CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1995, p. 219–224 Vol. 2, No. 2
1071-412X/95/$04.00+0

Long-Term Serologic Follow-Up of Hepatitis C Virus-Seropositive Homosexual Men

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Received 10 May 1994/Returned for modification 1 July 1994/Accepted 16 December 1994

Hepatitis C virus (HCV) infection may go undiagnosed and continue to present a source of community-acquired or transfusion-associated infection because of shortcomings in sensitivity, specificity, and reproducibility of serologic tests. This project was designed to longitudinally study persons who were HCV seropositive or were at risk for seroconversion to characterize the course of infection. Sequential serum samples obtained semiannually from 617 homosexual male volunteers were available for study from the Pittsburgh site of the Multicenter AIDS Cohort Study. Testing by anti-HCV enzyme immunoassay (EIA) was performed on baseline (1984 to 1985) and most-recent (censor date, August 1992) samples. Selected samples were also assayed for alanine aminotransferase and by recombinant immunoblot (RIBA II) and nested PCR. A total of 17 of 617 (2.8%) men were HCV seropositive at entry. Of the 600 seronegative men, 9 converted to HCV seropositive during the study interval. Parenteral sources of exposure could be identified in 6 of these 26 HCV-seropositive men. Four men were HCV seropositive at baseline and seronegative at their most recent visit. Of the 26 HCV-seropositive men, 12 were also seropositive for human immunodeficiency virus. EIA analysis of 298 longitudinal samples from the 26 men revealed three patterns of HCV seropositivity: persistent, intermittent, and rare. Nine men (35%) showed intermittent or rare seropositivity with periods of over 1 year between some seropositive samples. PCR was positive in 76% of the HCV EIA-positive and 84% of the RIBA-positive samples. Thus, a low but significant number of homosexual men were HCV seropositive with variable positivity over several years of follow-up. A portion of these men become HCV seronegative. Individuals who exhibit intermittent or rare seropositivity are a challenge to diagnosis.

In 1990, the U.S. Food and Drug Administration licensed the first anti-hepatitis C virus (HCV) enzyme immunoassay (EIA) test kit for commercial use. Clinical progress in the diagnosis and management of HCV infection has, however, not been straightforward. Factors which have contributed to the difficulty in diagnosis include the absence of an in vitro tissue culture system for HCV propagation. Furthermore, algorithms useful in the diagnosis of hepatitis B virus and human immunodeficiency virus (HIV) were not easily applicable to HCV.

Unlike the situation with hepatitis B virus infections, available markers do not reliably distinguish among acute, resolved, and chronic HCV infections (4, 13). Unlike the situation with HIV, a negative serostatus is not unusual in persons who are infected (2, 6, 9, 18). Seronegative infections may occur in up to 10% of HCV cases in the general population (2, 18). This gap in serologic detection contributes significantly to the continued problem of transfusion-associated hepatitis, 91% of which is caused by HCV (1). HCV cDNA in EIA-seronegative infections due to immunosuppression (14) and HCV types not detected by a particular assay (9, 18) can be detected by PCR with primers from the highly conserved 5′ noncoding region (11).

This study was undertaken to determine the serologic course of HCV infection in a large cohort of homosexual men. Sequential serum samples collected over 8 years were assayed by EIA. We wished to document HCV cases of persistent seropositivity, seroconversion, and seroreversion in the study participants. Better characterization of the serological response to the virus would be invaluable in devising testing strategies which consider the various serologic presentations of the infection.

(This material was presented in part at the IXth International Conference on AIDS, Berlin, Germany, June 6 to 11, 1993.)

MATERIALS AND METHODS

The Multicenter AIDS Cohort Study is a longitudinal study of the natural history of HIV infection in homosexual men (7, 8). This report focuses on Pittsburgh volunteers who were recruited between April 1984 and March 1985. The study population had an HIV seroprevalence of 19% and an average age of 32 years at baseline. Of the men, 95% were Caucasian and 97% had at least a high school education.

Semiannual physical examinations and collection of epidemiologic data and blood for a specimen bank were institutionally approved parts of the study (National Institutes of Health, Bethesda, Md., and University of Pittsburgh, Pittsburgh, Pa.). Serum and plasma were frozen at −20 or −70°C within 24 h of collection. A total of 617 of 1,062 enrollees were eligible for this study on the basis of multiple clinic visits for at least 3 years.

