The CCR5-Δ32 Mutation: impact on disease outcome in individuals with hepatitis C infection from a single source


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ABSTRACT

Background and Aims:
Chemokines are small polypeptides, a major function of which is lymphocyte recruitment and trafficking. This study aimed to assess the involvement of inherited variations in CCR2, CCR5 and the ligand RANTES in determining disease outcome in HCV infected individuals.

Methods:
283 women, all exposed to HCV genotype 1b from a single donor, and including those who had spontaneously cleared the virus and those chronically infected, were genotyped for CCR2, CCR5 and RANTES polymorphisms. The frequencies of these polymorphisms were then compared with disease activity and severity.

Results:
CCR5, CCR2 and RANTES genotypes were compared with HCV PCR status, ALT levels and liver histology. There was no significant relationship between the CCR2 or RANTES polymorphisms and disease outcome or severity. However, CCR5Δ32 heterozygotes were more likely to have spontaneous clearance of the virus than those without the mutation (42% PCR negative vs. 28.3% negative, p = 0.044, OR = 1.83, 95%CI = 1.1-3.6). Amongst the sub-group of DRB1*03011 negative individuals, previously found to be associated with more severe inflammation, the difference in the histological inflammatory score (CCR5WT/WT= 4.9 vs. CCR5Δ32/WT = 3.53, p = 0.043) was significant.

Conclusion:
Heterozygosity for CCR5Δ32 was shown to be significantly associated with spontaneous hepatitis C viral clearance and with significantly lower hepatic inflammatory scores in sub-groups within this cohort. Both the controls and the HCV population had similar heterozygosity frequencies.
INTRODUCTION

Chronic hepatitis C virus infection (HCV) has a prevalence of approximately 2% worldwide. \(^1\) Up to 70% of those exposed to HCV remain chronically infected. Many aspects of response to HCV are poorly understood, including the reasons for the wide variation in disease severity and for spontaneous clearance versus chronic infection. Viral genotype, age at exposure, and alcohol consumption are known determinants of disease severity but the role of genetic predisposition to chronic infection remains unclear. The population in this study, comprising 283 women infected by contaminated anti-D immunoglobulin in 1977, represent a unique opportunity to assess a large HCV population with minimal confounding issues. \(^2\) The Anti-D immunoglobulin was contaminated from a single source, therefore all women were infected with the same genotype, i.e. genotype 1b, minimising the varying effect that genotype could have on the study.

Chemokines are small polypeptides with a significant role in leukocyte recruitment and trafficking. Leukocyte recruitment during inflammation requires intercellular communication between infiltrating leukocytes, endothelial cells and parenchymal cells, mediated by early response chemokines. The migration of T cells is modulated in vitro and in vivo by conditioning with chemokines. \(^3\) CCR5 is a receptor for the pro-inflammatory chemokines MIP1α, MIP1β and RANTES, which have key roles in host responses to viruses in both human and murine disease. \(^4\) A 32 base pair (bp) deletion in CCR5 results in a protein that is not detectable at the cell surface. Homozygosity for this deletion is found in 1% of Caucasians and has been shown to be protective against HIV infection, while the heterozygote state, which is found in 10% of Caucasians leads to a slower disease progression. Its role in other viral infections remains to be determined.

CCR5 and other chemokines play an important role in T cell differentiation. CD4+ T cells can differentiate into Th1 or Th2 cells depending on their exposure to chemokines. CCR5 is expressed on Th cells and also on memory and activated T cells. The migration of antigen primed T cells is facilitated by CCR5. \(^5\) When human T cell clones were analyzed, CCR5 appeared to be expressed at higher levels on Th1 cells, whereas many Th2 clones had no expression of CCR5. Sallusto et al demonstrated that CCR5 expression depends on the activation state of T cells and that its expression is up regulated by IL-2. \(^6\) It has been proposed that in HCV, there is predominantly a Th1 response in the liver. \(^7\) Indeed progressive liver damage in HCV is associated with up-regulation of Th1 cytokines (IFN and IL-2), as shown by Napoli et al, where increased expression of IFNγ and IL-2 correlated with both fibrotic and portal inflammatory histological scores. \(^7\)

CCR5 along with CCR2 are two of a cluster of six chemokine receptor genes mapped to 3p21. CCR2 codes for a minor HIV receptor, for which a G to A coding sequence polymorphism resulting in a valine to isoleucine substitution, designated CCR264I, has been described. It appears that CCR2WT is in complete linkage disequilibrium with CCR5Δ32, which is 10kb away. Thus, in a study of 3000 individuals, Smith et al (1997) \(^9\) demonstrated that the Δ32 mutation is never seen on the same haplotype as the CCR264I mutation. The distribution of CCR2 and CCR5 in cells and tissues is very similar. CCR2 signalling also promotes Th1 development in infection.
models and studies using a CCR2 knockout mouse have shown that these mice have a 46% reduction in lymphocyte recruitment to sites of infection and inflammation and an 80% reduction in CD4+ T cells locally. (10) RANTES, the CCR5 ligand, is also critical for lymphocyte recruitment, as it attracts memory and activated T cells. An A to G mutation of RANTES has been described at position -403 resulting in an additional GATA transcription factor binding site, with the mutant promoter having up to an 8 fold higher constitutive transcriptional activity than the wild type. (11)

In normal liver, RANTES expression is restricted to a few scattered hepatocytes. However, in HCV infected livers its expression was significantly elevated, especially in periportal and lobular areas that had the most lymphocytic infiltration. (12) In view of this we postulated that genetic variation in either CCR5 receptors or in the chemokines binding to such receptors might have an impact on outcome of hepatitis C infection. The impact of such variation might be difficult to detect in populations in which there was heterogeneity in terms of ethnicity, viral genotype and source and dose of infection. Hence, we have undertaken a study of the genetic impact of both the CCR5Δ32 mutation and the RANTES position-403 mutation on the outcome of hepatitis C infection in a genetically homogenous population infected through a single source.

**Patients and Methods:**

**Study Population:**

The study population of 283 women was recruited from the outpatient hepatology units in St. James, St. Vincent’s and the Mater Misericordiae Hospitals in Dublin. All women attending these units who had been exposed to HCV via contaminated anti-D immunoglobulin in 1977 were invited to participate. The group includes those who are both chronically infected, persistently HCV RNA positive as determined by RT-PCR and those who have cleared the virus, i.e. remain HCV anti-body positive but RNA negative. None of the participants had any other risk factors for acquisition of viral hepatitis, e.g. blood transfusion or past history of intra-venous drug abuse. All had an alcohol consumption of less than 14U/wk and other forms of chronic liver disease were excluded in all cases. Of the 283 initially exposed to contaminated anti-D immunoglobulin, 196 remained chronically infected, i.e. RT-PCR(RNA) positive and 87 were anti-HCV antibody positive, but persistently RNA negative, i.e. they had cleared the HCV infection. The RNA negative individuals had an average of six RT-PCR reactions carried out at different time points to confirm spontaneous viral clearance. The majority of these subjects had already been genotyped for both class I and Class II HLA polymorphisms, and a significant association was found between viral chronicity and the presence of DRB1*03011 and DQB1*0201. (13) All subjects gave informed consent prior to participating in the study, which received ethical approval from the Research and Ethics Committees at all three institutions.

