Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 983–987, February 1990
Medical Sciences

Detection of antibody against antigen expressed by molecularly cloned hepatitis C virus cDNA: Application to diagnosis and blood screening for posttransfusion hepatitis

(non-A, non-B hepatitis/radioimmunoassay/seroepidemiology/hepatitis C virus carrier)

TATSUO MIYAMURA*, IZUMU SAITO*, TOHRU KATAYAMA†, SHU KIKUCHI‡, AKIRA TATEDA‡§, MICHAEL HOUGHTON¶, QUI-LIM CHOO¶, AND GEORGE KUO¶

*Department of Enteroviruses, National Institute of Health, 2-10-35, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan; †Tokyo National Chest Hospital, Kiyose, Tokyo 204, Japan; ‡Sendai National Hospital, Miyagino, Sendai 983, Japan; and ¶Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608

Communicated by William J. Rutter, October 6, 1989

ABSTRACT **A cDNA clone has been derived from the plasma of a chimpanzee with chronic non-A, non-B viral hepatitis (NANBH). We have assayed for antibodies reacting with the encoded antigen in sera from posttransfusion hepatitis patients (643 samples from 23 patients) and their corresponding donors collected during the past 10 years in Japan. The antibody was detected in 15 out of 17 (88.2%) posttransfusion NANBH (PT-NANBH) patients whose sera over time displayed multiple alanine aminotransferase (ALT) peaks. In general, the antibody was detected after several peaks of serum ALT elevations and, once detected, it persisted for years. In contrast to the patients of chronic hepatitis, the antibody was barely detected in patients with a single episode of ALT elevation (1 out of 6). Of the 15 well-defined cases of PT-NANBH that showed multiple ALT peaks and hepatitis C virus seroconversions, 11 (73.3%) were shown to be transfused with at least one unit of blood positive for the antibody. The retrospective analysis showed that all tested donor blood found to be positive for the antibody had been transfused to recipients who afterwards developed NANBH. These data strongly suggest that the cloned cDNA originated from an etiological agent of NANBH termed the hepatitis C virus. Furthermore, the present study demonstrates that had the screening been done with the anti-hepatitis C virus assay, 11 out of 17 (64.7%) cases of chronic PT-NANBH and 1 out of 6 (16.6%) acute PT-NANBH would have been prevented. The antibody assay thus can be used for diagnosis and blood screening for PT-NANBH.**

Even though donor blood is routinely screened for the hepatitis B virus (HBV) with sensitive assays, about 10% of blood transfusion recipients still develop viral hepatitis (refs. 1 and 2; T.K., unpublished data). Non-A, non-B hepatitis (NANBH) virus(es) is responsible for over 90% of the posttransfusion hepatitis (PTH) cases and causes a serious health problem throughout the world. NANBH is much more likely to become chronic than HBV-induced hepatitis and often progresses to chronic liver disease, including liver cirrhosis (3, 4). A role for NANBH in the development of hepatocellular carcinoma has also been suggested (5, 6).

The genome of a NANBH virus, termed hepatitis C virus (HCV), was cloned recently and shown to contain a positive-stranded RNA molecule (7). An assay for circulating viral antibodies was developed by using an HCV antigen purified from recombinant yeast clones to capture reactive antibodies. Results obtained using this assay indicated that HCV is a major cause of transfusion-associated NANBH throughout the world as well as community-acquired NANBH in which no previous parenteral exposure to the virus could be identified (8).

In this study, we assayed for HCV antibody in well-pedigreed transfusion-associated NANBH patients in Japan. The serum samples were from patients diagnosed on the basis of clinical presentation in the absence of markers indicating infection with HBV and hepatitis A virus (HAV). The recipient sera were a series of sequential samples collected after the onset of disease as well as pretransfusion sera. We also assayed sera from the corresponding blood donors.

