VIRAL HEPATITIS

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

C Goulding, A Murphy, G MacDonald, S Barrett, J Crowe, J Hegarty, S McKiernan, D Kelleher

Background and aims: Chemokines are small polypeptides, a major function of which is lymphocyte recruitment and trafficking. The aim of this study was to assess the involvement of inherited variations in CCR2, CCR5, and the ligand RANTES in determining disease outcome in hepatitis C virus (HCV) infected individuals.

Methods: A total of 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 polymerase chain reaction (PCR) status, alanine aminotransferase levels, and liver histology. There was no significant relationship between 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 v 28.3% negative; p=0.044, odds ratio 1.83 (95% confidence interval 1.1–3.6)). Among the subgroup of DRB1*03011 negative individuals, previously found to be associated with more severe inflammation, the difference in histological inflammatory score (CCR5WT/WT = 4.9 v CCR5Δ32/WT = 3.5; 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 subgroups within this cohort. Both controls and the HCV population had similar heterozygosity frequencies.

Chronic hepatitis C virus (HCV) infection has a prevalence of approximately 2% worldwide. 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 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.

Chemokines are small polypeptides with a significant role in leucocyte recruitment and trafficking. Leucocyte recruitment during inflammation requires intercellular communication between infiltrating leucocytes, endothelial cells, and parenchymal cells, mediated by early response chemokines. Migration of T cells is modulated in vitro and in vivo by conditioning with chemokines. CCR5 is a receptor for the proinflammatory chemokines macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and RANTES, which have key roles in host responses to viruses in both human and murine disease. 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 human immunodeficiency virus (HIV) infection, while the heterozygote state, which is found in 10% of Caucasians, leads to 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. Migration of antigen primed T cells is facilitated by CCR5. When human T cell clones were analysed, 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 upregulated by IL-2. It has been proposed that in HCV, there is predominantly a Th1 response in the liver. Indeed, progressive liver damage in HCV is associated with upregulation of Th1 cytokines (interferon (IFN) and interleukin (IL)-2), as shown by Napoli et al, where increased expression of IFN-γ and IL-2 correlated with both fibrotic and portal inflammatory histological scores.

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...
have a 46% reduction in lymphocyte recruitment to sites of using a CCR2 knockout mouse have shown that these mice 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 eightfold 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 included those who were chronically infected, persistently HCV RNA positive as determined by reverse transcription-polymerase chain reaction (RT-PCR), and those who had cleared the virus (that is, remained HCV antibody positive but RNA negative). None of the participants had any other risk factors for acquisition of viral hepatitis (for example, blood transfusion or past history of intravenous drug abuse). All had an alcohol consumption of less than 14 U/week 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 (that is, RT-PCR (RNA) positive) and 87 were anti-HCV antibody positive but persistently RNA negative. 10% of the samples were repeated in all of the above genotyping and 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*0301 and DQB1*0201.13 All subjects gave informed consent prior to participating in the study, which received ethics 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, California, USA) was then used as a confirmatory test.

RT-PCR testing

An RT-PCR assay (Amplisor; Roche Diagnostic Systems, New Jersey, USA) 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 QIAmp DNA midiprep kit, (Qiagen Ltd., Crawley, UK). During this process, all RNA was removed by incubating the digested preparation with 1.5 µl ribonuclease A (Boehringer Mannheim UK Ltd, East Sussex, UK) per 400 µl of nuclear lysate, according to the manufacturer’s instructions.

