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HCV-specific cellular immune responses in subjects exposed, but uninfected by HCV

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CD81 Sequence and Susceptibility to Hepatitis C Infection

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Several cell surface molecules have hepatitis C virus (HCV) binding properties and may serve as receptors facilitating viral entry into cells. The large extracellular loop (LEL) of CD81 has been shown to bind the HCV envelope protein E2 with several critical residues for the CD81-HCV-E2 interaction. It was hypothesised that variation in the CD81 LEL sequence may modify susceptibility to HCV infection. HCV RNA negative patients with spontaneous viral clearance (RNA −ve); HCV RNA positive cases, who are affected chronically (RNA +ve); and patients at high risk of HCV infection, exposed but uninfected patients (EU) were studied. Genomic DNA was extracted from whole blood samples and four exons of the CD81 LEL gene were amplified by PCR and sequenced. The cDNA derived from CD81 ($\approx$700 bp) was sequenced following RNA extraction from peripheral blood mononuclear cells. Patients, who are RNA positive, RNA negative, and exposed uninfected were sequenced for four DNA sections (A, B, C, and D). Sixty-two (43M:19F) patients, from all the patient cohorts, were sequenced and compared for the C section alone (which encompasses the important binding region of the molecule for envelope protein) including 21 (14M:7F) HCV RNA negative, 15 (10M:5F) HCV RNA positive and 26 (20M:6F) exposed uninfected and no sequence differences were observed. The entire CD81 sequence from cDNA was obtained in 23 cases— 11 RNA −ve, 5 RNA +ve and 7 EU. In 7 of the 23 cases, the nucleotides were confirmed with the genomic sequence (4 RNA −ve and 3 EU cases). No sequence variation was found in any of the patients studied by either method, including gene sections encoding the residues most important for CD81-HCV E2 binding. The LEL of CD81 is a molecule that is highly conserved. No differences in nucleotide sequence influencing susceptibility to, or outcome of HCV infection or evidence of methylation of the gene were found. **J. Med. Virol.**
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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, cirrhosis, and liver cancer occurring in up to 3% of the world's population [Alter, 1999]. Parenteral exposure to HCV is the major mode of transmission of infection and once established, infection will persist in up to 85% of individuals with only a minority of patients clearing viraemia. The apparent resistance to HCV infection is a phenomenon that has been described recently [Cramp et al., 1999; Freeman et al., 2005] whereby individuals have no evidence of infection despite prolonged injection drug use and likely repeated parenteral exposure to HCV. Resistance to viral infection can be mediated by altered cell membrane receptor binding and consequent inability of the virus to infect cells, as has been

described for HIV infection with the delta 5 CCR5 mutation [Huang et al., 1996]. The full mechanism by which HCV binds and gains entry to cells is not fully understood but it is thought that the HCV envelope glycoprotein, E2, is an important factor in the binding of the HCV virion to the host cell membrane, via the CD81 molecule on the cell surface. The binding of HCV E2 to CD81 is specific for the HCV strain and modulated by hypervariable regions 1 and 2 of E2 [Roccasecca et al., 2003]. T-cell, B-cell, and NK cell activation may be affected by E2 binding to CD81 as CD81 facilitates these events [Crotta et al., 2002; Deng et al., 2002; Levy and Shoham, 2005].

CD81 is a cell-surface tetraspanin that is widely distributed and participates in different molecular complexes on various cell types, including hepatocytes, B lymphocytes, T lymphocytes, and natural killer cells [Levy et al., 1998]. CD81 is a tetraspanin molecule that transverses the cell membrane four times creating two hydrophilic extracellular domains [Oren et al., 1990]. It is highly pleiotropic and has been associated with susceptibility to infection, metastasis of cancer cells, cell-to-cell interactions in the central nervous system and the fertilization of oocytes [Levy et al., 1998]. All cells of the immune system express tetraspanins [Tarrant et al., 2003] and it has been suggested that CD81 mediates HCV entry into host cells in concert with other cellular factors [Bartosch et al., 2003; Gardner et al., 2003; Yamada et al., 2005].

