Hepatitis C virus (HCV) specific immune responses in anti-HCV positive patients without hepatitis C viraemia

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Abstract

Background/Aims—Most patients infected with hepatitis C virus (HCV) develop chronic infection and persistent viraemia. The immune mechanisms responsible for resolution of viraemia remain poorly understood. HCV specific humoral and cellular immune responses in patients with and without viraemia were investigated.

Methods—In vitro T helper (T_H) lymphocyte responses to structural and non-structural HCV proteins were determined by means of proliferative response and cytokine production in 35 anti-HCV positive/HCV RNA negative patients and in 31 patients with chronic HCV infection and persistent viraemia. Humoral responses were determined by measuring HCV specific antibody quantity and specificity.

Results—A T_H response to two or more HCV proteins was present in 18 of 35 patients with serological viral clearance compared with just one of 31 viraemic patients ($p = 0.00001$). HCV specific interferon- γ production was increased only in the former group. In contrast, the antibody levels were significantly lower and directed at fewer HCV antigens in patients with undetectable HCV RNA.

Conclusions—Patients without viraemia after HCV infection frequently have strong T_H lymphocyte responses of the T_H1 type to multiple HCV antigens many years after the onset of infection, whereas antibody responses are less marked. These results suggest that control of HCV replication may depend on effective T_H lymphocyte activation.

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Hepatitis C virus (HCV) is a major cause of chronic liver disease and hepatocellular carcinoma world wide.¹ A striking feature of infection with HCV is the very high rate of chronicity with 80-90% of cases developing persistent viraemia.²⁻⁴ In these patients viraemia persists despite the presence of a humoral immune response with HCV specific antibody production and in some cases despite the additional presence of a virus specific cellular immune response from CD4 T helper (T_H) lymphocytes^{5,6} and CD8 cytotoxic T

lymphocytes.^{7,8} Details of the immune mechanisms involved in the minority of patients who are able to resolve HCV infection without developing chronic viraemia are not yet clear, although a better understanding of these immune responses may well provide important insights for the development of new treatment strategies and vaccines.

The humoral response to HCV infection is broadly targeted, with antibodies to both structural and non-structural proteins found in most cases.⁹ Although antibody mediated in vitro neutralisation of HCV infectivity has been shown experimentally, the protection afforded is highly strain specific.¹⁰ In vivo, antibody responses do not have an adequate neutralising effect, and multiple episodes of acute hepatitis C have been observed in both polytransfused thalassaemic children¹¹ and chimpanzees¹² despite the presence of anti-HCV antibodies.

T_H lymphocytes play a major role in directing effector immune responses to pathogens, largely by the cytokine profile produced in response to a stimulus, and can broadly be divided into two categories (T_H1 and T_H2).^{13,14} T_H1 cells produce interferon- γ (IFN- γ) and interleukin (IL)-2, promoting cellular immune responses and cytotoxic T lymphocyte activity, whereas T_H2 cells produce IL-4 and IL-10, promoting a humoral response. The nature of a T_H lymphocyte response has been shown to be important in influencing outcome in many infections including HIV,¹⁵ leprosy,¹⁶ and leishmania,¹⁷ and may also be important in HCV infection.¹⁸ The role of virus specific T_H lymphocytes in HCV elimination is not clear, although data from patients with acute HCV infection^{18,19} suggest that T_H lymphocyte responses to both structural and non-structural HCV proteins are important for the outcome.

To gain information on the host immune responses associated with successful control of HCV replication and a benign course of infection, we have analysed in detail the HCV specific cellular and humoral immune responses in two groups: firstly, in a group of well characterised asymptomatic patients who were repeatedly HCV RNA negative in serum with anti-HCV seropositivity, the only evidence of

Abbreviations used in this paper: HBV, hepatitis B virus; HCV, hepatitis C virus; NS3, HCV non-structural protein 3; NS4, HCV non-structural protein 4; NS5, HCV non-structural protein 5; PBMC, peripheral blood mononuclear cell; SI, stimulation index; T_H, T helper lymphocyte; IFN, interferon; IL, interleukin; ELISA, enzyme linked immunosorbent assay; FACS, fluorescein activated cell sorter.

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Table 1 Clinical characteristics of patient populations

	A (HCV RNA negative)	B (HCV RNA positive)
Number	35	31
Mean age (range)	38.6 (19–68)	38.2 (28–64)
Sex	23M, 12F	19M, 12F
Estimated duration of infection (years)	(not known in 4 cases)	(not known in 11 cases)
Maximum	17.5	14.3
Minimum	8.5	12.3
Route of infection		
IVDU	25 (71)	15 (48)
Blood/blood products	6 (17)	5 (16)
Sexual/familial	1 (3)	1 (3)
Unknown	3 (9)	10 (32)

Unless otherwise indicated, values in parentheses are percentages.

