donors (panel 3)

No. of donors	HCV-SP repeat reactive	RIBA-II	C-200/C-22 ELISA
1076	11	1	1

Only the 11 samples reactive in HCV-SP ELISA were tested by RIBA-II and C-200/C-22 ELISA.

Table 4. HCV-SP ELISA, C-100 ELISA, and RIBA-II testing of transfusion recipients and control subjects from TTVS–NHLBI (panel 4)

Group and diagnosis	No. of subjects	HCV-SP ELISA	C-100 ELISA	RIBA-II
Control				
No NANB	6	nr	nr	nr
NANB	3	nr	nr	nr
NANB	1	nr	r	r
Recipient				
HBV	2	nr	nr	nr
	1	nr	r	nr
	1	nr	r	i
HBV and NANB	2	r	r	r
NANB (type C)	8	r	r	r
NANB (type C)	1	r	nr	r
NANB (non C)	2	nr	nr	nr
	1	nr	nr	i

Controls were hospitalized patients who did not receive a blood transfusion and were diagnosed as having or not having viral hepatitis. Recipients were patients who received one or more blood transfusions and were diagnosed as having viral hepatitis. nr, Nonreactive; r, reactive; i, indeterminate; HBV, hepatitis B virus.

reactive with HCV-SP were further tested with RIBA-II and C-200/C-22 ELISA. One of the 11 sera was reactive by all three tests; 10 sera were repeat positives only by HCV-SP. At this stage, we do not know whether this reflects the rate of false positive tests (<1%) in HCV-SP ELISA or whether this particular ELISA can detect antibody to HCV that is not detected by the other tests.

Panel 4 (94 sera) obtained from serial bleeds and provided by the TTVS Repository at NHLBI (15) was tested with HCV-SP ELISA, HCV C-100 ELISA, and RIBA-II. The results on each of these samples were matched to the various transfused patients or control subjects in this panel (Table 4). Six control (nontransfused) subjects without evidence of hepatitis tested negative by all three assays. Four subjects (nontransfused) with a clinical diagnosis of NANB hepatitis tested negative by HCV-SP ELISA; three of these four subjects also tested negative by the two other tests. Of six transfusion recipients with clinical and laboratory-diagnosed hepatitis B, the two also diagnosed with NANBH tested positive for HCV by all three tests. The remaining four recipients with hepatitis B alone tested negative with HCV-SP ELISA. Nine of nine recipients with diagnosed NANB hepatitis who tested positive with HCV-SP were reactive on RIBA-II. In contrast, three other recipients with NANB hepatitis tested negative by HCV-SP ELISA and HCV C-100 ELISA; two of the three recipients were negative by RIBA-II, and one was indeterminate.

To document the time of appearance of HCV-specific antibody responses in certain recipients from the above TTVS–NHLBI study who developed NANB hepatitis, we used the HCV-SP ELISA to test a coded panel of serial serum samples from six recipients and one control subject (panel 5) and correlated these results with the alanine aminotransferase (ALT) levels. HCV-specific antibody was detected by HCV-SP ELISA in all six recipients and its appearance was found to coincide very well with the earliest elevation of ALT levels in five out of the six recipients; with the exception of recipient 10805, the other five recipients on average had