Recent studies show that recognition of HCV-infected hepatoma cells by plasmacytoid dendritic cells, involving CD81/CD9-only associated membrane microdomains, induces potent IFNα production during viral cell-to-cell contact with receptor mediated endocytosis [Zhang et al., 2012]. It is the LEL of CD81 that interacts with the major virus envelope protein E2 [Masciopinto et al., 2001]. Technical advances in RT-PCR and electron microscopy techniques have enabled the development of a model that replicates the early steps of the HCV life cycle. A number of research groups have shown that the envelope protein E2 binds to CD81 in humans and chimpanzees but the fact that it is expressed ubiquitously on human cells may indicate that it is not an important factor in the hepatocyte restricted tropism that occurs in HCV infection unless other liver specific cells are important cofactors with CD81 such as L-SIGN [Cole et al., 2004]. Mutations that alter use of the CD81 receptor also allowed the virus to escape neutralizing antibodies. These experiments replicate the mechanism of viral infection, mediating viral escape and leading to persistent infection in general [Fofana et al., 2012].

Recent studies have shown that the expression levels of CD81 receptors and others are modified by HCV exposure, not only in the classic but also in the occult infection [Roque-Cuéllar et al., 2012].

Several mutations in the 3′ non-coding region of CD81 cDNA have been observed exclusively in HCC tissue suggesting its possible role in hepatocarcino-genesis [Itakura et al., 2001]. It has been suggested that CD81 may play a role in the susceptibility to HCV infection. CD81 is exploited by one another human pathogen, malaria-causing *Plasmodium falciparum*. The infection is limited to hepatocytes and can be blocked by a CD81 monoclonal antibody. Mice lacking in CD81 cannot be infected by *P. falciparum* [Silvie et al., 2003]. The African Green Monkey is not susceptible to HCV infection and differs from humans in the binding region on the LEL of CD81 by four amino acids, thus it is postulated that a polymorphic difference in the binding region of the LEL of CD81 may alter the individual's susceptibility to HCV infection (Fig. 1).

CD81 is located on chromosome 11p15.5 and is in an imprinted region of the human genome, rich in CpG islands where methylation occurs frequently and it was considered that this might affect the cellular protein expression of the receptor so cDNA as well as genomic DNA was examined to ensure that the gene was expressed [Levy et al., 1998].

PATIENTS

Patients who were exposed to HCV but remained uninfected, patients who were chronically infected with HCV and those patients that resolved HCV were studied.

The group of patients that were exposed but uninfected, with apparent resistance to infection, was identified from intravenous drug users attending community drug services or in prison. HCV exposure risk was determined by reported injection behavior and history of sharing needles, syringes, or other drug injecting equipment on a repeated basis over a period of several years. A questionnaire was used to assess likely HCV exposure. The patient group that was chronically infected was both HCV antibody and HCV RNA positive. All patients found to be RNA positive had abnormal liver function tests and/or biopsy evidence of HCV related liver disease.

The patient group that resolved their HCV infection tested HCV antibody positive but repeatedly HCV RNA seronegative with persistently normal liver function tests over a period of at least 18 months with no clinical evidence of liver disease. None had received antiviral treatment at any stage and were considered to have spontaneously cleared virus and recovered from HCV infection.

Ethics committee approval and patient consent was obtained for all studies performed.