IVDU, intravenous drug use.

previous HCV infection, and secondly in a group of patients with persistent viral replication and chronic HCV related liver disease.

Methods

PATIENTS

Two groups of cases seen at the Institute of Liver Studies, King's College Hospital, London because of HCV antibody positivity were studied (table 1). Group A consisted of 35 patients identified from over 750 HCV antibody positive patients referred to us between 1992 and 1995. All had detectable HCV antibodies as evidence of previous exposure to HCV and were repeatedly HCV RNA negative, with persistently normal liver function tests over a period of at least 18 months and none had clinical evidence of liver disease. The majority (32 of 35) had risk factors for acquisition of HCV infection and none had received antiviral treatment at any stage. All 35 remained healthy many years after the likely time of infection and were considered to have spontaneously recovered from HCV infection. Group B consisted of 31 patients with chronic HCV infection. All 31 were HCV antibody and HCV RNA positive with persistently elevated serum transaminases and had biopsy proven chronic hepatitis. Sixteen had moderate to severe hepatitis, 13 mild to moderate hepatitis, and two had cirrhosis.

The two groups were comparable in terms of age, sex, route of infection, and duration of infection (table 1). Maximum and minimum duration of infection was estimated from the time of first and last exposure to blood products, or from the time of starting and discontinuing intravenous drug usage. HCV genotyping was performed on 27 patients from group B using the INNO-LiPA assay (Innogenetics, Ghent, Belgium), disclosing genotype 1a or 1b in 12 cases, type 2a in one, type 3a in 12, and type 4 in two. In group A, serotyping was attempted using genotype specific HCV non-structural protein 4 (NS4) antibody epitopes, but accurate determination was not possible because of low antibody titres.

The nature and purpose of this study was explained to all patients, and informed consent was obtained. The study was approved by the local ethics committee.

SEROLOGICAL TESTS FOR ANTI-HCV

HCV antibody was tested using second (Ab-bott IMx; Abbott Diagnostics, Maidenhead,

Berkshire, UK), third and fourth generation assays (INNOTEST HCV AbIII and IV; Innogenetics). In all cases from group A, anti-HCV specificity was confirmed using a third generation line immunoassay (INNO-LIA HCV Ab IIITM; Innogenetics) as an antibody confirmatory test.

DETECTION OF HCV RNA

In both groups the presence or absence of HCV RNA was determined by a commercially available assay sensitive to less than 1000 viral copies/ml (Amplicor; Roche, Basel, Switzerland),²⁰ and in group A the absence of HCV RNA was further confirmed in all cases by an in-house nested PCR (Innogenetics).

HCV SPECIFIC CELLULAR IMMUNE RESPONSES

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised fresh blood by density centrifugation (Lymphoprep; Nycgaard, Oslo, Norway), and the cellular responses analysed by proliferation assays and in vitro cytokine production.

Proliferation assays

PBMCs were cultured in 96 well flat bottomed microtitre plates (Nunc; Gibco-BRL, Glasgow, UK) using six replicates with 2×10^5 cells per well. Cells were cultured in buffered RPMI 1640 supplemented with 10% (v/v) heat inactivated human AB serum (lot 2054; Gmini Bio-Products Inc, Calabasas, CA, USA) at 37°C with 5% CO₂ and 100% humidity as previously described²¹ for six days in the presence or absence of HCV proteins at a concentration of 1 µg/ml. Four purified recombinant HCV proteins were used (Microgen, Munich, Germany): NS3, NS4, NS5, and core. These were derived from genotype 1a sequence, expressed in *Escherichia coli* and suspended in Tris/glycine buffer. Bacterial lipopolysaccharide content was <30 pg endotoxin/µg protein, with the acceptable limit for cell culture being <50 pg endotoxin/ml. Tetanus toxoid and anti-CD3 were used as positive control antigens. The proliferative response was evaluated by [³H]thymidine uptake and measured in cpm with a β counter (Matrix 9600; Packard Instruments, Groningen, The Netherlands). The stimulation index (SI) was calculated from the cpm found with antigen divided by that found without antigen. A significant SI in response to the HCV proteins was defined as being >2.5, which was greater than the mean SI plus two times the standard deviation obtained when responses in 15 healthy controls were tested.

