Mechanisms of disease

Protection against persistence of hepatitis C

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Summary

Background Neither previous hepatitis C virus (HCV) infection nor vaccination with HCV-derived antigens protects against reinfection. However, HCV infection and vaccination in chimpanzees has been shown to reduce the magnitude and duration of viraemia with re-challenge. We aimed to establish whether similar immunity could be achieved in man.

Methods From a study of injecting drug users, we identified 164 people who had no evidence of previous HCV infection and 98 individuals who had been previously, but were not currently, infected with HCV. We compared the incidence and persistence of HCV viraemia in these two groups over four consecutive 6-month periods.

Findings Of participants without previous infection, the incidence of HCV infection was 21% (35/164). By contrast, people previously infected were half as likely to develop new viraemia (12% [12/98]), even after accounting for risk behaviour (hazard ratio, 0·45; 95% CI 0·23–0·88). Furthermore, in HIV-1-negative people, those previously infected were 12 times less likely than people infected for the first time to develop persistent infection (odds ratio 0·05, 95% CI 0·01–0·30), and median peak HCV RNA concentration was two logs lower. HCV persisted in six of six HIV-1-positive people, even in one man who had previously cleared HCV infection when he was HIV-1 negative.

Interpretation There is an alarming frequency of HCV infection and persistence among injecting drug users. Our data suggest that immunity against viral persistence can be acquired, and that vaccines should be tested to reduce the burden of HCV-related liver disease.

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Introduction

Nearly 4 million people in the USA, and 170 million people worldwide, have been infected with hepatitis C virus (HCV).[1–3] About 85% of people infected with HCV develop persistent infection and are at risk of long-term complications, including liver cirrhosis and hepatocellular carcinoma.[1,4,5] Treatment of HCV with interferon-α and ribavirin is associated with a sustained response rate of nearly 40%,[6,7] and this rate is likely to improve in the next decade with the advent of more effective antiretroviral treatments. Although these therapies could substantially reduce the burden of HCV-related disease in more-developed countries, these new therapies might be too expensive for less-developed countries, where cirrhosis and hepatocellular carcinoma continue to be major health problems.

Vaccination is an important alternative way to control disease, as has been shown for hepatitis B virus infection.[8] However, the feasibility of developing an effective HCV vaccine has been questioned, mainly because immunity that protects against infection cannot be induced in chimpanzees by either vaccination[9] or previous HCV infection,[10,11] and reinfection of human beings has been reported.[12–14] Nonetheless, results of reports suggest that when chimpanzees have been previously infected or vaccinated (with a DNA vaccine), viral persistence and severity of subsequent HCV infections are strikingly diminished.[15,16] If similar immunity could be induced in man, then the frequency of HCV-related cirrhosis and hepatocellular carcinoma could be substantially reduced, since they only arise in people with persistent infection.

If human beings developed immunity to HCV infection like chimpanzees, then people previously infected should have brief episodes of low-concentration viraemia on reinfection, by contrast with the high-concentration viraemia that persists in 85% of people infected with HCV for the first time.[4] We tested this hypothesis in a cohort of injecting drug users in which a natural experiment of clearance and reinfection was taking place. We also did a retrospective study of stored liver tissue taken from people who had cleared infection to show that new instances of viraemia were because of reinfection rather than recrudescence of ongoing infection.

Participants and methods

Study population

Between 1988 and 1989, Vlahov and co-workers[17] recruited 2921 injecting drug users from Baltimore, MD, USA, into the AIDS Link to the Intravenous Experience (ALIVE) study, to analyse the natural history of HIV-1 infection. All participants acknowledged injection drug use within the past 10 years, were 18 years of age or older, and did not have AIDS at entry into the study. At enrolment, these researchers used a standardised questionnaire to gather information on demographics, medical care, and

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GLOSSARY

ENZYME IMMUNOASSAY
Detects antibodies to recombinant HCV antigens in serum or plasma.

RECOMBINANT IMMUNOBLOT ASSAY
Detects antibodies to individual recombinant HCV antigens in serum or plasma.