Virological Testing

In all groups the presence or absence of HCV antibodies and HCV RNA was determined by standard commercial assays (Abbott IMx, Abbott Diagnostics, Maidenhead, UK; Amplicor, Roche, Basel, Switzerland).
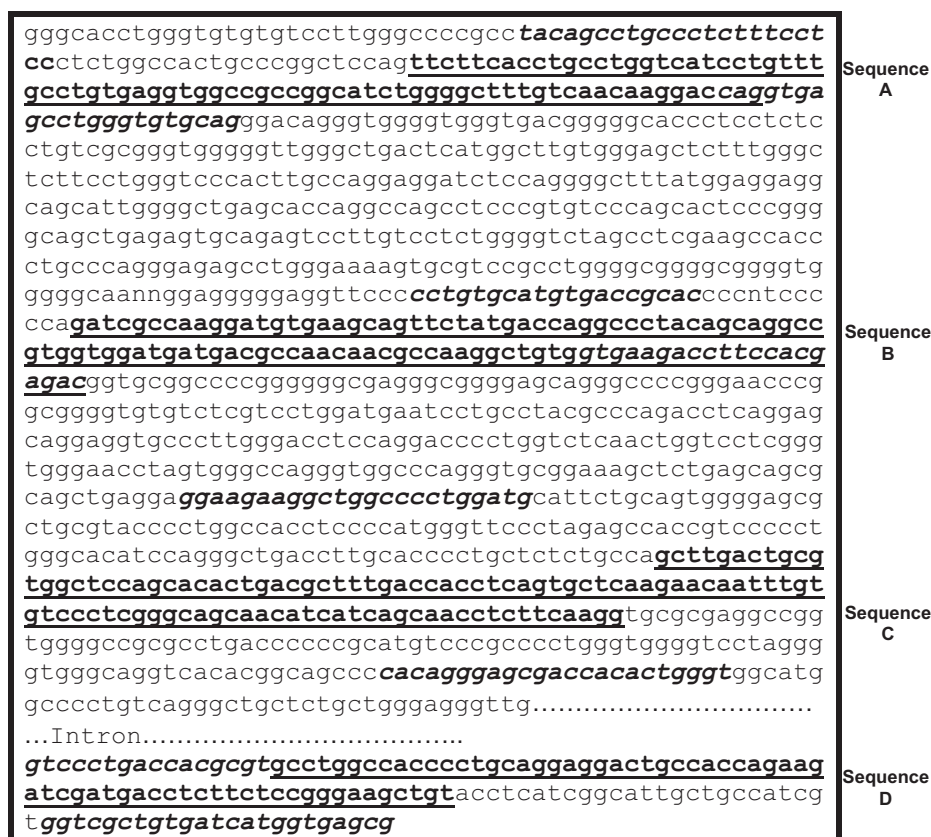
CD81 extra cellular loop genomic DNA

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Sequence A

Sequence B

Sequence C

Sequence D

Fig. 1. Nucleotide sequence of genomic CD81 and highlights the primers used to amplify the exonic sections.

Methods Employed

The intention of this investigation was to look for differences in the DNA sequence of CD81 (see Fig. 1) that may modify the disease outcome of HCV infection and may result in resistance to infection or spontaneous resolution of infection.

CD81 DNA Sequence

There were three different approaches to this analysis.

1. Amplifying and sequencing cDNA extracted from patient blood for the whole length of the transcribed part of the CD81 molecule using two primers (see Table I).
2. Amplifying and sequencing genomic DNA from four exonic sections of the CD81 gene corresponding to the transcribed part of the LEL from patient blood.

Each PCR was performed three or four times to ensure enough DNA for sequencing. Each amplified product needed to give bright bands on the agarose gel under UV light to be suitable for the sequencing process. The samples were sent to Bath University for sequencing analysis.

DNA Sequencing

A PEG precipitation method was used to clean the samples. The clean samples were then cycle sequenced with Big Dye terminators version 3.1 on an AB1 9600 thermal cycler, using standard cycling conditions as described in the Applied Biosystems Big dye 3.1 manual (Foster City, CA).

The samples were then ethanol precipitated and deionised formamide was added to the samples. The samples were denatured at 95°C for 2 min and run on a 3700 DNA Analyzer (Applied Biosystems).