CCR5-Δ32 genotyping

A PCR reaction consisting of PCR 1xbuffer (as supplied by the manufacturer), 25 µM MgCl2, 200 µM deoxynucleotide triphosphates, 0.5 µM of both forward and reverse primers, 15 ng of extracted DNA, 1 unit of Qiagen DNA polymerase Taq, and 14.3 µl of H2O was used to genotype both the subject and control groups. The primers flanking the CCR5-Δ32 mutation (sense 5′-CAA AAA GAA GGT CAT TAC ACC-3′; antisense 5′-CCT GGT CCT CTT CTC ATT TCG-3′) were used to amplify 189 bp (wild-type) and 157 bp (32 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′-TTG GTT TGG TGG GCA ACA TGA TGG-3′ and the reverse primer 5′-CAT TGC ATT CCC AAA GAC CCA CTC-3′ was performed to give a 173 bp amplicon. This was then digested with the enzyme Bsa BI (New England Biolabs, Beverly, Massachusetts, USA) to yield restriction fragments of 149 and 24 bp. When the wild-type sequence is present, the fragment remains uncut, thus giving a band of 173 bp.

RANTES genotyping

Applied Biosystems Ltd (Foster City, California, USA) designed a 5′ exonuclease assay using the Assay by Design service for TaqMan analysis to genotype the RANTES 403 polymorphism. Forward primer 5′-GAG GAC CCT CCT CAA TAA AAC ACT TTA TAA AT-3′, reverse primer 5′-ACT GAG TCT TCA AAG TTC CTC CCT CTG-3′ and the probes VIC CAT TAC AGA TCT TAC CTC CTG T and FAM CAT TAC AGA TCT TAT CTC CTT T 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 undergone liver biopsy as part of their initial clinical evaluation (that is, prior to the commencement of any treatment) and between 17 and 20 years post infection. Liver biopsies were all scored by a
The CCR5-D32 mutation and hepatitis C

Single histopathologist in each centre, blinded to the CCR5 genotype of the individual. Biopsies were scored according to the modified histological activity index (HAI 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 histological, inflammatory, and fibrotic scores, and alanine aminotransferase (ALT) levels, between the different subgroups classified according to patient genotypes. The association between viral clearance and polymorphism was assessed by the $\chi^2$ and Fisher's exact test. A $p$ value of 0.05 was deemed as significant for all of the above tests. The odds ratio (OR) $p$ values were calculated using the $\chi^2$ test, and odds ratio (OR) by Epi-Info.

RESULTS

The results of the genotyping for the three different polymorphisms were compared with HCV PCR status (table 1), histological scores, and ALT levels.

Genotypes associated with viral clearance

Heterozygote frequency for the CCR5A32 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 CCR5A32/A32 individual in each group. The presence of the CCR5A32/WT (wild-type) genotype was significantly associated with spontaneous viral clearance: 42.0% of those who were CCR5A32/WT were HCV PCR negative versus only 28.3% of CCR5WT/WT ($p = 0.044$, one sided Fisher's exact test, OR 1.9 (95% confidence interval (CI) 1.1–3.6)). Only one patient was homozygotic CCR5A32/A32 and she was HCV PCR negative. 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 (95% CI 0.23–1.6)) and RANTES ($p = 0.441$, OR 1.01 (95% CI 0.58–1.7)) genotypes failed to reveal any relationship with HCV clearance.

Relationship between genotypes and histological severity

There was no significant difference in hepatic inflammatory scores between heterozygotes for the D32 mutation and those without a copy of this mutation (HAI 3.82 v 4.53; $p = 0.098$) in this cohort. Furthermore, in the DRB1*03011 positive group, previously found to be associated with less severe inflammation, CCR5A32 had no further additive impact on histological severity, with a mean HAI of 4.16 for non-CCR5WT/WT and 3.80 for CCR5A32 heterozygotes ($p = 0.78$). In contrast, within the DRB1*03011 negative group, associated with more severe inflammation, CCR5A32 heterozygotes had significantly lower inflammatory scores than the CCR5WT/WT group (mean inflammatory score 3.53 v 4.91; $p = 0.043$) (table 2).

Relationship between genotypes and ALT levels

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 (table 3).