**Controls:**

To estimate the frequency of the CCR5Δ32 allele in the Irish population, a control group of 120 unselected, unrelated healthy volunteers were genotyped. These were health care workers and all of Irish descent.
Diagnosis of HCV Infection:

**HCV antibodies:**

A third generation enzyme immunoassay (ELISA) (Abbott Diagnostics, Germany) was used to test all subjects for HCV specific antibodies and a third generation recombinant immunoblot assay (RIBA 3) (Chiron Corp., Emeryville, CA) was then used as a confirmatory test.

**RT-PCR testing:**

An RT-PCR assay (Amplicor; Roche Diagnostic Systems, NJ) was used to test for HCV RNA in all subjects.

**DNA extraction**

A salting out technique was used to extract DNA from whole blood. (14) DNA was also extracted using the QLamp DNA midiprep kit, (Qiagen Ltd., Crawley, UK). During this process, all RNA was removed by incubating the digested preparation with 1.5µL ribonuclease A (Boehringer Mannheim UK Ltd., East Sussex, England) per 400µL of nuclear lysate according to the manufacturer’s instructions.

**CCR5-∆32 Genotyping:**

A PCR reaction consisting of PCR 1Xbuffer (as supplied by manufacturer), 25µM MgCl₂, 200µM deoxynucleotide triphosphates, 0.5µM of both forward and reverse primer, 15ng of extracted DNA, 1 unit of Qiagen DNA polymerase Taq and 14.3ul of H₂O was used to genotype both the subject and control groups. The primers flanking the CCR5-32 mutation (sense, 5’-CAAAAAAGAAGGTCTTCATTACACC-3’; antisense, 5’-CCTGTGCCTCTTCTTCTCATTTCG-3’) were used to amplify 189-bp (wild-type) and 157-bp (32bp deletion) fragments of the CCR5 gene, respectively. Following amplification the fragments were visualised on a 3% agarose gel.

**CCR264I Genotyping:**

Genotyping was performed by restriction digest of amplified fragments following electrophoresis as follows; a PCR reaction with the forward primer, 5’-TTGTTTGTGGGCAACATGATGG-3’ and the reverse primer 5’-CATTGCATTCCCAAGACCACCAC-3’ was performed to give a 173bp amplicon. This was then digested with the enzyme Bsa BI (New England Biolabs) to yield restriction fragments of 149 and 24bp . When the wild type sequence is present the fragment remains uncut, thus giving a band of 173bp.

**RANTES Genotyping:**

Applied Biosystems Ltd. designed a 5’ exonuclease assay using the ‘Assay by Design’ service for TaqMan analysis to genotype the RANTES 403 polymorphism. Forward primers 5’-GAGGACCTCCCTCAATAAAACACTTTATAAAT-3’, reverse primer 5’-ACTGAGTCTTCAAGGTCCTGCTT-3’ and the probes VIC CATTACAGATCTTACCTCTCCTTT and FAM CATTACAGATCTTACCTCTCCTT were used. As a quality control measure 10% of the samples were repeated in all of the above genotyping and all were in concordance. All of the above were read by one reader, who was blinded to the identification of the sample until all genotyping was completed and recorded.
Histological Evaluation:
All 196 RT-PCR positive women had had a liver biopsy taken as part of their initial clinical evaluation, i.e. prior to the commencement of any treatment, and between 17-20 years post infection. The liver biopsies were all scored by a single histopathologist in each centre, blinded to the CCR5 genotype of the individual. Biopsies were scored according to the modified histological activity index (0-18 for inflammation, 0-6 for fibrosis). HCV RNA negative subjects did not have a liver biopsy performed.

Statistical Analysis:
The Mann-Whitney U test was used to compare the histological, inflammatory and fibrotic scores and the alanine aminotransferase (ALT) levels, between the different subgroups classified according to patient genotypes. The association between viral clearance and polymorphism was assessed by the Chi-square and Fisher exact test. A p-value of 0.05 was deemed as significant for all of the above tests. The odds ratio for each of the different polymorphisms and disease association was also calculated by Epi-Info. The results were also analyzed taking the previous genotyping for HLA DR, DQ loci into account. All data was entered into the statistical package SPSS (SPSS Inc, Chicago, Illinois, USA) and Epi-Info.

Results:
The results of the genotyping for the three different polymorphisms were compared with HCV PCR status, histological scores and alanine aminotransferase (ALT) levels.

Genotypes associated with viral clearance
The heterozygote frequency for the CCR5Δ32 mutation in the general population was similar to that of the HCV study group (17.9% and 17.6%, allele frequency 0.193 and 0.186 respectively). There was only one CCR5Δ32/Δ32 individual in each group. The presence of the CCR5Δ32/WT (wild type) genotype was significantly associated with spontaneous viral clearance. 42.0% of those who were CCR5Δ32/WT were HCV PCR negative, versus only 28.3% of CCR5WT/WT (p= 0.044, one-sided Fisher’s exact test, Odds Ratio (OR) = 1.9, 95% CI = 1.1-3.6). Only one patient was homozygotic CCR5Δ32/Δ32 and she was HCV PCR negative. The allele frequency was in Hardy-Weinberg equilibrium for both patient and control groups. When the association of CCR5 genotype and viral clearance was looked at in the DRB1*03011 and DQB1*0201 negative groups, none was found (p=0.563 and 0.68, respectively). Analysis of the CCR264I (p=0.327, OR =0.66, CI =0.23-1.6) and RANTES (P=0.441, OR= 1.01, CI=0.58-1.7) genotypes failed to reveal any relationship with HCV clearance. See Table 1.
Table 1: Summary of polymorphism distribution and HCV PCR status.

<table>
<thead>
<tr>
<th>Genotyping</th>
<th>HCV PCR +ve (%)</th>
<th>HCV PCR –ve (%)</th>
<th>P-Value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5WT/WT</td>
<td>167 (85.2%)</td>
<td>66 (75%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5Δ32/WT</td>
<td>29 (14.8%)</td>
<td>21 (23.9%)</td>
<td>p = 0.044</td>
<td>1.9 (1.1-3.6)</td>
</tr>
<tr>
<td>CCR5Δ32/Δ32</td>
<td>0</td>
<td>1 (1.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR2WT/WT</td>
<td>125 (90.6%)</td>
<td>73 (93.6%)</td>
<td></td>
<td>0.66 (0.23-1.6)</td>
</tr>
<tr>
<td>CCR264I/WT</td>
<td>11 (7.8%)</td>
<td>5 (6.4%)</td>
<td>p = 0.327</td>
<td></td>
</tr>
<tr>
<td>CCR264I/64I</td>
<td>2 (1.6%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTESWT/WT</td>
<td>90 (63.8%)</td>
<td>54 (63.5%)</td>
<td>p = 0.441</td>
<td>1.01(0.58-1.7)</td>
</tr>
<tr>
<td>RANTES403/WT</td>
<td>45 (32%)</td>
<td>29 (34.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES403/403</td>
<td>6 (4.2%)</td>
<td>2 (2.4%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Shows the number of individuals with each genotype for the 3 different polymorphisms tested, divided according to their HCV PCR status. The figures in parenthesis are the percentage of those HCV PCR positive or negative, according to their genotype. P values were calculated using chi-square test, Odds ratio by Epi-Info.