RESULTS

Kings College Hospital Patient DNA

The King's College Hospital, London patient DNA (30M:18F, n = 48) included RNA positive with a mean age of 49.1 ± 1.69 SEM years (10M:5F, n = 15), RNA negative mean age 43.57 ± 3.25 SEM years (10M:6F, n = 16) and patients exposed to HCV but resistant to

TABLE I. Table of Primers for CD81 Sequencing[a]

DNA/Primers	A	B	C	D
cDNA *Jun Itakura et al J Medical Virol 63:22-28 (2000)*	5' GTG CTG GAA AGG CGC GCA ACG GCG G 3'F* 5' CAG CTC CAG ATA GAG GTT GGT G 3'R**	5' GGG AGT GGA GGG CAC CAA GT 3'F 5'GGG AGT GGA GGG CTG CAC CAA GT 3'R	5' GCT GGG GAC GTT CTT CAC CTG C 3'F 5' CTC AGT ACA CGG AGC TGT TCC GG3'R	5' CTT CGA GAT GAT CCT GAG CAT GGT G 3'F 5' TTA TTA AAT GAC GGA GTC AGG T 3'R
	Nested 5' AAC GGC GGC GAC GGC GGC GA 3'F 5' TGG TGG TCT GCG GGT CAT GG 3'R	**Nested** 5' CTG CTT CAC ATC CTT GGC GAT CT 3'R		**Nested** 5'GCA TGG TGC TGT GCT GTG GC 3'F 5' CAG GAT GTT GTG ATT ACA GT 3'R
cDNA (CD81D) *Shoshana Levi*	5' CTG CAC CAA GTG CAT CAA GTA 3'F 5'CAC AGC ACC ATG CTC AGG 3'R	→		
Genomic DNA *Annwyne Houldsworth*	5'TAC AGC CTG CCC TCT TTC CTC C 3'F 5'CTG CAC ACC CAG GCT CAC CTG 3'R (CD81A)	5' CCT GTG CAT GTG ACC GCA C 3'F 5' CGT CTC GTG GAA GGT CTT CAC 3'R (CD81B)	5' GGA AGA AGG CTG GCC CCT GGA 3'F 5' ACC CAG TGT GGT CGC TCC CTG TG 3'R (CD81C)	5' GTC CCT GAC CAC GCG TGC CTG 3'F 5' CTG TCA CCA TGA TCA CAG CGA CCA 3'R (CD81D)

*'F' denotes a forward primer.
**'R' denotes a reverse primer.
[a]Primers used for the different sequencing procedures.

infection mean age 41.8 ± 1.84 SEM years (10M:6F, n = 16).

South West Clinic HCV Antibody Positive Patients

Patients that are positive for the HCV antibody were recruited from the HCV clinic at Derriford Hospital, Plymouth (8M:4F, n = 12) with a mean age of 42.1 ± 2.72 SEM years. cDNA was synthesized from patients that are HCV RNA negative, mean age 41.9 ± 3.51 SEM (5M:2F, n = 7) and patients that are RNA positive mean age $42.5.0 \pm 4.45$ (3M:2F, n = 5).

South West Patients Exposed to HCV But Resistant to Infection

South West patients that were antibody HCV negative and RNA negative (10M:1F, n = 11) with a mean age of 29.8 ± 1.11 SEM years were recruited from prisons (Dartmoor and Channingwood) and drugs rehabilitation programs based at Plymouth and included patients with reported injection behaviour and a history of sharing needles, syringes, or other drug injecting equipment on a repeated basis over a period of several years (Horne et al., 2004).

South West cDNA Patients

HCV RNA was isolated from 23 of South West patients in all, including HCV RNA negative (8M:3F,

n = 11), HCV RNA positive (3M:2F, n = 5), and exposed uninfected (6M:1F, n = 7) and the whole CD81 molecule was sequenced from cDNA.

South West Genomic DNA

Genomic DNA was extracted from the PBMCs of 11 of the South West patients who are exposed to HCV but uninfected (HCV antibody negative, RNA negative) (10M:1F) and from 5 of the South West patients that were found to be RNA negative (5M:0F).

Exonic sequences of CD81 of the large extracellular loop (LEL) and CD81 cDNA were compared between patient groups by analyzing sequence data (see Table II for all patients studied).

Comparison of Kings College Hospital Patient Genomic Sequences

The 46 patients from Kings included patients with no HCV RNA (n = 11), patients who test positive for HCV RNA (n = 9) and also patients who remain HCV RNA negative despite exposure to the virus (EU) (n = 16) were compared for their genomic sequences in all four exons. There were no differences observed between the genomic sequences in any of these groups including the important HCV-binding section of the gene encoding the 163 and 186 amino acid residues which in mutational studies have been found to change the nature of the E2 binding.