BRANCHED DNA ASSAY
Quantifies HCV RNA load in serum or plasma by chemilluminescent signal amplification.

injection practices. They used a modified questionnaire at biannual follow-up visits, and obtained serum samples at all visits. Although these researchers gave counselling to individuals at all visits to reduce drug use, and referred participants for treatment, many people continued to inject drugs during follow-up. In 1994, the researchers recruited an additional 335 people, who were HIV-1 seronegative.

To detect people at risk for reinfection, we assessed 1344 people from this cohort who were HCV-antibody positive at enrolment and who had a follow-up visit between January, 1995, and March, 1996, at which time we did HCV-RNA testing. We detected HCV RNA in at least one serum sample from 1246 people, who we did not analyse further. Thus, we could not detect HCV RNA in 98 people at two consecutive visits, a group we subsequently refer to as previously infected. All but one of these 98 individuals had an alanine aminotransferase concentration less than 50 U/L (median 16 U/L [IQR 11–21]) at these nonviraemic visits, which further shows that they were not infected with HCV. In addition, we assessed all individuals (n=164) who did not have HCV antibodies at enrolment, a group we subsequently refer to as previously uninfected. We compared subsequent occurrence and persistence of viraemia in these two groups over four consecutive visits at 6-month intervals after the index visit (figure 1). The index visit was the first visit for participants in the group without previous infection, and was the visit after viraemia was excluded in those in the previously infected group.

During follow-up, we classified individuals as infected if we detected viraemia. We further classified infections as persistent if we detected HCV RNA at the next consecutive visit, and as clearance if viraemia was not sustained at this next visit. For people in whom HCV RNA was detected at the fourth visit, we tested sera from the next visit to resolve the outcome.

We obtained informed consent from all participants, with a protocol approved by the institutional review boards of the Johns Hopkins Schools of Public Health and Medicine.

Laboratory testing

We initially tested individuals in the two groups we included in our investigation by second-generation or third-generation ENZYME IMMUNOASSAY (Ortho Diagnostics, Raritan, NJ, USA). We repeated testing at successive visits in participants who had a negative enzyme immunoassay at enrolment, until seroconversion was noted, as described.[4,18] For every individual, we also tested at least one positive enzyme immunoassay sample by RECOMBINANT IMMUNOBLOT ASSAY (Ortho Diagnostics). We considered people reactive by enzyme immunoassay and recombinant immunoblot assay to be HCV-antibody positive.

In the previously infected group, we initially did HCV RNA testing with the second-generation BRANCHED DNA ASSAY (Chiron, Emeryville, CA, USA). We further tested samples from participants with at least one branched DNA result below the linear range (200 000 mEq/mL) with the qualitative Cobas amplicor assay (Roche Diagnostic Systems, Branchburg, NJ, USA). When a specimen was negative by this assay, we tested the sample taken at the visit immediately before or after the negative visit. We classified people as clearance if we could not detect HCV RNA with the qualitative Cobas amplicor assay for two consecutive visits.

To detect new instances of viraemia and subsequent viral persistence in the previously infected group, we