Blood samples from the first (termed baseline or entry) and most-recent (censor date, August 1992) visits were tested for antibody to HCV (anti-HCV) by EIA (Abbott HCV 2.0; Abbott Laboratories, North Chicago, Ill.). This was expected to divide the study participants into four groups: persons who were seronegative at both visits, persons who were seropositive at both visits (seropersistent), persons who were seropositive at baseline but seronegative at the most-recent visit (seroreverters), and persons who were seronegative at baseline but seropositive at the most-recent visit (seroconverters). Anti-HCV EIA-reactive samples were repeated in duplicate. When at least one of the duplicates was reactive, the sample was considered positive and the samples from the interval

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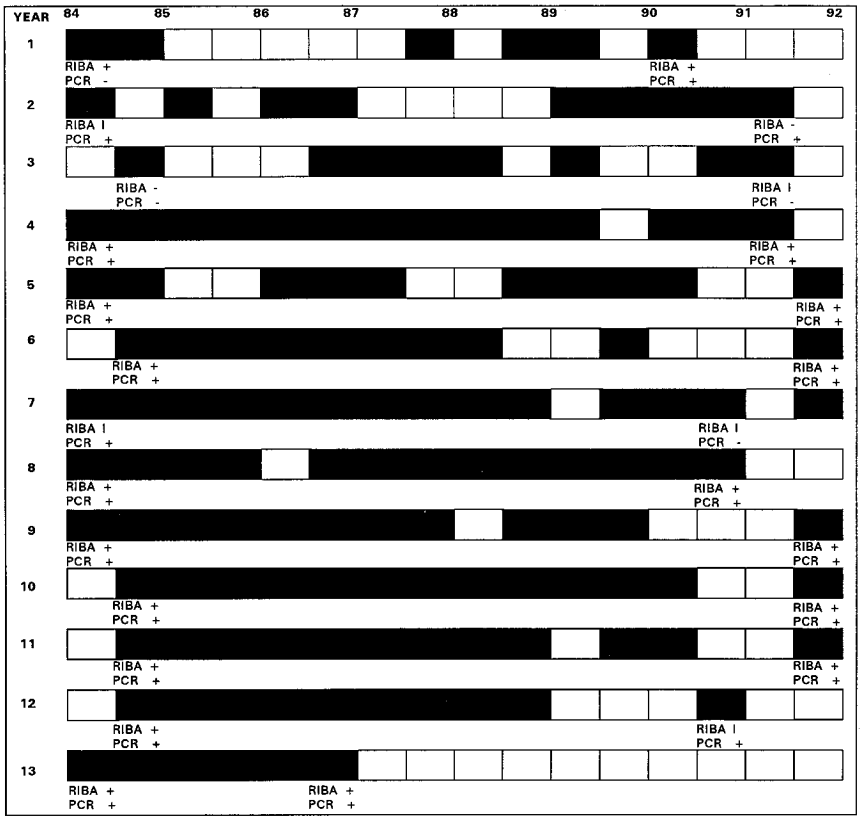

FIG. 1. Anti-HCV EIA results for 13 seropersistent men (identified by number at left) over 8 years. Sixteen half-yearly visits from 1984 to 1992 are represented. The filled bars indicate positive HCV EIAs over the interval, while the open bars indicate that the visit was missed or that serum was unavailable for testing. RIBA and PCR were performed on baseline and final-visit samples. Serum from subject 13 from the final visit was not available for performance of RIBA and PCR; the indicated RIBA and PCR results are from the penultimate visit. Results of testing are indicated as positive (+), indeterminate (I), or negative (−).

between the baseline visit and the most-recent visit (interval samples) were also tested by EIA for anti-HCV. An anti-HCV supplemental recombinant immunoblot assay (RIBA) (RIBA II; Ortho Diagnostics, Raritan, N.J.) and HCV nested PCR were performed on the baseline-visit and most-recent-visit samples of patients with positive EIA.