TABLE II. Complete Nucleotide Sequences of CD81 in HCV Infected Cases and HCV Exposed Cases[a]

Cases	Mean age/tears ± SEM	Sex M:F	DNA fragment A	DNA fragment B	DNA fragment C	DNA fragment D	cDNA
SW EU	29.8 ± 1.11	10:1	10	8	10	11	7
SW −ve	41.9 ± 3.51	4:1	4	3	5	4	11
SW +ve	42.5 ± 4.45	0:0	0	0	0	0	5
Kings EU	41.8 ± 1.84	10:6	16	16	16	16	0
Kings −ve	49.1 ± 1.69	10:6	14	13	16	15	0
Kings +ve	43.57 ± 3.25	10:5	15	13	15	12	0
Total			59	53	62	58	23

[a]The number of complete exons of the large the extra cellular loop of the CD81 molecule from the genomic DNA sequence. The patients were characterized by their mean age and standard error of the mean and the male to female ratio in the group. cDNA sequences are also recorded. The patient groups included HCV antibody positive/RNA positive (RNA +ve), antibody positive/RNA negative (RNA −ve) and patients that were exposed but remained uninfected were antibody negative/RNA negative (EU).

Comparison of cDNA in South West Patients

The sequencing of cDNA for the whole LEL molecule (≈ 700 bp), synthesised from RNA that was extracted from the PBMCs of patients infected with HCV, recruited from Derriford hospital HCV clinic, the drug rehabilitation centres, and prisons (11 RNA negative patients, 5 patients with positive RNA, and 7 individuals that were exposed but uninfected showed no differences in nucleotide sequence.

The 6 (5M:1F) EU patients and the 4 (4M:0F) RNA negative patients were compared for both genomic DNA and cDNA showing no differences in the sequences.

Comparison of All DNA Encoding the Envelope Protein Binding Site of CD81

Sixty-two (43M:19F) patients, from all the cohorts used in the study, were sequenced for the C section alone (which encompasses the important binding region of the molecule for envelope protein) including 21 (14M:7F) HCV RNA negative, 15 (10M:5F) HCV RNA positive as well as 26 (20M:6F) patients who were exposed but uninfected and no sequence differences were observed.

Three of the DNA and cDNA sequences were duplicated as an internal standard to ensure that the method had reproducible results.

The sequencing was performed by Dr. Paul Wilkinson at Bath University Genomics Laboratory.

DISCUSSION

It is clear that CD81 is an important molecule involved in the pathogenesis of HCV infection but the exact mechanism of infection is not fully understood. In the absence of CD81 expression, although B cell proliferation is impaired, the proliferative response of T cells is enhanced [Miyazaki et al., 1997]. However, CD81−/− mice do not develop allergen-induced hyper-reactivity and show impairment in Th2 cell responses [Oren et al., 1990].

There is evidence that CD81 is also expressed on CD8 +ve lymphocytes, and that this expression is variable. High binding affinity of HCV-E2 is associated with high CD81 expression on CD8 +ve lymphocytes [Kronenberger et al., 2004].

T cells in general but CD8+ cells in particular have also shown a decrease in CCR5 expression when PBMCs were exposed to HCV E2. Downregulation of the CCR5 cell surface expression on T cells by the presence of HCV E2 proteins and their association with CD81 may result in important consequences for an HCV antiviral immune response in patients infected with HCV, including the impaired recruitment of T cells into the liver [Nattermann et al., 2004].