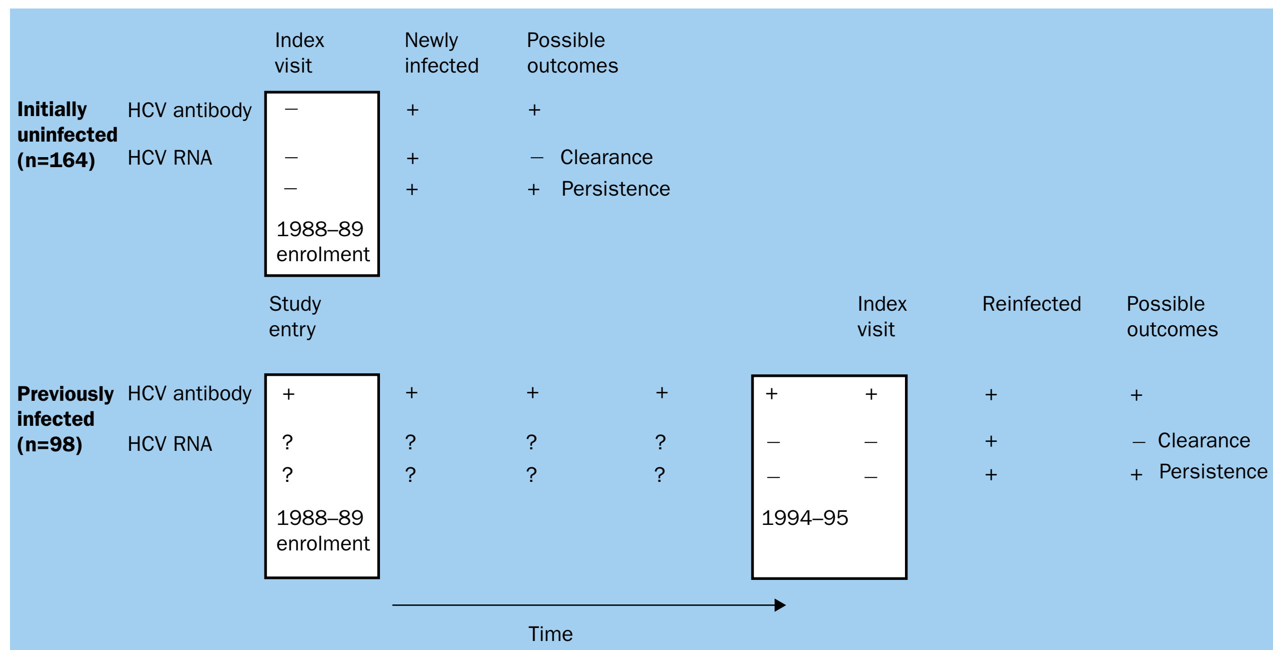

Figure 1: **Classification and testing scheme**
HCV-RNA testing was done at follow-up visits between January, 1995, and March, 1996.

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tested for HCV RNA with the qualitative Cobas amplicor assay. For samples in which HCV RNA was detected, we compared the concentration of HCV RNA with a previously unthawed sample, with the quantitative Cobas amplicor monitor assay (Roche Diagnostic Systems), and reported this concentration as IU/mL.

In the previously uninfected group, we did HCV-RNA testing on sera obtained at least 6 months before seroconversion (for median 72 months [range 14–93]) from all people who developed HCV antibodies. For this test we used the quantitative amplicor HCV monitor test (Roche Diagnostic Systems), which was the assay available at the time this testing was done; we reported HCV-RNA concentration as copies/mL. Since this quantitative test was less sensitive than others, we also did HCV-RNA testing on samples below the level of detection, with a commercially available qualitative HCV-RNA assay (amplicor HCV detection kit, Roche Diagnostic Systems) or an in-house nested PCR test, as described.[4] All positive HCV-RNA tests were verified by testing of another previously unthawed serum sample.

To compare the results of the old amplicor HCV monitor assay and the new Cobas amplicor monitor assay we tested sera from ten HCV-infected individuals by both methods in our laboratory, and the average difference was 2·5 copies/mL (that is, 2·5 copies/mL= 1 IU/mL, similar to the conversion of 2·7 recommended by the manufacturer). However, because it was more conservative (and did not change results), we converted concentrations of viraemia—reported as copies/mL by the amplicor HCV monitor assay—to IU/mL, by dividing by 6·6, as suggested by Saldanha and co-workers.[19]

HCV genotypes were obtained by examination of E1 sequences as described.[20] We obtained prototype sequences from GenBank. The core/E1 primers we used were as follows: outer, 493S_H77 (493), 5′-GCAACAG GGAACCTTCCTGGTTGCTC-3′, and 987R_H77 (987), 5′-CGTAGGGGACCAGTTCATCATCAT-3′; inner, 5′-502S_H77 (502), 5′-AACCTTCCTGGTTG CTCTTTCTCTAT-3′, and 975R_H77 (975), 5′-GTT CATCATCATATCCCATGCCAT-3′ (nucleotide positions are relative to the 5′ base of the Hutchinson 77 genome). We also assessed E1 sequences in people in whom HCV RNA was repeatedly detected and clustered phylogenetically to establish whether repeated viraemic visits were because of reinfection or viral persistence.