METHODS

Patients and Serum Selection. Serum samples were obtained from 14 patients in the Surgery Unit at the Tokyo National Chest Hospital and from 9 patients in the Sendai National Hospital. These patients were diagnosed as having posttransfusion NANBH (PT-NANBH) by one of the authors and were followed at regular intervals in outpatient clinics. All of them fulfilled the following criteria proposed by the Japanese Society of Gastroenterology in 1985: (*i*) liver enzymes were normal before the transfusion; (*ii*) the alanine aminotransferase (ALT) values started to increase with an incubation period of at least 2 weeks posttransfusion; (*iii*) the ALT peak values were >5 times the upper normal range; (*iv*) hepatic failures directly due to the surgical operation, drug, or anesthesia were excluded; (*v*) at least 6 months of follow-up blood tests were performed; and finally (*vi*) known viral infections were ruled out. The involvement of HAV infection was excluded epidemiologically by the clinical course and decisively by the absence of anti-HAV by a HAVAB radioimmunoassay kit (Abbott). We also excluded patients positive for hepatitis B surface antigen (reversed passive hemagglutination test) or antibody to hepatitis B surface antigen (passive hemagglutination test) in this study. The authors can provide detailed clinical, biochemical, and histopathological data on each of the patients upon request. We also tested 802 serum samples from donors whose blood had been transfused into the 23 patients described above.

In addition, a series of sera from three equivocal NANBH patients and from six patients with abnormal ALT values were examined. Although the former group developed hepatitis after the transfusion, either ALT elevations were detected too early or their ALT values were not high enough to satisfy the criteria for definite PT-NANBH. The latter six patients had surgery and showed transient ALT elevations but received no blood transfusions. Sera from sporadic

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NANBH, non-A, non-B hepatitis; PTH, posttransfusion hepatitis; PT-NANBH, posttransfusion NANBH; ALT, alanine aminotransferase; HCV, hepatitis C virus; HBV, hepatitis B virus; HAV, hepatitis A virus.
§Deceased, Sept. 27, 1986.

hepatitis A patients (during convalescence) and from chronic hepatitis B patients were also tested.

We also assayed sera from 800 "normal" donors. The absence of infectious NANBH virus in those donors was retrospectively proven by the fact that none of the recipients had developed PTH, as determined both clinically and by ALT monitoring.

Antibody Assay. A cDNA fragment from the HCV genome was ligated downstream of the human superoxide dismutase gene (9). The fusion polypeptide (C100-3) comprising 363 amino acids of HCV was efficiently expressed in *Saccharomyces cerevisiae* (8). After solubilization and extensive purification through successive Q-Sepharose and Sephacryl S-300 (Pharmacia) columns, the antigen C100-3 was coated onto microtiter plates to detect circulating anti-HCV antibodies in serum samples. The captured antibody was quantitated by binding of ^{125}I-labeled anti-human IgG sheep sera (1 μCi/ml; 1 Ci = 37 GBq; Amersham). Serum samples containing >4000 cpm in the radioimmunoassay were considered positive.

RESULTS

Antibody Detection Among NANBH Patients. Among 23 patients who developed typical NANBH after blood transfusion, we could detect HCV antibody in 16 patients (70%; patients 1–15 and 18; Table 1). We then analyzed in detail the time course of antibody development and its relationship with the ALT levels in each case.

The antibody was positive in 15 out of 17 patients (88%; patients 1–17) who developed chronic hepatitis with multiple peaks of serum ALT elevation. A typical profile of the antibody and ALT values in a chronic PTH patient are shown in Fig. 1*A* for patient 1. The antibody was first detected between 2 weeks (patient 3) and 10 months (patients 1 and 8) after the first peak of ALT elevation. The mean period between the first ALT peak and the antibody rise was 4.3 months. Once acquired, antibodies continued to be detectable over years (for example, for more than 9 years in patient 6). When it was possible to examine the serum samples very frequently, as in patient 11 (every other week for 1.5 years), we observed fluctuations in the antibody level, although antibody was always detectable following the initial seroconversion. In patients 14 and 15, the antibodies were already detectable at the time of blood transfusion, indicating that these patients had already been infected prior to the transfusion. Although their antibody level apparently fluctuated, the antibody persisted until the patient died (patient 14: Fig. 1*B*) or, at present, for more than 5 years (patient 15). From a total of 17 patients showing multiple ALT peaks, only 2 patients (patients 16 and 17) were found negative for antibody. It should be noted, however, that the ALT levels of these two patients were lower than those of other chronic cases described above (Table 1).