**Relationship between genotypes and histological severity**

There was no significant difference in hepatic inflammatory scores between hetereozygotes for the Δ32 mutation and those without a copy of this mutation (HAI; 3.82 vs. 4.53, p=0.098) in this cohort. Furthermore, in the DRB1*03011 positive group, previously found to be associated with less severe inflammation, CCR5Δ32 had no further additive impact on histological severity; with a mean HAI of 4.16 for non-CCR5WT/WT and 3.80 for CCR5Δ32 heterozygotes, (p=0.78). In contrast, within the DRB1*03011 negative group, associated with more severe inflammation, CCR5Δ32 heterozygotes had significantly lower inflammatory scores than the CCR5WT/WT group (mean inflammatory score = 3.53 vs. 4.91, p= 0.043). See Table 2.
Table 2: Summary of CCR5 Results.

<table>
<thead>
<tr>
<th></th>
<th>CCR5 WT/WT (%)</th>
<th>CCR5Δ32/WT (%)</th>
<th>CCR5Δ32/Δ32</th>
<th>P- Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV PCR +ve</td>
<td>167 (71.7%)</td>
<td>29 (58%)</td>
<td>0</td>
<td>p = 0.044</td>
</tr>
<tr>
<td>HCV PCR - ve</td>
<td>66 (28.3%)</td>
<td>21 (42%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>4.53</td>
<td>3.82</td>
<td>p = 0.098</td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td>1.20</td>
<td>1.05</td>
<td>p = 0.503</td>
<td></td>
</tr>
<tr>
<td>DRB1*03011-ve inflam, n=81</td>
<td>4.91</td>
<td>3.53</td>
<td>p = 0.043</td>
<td></td>
</tr>
<tr>
<td>DRB1*03011+ve inflam, n=55</td>
<td>4.16</td>
<td>3.80</td>
<td>p = 0.78</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: details the impact of the CCR5Δ32 mutation on HCV PCR status, HAI scores for the entire group, and also for the sub-groups of DRB1*03011 positive and negative individuals. The p-value for the affect of PCR status was given by chi-square, and for HAI scores, as per Mann-Whitney U test.

Relationship between genotypes and ALT levels
The ALT levels were slightly higher in the CCR5 and CCR2 wild type group, compared with the heterozygotes, while the opposite was observed for the RANTES group. However none of these differences reached statistical significance. See Table 3.

Table 3: Summary of mean ALT levels and the polymorphisms examined.

<table>
<thead>
<tr>
<th></th>
<th>ALT levels</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5WT/WT</td>
<td>52.76</td>
<td>0.419</td>
</tr>
<tr>
<td>CCR5Δ32/WT</td>
<td>44.69</td>
<td></td>
</tr>
<tr>
<td>CCR2WT/WT</td>
<td>51.03</td>
<td>0.826</td>
</tr>
<tr>
<td>CCR264I/WT</td>
<td>40.67</td>
<td></td>
</tr>
<tr>
<td>RANTESWT/WT</td>
<td>49.07</td>
<td>0.535</td>
</tr>
<tr>
<td>RANTES403/WT</td>
<td>54.46</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Gives the mean alanine aminotransferase levels for all individuals with the above genotypes, and the associated p-values. P-value level of significance by Mann-Whitney U test.
Discussion:

This study shows significantly higher spontaneous HCV viral clearance in the CCR5Δ32/WT over the CCR5WT/WT group (p=0.044). This association was not found in Hellier’s\(^{(16)}\) or Promrat’s\(^{(17)}\) studies, both of which had several confounding issues in relation to HCV genotype, gender, and ethnicity. Specifically, Hellier’s study comprised individuals from multiple European populations and contained a lower percentage of viral negative patients. HCV genotype was not specified in this study. In addition, the infection came from multiple sources suggesting a high degree of HCV genetic heterogeneity. Hence a number of confounding variables are inherent to these studies that may have hindered the ability to detect changes in viral clearance. Our study population has a number of relatively unique features, principally that (i) all subjects are female and Caucasians of Irish descent; and (ii) all were infected by a single inoculum of HCV genotype 1b through anti-D immunoglobulin in 1977.\(^{(2)}\) They have no other risk factors for liver disease and no other significant co-morbid illnesses.

The CCR5Δ32 mutation arises from a 32bp deletion causing a frame shift mutation and premature termination of the protein. The resultant CCR5 mutant protein is likely to be functionally inert since it not only lacks the last three of seven putative transmembrane regions, but also lacks the domains involved in \(G\) protein coupling and signal transduction.\(^{(18)}\) Indeed, Liu et al showed that the resultant protein was not detectable on the surface of cells that would normally express it, and therefore cannot act as a receptor.\(^{(19)}\) However the role of the CCR5 mutation in HCV is not the same as for HIV since the method by which HCV gains entry into the cell is unknown, but unlike HIV, it is generally not believed to be related to the CCR5 receptor.

The heterozygous genotype was present in 17.6% of the HCV population and 17.9% of the control population, with the CCR5Δ32/Δ32 genotype found in 0.32% and 0.69% respectively, one homozygote in each group. This is one of the highest carrier rates reported for any European population and is consistent with the results of Libert et al who investigated the gene frequency in 18 European countries and found a North / South gradient, the highest frequencies being in Finland (16%) and the lowest in Sardinia (4%).\(^{(20)}\) Controversially, in 2002, Woitas et al reported that CCR5Δ32 homozygosity occurred three times more frequently in anti-HCV antibody positive, HIV negative individuals.\(^{(21)}\) The fact that this group remained HIV negative, despite multiple exposure would suggest that they were a selected population, most probably on the basis of the CCR5Δ32 genotype. Hence, the increased CCR5Δ32 heterozygosity most likely reflected resistance to HIV, rather than increased risk of HCV infection.

In explaining how the CCR5Δ32 polymorphism could alter HCV clearance, we must consider that the effect of CCR5 heterozygosity in acute HCV may not be representative of what happens in chronic HCV infection. In acute HCV infection, clearance is associated with a strong T cell response to a wide range of HCV specific antigens.\(^{(22)}\) Counter-intuitively, lack of CCR5 may actually lead to increased T cell expansion. This was demonstrated using an acute lymphocytic choriomeningitis (LCM) infection model in CCR5 knock-out mice, where clonal expansion of antigen specific T cells was increased, not decreased, among CD8+ and CD4+ T cells.\(^{(23)}\) Likewise, lack of
CCR5 has been associated with increased T cell production of IFNγ leading to the suggestion that CCR5 might be part of a negative regulatory feedback loop on acute T cell activation.\(^{(24)}\) In CCR5 deficient mice infected with mouse hepatic virus (MHV) there was reduced T cell infiltration at day7, but by day 12, T cell infiltration was similar to wild type and this study also suggested that IFN production may be increased in the CCR5-/- group.\(^{(25)}\) Infection with Leishmania donovani showed a shift from an initial low to an exaggerated antigen specific IFN response at 8 weeks post infection in CCR5-/- mice, suggesting that perhaps the impact of CCR5 alters during the course of an infection.\(^{(26)}\) In contrast to the above, a study by Belnoue et al showed that CCR5-/- mice infected with cerebral malaria had significantly reduced T cell cerebral infiltration.\(^{(27)}\) These contrasting results may reflect CCR5 interaction with parasitic rather than viral infection.