Additional tests were performed on samples of persons who were HCV EIA positive at the baseline visit or the most-recent visit. These included alanine aminotransferase (ALT) (Kodak Ektachem 700; Kodak, Rochester, N.Y.) and aspartate aminotransferase (AST) (Kodak Ektachem 700).

Nested PCR was performed as previously described (10, 11). The following set of primer sequences were used: outer sense (nucleotides 2 to 20), 5′-GCGA CGCTCCACCATAGAT-3′; outer antisense (nucleotides 305 to 324), 5′-GGT GCACGGTCTACGAGACC-3′; inner sense (nucleotides 10 to 27), 5′-CCAC CATAGATCACTCC-3′; and inner antisense (nucleotides 271 to 292), 5′-GCA AGCACCCTATCAGGCAGT-3′. Reverse transcription of HCV RNA was performed in a reaction mixture of 20 μl of the template, 40 U of RNase inhibitor (Promega, Madison, Wis.), 300 pg of antisense external primers, 50 U of reverse transcriptase (Bethesda Research Laboratories, Bethesda, Md.), 200 μmol of each deoxynucleoside triphosphate (Perkin-Elmer Cetus, Norwalk, Conn.), 10× buffer II (final concentration, 1×), and 5 mmol of magnesium chloride per liter. The reaction mixture was incubated at 40°C for 30 min. Reverse transcriptase was inactivated at 96°C for 10 min; the mixture was then cooled to 4°C and amplified in a total volume of 100 μl. A mixture of 180 pg of sense external primer, $MgCl_2$ (final amount, 2 mmol), 10× buffer II (final concentration, 1×), and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) was added. The reaction mixture was overlaid with 80 μl of mineral oil (Sigma Chemical Co., St. Louis, Mo.). PCR was performed for 35 cycles, with denaturing at 95°C for 1 min, primer annealing at 37°C for 2 min, and primer extension at 72°C for 2 min. After two such cycles, the annealing temperature was changed to 50°C and amplification was continued for another 33 cycles. A second round of amplification was performed on 10 μl of the first reaction mixture. In this round of amplification, all PCR conditions remained the same, except that the outer primer pair was omitted and an inner set of primers was included. PCR products from both rounds of amplification were analyzed by gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

All sera were analyzed in parallel with known positive and negative controls of human sera. A human serum sample that was repeatedly reactive for anti-HCV and RIBA and that had consistently detectable HCV RNA was used as the PCR-positive control. Also, a human serum sample that was negative for HCV RNA and nonreactive for anti-HCV served as the PCR-negative control. To exclude false-positive results caused by contamination of reagents with previously made PCR products, samples were assayed with all components except nucleic acid present; a negative reaction was part of the assay validation procedure.

By using 10-fold serial dilutions of HCV RNA synthesized from the cloned HCV genome, the sensitivities and specificities of the nested primers were analyzed. HCV sequences were detected at a 10^{-8} dilution. The estimated copy number of HCV RNA at a 10^{-8} dilution was about 20 to 50 copies. Positive and negative results were validated only if they were consistent in repeat experiments. The specificity of the PCR products was confirmed by liquid hybridization with an HCV-specific, ^{32}P-end-labelled oligonucleotide probe that spans the region between the internal primers, excluding the primer sequence.

Hepatitis testing sites. HCV 2.0 EIA was performed by a Food and Drug Administration licensed blood donor facility (Central Blood Bank, Pittsburgh, Pa.) with sample mix-up safeguards in place for donor testing. RIBA (done in duplicate) and nested reverse transcriptase PCR were performed at the Hepatitis Laboratory, Division of Transfusion Sciences, Food and Drug Administration, Rockville, Md.

Multicenter AIDS Cohort Study database. Data from the Multicenter AIDS Cohort Study available for review included demographics, intravenous drug use, transfusion history, HIV type 1 (HIV-1) antibody status (by enzyme-linked immunosorbent assay [Genetic Systems, Seattle, Wash.]), anti-HIV-1 confirmation (immunoblot; Bio-Rad, Richmond, Calif.), and $CD4^+$ cell counts (15).

Controls for aminotransferases. To exclude the effects of HCV disease and control for behavioral changes in the cohort over time, 68 entry samples and 63 most-recent-visit samples from 70 HCV-seronegative men in the cohort were assayed for aminotransferases.