In contrast to these chronic PTH patients, the antibody was generally not detectable among patients who showed an acute, resolving clinical course. Patients 18–23 all involved a single ALT peak, indicating just a transient period of liver cell damage. The duration of the liver cell enzyme abnormality was less than 3 months (Table 1). Among these six patients, HCV antibody was detected in only one (patient 18: Fig. 1*C*). In this case, the ALT and the antibody elevation occurred at about the same time. Slight antibody elevations were observed in patients 19 and 20, but these elevations were transient. In the other 3 patients (patients 21–23), the antibody was not detected, even though we examined sera from these patients once a week for 1–4 years (for example, see Fig. 1*D*).

We could not detect the antibody in the sera from three equivocal PT-NANBH patients. Six patients who developed transient liver cell damage but received no blood transfusion were also shown to be antibody negative. We also examined sera from 10 patients convalescing from HAV infection and

Table 1. Clinical and biochemical profile of 23 patients with PT-NANBH

Patient no.	Age/ sex	Clinical characteristics: Original disease	Incubation period,* wk	ALT peak†	Duration of abnormal ALT	Jaundice	Units positive for HCV antibody/ total units transfused	Anti-HCV antibody in recipient
1	36/M	Bronchial fistula	7	727	>3 yr	−	1/28	+
2	61/M	Lung cancer	7	456	>4 yr	−	1/29	+
3	77/M	Thoracic empyema	4	506	>2 mo	−	1/10	+
4	65/M	Lung cancer	5	607	>2 yr	−	2/70	+
5	59/M	Thoracic empyema	9	738	>3 yr	−	1/14	+
6	67/M	Gastric cancer	6	600	>9 yr	−	1/12	+
7	72/M	Duodenal ulcer	8	1510	>2 yr	−	1/12	+
8	62/M	Rectal cancer	3	1110	>5 yr	−	1/5	+
9	52/M	Gastric cancer	2	610	>2 yr	+	1/7	+
10	44/F	Ovarian cancer	4	935	>3 yr	+	1/12	+
11	62/M	Thoracic empyema	8	949	>1.5 yr	−	1/15	+
12	64/M	Gastric cancer	4	1380	>1 yr	−	0/20	+
13	54/M	Pulmonary abscess	4	688	>1 yr	−	0/15	+
14	60/M	Lung cancer	5	400	>4 yr	−	0/3	+‡
15	58/M	Thoracic empyema	9	489	>5 yr	−	0/88	+‡
16	63/M	Gastric cancer	1	430	>2 yr	−	0/3	−
17	38/F	Gastric cancer	2	260	>2 yr	−	0/3	−
18	69/M	Spontaneous pneumothorax	4	405	3 mo	−	2/8	+
19	32/F	Gastric cancer	1	500	2 mo	−	0/3	−
20	45/M	Pulmonary abscess	4	280	1 wk	−	0/20	−
21	58/F	Thoracic empyema	6	1971	2 mo	+	0/7	−
22	49/F	Breast cancer	9	317	2 mo	−	0/7	−
23	50/F	Thoracic empyema	4	881	1 mo	+	0/11	−

*Interval between blood transfusion and the first ALT elevation.
†ALT value expressed in Karmen units.
‡HCV carrier.