This study also shows a trend towards less severe hepatic inflammatory scores in CCR5WT/∆32 vs. CCR5WT/WT individuals. In a previous study we identified HLA DRB1*03011 positivity as being associated with reduced hepatic inflammation in this cohort. We did not observe an additive effect of CCR5Δ32 in DRB1*03011 positive individuals suggesting a dominant role for this HLA allele. However we observed significantly lower hepatic inflammatory scores for the CCR5Δ32/WT groups who were DRB1*03011 negative (p= 0.043). In a recent publication by Hellier et al, a significant decrease in portal inflammation, but not in overall necro-inflammatory score was found amongst CCR5Δ32 heterozygotes.\(^{(16)}\) CCR5Δ32 is associated with reduced migration of circulating lymphocytes in response to ligands such as MIP-1 alpha\(^{(19)}\) and we also observed this in vitro in CCR5Δ32 heterozygotes (data not shown).

In HCV there is predominantly a Th1 response in the liver. CCR5 is expressed on Th1 cells and facilitates the migration of T cells primed by antigen. Although the number of HCV specific cytotoxic lymphocytes (CTL’s) in the liver is low during infection, there are many activated / memory T cells present, most of which express CCR5. It has been reported that in HCV patients, liver-infiltrating lymphocytes (LIL) showed increased expression of CCR5,\(^{(28)}\) which correlated with histological severity.\(^{(29)}\) Similarly, animal studies have shown a key role for CCR5 in hepatic lymphocyte migration.\(^{(30)}\) Hence reduced expression of CCR5, associated with heterozygosity for CCR5Δ32, could be mechanistically associated with less hepatic inflammation, due to reduced migration of CCR5 expressing cells.

Both Hellier and Promrat found an association with the RANTES -403 promoter polymorphism and reduced hepatic inflammation in a subgroup of patients, which was not found in this study. It is possible that ethnic variation in the RANTES polymorphism and the patient numbers may partly explain differences between these studies. There is clearly much work yet to be done in this very exciting area, particularly detailed functional studies. While there is a detectable effect on HCV clearance seen in this study, further studies are required to determine whether such data are generalisable to the broader HCV-infected population. Such studies will require large numbers of patients and will also require either genetic homogeneity regarding ethnic origin or stratification given the wide diversity in allele frequency for this polymorphism even in Caucasian European populations. The effect that this mutation may have on HCV clearance and severity may be not only important in relation to those solely infected with HCV, but also of vital
importance to the vast numbers who are co-infected with HIV, particularly as anti-CCR5 directed medications are already being investigated for the treatment of HIV. (31-32)
REFERENCES


Bacterial DNA induces a proinflammatory immune response in patients with decompensated cirrhosis

We read with interest the study of Thalheimer et al (Gut 2005;54:556–63) in which they reviewed actual knowledge regarding the influence of infection on haemodynamics, variceal haemorrhage, hepatic effects, liver damage, and other effects. We agree with these assumptions and would like to add information not quoted in the paper that may help explain some of the immune abnormalities usually found in patients with advanced decompensated cirrhosis. As the authors detailed in their paper, our group has reported on the detection of bacterial DNA in a significant proportion of patients with cirrhosis and culture negative non-neutrocytic ascites, and has also shown that these fragments may last in blood for variable periods of time.\(^1\) In our opinion, the presence of bacterial DNA is not only representative in itself of the presence of bacteria (either viable or non-viable) in our patients, but induces similar immunological changes as endotoxin or viable bacteria. The question of whether bacterial DNA also induces haemodynamic disturbances is currently under investigation.

Bacterial DNA contains a series of CpG motifs that join toll-like receptor 9 and activates a series of intracellular mechanisms leading to the synthesis of proinflammatory cytokines.\(^2\) We therefore observed that peripheral white cells obtained from ascitic fluid in patients with the presence of bacterial DNA showed a marked activation pattern when the intracellular presence of cytokines involved in a type 1 immune response by means of flow cytometry was analysed, and also an increased ability to secrete this type of cytokines when cultured.\(^3\) Importantly, white cells in culture also displayed a significantly higher ability to secrete nitric oxide than cells obtained from patients without the presence of bacterial DNA, and nitric oxide levels showed a direct and significant relationship with the inducible form of nitric oxide synthase,\(^4\) suggesting that in this setting, ascitic fluid nitric oxide synthesis is, at least in part, induced by this isoform. Nitric oxide is a key agent in the pathogenesis of haemodynamic disturbances present in patients with advanced cirrhosis, and its levels are further increased in patients with hepatorenal syndrome.\(^5\) Ascitic fluid nitric oxide levels are independently related to the development of renal impairment in patients with spontaneous bacterial peritonitis.\(^6\)

The relation between the presence of bacterial DNA in blood and the ability to secrete proinflammatory cytokines and nitric oxide by cells of the immune system in patients with decompensated cirrhosis suggests that endotoxin and viable bacteria should not only be taken into account in the design of new research protocols, but also bacterial DNA, or similar molecules, as demonstration of the presence of bacteria in patients with advanced cirrhosis.

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Conflict of interest: None declared.

References

Author’s reply
We are grateful to Such et al for their comments on our review. As we had outlined, the influence of bacterial infection on the pathophysiology of cirrhosis is indeed an important one and Such et al have contributed significantly to this topic.\(^\ast\) We were aware of their data, but unfortunately some of it could not be retained in the final version of our paper due to editorial restrictions. Nevertheless, we agree that the presence of bacterial DNA, in the absence of viable bacteria or endotoxemia, might be an additional step in the sequence of events outlined in fig 2 of our review, maybe even preliminary to endotoxemia.

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Perinatal passive smoke exposure may be more important than childhood exposure in the risk of developing childhood IBD

The large case control study of patients with inflammatory bowel disease (IBD) in the French paediatric population by Baron et al has clarified the role of well established genetic and environmental risk factors, as well as suggesting novel environmental risk factors (Gut 2005;54:357–64). However, we caution the authors on dismissal of the role of passive smoking in the risk of IBD development in childhood. Our own data would suggest that analysing smoking data during pregnancy and at birth is more important in the development of childhood IBD, rather than assessing smoking during childhood and at disease onset, as performed in this current study.

We have performed a case control study in South East Scotland of children with early onset IBD, matching cases of IBD diagnosed at less than 16 years of age with same sex and age (±1) year controls attending the same general practice.\(^\ast\) In total, we matched 62 pairs of cases and controls, with a median age of disease onset in cases of 10.6 years. We demonstrated that parental smoking during pregnancy and around the time of birth was more common in parents of IBD cases, at 54% compared with control parents at 29% (p = 0.01; odds ratio (OR) 2.87 (95% confidence interval (CI) 1.23–6.66)). Maternal smoking during pregnancy and at birth was also more common in IBD cases than in controls, at 23% versus 6.2% (p = 0.04; OR 4.46 (95% CI 1.16–17.1)), and in mothers of patients with Crohn’s disease, at 27.8% versus control mothers at 8.3% (p = 0.03; OR 4.23 (95% CI 1.05–16.97)). There was no significant effect seen when paternal smoking in pregnancy and at birth was analysed in IBD cases versus controls (p = 0.27). These...
data replicate the publication by Lashner and colleagues who studied 72 IBD cases and controls and found a similar relationship to smoking at birth—this was increased in children who later developed IBD in childhood (OR 3.02) and CD in childhood (OR 5.32). The authors of this study also demonstrated that maternal smoking at birth was important in the development of IBD and CD.