Statistical analysis. Chi-square analysis was used to determine statistical significance. A P value of <0.05 was considered significant.

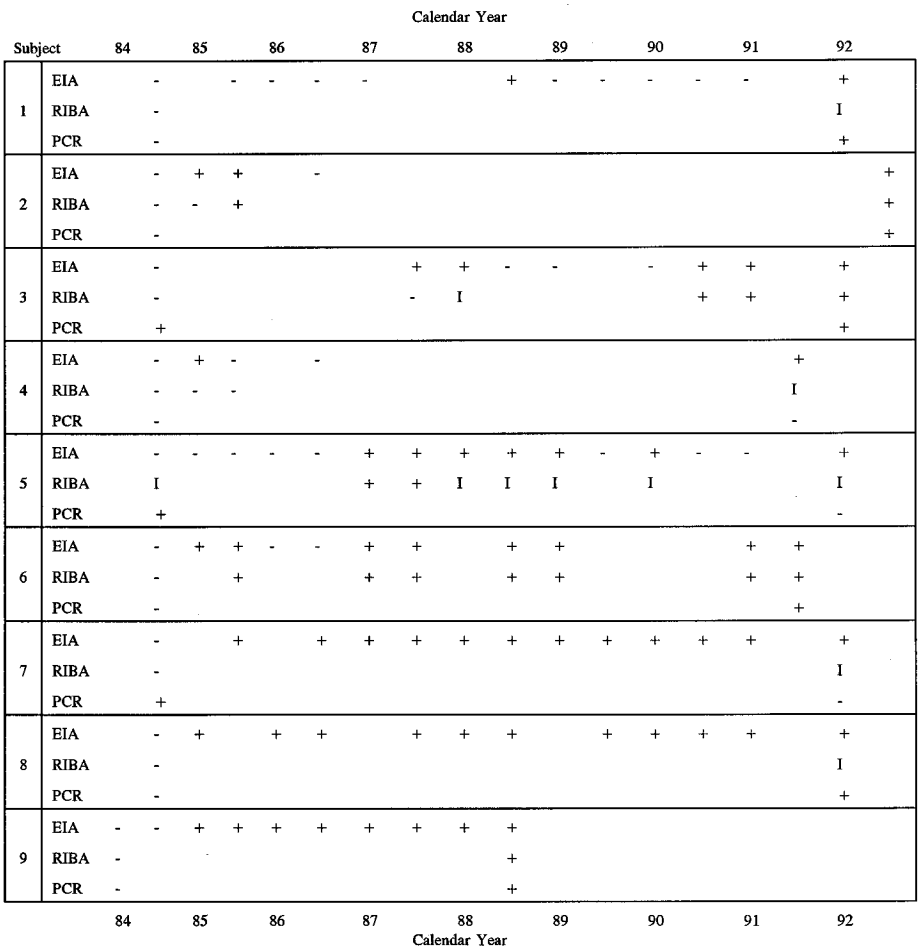

FIG. 2. Anti-HCV EIA, RIBA, and PCR results for nine seroconverters over 8 years. Sixteen half-yearly visits from 1984 to 1992 are represented. RIBA and PCR were performed on baseline and final-visit samples. RIBA on anti-HCV EIA-positive samples from persons with fluctuating EIA results was also performed. Results of testing are indicated as positive (+), negative (−), or indeterminate (I). A blank space indicates that information for an interval is unavailable.

RESULTS

HCV seropatterns. Of the 617 men, 26 (4.2%) were HCV seropositive either at the baseline visit or at the most-recent visit. Six of them had a history of intravenous drug abuse. None of the men had a history of blood transfusion. Ten men (38%) were HIV seropositive at the baseline visit, compared with 19% of the cohort as a whole. Two men seroconverted to HIV positivity between the baseline and the final visits.

Of the 26 HCV-seropositive men, 13 were HCV seropositive both at the baseline visit and at the most-recent visit (seropersistent [Fig. 1]), 9 were seropositive at the last visit and not at the baseline visit (seroconverters [Fig. 2]), and 4 were seropositive at the baseline visit but not at the final visit (seroreverters [Fig. 3]).