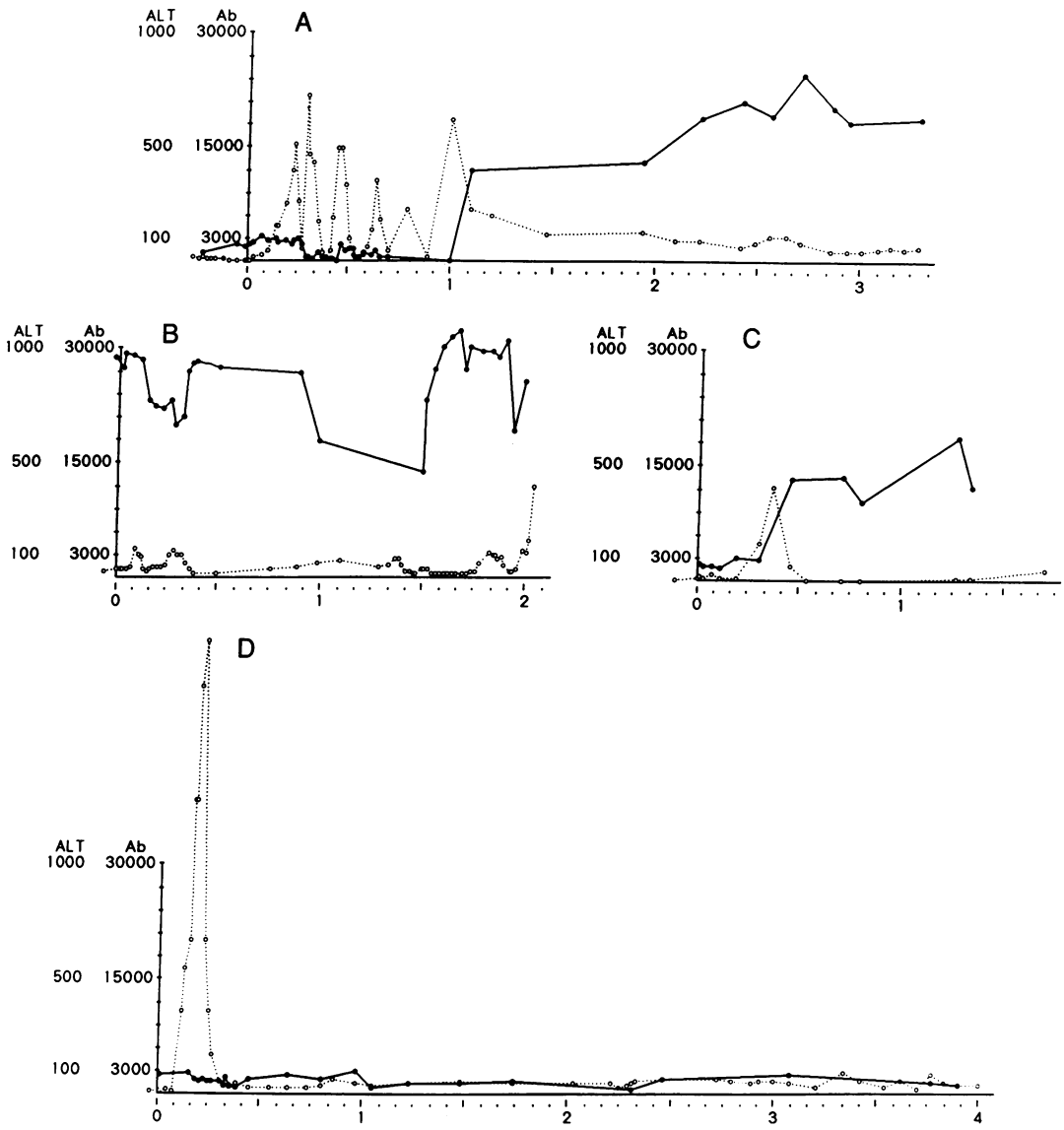

FIG. 1. The profiles of serum ALT and anti-HCV antibody levels. The dotted line represents ALT values in Karmen units and the solid line represents the HCV antibody values (in cpm). The abscissa represents the time after transfusion (in years). (*A*) Patient 1. (*B*) Patient 14. (*C*) Patient 18. (*D*) Patient 21.

sera from 15 hepatitis B patients at various stages of infection. All these samples were negative for the antibody.