Liver histology

We analysed stored liver tissue for HCV RNA in a subset of individuals who cleared virus after previous infection. We took liver biopsy specimens between 1996 and 1998 with a protocol that enrolled people on the basis of HCV-antibody and alanine aminotransferase status, without knowledge of HCV-RNA test results. A hepatopathologist assessed liver histological findings and scored them according to the Ishak modified hepatic activity index (MHAI).[21] Immediately after the biopsy, we placed about 2 mg of liver tissue in RNAzol and quickly froze this specimen in liquid nitrogen for storage.

At completion of testing for HCV RNA in serum for this study, it was apparent that 21 participants in the previous infection and HCV clearance group had been included in the liver-biopsy protocol. For these individuals, liver tissue was thawed and immediately divided into two equal samples that were tested in five one-log dilutions. Into one series, we added in-vitro transcribed HCV RNA at known concentration, and then we extracted and amplified it in parallel with the other series to control for sensitivity of the reaction. We did preliminary experiments with other stored liver tissue samples, which showed equivalent sensitivity of RNA extractions when done in a liquid nitrogen bath and at room temperature. We extracted RNA immediately after thawing at room temperature with Trizol, then we did reverse transcription and cDNA amplification by nested PCR, with primers for HCV 5′ untranslated regions and human glyceraldehyde-3-phosphate dehydrogenase. We visualised reverse transcription-PCR products after electrophoresis with SYBR green plus, and further verified these products by Southern-blot analysis.[22] We detected glyceraldehyde-3-phosphate dehydrogenase at liver RNA dilutions of 10^4–10^5 in all 21 people, and HCV RNA at 20 copies/mL, in the positive-control reactions that were run along with many negative controls for every reaction.

Statistical analysis

We compared characteristics of previously infected and uninfected people by Fisher's exact tests, and the occurrence of viraemia in the two groups by Cox's proportional-hazards regression, in which risk behaviours (eg, injecting drug use, needle sharing) were included as time-dependent covariates. We stratified HCV persistence analyses by HIV-1 status because HCV persistence differed in accordance with HIV-1 status in other studies and in this analysis. We did multivariate logistic regression analysis to establish whether the association of previous HCV infection with viral persistence in HIV-1 negative people was independent of other factors associated with viral persistence. Potential confounders that we considered included age, ethnic origin, and sex. We compared peak concentrations of viraemia by Wilcoxon's rank-sum test, with adjustment for tied values. We excluded HIV-1-positive people from this analysis because of results of studies in which HIV-1 was shown to affect HCV viral load. We did statistical analysis with STATA version 7.0 (College Station, TX, USA) and SAS version 6.12 (Cary, NC, USA).

Role of the funding source

The sponsor had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

98 people previously infected with HCV were followed up for median 2·14 years (IQR 1·90–2·59) beyond the index visit, whereas 164 who had not been infected with HCV before were followed up for median 2·35 years (1·95–2·97). The two groups of participants were similar with respect to ethnic origin; however, compared with people who were previously infected with HCV, those

Characteristic	HCV antibody+ HCV RNA–	HCV antibody– HCV RNA–	p*
Age (years, mean [SD])	41·1 (6·3)	31·5 (7·0)	<0·0001
Male sex	58 (59%)	121 (74%)	0·01
African-American race	87 (90%)	146 (90%)	0·91
HIV-1 positive	36 (37%)	17 (10%)	<0·0001
Injection drug use	64 (65%)	129 (79%)	0·02
Frequency of drug use			
None	34 (35%)	35 (21%)	<0·0001
Light (<1/day)	32 (33%)	94 (57%)	
Heavy (⩾1/day)	32 (33%)	35 (21%)	
Needle sharing	19 (19%)	71 (44%)	<0·0001

Data are number (%) unless stated. *Statistical comparisons made with Student's *t* test for age and χ^2 test for all other variables.