The 26 HCV-seropositive men had 298 longitudinal samples obtained during the study period (mean, 11.5; range, 6 to 16). The HCV EIA results showed three patterns of seropositivity over time: persistent, intermittent, and rare. Of the 26 men, 16 had persistent seropositivity, which was defined as having at least six consecutive positive anti-HCV EIAs leading up to the final visit without a fluctuation of EIA serostatus after the first positive test. This group included all 13 of the seropersistent men (Fig. 1) and three of the HCV seroconverters (Fig. 2, subjects 7, 8, and 9). Intermittent seropositivity was characterized by one or more intervals of EIA seronegativity in persons who were otherwise EIA seropositive. Subjects 2, 3, and 5 in Fig. 2 and subjects 1 and 4 in Fig. 3 have characteristic EIA profiles. The rarely seropositive had predominantly negative anti-HCV EIAs interspersed with occasional positive serology. This pattern is exemplified by subjects 1 and 4 in Fig. 2. Thus, while the seropersistent men were consistently HCV seropositive, the seroconverters and seroreverters had various patterns.

The demonstration of periods of antibody loss flanked by periods of HCV seropositivity in a good proportion of the selected men was unexpected, warranting exclusion of the possibility of gross sample mix-up. The interval samples which were tested by HCV 2.0 EIA were ABO typed with A_1, B, and A_2 erythrocytes. A total of 269 samples from 25 men were available. The observed ABO types were internally consistent. Furthermore, initially reactive samples are pulled and retested in duplicate as a matter of protocol. Thus, the rare EIA-positive sample in a run is EIA positive. The negative sample, on the other hand, is not retested. Review of Fig. 2 and 3, with the results from persons with intermittent and rare seropositivity, does not reveal isolated negative results (as would be expected with sample mix-up) but reveals a series of negative samples, in every case. Missampling of such magnitude and predictability is unlikely.

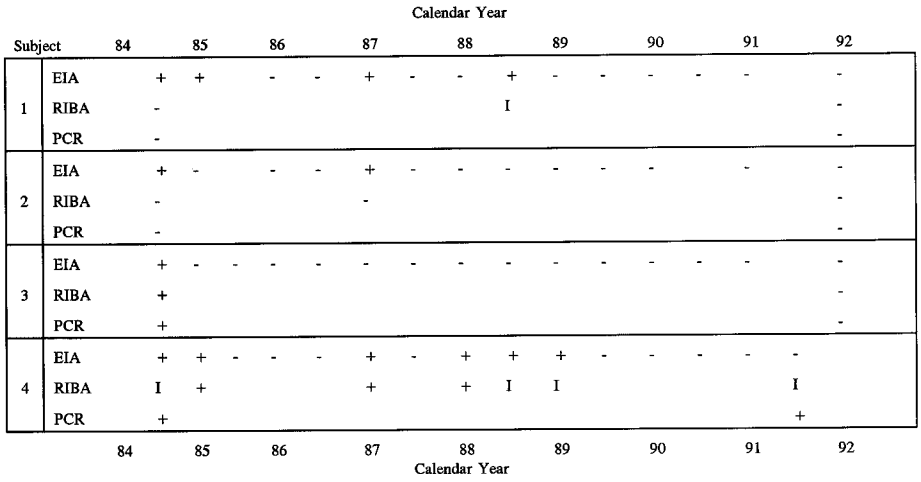

FIG. 3. Anti-HCV EIA, RIBA, and PCR results for four seroreverters over 8 years. Sixteen half-yearly visits from 1984 to 1992 are represented. RIBA and PCR were performed on baseline and final-visit samples. RIBA on anti-HCV EIA-positive samples of persons with fluctuating EIA results was also performed. Results of testing are indicated as positive (+), negative (−), or indeterminate (I). A blank space indicates that information for an interval is unavailable.

Samples from persons with persistent HCV seropositivity were PCR positive 76% of the time, while those from intermittently and rarely seropositive persons were PCR positive 50 and 33% of the time, respectively. Six seroconverters became seropositive within 18 months of baseline, and none seroconverted after 1989. Similarly, the four seroreverters were consistently seronegative by 1989. Five of the six intravenous drug abusers were persistently seropositive (Fig. 1, subjects 1, 2, 5, 8, and 12), and one was intermittently seropositive (Fig. 2, subject 2).