Antibody Detection Among Corresponding Donors. Each recipient received different amounts of blood ranging from 3 to 88 units (average, 17 units; one unit is 200 ml). We assayed for antibody in those donor serum samples collected at the time of each transfusion. Antibody was detected in at least one unit of blood transfused into 11 of the 17 patients who developed chronic NANBH (Table 1, patients 1–11). All of these 12 blood units (in patient 4, 2 out of 70 units were positive) were transfused into patients who seroconverted to the antibody during the course of NANBH. The antibody was not detected in blood donor sera transfused into the remaining 6 patients (patients 12–17) who developed NANBH. However, two (patients 14 and 15) had HCV antibody prior to the transfusion and another two (patients 16 and 17) had no detectable levels of the antibody after the transfusion.

The situation seemed to be different again in those cases with self-limited hepatitis infections. Among 56 donor sera tested, which were transfused into the six recipients that later showed single ALT peaks, 2 were positive for the antibody. Both of them were transfused to patient 18, the only case in which seroconversion had occurred among this group. All the serum samples from donors transfused into the three equivocal cases were also negative for the antibody.

HCV Antibody Among Donor Sera. To investigate the incidence of HCV antibody in the blood donor population, we analyzed sera from 1365 donors used for blood transfusion at the Tokyo National Chest Hospital between 1983 and 1988. Of these, 800 samples were transfused to recipients who did not show any obvious clinical manifestation of PTH. The remaining 565 samples were, as previously described, from donor blood transfused into recipients who afterwards developed either definite or equivocal PTH. Since each recipient had received multiple units of blood, samples from implicated donor blood were considered to be included among these 565 samples. The antibody values (cpm) showed a logarithmic normal distribution skewed to the right (Fig. 2). Therefore, the mean and SD were calculated in a logarithmically corrected distribution. The mean was 532 cpm and cut-off value we had set previously (4000 cpm) was the mean + 2.7 SD. Since all of these blood donations were eventually used for blood transfusion, ALT values were all normal (below 34 Karmen units).

Seventeen serum samples out of 1365 donors (1.2%) gave values higher than 4000 cpm. Among them, 2 samples were

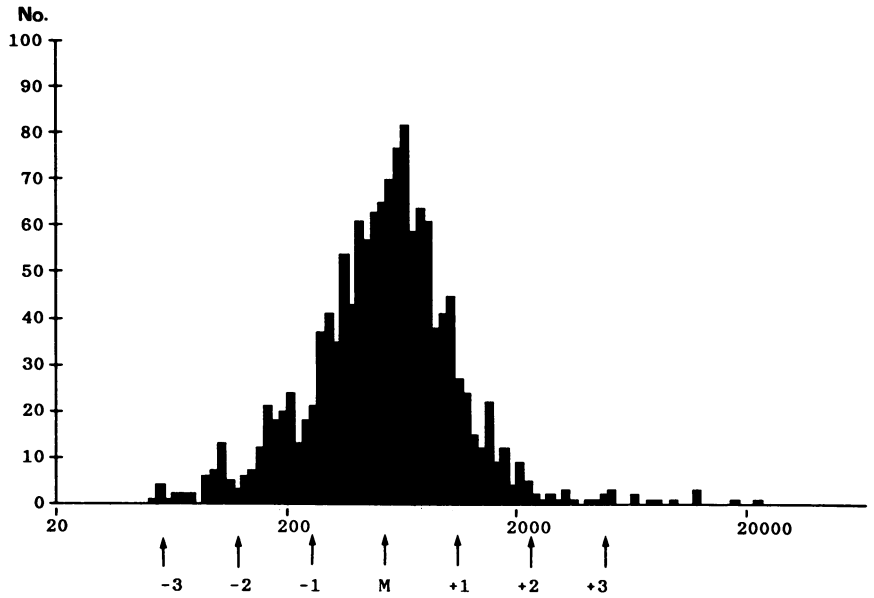

FIG. 2. Geometric distribution of the HCV antibody values (in cpm). Antibody assays were performed by radioimmunoassay. The antibody values of 1365 blood donors are shown. Of these, 800 were transfused to "normal" recipients and 565 were transfused to those who developed definite or equivocal PT-NANBH. The latter group was considered to contain samples from some "implicated" donors. M, mean. The numbers below the arrows represent standard deviations.