Baseline characteristics

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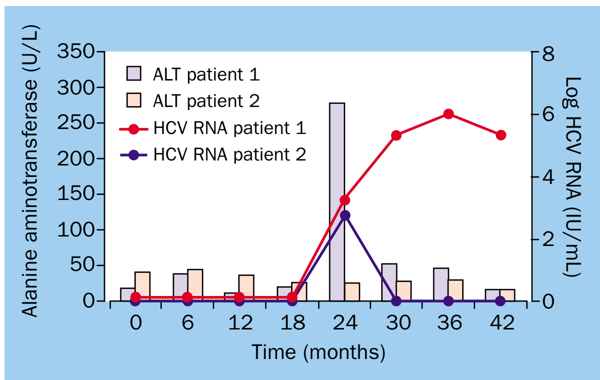

Figure 2: **HCV RNA and serum alanine aminotransferase (ALT) concentrations**
Patient 1 was previously uninfected who acquired HCV infection; patient 2 was previously infected who was reinfected.

who had not been infected before were younger and more likely to be male, HIV-1 negative, and more active drug users (table).

Occurrence of viraemia was lower in the previously infected group (12/98 [12%]) than in the initially uninfected group (35/164 [21%]), though this difference was not significant (p=0·07). Although less drug use-related risk behaviour was reported in the previously infected group than in the previously uninfected group, time to first infection was significantly longer in those previously infected than in those previously uninfected, even after these behaviours (drug use practices) were accounted for as time-dependent covariates with Cox's proportional-hazards regression model (hazard ratio 0·45, 95% CI 0·23–0·88, p=0·02).

In HIV-1-negative people, the frequency of viral persistence was lower in those who were previously infected (3/9 [33%]) than in those who were infected for the first time (27/32 [84%]; odds ratio 0·09, 95% CI 0·02–0·50, p=0·006; figure 2). By contrast, all HIV-1-positive people (n=6) in whom viraemia was detected developed persistent infection, irrespective of previous HCV infection (three of six had been previously infected), including one individual who cleared HCV previously when he was HIV-1-negative. In view of this heterogeneity, HIV-1-positive people were excluded from all subsequent analyses. In multivariate analysis, when previous HCV infection was assessed as a covariate with potential confounders (age, sex, and ethnic origin), people previously infected with HCV were 12 times less likely to develop persistent HCV infection than those infected for the first time (odds ratio 0·08, 95% CI 0·01–0·46, p=0·002).

In HIV-1-negative people, peak concentrations of viraemia were significantly lower in previously infected people (median 2·8 log IU/mL [IQR 2·8–3·6]) than in those infected for the first time (4·6 log IU/mL [4·0–4·7]; p=0·02; figure 3). Furthermore, we repeated the analysis with HIV-1 patients included and with transformations of up to 1 log, without any substantial difference in the findings. Six (75%) of eight reinfected people were infected with HCV genotype 1 (proportion was the same for reinfected people whose infection persisted and those whose infection cleared), and the median alanine aminotransferase concentration at the time of reinfection was 38 U/L (IQR 26–119).

To exclude the possibility that some instances of viraemia indicated recrudescence of a previous infection, HCV RNA was analysed in liver tissue for 21 individuals

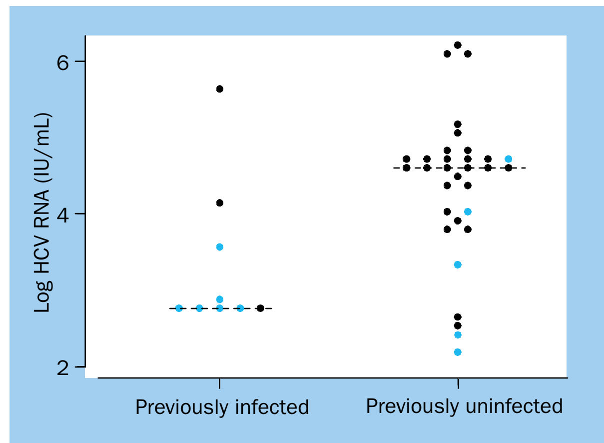

Figure 3: **Peak concentrations of HCV viraemia among previously infected and initially uninfected individuals**
Black circles represent people who developed persistent infection and blue circles represent people who cleared. Dashed lines represent medians.