We agree with the findings of Baron et al that parental/passive smoke exposure outside of the perinatal period, including at the time of pregnancy and at birth with the risk of developing IBD in children (p = 0.18). This lack of association between passive smoke exposure in childhood and development of childhood IBD has also been replicated by Lashner and colleagues. It is important to note that the other studies quoted by Baron et al in relation to the risk of passive smoking in IBD patients relate to the risk of adult onset IBD after passive smoke exposure during childhood, not the risk of developing IBD as a child. The mechanism by which smoke exposure during pregnancy and at birth leads to an increased risk of childhood IBD can only be a subject for speculation, but it is interesting to note a recent study has demonstrated chromosomal abnormalities in fetal epithelial cells in women who smoke during pregnancy.

In conclusion, our study agrees with previously published data to suggest a role between passive smoke exposure during pregnancy and at birth with the risk of childhood development of IBD. When assessing passive smoking in relation to childhood onset IBD, investigators should survey smoke exposure in the perinatal period and during childhood.

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Reference

Author’s reply
We thank Russell et al for their interest in our study, concerning the link between passive smoking and the risk of IBD in children.

We agree that it is important to take into account the role of passive smoking not only during childhood and at disease onset but also during the perinatal period. We also looked at this point in our study but came to different conclusions: 9.6% of mothers of IBD patients smoked during pregnancy versus 9.2% of control mothers (odds ratio (OR) 0.95 (95% confidence interval (CI) 0.53–1.72); p = 0.87). Moreover, concerning passive smoking during pregnancy, the findings were 14.2% and 12.8% for IBD patients and controls, respectively (OR 0.87 (95% CI 0.52–1.46); p = 0.60) and 15.3% for Crohn’s disease patients versus 14.4% for controls (OR 0.92 (95% CI 0.53–1.61); p = 0.77).

Due to the high number of questions and findings in our case control study, we only reported positive findings and what we considered as being the most important negative results. In conclusion, we confirm that in our study we found no link between IBD and passive smoking, including exposure during pregnancy and at birth.

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An alternative to prophylactic colectomy for colon cancer prevention in HNPCC syndrome

The surgical option for treatment of a patient with screen detected colorectal cancer (CRC) from a family with hereditary non-polyposis colorectal cancer (HNPCC) is subtotal colectomy or segmental resection. Using decision analysis, we showed that subtotal colectomy performed at a young age leads to an increased life expectancy (LE) of 1–2.3 years. Based on these results and the high risk of developing a second CRC, we concluded that if CRC is detected in a young patient participating in a surveillance programme, colectomy with ileorectal anastomosis seems to be the treatment of choice.

A French Committee on HNPCC commented on our study. Firstly, they stated that using quality adjusted LE would be a more accurate approach for comparing outcomes. Completely but studies on quality of life (QOL) did not specifically consider HNPCC patients. In HNPCC, QOL after segmental resection may be decreased by the need for colonoscopy (versus rectoscopy after colectomy) and the fear of a second tumour. Secondly, the committee considered our five year survival rates optimistic. The five year survival rates for HNPPC patients with Dukes’ B cancer varied in the literature from 70% to 91% and those for patients with Dukes’ C from 19% to 70%.

These survival rates are comparable to those used in our analysis. Thirdly, the committee mentioned that the overall five year survival of patients with CRC in HNPCC is approximately 55%. They stated that if the decision was for an extended follow up before the pathological staging of the tumour is known, 45% of patients will sustain a substantial decrease in QOL with no counterpart in quantity (that is, LE). The committee referred to the survival (5%) of symptomatic CRC in HNPCC. In our study, we discussed the surgical options for patients with CRC detected during surveillance. In our table 1, we showed the stage distribution of screen detected CRC based on our study and the Finnish series. As 86% had local cancer, the five year survival will be higher than 55%. Fourthly, the committee indicated that only a very small proportion of patients will be identified with CRC by the age of 27 years and that the increased LE for patients with CRC diagnosed at age 47 years was only one year. Half of the patients with screen detected CRC will be diagnosed before the age of 50 years and will have a substantial increase of LE of 1–1.2 years. Fifthly, the committee stressed that different indications should be made in men and women because of their different risks for metastrophic cancer as well as for the competing risk of endometrial cancer. Although female mutation carriers may have a lower risk of CRC than male carriers, it has not been shown that they also have a lower risk of a second CRC. In fact, among HNPCC patients that developed a second tumour, we found more female mutation carriers. Female mutation carriers do indeed have a high risk of developing endometrial cancer but this cancer is only a rare cause of death in HNPCC.

As stated by the committee, it is difficult for a patient diagnosed with CRC to decide between an increase in LE and a potential decrease in their QOL. An increased LE is a somewhat theoretical concept that entails additional years at the end of one’s life while the negative impact on QOL of subtotal colectomy will start from the first post-operative day. On the other hand, it may be even more difficult for a physician to explain to a patient that has developed CRC under surveillance that after segmental resection, surveillance of the remaining colon will prevent cancer development. It is possible that this patient will be happy after removal of the colon as now they are at a substantially lower risk of developing a second CRC. We agree that the patient’s choice is pivotal in decisions on prophylactic surgery, after being fully informed of the pros and cons of the surgical options.

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There is however a far easier and well validated method available for the study of human tissue. This is the so-called microdissection technique in which small pieces of stained material are teased apart and mitotic figures scored. This literally allows one to score over 100 crypts (if so wished) and as the results are expressed per crypt the effects of changes in denominator are automatically accounted for.

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References


Author’s reply

We thank Goodlad for his interest in our article. In our study (Gut 2004;53:1610-16), we assessed expression in the three markers most commonly used to indicate cell cycle entry in tissue sections. Importantly, there was no difference in the data obtained for all three. We agree that proliferating cell nuclear antigen is of limited value for the reasons mentioned by Goodlad and also the fact that the protein has a role in DNA repair, which reduces its specificity as a cell cycle marker. Similarly, Ki67 is not expressed by all cycling cells, may be downregulated by nutritional deprivation, and may also be involved in non-cell cycle related processes, such as ribosomal biosynthesis.

We consider that the most useful markers of cycling cells are the minichromosome maintenance (MCM) proteins, which are abundant at all phases of the cell cycle and are downregulated following exit into quiescence, differentiation, or senescence.1 MCMs therefore provide a sensitive and specific indication of cell cycle entry. In our opinion these markers are preferable to counting mitotic figures, which is a subjective and error prone exercise that by definition provides a limited phase specific indication of cell cycle state in histological sections.

We agree that proliferation indices can be misleading and that when assessing large bowel crypts it is important to determine the number of labelled cells per crypt.7 We confirm that the mucosa in all subjects in our study was microscopically normal, as well as macroscopically normal, as stated. In particular, there was no difference in crypt length and number of cells per crypt between the study groups. The labelling indices determined were therefore valid indicators of cell cycle entry in the samples investigated.