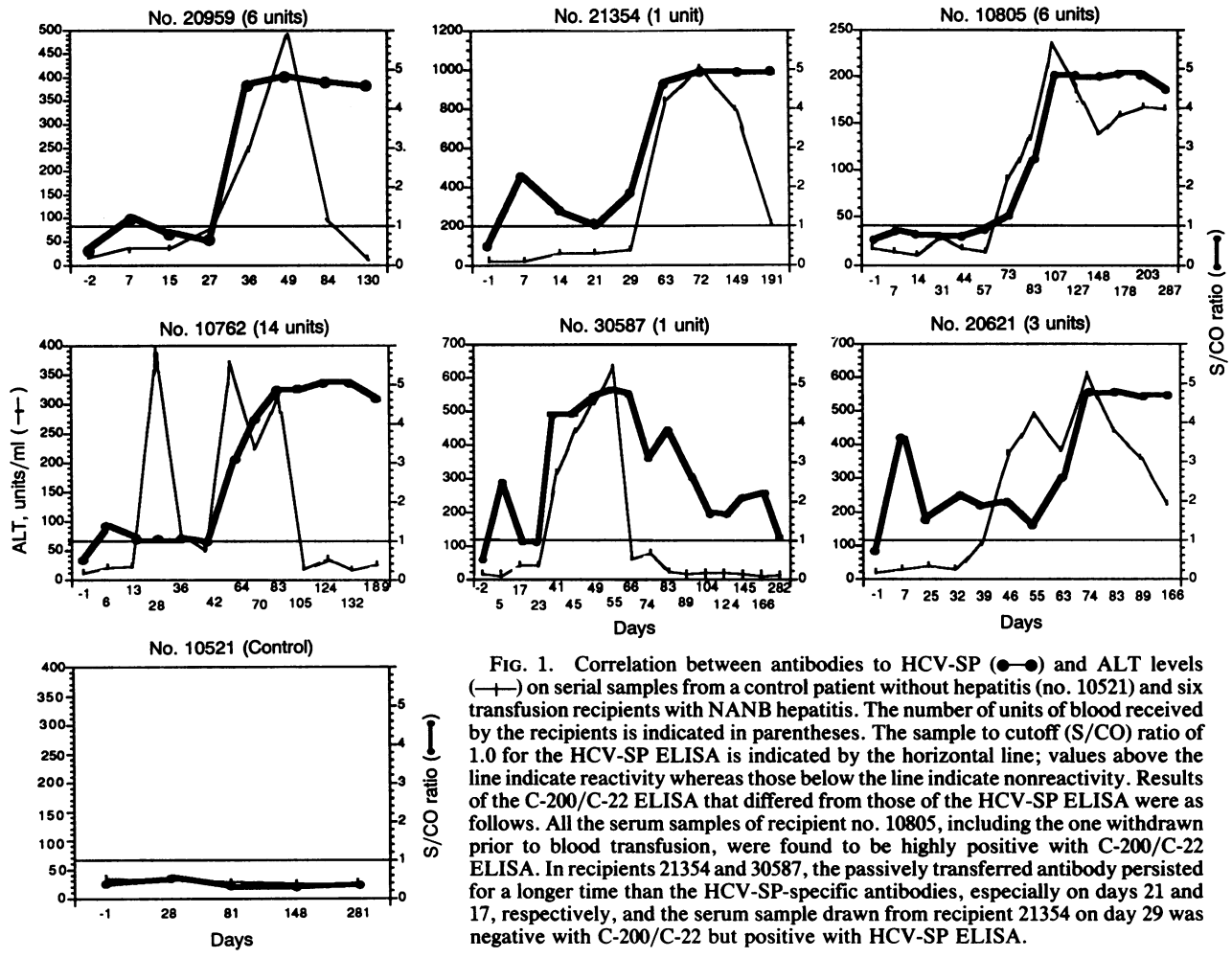

FIG. 1. Correlation between antibodies to HCV-SP (●—●) and ALT levels (—+—) on serial samples from a control patient without hepatitis (no. 10521) and six transfusion recipients with NANB hepatitis. The number of units of blood received by the recipients is indicated in parentheses. The sample to cutoff (S/CO) ratio of 1.0 for the HCV-SP ELISA is indicated by the horizontal line; values above the line indicate reactivity whereas those below the line indicate nonreactivity. Results of the C-200/C-22 ELISA that differed from those of the HCV-SP ELISA were as follows. All the serum samples of recipient no. 10805, including the one withdrawn prior to blood transfusion, were found to be highly positive with C-200/C-22 ELISA. In recipients 21354 and 30587, the passively transferred antibody persisted for a longer time than the HCV-SP-specific antibodies, especially on days 21 and 17, respectively, and the serum sample drawn from recipient 21354 on day 29 was negative with C-200/C-22 but positive with HCV-SP ELISA.

detectable HCV-specific antibody within a month of transfusion transmitted infection (Fig. 1). Significant levels of HCV-specific antibody, probably passively transferred from a donor, were detected during the first week after transfusion for three recipients (nos. 30587, 21354, and 20621) with marginal presence in two other recipients (nos. 20959 and 10762). This same coded panel was tested subsequently under code with the C-200/C-22 ELISA at the Sacramento Medical Foundation Blood Center. With the exception of a few test results, the data coincided with the HCV-SP-specific antibody response shown in Fig. 1.