who were noted to have cleared HCV RNA from serum. In these people, median MHAI score was 3 (IQR 2–4), median fibrosis score was 0 (0–1), and median alanine aminotransferase concentration at the time of biopsy was

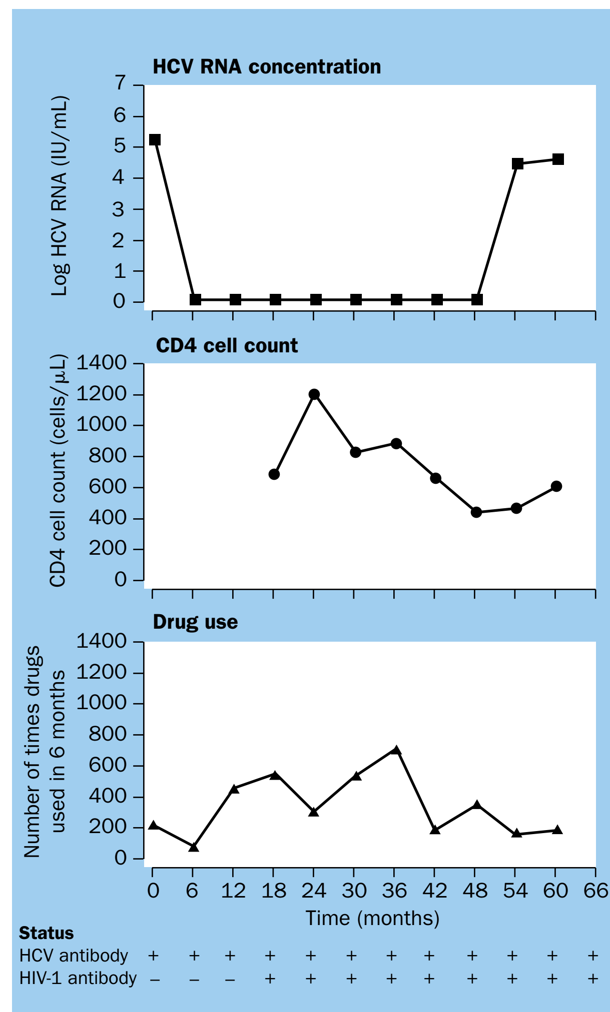

Figure 4: **HCV RNA concentration, CD4 cell count, drug use, and HIV-1 and HCV antibody status in a previously infected injection drug user who became reinfected after acquiring HIV-1 infection**

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29 U/L (20–43). HCV RNA was detected in only one of the 21 tissue samples, with an assay that always detected at least 20 copies/mL of HCV RNA. The one individual with detectable HCV RNA in his liver had cleared HCV infection when he was HIV-1 negative, but then became HIV-1 positive after high-risk drug use (daily needle sharing; figure 4). Serum samples were not available at the time the liver biopsy specimen was taken, but the same HCV RNA sequences were recorded at later study visits (data not shown).

Discussion

Our results show an alarming incidence of HCV infection (21%) and reinfection (12%) in injecting drug users. Infection happened despite efforts to treat drug use, which underscores the importance of development of vaccinations to prevent hepatitis C. In this respect, it is encouraging that there were fewer instances of viraemia in previously infected people than in previously uninfected individuals (despite continuing injecting drug use), and that most instances of viraemia were of low magnitude and resolved. Our findings suggest that human beings can acquire immunity which protects against HCV persistence, as has been shown in chimpanzees.[15,16]

The difference in viral persistence that we saw between the groups might also indicate underlying host genetic differences—ie, people who cleared infection once are most likely to clear it again. Although this conjecture cannot be directly assessed in man, the importance of induced immunity is clear from chimpanzee studies, in which animals have been challenged repeatedly or after vaccination.[15] Furthermore, in both human beings and chimpanzees, the nature of reinfection is different from first infection, and is characterised by lower levels of virus. Showing that human beings who previously cleared HCV infection when they were HIV-1 negative subsequently developed persistent infection in association with HIV-1-related immune suppression suggests that a protective immune response can be not only acquired but also lost. Collectively, these findings suggest that at least some of the difference in persistence noted in our study are an acquired immune response.