T_H lymphocyte restriction of lymphoproliferative responses was confirmed by CD4 cell depletion using immunomagnetic separation (Dynabeads; Dynal, Oslo, Norway). T lymphocyte activation was determined by IL-2 receptor (CD25) expression and assessed by fluorescence activated cell sorter (FACS) (FACScan; Becton and Dickinson, Oxford, UK).

Figure 1 Presence of significant T_H cell proliferative response to hepatitis C virus (HCV) antigens in patients with viral clearance (group A) compared with that in patients with viral persistence (group B). (A) Percentage of patients in groups A and B with a significant response to two or more HCV proteins. Numbers above bars are numbers responding/numbers tested. p value was calculated using the χ^2 test. (B) Number of patients in each group with a significant response to various numbers of HCV proteins.

In vitro cytokine production

In parallel with the proliferation assay, PBMCs were cultured with or without HCV proteins, and IL-4, IL-10, and IFN- γ levels were then measured in the cell culture supernatant after four days. Cytokine production from cells cultured in the absence of antigen and antigen specific cytokine production in response to the HCV proteins were measured blindly in 11 patients from group A and nine from group B using a previously described sandwich enzyme linked immunosorbent assay (ELISA) for IL-4 and IFN- γ ²² and by a commercially available ELISA for IL-10 (Medgenix, Fleurus, Belgium).

HCV SPECIFIC HUMORAL IMMUNE RESPONSES

The serum levels of total anti-HCV antibody and antibodies to individual HCV antigens were quantified in all 35 cases from group A and for 25 of the 31 cases from group B. Total HCV antibody was quantified using a third and fourth generation screening assay (INNO-TEST HCV Ab III and IV; Innogenetics). Specific antibody responses against synthetic peptides from two non-overlapping regions of HCV core (amino acids 1–32 (core 1) and 31–74 (core 2)), NS4 (amino acids 1696–1739 and 1916–1944), NS5A (amino acids 2263–2318), and envelope 2/hypervariable region 1 (amino acids 386–409), and against recombinant subtype 1b NS3 protein (amino acids 1188–1465) were quantified using a third generation confirmatory assay (INNO-LIA HCV Ab III; Innogenetics) combined with the LIA-Scan automated package (Innogenetics). In

Figure 2 Effect of CD4 cell depletion on response to hepatitis C virus (HCV) non-structural protein 4 (NS4); confirmation that the proliferative response to HCV antigens is restricted to CD4 lymphocytes. Progressive depletion of CD4 lymphocytes from the pool of peripheral blood mononuclear cells progressively reduces the stimulation index (SI) to NS4 in a typical case.

addition, anti-envelope antibodies were quantified separately by enzyme immunoassays containing recombinant E1 and E2 proteins produced in mammalian cells. These proteins are glycosylated and contain all major conformational E1 and E2 epitopes.

STATISTICAL ANALYSIS

The results were analysed with non-parametric tests (χ^2 , Wilcoxon, Mann-Whitney U, and Spearman rank correlation tests) as appropriate. All calculations were performed on a personal computer with SPSS software (SPSS Inc, Chicago, IL, USA).

Results

PROLIFERATION ASSAYS

A significant T_H lymphocyte proliferative response to at least one HCV protein was found in 23 of the 35 patients without hepatitis C viraemia (group A) compared with five of the 31 patients seropositive for HCV RNA (group B) ($\chi^2 = 16.5$, $p = 0.00005$). A multispecific T_H lymphocyte response to two or more HCV proteins was found in 18 patients from group A compared with one patient only from group B ($\chi^2 = 19.3$, $p = 0.00001$) (fig 1). HCV core protein proved the most immunogenic, with 34.8% of all patients (both group A and B) having a significant proliferative response. Among responders in group A, the mean SI was lowest to NS5 (7.8, range 2.6–16.7), with NS3, NS4, and core all having a mean SI > 20 (NS3 = 26.2, range 2.6–142.3; NS4 = 22.5, range 2.6–79.9; core = 25.4, range 3.8–141.8). For group B, the four responders to core had a mean SI of 10.9 (range 2.6–27.2).

Patients in group A without a lymphoproliferative response ($n = 12$) were comparable with those with significant T cell reactivity to HCV ($n = 23$) in terms of age, sex, route of infection, and estimated duration of infection.