DISCUSSION

We have identified an epitope, HCV-SP, in the structural region of the putative polyprotein of HCV that is capable of detecting circulating antibodies to HCV. Anti-HCV specific antibodies detected by HCV-SP ELISA correlate well with the presence of apparently true positive anti-C100 or anti-C-200/C-22 antibody as defined by positive ELISA and RIBA tests, respectively. Furthermore, HCV-SP ELISA detected antibodies in two sera that were nonreactive with the licensed C-100 ELISA (Table 1, 2*) and in another sample, which was nonreactive with both C-200/C-22 ELISA and RIBA-II (Table 2, 1r†). Testing of additional serum samples collected subsequently to the sample already assayed from the latter patient may confirm whether or not this particular sample was a false positive by HCV-SP ELISA or whether this newly developed test is capable of detecting HCV antibody at an earlier stage of infection. Two sera (Table 2, 2nr‡) that were nonreactive when tested under code with HCV-SP were reactive upon retesting in duplicate with the same test. Finally, of those sera with reactivity in the C-100 ELISA and in the newer C-200/C-22 ELISA, those which did not appear to be specific when evaluated by confirmatory tests were usually not reactive in the HCV-SP assay. Recent studies showed that the use of synthetic peptides whose sequences were derived from the HCV capsid protein led to improvements in the serodiagnosis of HCV infection (16, 17). The HCV-SP assay for HCV antibodies may also be more sensitive and specific than the newer C-200/C-22 ELISA, especially when confirmatory testing (RIBA-II) defines a probable true positive for antibody to HCV compared with a false negative result (Table 2).

In our final assessment of the HCV-SP ELISA, we evaluated a coded panel (panel 4) provided to us by the TTVS–NHLBI Repository Use Committee. In summary, 11 of 11 (100%) blood-transfusion recipients with NANB hepatitis whose sera were reactive with RIBA-II were also positive with HCV-SP ELISA. On the other hand, one nontransfused control NANB subject whose serum was reactive with both C-100 ELISA and RIBA-II was negative with HCV-SP ELISA. Using serial specimens from patients with transfusion-associated NANBH (panel 5), we can infer that HCV-specific antibodies are detected by HCV-SP ELISA as early as 1 month after the initial transfusion-initiated HCV infection and remain at a relatively high level for several months (Fig. 1). Moreover, the appearance of anti-HCV-SP coincided with the first elevation of ALT level in five of six recipients, making it a marker for detecting acute HCV infection. Thus, a single, short polypeptide derived from the structural region of HCV may be adequate for reliable, early detection of HCV infection. Addition of antigens from the nonstructural region of HCV may not improve the sensitivity of the second-generation ELISA; instead, their presence might cause a significant decrease in the specificity of that assay.

Note Added in Proof. In a recent report (18), a larger polypeptide, designated CP10 and derived entirely from the structural region of the HCV genome, was used in an ELISA to detect HCV-specific antibodies. Unlike HCV-SP, CP10 lacks a cysteine at the NH_2 terminus and a tyrosine at the COOH terminus. Fifteen of 20 patients (75%) at the onset of acute NANB hepatitis were reactive for anti-CP10, whereas 9 of these patients (45%) were reactive for anti-C100-3. In contrast, by using HCV-SP ELISA we were able to detect specific antibodies in 6 of 6 (100%) recipients at the onset of acute NANB hepatitis and also were able to detect passively transferred antibodies from the donors (Fig. 1). Moreover, our results were confirmed by the C-200/C-22 ELISA, which was recently approved by the Food and Drug Administration (FDA) and which has been found to be more specific than the previously FDA-approved C-100 ELISA.