Prebiotic carbohydrates, such as those used in our study, are completely fermented in the large bowel and none is excreted in faeces. The principal products of this fermentation are short chain fatty acids (SCFA). While SCFA have been associated with increased cell proliferation in some animal models, it is hard to believe that what are the major contributors to all mammalian species should enhance the risk of cancer, particularly since one of these fatty acids, butyrate, is thought to be a differentiating agent. Fermented carbohydrates, such as dietary fibre, when measured properly in the diet, appear to protect against colorectal cancer in observational studies.4 The observed lack of effect of prebiotic carbohydrates on colorectal cancer proliferation in our study suggests that a substantial increase in fermentable carbohydrate intake, as provided by these prebiotics, does not enhance proliferation, as shown in some animal models, and thus might be regarded as adding to the protective role of the fermentable non-starch polysaccharides (fibre).

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References


Author’s reply

Further to Cummings and Coleman’s reply to my letter above, I would like to question the advocacy of minichromosome maintenance (MCM) proteins as proliferative markers, as the number of MCM positive cells can greatly exceed other labels and they are widely distributed on unreplicated chromatin. They would appear to be more of an indicator of replication potential and, as such, are likely to be useful markers of dysplasia.2 In addition, scoring immunohistochemical labelled cells is just as, if not more, ‘‘subjective and error prone’’ than scoring mitotic figures (which are far easier to score in ‘‘squash’’ preparations than in sections). My main
Concern still stands, as scoring histological sections of human biopsies, unlike squash preparations, leads to the sampling of a very limited number of crypts (2–4 in the present study) which prevents credence of the “observed lack of effect” of prebiotic carbohydrates.

Finally, I think that the jury is still out on the “protective role” of fermentable non-starch polysaccharides (fibres) as while the EPIC study showed a dramatic effect of intrinsically high fibre diets,1,2 many others have shown null effects and some of these, especially the intervention ones, demonstrated adverse effects. For example, wheat bran supplementation increased polyp recurrence in women3 and ispaghula had a more general adverse effect on polyps.4

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References

Author’s reply

We sought to identify cells at any point of the cell cycle, regardless of the rate of cycling or the duration of particular cell cycle phases. We therefore selected not to assess individual cell cycle phases in our samples, either by immunostaining or by counting mitotic figures. While additional roles for minichromosomes (MCMs) have been suggested, there is strong evidence that they function as essential replication factors.1 MCMs are displaced from chromatin following DNA replication, yet remain abundant in the nucleus throughout the cell cycle.2 Importantly, several groups have shown that MCMs are lost following cell cycle exit (into differentiation, quiescence, or senescence).3-6 MCMs are therefore useful immunohistochemical markers of cell cycle state. It is not surprising that MCMs are more abundant than Ki67 and proliferating cell nuclear antigen (PCNA), as the latter markers are not detectable in all cycling cells.

Objectivity in reproducibility in the interpretation of immunohistochemical staining are functions of the marker used. Some markers, such as PCNA, produce substantial variation in staining intensity and cause difficulty in slide interpretation. However, our MCM antibodies have not provided us with such difficulties, resulting in low interobserver variation in numerous studies to date.7

Interpreting observational and intervention studies of fibre has filled many journal pages in recent years. There are numerous problems which, in the context of the present discussion, relate primarily to people treating all sources of fibre as being equal, thinking that fibre supplements will have the same effect as fibre present in whole foods in the diet and the amounts of fibre considered to be protective. With regard to the study by Alberts and colleagues,1 the fibre was provided as a supplement and was only of wheat bran. As Goodlad and Alvarez correctly note, the EPIC study showed a protective effect for fibre when intrinsically part of the diet, and from mixed sources. In other words, it is a high fibre diet that protects. The Bonithon-Kopp study2 used a fibre supplement, ispaghula, not found in most diets of the world, and at a very small dose of only about 3 g/day.

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References

Recurrent of exhausting hiccup in a patient treated with chemotherapy for metastatic colon cancer

A 61 year old man was surgically treated for a pT3 N1-G2 MO adenocarcinoma of the colon in February 2003. Immediately after surgery, an enteric fistula occurred that caused a delay in administration of adjuvant treatment. At the start of adjuvant chemotherapy (CT) in May 2003, CEA level was 18.2 ng/ml and a new work-up with computed tomography scan of the thorax and abdomen revealed the early appearance of two metastatic lesions in the liver. The patient underwent liver metastasectomy and in July 2003 was started on post-surgical CT and radiotherapy with the FOL-FIRI (leucovorin, 5-fluorouracil, oxaliplatin) regimen. After day 1 of CT, recurrence of an exhausting hiccup was observed that continued for nine days after therapy. No benefit from the re-use of chlorpromazine was obtained.

Notably, while undergoing the two CT regimens, the patient had received intravenous ondansetron (8 mg) plus intravenous dexamethasone (8 mg), which was used for prophylaxis of delayed emesis. In order to identify the causative drug of hiccup and taking into consideration previous reports indicating dexamethasone as a possible cause of hiccup,4 during the following cycles of CT this drug was omitted. This approach allowed the patient to continue CT without recurrence of hiccup.

This strong temporal relation between dexamethasone administration and occurrence of hiccup indicated that this drug was the cause of the patient’s hiccup. Moreover, discontinuing dexamethasone was sufficient to achieve disappearance of hiccup without any further pharmacological intervention.

The mechanism of corticosteroid induced hiccup is unknown, although some hypotheses have been proposed.3,7 For example, it has been suggested that there is a hiccup centre in the midbrain that receives input from the thoracic sympathetic nerves and the pharyngeal plexus. It has been proposed that stimulation of the midbrain or these various pathways may be responsible for production of hiccup. Moreover, animal studies suggested that corticosteroids may reduce the synaptic transmission threshold in the midbrain and affect the metabolism of brain neurotransmitters.7,8

We reported our case to make oncologists aware that a symptom appearing during CT treatment (hiccup in our case) should not always be ascribed to the use of antineoplastic drugs. It is also true that some cytotoxic drugs, such as irinotecan and oxaliplatin, have been implicated as a cause of hiccup.9-11 In particular, the incidence of hiccup after treatment with irinotecan was reported in 49/16518 patients and, as for other cytotoxic drugs, almost exclusively in men (49/9313).9

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Laterally spreading tumour in which interstitial deletion of β-catenin exon 3 was detected

Laterally spreading tumours (LSTs) of the colon and rectum are defined as lesions greater than 10 mm in diameter with a low vertical axis that extend laterally along the luminal wall. As most LSTs remain as adenomas or early invasive cancers, LSTs have been thought to have relatively little malignant potential. LSTs are divided into two macroscopic subtypes: flat (F)-type, which is composed of superficially spreading lesions with flat and smooth surfaces, and granular (G)-type, which is composed of superficially spreading aggregates of nodules. Although distinctive biological behaviours of LSTs, only a few genetic alterations have been reported, such as K-ras and p53 mutations and β-catenin overexpression. A 62-year-old Japanese woman was referred to our hospital for treatment of a colorectal tumour. Colonoscopy in our hospital showed an F-type LST with a central depression surrounded by a flat elevated area with a smooth surface in the caecum (fig 1A). Microscopically, the tumour consisted of a well-differentiated adenocarcinoma with a tubular adenoma and had invaded the submucosal layer. After obtaining informed consent from the patient, genetic analysis was carried out. No genetic alterations were found in APC, K-ras, or p53 genes. To clarify relevant alterations in gene expression, we analysed the gene expression profiles by a cDNA array. Among 550 colon cancer-related genes, bone morphogenic protein 4 (BMP4) was one of the most differentially expressed genes in the tumour tissues and matched normal tissues (fig 2). Three base inverted repeats, AGC and GCT, were found in the sequences flanking the interstitial deletion. Short nucleotide sequences at both ends of the deletion were complementary, suggesting that inversely repeated sequences were involved in the somatic rearrangements. These results suggest that β-catenin deletion played an important role in the early stage of tumorigenesis in the present case. Abnormalities of β-catenin may play a crucial role in the morphological features of LSTs, as β-catenin is involved in cell adhesion. It would be interesting to investigate the frequency of β-catenin and APC alterations in a number of LST cases.