from the 800 normal donors. One with 6450 cpm was, however, transfused to a patient who already had severe hepatic cirrhosis at the time of transfusion. The other, with 8250 cpm, was transfused to a possible asymptomatic carrier of HCV (see *Discussion*). The remaining 15 blood samples with >4000 cpm were all transfused to recipients who afterwards developed typical NANBH. This suggests that the existence of antibody is closely associated with the infectivity of HCV.

DISCUSSION

Using the highly purified antigen expressed from a partial cDNA of the HCV genome, we assayed serum from various NANBH patients in Japan for reactive antibody. From this seroepidemiological analysis, we obtained the following important results. First, we confirmed that the cDNA cloned by immunoscreening (7) was really derived from the genome of an agent implicated in PT-NANBH. The specific reactivity of antibodies in NANBH patient sera collected from patients in different parts of the world suggests that HCV is the major etiological agent of NANBH (8).

Second, it is clear that the HCV antibody assay is important in the diagnosis of PT-NANBH. The antibody was detected in the sera of 88% (15/17) of the PT-NANBH patients who developed chronic hepatitis and whose sera had multiple peaks of ALT elevation. The antibody was also retrospectively detected in the corresponding donor sera in 73% (11/15) of these patients. In the present study, two recipients (patients 14 and 15) were considered to be HCV carriers who did not have antibody-positive blood transfusions. On the contrary, the seroconversion occurred in only one of six patients (17%) who showed a single ALT peak, and this was the only case involving a positive blood donor. Data have accumulated suggesting that there might be more than one type of NANBH virus associated with blood transfusion (10). They include (*i*) clinical manifestations (11), (*ii*) electron microscopic findings of infected chimpanzee livers (12), (*iii*) experimental cross challenges in chimpanzees (13, 14), and (*iv*) physicochemical properties of the agents (15). Our data suggest that HCV seroconversion is closely associated with NANBH patients whose sera display multiphasic enzyme elevations for long periods of time. Those patients with an elevated but constant ALT level (patients 16 and 17) and those patients with a single ALT peak may be associated with a different type of NANBH agent(s) for "non-C" hepatitis. However, it is possible that persistent stimulation of the immune system resulting from chronic infection is generally required for detectable seroconversion to take place. This may explain the failure of such antibodies to arise in cases of acute, self-limited PTH among recipients of antibody-positive blood. Conversely, the failure to find such cases might imply that HCV rarely or never fails to develop to chronic infection in itself. A nonviral cause of transient liver injury cannot be also excluded.

Third, the presence of the antibody is closely associated with the presence of infectious HCV. Most probably, the antigen encoded by this partial HCV cDNA clone does not contain neutralizing epitopes of the HCV particle. In those cases in which both donor and recipient sera were positive for the antibody, the seroconversion occurred after several peaks of ALT elevations. During this time, HCV viral antigen may be released into the blood stream from infected hepatocytes along with the liver-specific enzymes like ALT. Since our antibody assay depends on the quantitative determination of the binding activity of the antibody to the expressed C100-3 antigen, the C100-3-related antigen may coexist with the antibody in the serum and may hamper the binding capacity of the antibody. This may explain the observed corresponding fluctuations in the ALT and antibody profiles from virus carriers (Fig. 1*B*). One possible explanation for the reason why chronic infection is required for seroconversion may be that the amount of antigen released into the blood stream is so low that it has to accumulate to induce the antibody production.