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1. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989) *Science* **244**, 359–362.
2. Kuo, G., Choo, Q.-L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Mitamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W. S., Kuo, C., Berger, K., Shuster, J. R., Overby, L. R., Bradley, D. W. & Houghton, M. (1989) *Science* **244**, 362–364.
3. Choo, Q.-L., Weiner, A. J., Overby, L. R., Kuo, G. & Houghton, M. (1990) *Br. Med. Bull.* **46**, 423–441.
4. van der Poel, C. L., Cuypers, H. T. M., Reesink, H. W., Weiner, A. J., Quan, S., di Nello, R., van Boven, J. J. P., Winkel, I., Mulder-Folkerts, D., Exel-Oehlers, P. J., Schaasberg, W., Leentvaar-Kuypers, A., Polito, A., Houghton, M. & Lelie, P. N. (1991) *Lancet* **337**, 317–319.
5. van der Poel, C. L., Reesink, H. W., Schaasberg, W., Leentvaar-Kuypers, A., Bakker, E., Exel-Oehlers, P. J. & Lelie, P. N. (1990) *Lancet* **335**, 558–560.
6. Ebeling, F., Naukkarinen, R. & Leikola, J. (1990) *Lancet* **335**, 982–983.
7. Zuck, T. F., Rose, G. A., Dumaswala, U. J. & Geer, N. J. (1990) *Transfusion* **30**, 759–761.
8. Cotton, P. (1991) *J. Am. Med. Assoc.* **265**, 312.
9. Garson, J. A., Tedder, R. S., Briggs, M., Tuke, P., Glazebrook, J. A., Trute, A., Parker, D., Barbara, J. A. J., Contreras, M. & Aloysius, S. (1990) *Lancet* **335**, 1419–1422.
10. Kubo, Y., Takeuchi, K., Boonmar, S., Katayama, T., Choo, Q.-L., Kuo, G., Weiner, A. J., Bradley, D. W., Houghton, M., Saito, I. & Miyamura, T. (1989) *Nucleic Acids Res.* **17**, 10367–10372.
11. Weiner, A. J., Kuo, G., Bradley, D. W., Bonino, F., Saracco, G., Lee, C., Rosenblatt, J., Choo, Q.-L. & Houghton, M. (1990) *Lancet* **335**, 1–3.
12. Ulrich, P. P., Romeo, J. M., Lane, P. K., Kelly, I., Daniel, L. J. & Vyas, G. (1990) *J. Clin. Invest.* **86**, 1609–1614.
13. Okamoto, H., Okada, S., Sugiyama, Y., Yotsumoto, S., Tanaka, T., Yoshizawa, H., Tsuda, F., Miyakawa, Y. & Mayumi, M. (1990) *Jpn. J. Exp. Med.* **60**, 167–177.
14. Kotwal, G. J., Rustgi, V. K. & Baroudy, B. (1992) *Digest. Dis. Sci.* **37**, in press.
15. Hollinger, F. B., Mosley, J. W., Szmuness, W., Aach, R. D., Melnick, J. L., Afifi, A., Stevens, C. E. & Kahn, R. A. (1982) in *Viral Hepatitis: 1981 International Symposium*, ed. Szmuness, W., Alter, H. J. & Maynard, J. E. (Franklin Inst., Philadelphia), pp. 361–376.
16. Hosein, B., Fang, C. T., Popovsky, M. A., Ye, J., Zhang, M. & Wang, C. Y. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3647–3651.
17. Okamoto, H., Munekata, F., Tsuda, F., Takahashi, K., Yotsumoto, S., Tanaka, T., Tachibana, K., Akahane, Y., Sugai, Y., Mitakawa, Y. & Mayumi, M. (1990) *Jpn. J. Exp. Med.* **60**, 223–233.
18. Okamoto, H., Tsuda, F., Machida, A., Munekata, E., Akahane, Y., Sugai, Y., Mashiko, K., Mitsui, T., Tanaka, T., Miyakawa, Y. & Mayumi, M. (1992) *Hepatology* **15**, 180–186.