Supplemental HCV testing (RIBA II) was performed on the HCV EIA-positive interval samples of persons who were intermittently or rarely seropositive (Fig. 2 and 3); 27 of the 31 EIA-positive samples from the 10 participants were available for testing. There were 14 RIBA-positive, 8 RIBA-indeterminate, and 5 RIBA-negative samples. The RIBA-indeterminate samples were only c22-3 positive. Three of the four participants with RIBA-indeterminate interval samples previously or subsequently had positive RIBAs (Fig. 2 and 3).

Entry-visit and final-visit supplemental test and viral RNA amplification studies. A total of 51 results were available out of 26 sample pairs (baseline and most recent) targeted for RIBA and nested PCR. The quantity of the most-recent-visit sample from one seropersistent subject was insufficient for RIBA and PCR analysis. Of the samples which were EIA positive, 92% (35 of 38) had indeterminate or positive RIBAs (Table 1). Two samples were RIBA indeterminate and EIA negative. One RIBA-positive sample was PCR negative, and two RIBA-negative samples were PCR positive. HCV was detected by PCR in 76% of the HCV EIA-positive samples compared with 84% of RIBA-indeterminate or -positive samples. Only 4 of the 26 men were PCR negative at both the entry and most-recent visits. This included one seropersistent individual.

Aminotransferase levels as a predictor of viremia. Aminotransferases were assayed on 68 entry-visit and 63 final-visit samples from 70 randomly selected men in the cohort (Table 2). Of note, the aminotransferase levels at baseline were higher than those at the final visit. Not shown is the fact that ALT level elevation (>40 U/liter) correlated with HCV seropositivity in 15 of the 17 entry-visit and in 17 of the 22 final-visit samples. In addition, the ALT levels were elevated 17 of the 33 times that PCR was positive and in only 6 of the 131 control samples. Similarly, the number of elevated ALT levels in the HCV EIA-seropositive and PCR-negative populations was significantly different from that of the control population ($P < 0.05$).

HIV coinfection. Of the 17 men who were HCV seropositive at baseline, 3 were HIV infected, as were 7 of the 9 men who seroconverted for HCV. Three of the four HCV seroreverters were HIV seronegative, and one seroconverted for HIV be-

TABLE 1. Concordance of PCR, RIBA, and HCV EIA serostatuses of entry-visit and final-visit samples

EIA serostatus[a]	RIBA serostatus[b]	No. of samples of indicated serostatus	
		PCR+ (n = 33)	PCR− (n = 18)
+	+	22	1
	I	7	5
	−	0	3
−	+	0	0
	I	2	0
	−	2	9

[a] +, positive; −, negative.
[b] I, indeterminate.

TABLE 2. Mean ALT and AST levels in control and HCV-seropositive patients

Sample type	No. of samples (entry, final)	Mean levels of ALT and AST in indicated sample (U/liter)[a]	
		Entry	Final
Control population[b]	68, 63	29 (15), 42	20 (7), 53
Study population[c]	17, 22	94 (134), 76	36 (11.6), 57
PCR+[d]	16, 17	169, 118	35, 60
RIBA+[e]	11, 12	185, 113	35, 61

[a] The reference ranges for both ALT and AST were <40 U/liter. Values in parentheses are standard deviations of the ALT values.
[b] Anti-HCV EIA-negative samples.
[c] Anti-HCV EIA-positive samples.
[d] PCR-positive samples.
[e] RIBA-positive samples.

tween the baseline visit and the final visit. There was no clear relationship between HCV seropatterns and final-visit $CD4^+$ cell counts. The mean $CD4^+$ cell counts for the rarely, intermittently, and persistently seropositive groups were 962, 686, and 852 cells per mm^3, respectively.

DISCUSSION

The data indicate that HCV viremia is highly correlated with persistent anti-HCV EIA seropositivity. In addition, cross-sectional and longitudinal examination of samples from the HCV-seropositive men in this cohort indicates that the interval between positive samples from intermittently and rarely seropositive persons can be in excess of 1 year. One man was HCV seropositive on just one occasion. The sample was, however, also RIBA II and PCR positive, a clear indication that the volunteer was HCV infected at the time.