Virus specific lymphoproliferation was confirmed to be T_H restricted in two ways. Firstly, progressive CD4 cell depletion resulted in complete loss of cell proliferation to NS4 in patients with high SI (fig 2). Secondly, FACS

Figure 3 Comparison of *in vitro* production of interferon- γ (A) and interleukin-10 (B) in response to hepatitis C virus (HCV) proteins in patients with viral clearance (group A) and patients with viral persistence (group B). Bars indicate mean concentration of cytokine produced by lymphocytes cultured in the absence of HCV proteins (no antigen) or in the presence of HCV proteins (with antigen). Vertical lines represent standard error. *p* values were calculated using the Wilcoxon test on all values.

analysis of PBMCs cultured in the presence of NS4 showed increases in both CD4 cells and the proportion of cells expressing the T lymphocyte activation marker CD25 when compared with cells cultured in medium alone, with 90% of the cells expressing CD25 being CD4 positive.

IN VITRO CYTOKINE PRODUCTION

Mean levels of IL-10 and IFN- γ production from PBMCs cultured in the absence of any HCV protein were similar in the two groups (fig 3).

Figure 4 Comparison between groups A (shaded bars) and B (solid bars) of antibody responses to individual hepatitis C virus (HCV) antigens as measured by LIA-Scan. (A) The percentage of patients with detectable antibody to each HCV antigen. *p* values were calculated using the χ^2 test. (B) Quantification of serum antibody levels in arbitrary units to HCV non-structural protein (NS)3, NS4, NS5, core 1, core 2, and E2. Bars represent mean values from all those with detectable antibody, excluding values of zero or below the cut off value. Vertical lines represent standard error. *p* values were calculated using the Mann-Whitney U test for unpaired samples. Core 1 and core 2 are non-overlapping epitope clusters from HCV core. E2 refers to envelope 2/hypervariable region 1.

In group A, mean IFN- γ production was significantly increased in response to all four proteins tested ($p < 0.01$ in each case using Wilcoxon test). In group B, mean IFN- γ production was increased only in response to NS4 ($p < 0.01$), but not to NS3, NS5, or core. IL-10 production was slightly enhanced only by HCV core in group A ($p = 0.03$) and by none of the proteins in group B. No patient from either group produced detectable amounts of IL-4 except to control antigen. For individuals in group A there was a close correlation between the presence of a lymphoproliferative response to an HCV protein and an increase in *in vitro* IFN- γ , but not IL-10, production in response to the same protein. In addition, the strength of the T_H lymphocyte proliferative response to the HCV protein correlated closely with the magnitude of the increase in IFN- γ production in response to the same protein. For responses to HCV core, correlation coefficient $r = 0.86$ ($p = 0.0003$); for NS3, $r = 0.77$ ($p = 0.003$); for NS4, $r = 0.70$ ($p = 0.01$); and for NS5, $r = 0.63$ ($p = 0.06$).

HUMORAL IMMUNE RESPONSE

Total HCV antibody levels were significantly lower in the group A cases by both third and fourth generation screening tests (mean (SE) = 1445 (98) and 1594 (103) units of absorbance in group A and 1942 (41) and 2078 (35) in group B; $p = 0.0001$ and 0.00006 respectively). For antibody responses HCV core antigens again proved most immunogenic, with all 25 patients from group B and 34 and 33 patients from group A producing antibodies to core 1 and core 2 respectively. Antibody responses, as analysed by LIA-Scan, to all other antigens were found significantly less frequently in group A patients (fig 4A). In addition, even when detectable antibody responses to individual antigens were present in group A patients, the quantity of antibody produced to these regions was lower than in group B patients, reaching statistical significance for antibodies to NS4, NS5, and core 2 (fig 4B). Measurement of envelope antibodies by enzyme immunoassay showed anti-E2 antibodies in 11/35 patients from group A in comparison with 17/25 from group B ($p = 0.005$) and E1Ab in 15/35 in group A and in 14/25 in group B ($p = ns$).

Discussion

The present series comprises the largest group of patients yet reported who are seropositive for antibodies to HCV but repeatedly negative for HCV RNA in the serum, most of whom have been studied many years after the likely time of first exposure to HCV. Comparison of the HCV specific humoral and cellular immune responses found in this group with those found in patients with chronic hepatitis C and persistent viraemia showed that the presence of a strong and multispecific T_H lymphocyte response to a range of HCV proteins is strongly associated with spontaneous control of hepatitis C viraemia. In contrast, virus specific antibody responses in patients with absent viraemia were found to be both weaker and targeted at fewer antigens than in patients with serological evidence of continuing HCV replication.

The demonstration of powerful T_H cell responses in patients seropositive for HCV antibody but with undetectable HCV RNA in this study is supported by similar findings reported in a smaller series of cases.²³ By analysing the cytokine profile produced in response to HCV proteins, we have in addition been able to define the T_H cell response in these patients to be predominantly T_H1 in nature. This would serve to promote cellular effector mechanisms rather than humoral responses¹⁴ and is in keeping with the dissociation found between T_H cell responses and antibody responses in patients who remain without detectable viraemia.