A chemical messenger involved in chemotaxis called "regulated on activation, normal T-cell expressed and secreted" (RANTES) binds to the CCR5 receptor. Two other chemokines selectively stimulate T-cells, macrophage inflammatory protein-1β and 1α (MIP1β, MIP1α). T cell recruitment via chemotaxis (MIP-1α, MIP-1β, and RANTES) is impaired as a result of PBMC exposure to E2. It has been postulated that HCV-CD81 binding may induce this impairment as CD81 increases RANTES secretion by CD8+ T cells and down-regulates CCR5 expression [Nattermann et al., 2004]. HCV-E2-The engagement of CD81 has also been reported to reduce the activation threshold of B- and T- cells and to inhibit T-cell function [Wack et al., 2001].

Both genomic and cDNA was amplified and sequenced from PBMCs in a number of subjects including healthy controls, patients seropositive for the replication of HCV and patients that were seronegative for HCV, despite having subjected themselves to parenteral exposure to the HCV virus showed no genomic differences between the groups. The group of individuals that were exposed but remained uninfected had not been examined in this way before and their CD81 sequence was compared to that of patients infected with HCV.

Another research group have compared the exons of CD81 encoding the LEL in 35 patients with HCV infection but they did not find any novel polymorphisms [Hennig et al., 2002].

Investigations into the sequence of the LEL of CD81 confirmed that it is a molecule that is highly

Protein sequence for CD81

MGVEGCTKCIK *YLLFVFNFVFWLAGGVILGVALWL* **RHDPQTTNLLYLEL**

GDKPAPNT *FYVGIYILIAVGAVMMFVGFLGCYGAI* **QESQ** *CLLGTFFTCLVI*

LFACEVAAGIWG **FVNKDQIAKDVKQFYDQALQQAVVDDDANNAKAVV**

KTFHETLDCCGSSTLTALTTSVLKNNLCPSGSNIISNLFKEDCHQKIDDL

FSGKLY *LIGIAAIVVAVIMIFEMILSMVLCCGI* **RNSSVY----**

→ SHORT EXTRACELLULAR   → *TRANSMEMBRANE*

→ **INTRACELLULAR LOOP**   → **LONG EXTRACELLULAR LOOP (LEL)**

Fig. 2. Protein sequence for CD81 molecule related to its tertiary structure.

conserved between individuals (Fig. 1). Conservation of the CD81 molecule is important as it is involved in so many cellular functions. It was hoped that a nucleotide base change would be found that may be associated with susceptibility to infection with particular interest in the folding region sulphide-bridged by the cysteine residues at the binding site of CD81.

A comparison of the sequences of genomic DNA that are untranslated between the patient groups with different clinical outcomes would be an interesting extension to this study. One research group has identified several 3′UTR mutations in hepatocellular carcinoma tissue that was related to HCV infection suggesting a possible role in carcinogenesis for CD81 [Itakura et al., 2001]. HCV has been found to protect B cells from Fas-mediated apoptosis via E2 CD81 engagement [Chen et al., 2011]. Infection with HCV is often associated with B-cell lymphoproliferative disorders such as mixed cryoglobulinaemia and non-Hodgkin lymphoma [Weng and Levy, 2003)]. Introns from polymorphic areas of the genome may alter the folding or expression of the molecule which could affect the binding regions in some way, however, in the 23 patients where the cDNA was compared for the whole CD81 molecule there were no nucleotide differences between any of the patients whether they were HCV RNA positive, RNA negative and exposed uninfected.

The tertiary structure of the CD81 molecule may prove an interesting area of study looking for aberrant folding, particularly in the binding regions as well as examination of post-translational transformation, searching for glycosylation, and other post-translational alterations. The use of monoclonal antibodies to the binding sites of CD81 may reveal differences in protein conformation due to post-translational changes or aberrant folding that may affect CD81 binding (Fig. 2).

CD81 is expressed ubiquitously but is more highly expressed in the liver. Factors that influence CD81 expression may differ between individuals. E2 binding to CD81 during HCV infection can alter the expression of CD81. The examination of CD81 expression levels in patients infected with HCV from both peripheral blood and liver biopsies may reveal differences between individuals. Assays that measure the protein levels and mRNA of CD81 in individuals with different disease outcomes would be an interesting area to investigate.

Clearly there are many more investigations needed to clarify that CD81 changes in structure or function are responsible for individuals that are exposed but uninfected.

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