Microsatellite instability (MSI) due to defective DNA mismatch repair occurs in the majority of hereditary non-polyposis colorectal cancers (HNPCC) and in 10–15% of sporadic colorectal cancers. It has been reported that β-catenin mutations occur more often in MSI positive colorectal cancers. However, tumour tissues in the present case were MSI negative. Samowitz and colleagues reported that β-catenin exon 3 mutations are MSI negative and in HNPCC sporadic adenomas (1/83, 1.2%), HNPCC adenomas (1/37, 2.7%), and in both MSI positive (9/34) and MSI negative (9/78) sporadic colorectal cancers. In contrast, a significantly increased frequency (8/44, 18.2%) was found in HNPCC cancers.

![Image](image_url)

**Figure 1** (A) Endoscopic picture with indigocarmine dye spraying showing an F-type laterally spreading tumour with a central depression surrounded by a flat elevated area in the caecum. (B) cDNA array hybridisation image of the tumour and non-tumour tissues. Bone morphogenetic protein 4 (BMP4) was one of the most differentially expressed genes in the tumour tissues and matched normal tissues. (C) Intense nuclear expression of β-catenin immunohistochemically seen within the nuclei of tumour cells. (D) Interstitial deletion examined by polymerase chain reaction spanning the genomic region flanking exon 3 and the surrounding introns. A shorter band was detected in both carcinoma and adenoma tissues compared with the normal size of 931 bp. CA, carcinoma tissue; TA, tubular adenoma tissue; N, normal tissue.
present patient had no past history or family history of cancer. It would be interesting to investigate whether β-catenin mutation positive HNPCC cancers have any specific morphological features.

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References

Functional role of the 503F variant of the organic cation transporter OCTN1 in Crohn’s disease

Several susceptible gene loci were identified as being involved in the aetiology of Crohn’s disease (CD). Recently, a non-synonymous single nucleotide polymorphism in the SLC22A4 gene encoding the organic cation transporter OCTN1 has been linked with CD in Caucasian populations (a 1672CT transversion, resulting in the amino acid substitution L503F). However, the functional consequences of this alteration are unclear as yet.

We have now discovered that L-ergothioneine (ET, 2-mercaptopropionic acid thio-ether), a naturally occurring water soluble thiol compound of dietary origin, is the physiological substrate of OCTN1. Analysis of the concentration dependence of ET transport in OCTN1 transfected HEK293 fibroblasts by liquid chromatography tandem mass spectrometry revealed that the 503F variant was associated with a threefold higher substrate affinity (1/Km) and a two-fold lower maximal transport velocity (Vmax), which resulted in a 50% higher initial transport capacity (Vmax/Km (503F) = 1.5 × Vmax/Km (503L)) at low ET levels (<10 μmol/l) (fig 1A). Analysis of the time course of ET transport showed a higher clearance for the 503F variant (CL (503F) = 1.65 × CL (503L)) at an ET concentration of 10 μmol/l (fig 1B). ET transport by 503L and 503F was sodium

![Figure 1](https://www.gutjnl.com)

**Figure 1** Ergothioneine and OCTN1. Concentration dependence, Km, and Vmax of specific ergothioneine (ET) uptake in HEK293 cells constitutively expressing the 503L variant or the 503F variant after one minute of loading (A); specific uptake and clearance (CL) over a time course after one minute of loading with 10 μmol/l ET (B); effects of sodium (C) and pH (D) on specific uptake after one minute of loading with 10 μmol/l ET. In sodium reduced transport buffer, NaCl was isotonically replaced with choline chloride (which did not interfere with ET transport). An equal expression level (relative to the housekeeping gene GAPDH, lowest expression was set to 1) in eight healthy volunteers that were homozygous carriers of the 503L variant (E). MTT assayβ of the proliferation of Caco-2 colon tumour cells with and without OCTN1 mRNA expression after 24 hours of incubation with ET or glutathione. Resulting formazan formation was determined by absorbance at 568 nm (F). Data are mean (SEM) of three (A–D) or 8–16 (F) independent experiments. *p<0.05, **p<0.01, ***p<0.001: significant differences between OCTN1 variants (A–D); significant differences compared with buffer controls (F), as determined by one way ANOVA with Holm-Sidak correction (α = 0.05).
and pH dependent; only at unphysiologically low Na+ and pH values were the differences in transport activity between both variants lost. Considering that maximal levels of ET found in tissues and in common foods are in the nanomolar to low micromolar range, our data suggest that carriers of the 503F allele accumulate higher ET concentrations in OCTN1 expressing cells compared with carriers of the wild-type 503L allele. Therefore, high tissue levels of ET may constitute a possible risk factor for CD.

The involvement of OCTN1 in the inflammatory process is further supported by observations that OCTN1 is strongly expressed in intestinal epithelial and immunological cells, particularly in CD14+ monocytes/macrophages playing a key role in the immunopathogenesis of CD, as well as by the finding that levels of SLC22A4 mRNA were upregulated by proinflammatory cytokines such as tumour necrosis factor α. Moreover, we found transcriptional regulation of SLC22A4 to determine essentially ET uptake: in CD14+ monocytes homozygous for the 503L variant, expression levels of SLC22A4 mRNA showed high interindividual heterogeneity and were directly proportional to cellular ET content (fig 1E). Accordingly, in CD4+ and CD8+ lymphocytes lacking OCTN1 expression, we detected no ET (data not shown).

The physiological or pathophysiological functions of ET are as yet unknown. We tested the effect of ET on proliferation of the colon cancer epithelial cell line Caco-2 that was shown to be homozygous for the susceptible 503F allele and to express high levels of OCTN1 mRNA. Cell proliferation was enhanced in a dose dependent manner after exposure to ET concentrations above 20 μmol/l for 24 hours: at 200 μmol/l, proliferation was increased to 120 (3%) of the buffer control and intracellular ET concentration reached 6.7 (0.3) nmol/mg protein. In contrast, no stimulation of proliferation was seen when a Caco-2 variant without OCTN1 expression was employed; consequently, after treatment with 200 μmol/l ET, only diffusion controlled ET uptake to 0.67 (0.03) nmol/mg protein occurred. When incubated with glutathione, both Caco-2 cell lines exhibited an antioxidant typical inhibition of proliferation that was independent of OCTN1 expression (fig 1F). In contrast, rather than antioxidant activities, stimulatory effects on cell proliferation appear to constitute the functional role of ET. ET may accelerate the inflammatory process by transcriptional activation of proinflammatory cytokines such as TNF-α, IL-1 and IL-6, and consequently died. Atypical presentation among reported Thai patients and the correlation with outcome. A literature review on papers concerning human bird flu infection in Thailand was performed using databases of published works cited in Index Medicus and the Science Citation Index. I also reviewed published books and 256 local Thai journals, which are not included in the international citation index, for reports of human bird flu infection in Thailand. Studies that contained incomplete data were excluded from further analysis. Six reports* of 12 Thai patients with a confirmed diagnosis of bird flu were found. Of 12 infected cases, respiratory symptoms were seen in all cases and diarrhoea was detected at presentation in five cases (41.7%). Considering the five diarrhoeal cases, acute diarrhoeal distress syndrome (ADDS) was detected in four cases and there were three deaths. Concerning the seven non-diarrhoeal cases, ARDS was detected in five cases and there were five fatalities. There was no significant correlation between presentation of diarrhoea and development of ARDS (p=0.05) or mortality (p=0.05) but there was a significant correlation between the development of ARDS and mortality (p = 0.001).