Finally, the possibility of screening blood donors with a specific test for NANBH has materialized. All donor blood with an antibody value over 4000 cpm was found to transmit NANBH to the recipient. There were two exceptions involving donors with high antibody values whose blood was transfused to those not enrolled in our initial study. One unit was transfused to a patient who had chronic liver disease already at the time of transfusion and the other unit was to a patient who himself was considered to be an HCV carrier.

Since there were patients who developed NANBH after receiving antibody-negative blood (Table 1), blood shown to be negative in the current assay is not, of course, always free of NANBH virus. However, it is clear that antibody-positive blood should not be used for transfusion. A preliminary survey indicated that 1–2% of blood donated for transfusion in Japan is positive for the HCV antibody.

We are indebted to Drs. W. J. Rutter and H. Shimojo for a critical review of the manuscript; to the members of the Hepatitis Research Liaison Council of the Ministry of Health and Welfare, Japan, particularly to Dr. T. Oda, for his helpful advice and continuous encouragement; to Dr. T. Nojiri at Hachioji Red Cross Blood Center for helping to collect serum samples; to Dr. Y. Moritsugu for the generous gift of the HAV convalescent patient sera; and to Mr. Wang Chang, Mrs. Y. Kaminuma, and Miss F. Sakurai for expert assistance. This study was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control and by a Grant-in-Aid for Overseas Scientific Research from the Ministry of Education, Science and Culture, Japan.

1. Prince, A. M., Brotman, B., Grady, G. F., Kuhns, W. J., Hazzi, C., Levine, R. W. & Millian, S. J. (1974) *Lancet* **ii,** 241–246.
2. Feinstone, S. M., Kapikian, A. Z., Purcell, R. H., Alter, H. J. & Holland, P. V. (1975) *N. Engl. J. Med.* **292,** 767–770.
3. Knodell, R. G., Conrad, M. E. & Ishak, K. G. (1977) *Gastroenterology* **72,** 902–909.
4. Realdi, G., Alberti, A., Rugge, M., Rigoli, A. M., Tremolada, F. & Schivazappa, L. (1982) *Gut* **23,** 270–275.
5. Resnick, R. H., Stone, K. & Antonioli, D. (1983) *Dig. Dis. Sci.* **28,** 908–911.
6. Kiyosawa, K., Akahane, Y., Nagata, A. & Furuta, S. (1984) *Am. J. Gastroenterol.* **79,** 777–781.
7. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989) *Science* **244,** 359–362.
8. Kuo, G., Choo, Q.-L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Miyamura, 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.
9. Hallewell, R. A., Masiarz, F. R., Najarian, R. C., Puma, J. P., Quiroga, M. R., Randolph, A., Sanchez-Pescador, R., Scandella, C. J., Smith, B., Steimer, K. & Mullenbach, T. G. (1985) *Nucleic Acids Res.* **13,** 2017–2034.
10. Alter, H. J. (1988) in *Viral Hepatitis and Liver Disease*, ed. Zuckerman, A. J. (Liss, London), pp. 537–542.
11. Mosley, J. W., Redeker, A. G., Feinstone, S. M. & Purcell, R. H. (1977) *N. Engl. J. Med.* **296,** 75–78.
12. Shimizu, Y. K., Feinstone, S. M., Purcell, R. H., Alter, H. J. & London, W. T. (1979) *Science* **205,** 197–200.
13. Hollinger, F. B., Mosley, J. W., Szmuness, W., Aach, R. D. & Peters, R. L. (1980) *J. Infect. Dis.* **142,** 400–407.
14. Yoshizawa, H., Itoh, Y., Iwakiri, S., Kitajima, K., Tanaka, A., Nojiri, T., Miyakawa, Y. & Mayumi, M. (1981) *Gastroenterology* **81,** 107–113.
15. Bradley, D. W., McCaustland, K. A., Cook, E. H., Schable, C. A., Ebert, J. W. & Maynard, J. E. (1985) *Gastroenterology* **88,** 773–779.