Studies of patients with acute hepatitis C have suggested that the strength of the T_H cell responses during the early stages of infection may be a critical determinant of disease resolution and control of infection.^{18, 19} Our findings extend these observations by showing that a powerful T_H response can still be shown in most patients who are able to control HCV replication and remain persistently negative for HCV RNA in the serum many years after the time of first exposure to HCV. Furthermore, our results indicate that it is the breadth of the T_H cell response to a range of HCV structural and non-structural proteins that most distinguishes patients who have cleared virus from those with chronic hepatitis C and persistent viraemia.

The maintenance of such a strong and broadly targeted T_H cell response many years after the likely time of infection is comparable with the recent demonstration of vigorous T lymphocyte responses to hepatitis B virus (HBV) antigens many years after acute self limited HBV infection.^{24, 25} Trace amounts of HBV DNA can be found in serum or PBMCs in some of these patients,^{24, 26} and it is possible that occult HCV infection persists in the patients we studied and is responsible for maintaining the immune response. A recent study on intrahepatic HCV RNA detection and quantification in liver biopsy specimens from patients with HCV infection included 12 patients who were repeatedly seronegative for HCV RNA, and low levels of intrahepatic HCV RNA were found in 10 of these 12 cases.²⁷

Although some of these patients had abnormal liver enzymes, unlike any of the HCV RNA seronegative cases included in our study, it suggests that the liver may well have been acting as a reservoir of occult infection in some of the cases we studied.

In contrast with the T_H cell reactivity, the humoral immune responses to HCV antigens are weaker and less frequently detectable in HCV RNA negative patients. Anti-envelope protein antibodies can be protective against viruses related to HCV,^{28, 29} as this region may be important for cell attachment and entry, and there is some evidence that antibodies to the hypervariable region 1 of the HCV protein E2 are neutralising.³⁰ However, we found that E2 antibodies were present significantly less frequently in patients with serological viral clearance compared with those with viral persistence, which is in keeping with other work showing that anti-envelope antibody titre correlates with HCV viraemia.^{31, 32}

The differences in T cell reactivity in the two patient groups we studied are unlikely to result from different HCV genotype distribution, as, although the recombinant proteins used are derived from HCV genotype 1a, significant T cell responses were also detected in patients infected with genotypes other than 1a. Similar cross reactivity between genotypes at the level of T cell recognition has been reported by other groups using the same HCV proteins.^{6, 23} Compartmentalisation of HCV-specific lymphocytes within the liver of patients with chronic HCV infection has been shown³³ and could explain the finding of weaker T cell responses from the peripheral blood lymphocytes of chronically infected cases. However, Minutello *et al*³³ were only able to show enrichment of NS4 specific T_H cells within the liver and did not find evidence for compartmentalisation of T_H cells specific for any other region. Furthermore, patients with acute self limited HCV infection have been shown to have strong peripheral blood lymphocyte responses even at the peak of serum transaminases^{18, 19} when compartmentalisation would be expected to be maximal. Variability in immune response is often associated with HLA polymorphism, and HLA DR alleles have been associated with clearance of HBV infection.³⁴ We have recently shown a significantly higher frequency of HLA DQB1*0301 in patients with viral clearance compared with those with chronic infection,³⁵ and the role of this allele in influencing T_H cell responses to HCV antigens is currently under investigation.

Most of the patients studied who were seronegative for HCV RNA had potential for repeated inoculation with HCV through intravenous drug use over many years, and one patient had repeated clotting factor concentrate infusions before 1985 and the introduction of heat treatment, when each batch (produced from a pool of 20 000 donors) would be expected to be contaminated with HCV (J A J Barbara, National Blood Service, London, UK, personal communication), and despite this they did not develop chronic viraemia. This suggests that the immune responses

that we have shown in these patients may also have a role in protection from reinfection with HCV. To date research into vaccine design and immunotherapy for established HCV infection has focused on HCV core as the most conserved HCV antigen. Our findings in the patients able to control HCV replication suggest that a successful vaccine will need to induce a powerful and multispecific T_H cell response to a number of HCV proteins. Naked DNA vaccines³⁶ that achieve this aim may well be possible in the near future, and, using a similar approach, a DNA vaccine that encodes several HIV proteins has recently been shown to induce both humoral and cellular immune responses and protect chimpanzees from HIV infection.³⁷

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