The actual differences in incidence and persistence of viraemia that can be attributed to previous infection are probably greater than what was seen in our study. People without a history of previous infection were younger and more likely to be HIV-1 negative than were those with a history of infection—factors that are associated with viral clearance that could have diminished the differences between the groups. Furthermore, use of the 6-month sampling protocol probably missed instances of brief, self-limited viraemia in injecting drug users, making the actual frequency of viral clearance in people who were previously infected greater than what we recorded. Since infections in people who had not previously been infected were associated with HCV seroconversion and higher concentrations of viraemia, that any were missed is unlikely.

Since HCV antibodies can decline to concentrations below the level of detection of commercially available assays in some people who recover from HCV infection, it is also possible that some of our uninfected group were previously infected.[23] Although this misclassification could further diminish the noted differences between our groups, we do not believe that there was substantial misclassification, since we previously showed that HCV antibody titres of people in this cohort who seroconvert remain above the level of detection of the second-generation enzyme immunoassay for 6 or more years.[4]

An important alternate explanation for these transiently detected low concentrations of viraemia in people who cleared infection is that they were because of recrudescence of persistent infection (rather than abbreviation of a new instance of viraemia). However, we did not detect HCV RNA in liver tissue from any HCV-antibody-positive individual in whom HCV RNA could not be detected in serum. The one participant in whom HCV RNA was detected in liver tissue seemed to have acquired a new HCV infection, with high concentrations of viraemia after becoming HIV-1 positive. Thus, even though he had previously cleared HCV RNA from two consecutive serum samples, he was most probably viraemic at the time of the biopsy. HCV infection persisted not only in this individual who acquired HIV-1 infection after initially clearing HCV but also in the other people who were HIV-1 positive. These data lend support to the finding that many HIV-1-infected individuals in the entire cohort are more likely to have persistent HCV infection than those without HIV-1 infection.[24]

In our cohort, HCV infections generally arose years before HIV-1 infections did.[25] Thus, greater HCV persistence in HIV-1-positive people than in those negative for HIV-1 showed either that individuals who cleared viraemia had a reservoir of HCV, which recrudesced after HIV-1-related immunosuppression, or that HCV infection most frequently persisted when people who previously cleared HCV infection became reinfected with HCV after acquiring HIV-1. Although definitive conclusions cannot be based on so few people, our data lend support to the latter hypothesis.

Recommendations that HCV infections should not be treated in people injecting drugs are partly based on the supposition that reinfection would diminish the benefit of treatment.[26] We are not aware of data about the occurrence of reinfection after HCV recovery associated with treatment. However, if there is also a lower frequency of viral persistence when viraemia is cleared by treatment, as was noted by us after natural recovery, reinfection could represent less of a concern when weighing the risks and benefits of treatment for an individual.

The high rates of HCV infection in injecting drug users underscore the importance of prevention of HCV infection. Since immunity can be acquired to protect against viral persistence, vaccines should be tested to reduce the medical results of HCV infection among people at highest risk.

Contributors

S Mehta and D Thomas were responsible for study design, statistical analysis, interpretation of data, and writing the report. A Cox assisted with liver biopsy specimen analysis and did immunological experiments. D Hoover was responsible for study design and statistical analysis. X-H Wang did HCV RNA testing. Q Mao did the liver biopsy specimen analysis. S Ray assisted with laboratory and HCV genotype analysis. S Strathdee and D Vlahov were responsible for study design, organisation, and data collection. All authors wrote and edited the final report.

Conflict of interest statement

None declared.

Acknowledgments

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