It is not clear why there were no new cases of HCV after 1989. This may represent an artifact of the selection process or behavioral changes in the cohort. It is evident that the cohort as a whole had less liver dysfunction (as noted by the normalization of ALT levels) at the end of the study. AIDS-related educational efforts, which tempered the transmission of HIV, could similarly have reduced the spread of HCV in the men. This hypothesis presupposes shared nonparenteral modes of transmission. It is consistent with the observation that seven of the nine seroconverters were HIV seropositive. There is evidence that oral and anal receptive intercourse (12) and the lifetime number of sexually transmitted diseases (16) are statistically associated with HCV infection in homosexual men.

The long interval between seropositive samples in 35% of the HCV-seropositive volunteers could be attributed to reinfection. Chimpanzee studies have shown that recovery from HCV infection does not necessarily confer immunity on the individual (5). Infection with the same or a different strain is therefore a possible explanation. Ragni et al. (14) showed that seronegative HCV infection in hemophiliacs is associated with HIV-related immunodeficiency and a low $CD4^+$ cell count. Though this is an attractive explanation for the seronegative intervals in persons studied here, $CD4^+$ cell counts did not appreciably differ among the persistently, intermittently, and rarely seropositive groups. Furthermore, a comparison of PCR results with seropatterns indicates that HCV viremia (represented by positive PCR) is most prevalent in the persistently HCV-seropositive persons. This suggests that seronegative intervals are truly aviremic and not a result of immunoincompetence.

A hepatic replicative phase has been advanced to explain negative cDNA PCR results for RIBA-seropositive persons (3). Whatever the case, it is clear that a long interval between seropositive samples decreases the likelihood of confirming or detecting HCV infection. To avoid this pitfall, EIA should be repeated for at least 1 year and supplemented by PCR to rule out active infection.

We postulate that the antibody response to HCV viral antigens fluctuates late in the disease course. This would explain the HCV EIA 2.0 and RIBA II seronegativity in some persons with long-standing disease. Yuki et al. described 59 patients with chronic HCV, 5 of whom were HCV seronegative (18). In their longitudinal study of ALT, anti-HCV EIA, and HCV RNA in five HCV-infected persons, Farci et al. (6) found one patient who exhibited persistent viremia (HCV RNA positive by PCR) with an antibody pattern that fluctuated between "borderline negative" and weakly positive during the final 8 years of the 13-year follow-up. It is not clear whether the 10% of HCV PCR-positive persons with acute presentation of HCV disease who are EIA seronegative (2) become seropositive or remain seronegative over time; viremia precedes serologic demonstration of HCV disease by at least 10 weeks (6). Of note, three of the nine seroconverters in this study were HCV PCR positive at entry. In addition, we document transitions between RIBA-indeterminate and -positive status interspersed with RIBA-negative status over 8 years in persons with indeterminate c22-3-reactive RIBA II results. In a study with a much shorter follow-up, c22-3-reactive RIBA II-indeterminate blood donors (17) were invariably RIBA II and PCR negative.

On the basis of this study, we infer that it is not possible to serologically pinpoint the onset of HCV disease in individuals without an acute symptomatic presentation. Three of the nine seroconverters in our study were consistently seropositive, while the majority were intermittently so. The frequent observation of fluctuating seropositivity limits the usefulness of seroconversion as an epidemiological marker in HCV disease. The fact that the intermittently and rarely seropositive persons were not more likely to have low $CD4^+$ cell counts suggests that fluctuating HCV serology is more common in the general population than has heretofore been recognized.

ACKNOWLEDGMENTS

This research was supported by NIH Contract N01-AI-72632 and Cooperative Agreement U01-AI-35041, by the Central Blood Bank, Pittsburgh, Pa., by the Pathology Education and Research Foundation, Pittsburgh, Pa., and by Abbott Laboratories.

The authors acknowledge the significant contribution of the participants and staff of the Pitt Men's Study, without whom this study would not have been possible. Alison Logar, Pat Wehman, and Carol Perfetti (University of Pittsburgh) were instrumental in data and sample processing. Gayle Longstreth (Central Blood Bank, Pittsburgh) contributed to the graphical presentation of the results.

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