There are some reports of diarrhoea in severe bird flu infection. Poovorawan recently proposed that diarrhoea was an important presentation of bird flu and could imply a poor prognosis. Here, I attempted to assess the magnitude of diarrhoea among Thai infected cases and its correlation with infection outcome. According to this study, the prevalence of diarrhoeal presentation was high, similar to a recent study in Vietnam (approximately 70%). Therefore, I therefore concluded that diarrhoeal presentation had a poor correlation with outcome among our subjects.

References


High levels of disease related prion protein in the ileum in variant Creutzfeldt-Jakob disease

Disease related prion protein (PrPSc) is readily detectable in lymphoreticular tissues in variant Creutzfeldt-Jakob disease (vCJD) but not in other forms of human prion disease. This distinctive pathogenesis together with the unknown population prevalence of asymptomatic vCJD infection* has led to significant
concerns that secondary transmission of vCJD prions will occur through a wide range of surgical procedures. Risk assessment for intestinal endoscopy, biopsy, and surgery is currently limited by a lack of knowledge about relative PrPSc levels and prion titres within intestinal tissues in vCJD patients. Because of its high content of lymphoid follicles, terminal ileum is regarded as the intestinal tissue having the highest potential for iatrogenic transmission of vCJD prions. Here we provide the first report of relative PrPSc concentrations in vCJD terminal ileum.

Tissues ileum obtained at autopsy with consent from relatives from four patients with neuropathologically confirmed vCJD and two patients with neuropathologically confirmed sporadic CJD (both PRNP codon 129MM with type 2 PrPSc in brain). Terminal ileum was analysed for PrPSc by high sensitivity immunoblotting and for abnormal PrP immunoreactivity by immunohistochemistry. Using these methods, terminal ileum from all four vCJD cases showed high levels of detectable PrPSc (fig 1A). In three vCJD cases, 2/2 homogenates prepared from each ileum specimen were positive for PrPSc whereas 2/4 ileum homogenates were positive in the other vCJD case. The glycoform ratio of protease resistant fragments of dimono- and non-glycosylated PrP in terminal ileum appeared to be closely similar to the type 41 PrP pattern seen in vCJD tonsil. Although there was variation in PrPSc concentration between different homogenates of vCJD terminal ileum, PrPSc levels in positive samples were typically in the range 0.1–1% of that present in vCJD brain (fig 1B). With respect to both sampling variation and PrPSc concentration, terminal ileum appears to be closely similar to lymph nodes in vCJD. These findings, together with our previous studies, show that PrPSc deposition within the intestine is not uniform in vCJD. From the four cases of vCJD with PrPSc positive terminal ileum studied here, 0/2 cases with available tissue had detectable PrPSc in the appendix and only 1/3 cases had detectable PrPSc in the rectum. In contrast with findings with vCJD terminal ileum, no detectable PrPSc was found in homogenates of terminal ileum prepared from sporadic CJD patients (fig 1A). The lack of detection of PrPSc in sporadic CJD terminal ileum extends our previous findings for one of these cases in which we have previously reported a lack of detectable PrPSc in tonsil, rectum, and appendix.

In agreement with findings from immunoblotting, immunohistochemistry showed abnormal PrP deposition in the terminal ileum in vCJD (fig 1C) but not in sporadic CJD (data not shown). The irregular distribution of abnormal PrP positive lymphoid follicles seen in vCJD terminal ileum is consistent with variation in PrPSc concentration detected in different terminal ileum samples by immunoblotting. Although from necessarily limited numbers investigated, the uniform presence of PrPSc in vCJD terminal ileum, at concentrations of up to 1% of those found in vCJD brain, reinforces concerns that iatrogenic transmission of vCJD prions might occur through contaminated intestinal endoscopic, biopsy forces, or surgical instruments. These findings should assist policy makers in the UK and elsewhere in risk assessments about the use of disposable forces for intestinal biopsy. Alternative approaches to risk reduction may now be possible as practical means of prion decontamination for endoscopes and surgical instruments are now feasible using enzymatic methods.

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References

3 Wadsworth JDF, Joiner S, Hill AF, et al. Tissue distribution of protease resistant prion protein in variant CJD using a highly sensitive


**Chronic inflammatory intestinal diseases and bone loss**

We were very interested in the recent article by Moschen et al on activation of the RANKL/OPG system in inflammatory bowel disease (IBD) (*Gut* 2005; 54:479–87). Until recently, osteoporosis secondary to gastrointestinal diseases was mainly considered a direct consequence of malabsorption.1 The article of Moschen et al and a previous one of our group on bone loss in coeliac disease,2 a disorder similarly characterised by intestinal inflammation, offer a new perspective on the pathogenesis of bone loss and reveal a more complex picture. Moschen et al demonstrated overproduction of OPG in the cells of colonic mucosa in IBD whereas Taranta and colleagues3 showed the direct role of the soluble cytokines in the serum of coeliac patients on bone cells. In fact, they found an increased RANKL/OPG ratio in untreated coeliac patients and different effects of the sera of untreated coeliac patients with respect to those on a gluten free diet, on cultured bone cells. These effects included increased in vitro osteoclastogenesis, and lower interleukin 18 and OPG expression in osteoblasts.

In both studies, these biochemical observations were translated in a reduction of bone mass. Moschen et al found a negative correlation between OPG plasma levels and spine and femoral neck bone mineral density (BMD). Taranta and colleagues observed a significant negative correlation between BMD z score and interleukin 6 levels and RANKL/OPG ratio. In the discussion, Moschen et al observed that “studies of OPG/RANKL and BMD are required to validate” his model.

We believe that our study may be a first step towards understanding, at least in part, the relevance of inflammation to bone loss in intestinal diseases. These results are also in accordance with recent studies on primary osteoporosis, which are beginning to show a relevant role of local and systemic factors on bone cell activity.**”** Finally, these studies may also open the way to different therapeutic approaches—namely, drugs specifically acting on cytokines release and/or activity—for bone loss secondary to “inflammatory intestinal diseases”.

**References**


**Acid Related Diseases: Biology and Treatment**


This textbook by Irvin Modlin and George Sacks is a welcome addition to the increasingly important and rapidly evolving field of gastroenterology and the authors ought to be congratulated for their efforts. Would I buy it? Probably yes, but only if I did not have a copy of the first edition. I would certainly recommend it as a departmental book as, among its many virtues, it provides useful titbits to amuse the audience during presentations.

A Mahmood