Table 1 Summary of polymorphism distribution and hepatitis C virus (HCV) polymerase chain reaction (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 (71.7%)</td>
<td>66 (75%)</td>
<td>0.044</td>
<td>1.9 (1.1–3.6)</td>
</tr>
<tr>
<td>CCR5A32/WT</td>
<td>29 (14.8%)</td>
<td>21 (23.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR5A32/A32</td>
<td>0</td>
<td>1 (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR2WT/WT</td>
<td>125 (90.6%)</td>
<td>73 (93.6%)</td>
<td>0.327</td>
<td>0.66 (0.23–1.6)</td>
</tr>
<tr>
<td>CCR2A64/64I</td>
<td>11 (7.8%)</td>
<td>5 (6.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR2A64I/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>0.441</td>
<td>1.01 (0.58–1.7)</td>
</tr>
<tr>
<td>RANTES64I/WT</td>
<td>45 (32%)</td>
<td>29 (34.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES64I/64I</td>
<td>6 (4.2%)</td>
<td>2 (2.4%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Impact of the CCR5A32 mutation on hepatitis C virus polymerase chain reaction (PCR) status, histological activity index (HAI) scores for the entire group, and also for the subgroups of DRB1*03011 positive and negative individuals. The $p$ value for the effect of PCR status was given by $\chi^2$, and for HAI scores as per the Mann-Whitney U test.

Table 2 Summary of CCR5 results

<table>
<thead>
<tr>
<th></th>
<th>CCR5WT/WT (%)</th>
<th>CCR5A32/WT (%)</th>
<th>CCR5A32/A32 (%)</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>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>0.098</td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td>1.20</td>
<td>1.05</td>
<td>0.503</td>
<td></td>
</tr>
<tr>
<td>DRB1*03011 –ve inflam (n = 81)</td>
<td>4.91</td>
<td>3.53</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td>DRB1*03011 +ve inflam (n = 55)</td>
<td>4.16</td>
<td>3.80</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

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Table 3 Summary of mean alanine aminotransferase (ALT) levels and the polymorphisms examined

<table>
<thead>
<tr>
<th>1</th>
<th>ALT levels</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5WT/WT</td>
<td>52.76</td>
<td>0.419</td>
</tr>
<tr>
<td>CCR5A32/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>40.07</td>
<td>0.535</td>
</tr>
<tr>
<td>RANTES403/WT</td>
<td>54.46</td>
<td></td>
</tr>
</tbody>
</table>

Mean ALT levels for all individuals with the above genotypes, and the associated p values; p values were calculated using the Mann-Whitney U test.

DISCUSSION

This study showed significantly higher spontaneous HCV viral clearance in the CCR5A32/WT over the CCR5WT/WT group (p = 0.044). This association was not found in Hellier's or Promrat's studies, both of which had several confounding issues in relation to HCV genotype, sex, 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 in these studies that may have hindered the ability to detect changes in viral clearance. Our study population had a number of relatively unique features, principally that (i) all subjects were 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. They had no other risk factors for liver disease and no other significant comorbid illnesses.

The CCR5A32 mutation arises from a 32 bp deletion causing a frame shift mutation and premature termination of the protein. The resultant CCR5 mutant protein is likely to be functionally inert as it not only lacks the last three of seven putative transmembrane regions but also the domains involved in G protein coupling and signal transduction. 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. However, the role of the CCR5 mutation in HCV is not the same as for HIV as 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 in 17.9% of the control population, with the CCR5A32/A32 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 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%).

Controversially, in 2002, Woitas et al reported that CCR5A32 homozygosity occurred three times more frequently in anti-HCV antibody positive HIV negative individuals. 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 CCR5A32 genotype. Hence the increased CCR5A32 homozygosity most likely reflected resistance to HIV, rather than increased risk of HCV infection.

In explaining how the CCR5A32 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. Counterintuitively, lack of CCR5 may actually lead to increased T cell expansion. This was demonstrated using an acute lymphocytic choriomeningitis infection model in CCR5 knockout mice where clonal expansion of antigen specific T cells was increased, not decreased, among CD8+ and CD4+ T cells. Likewise, lack of CCR5 has been associated with increased T cell production of IFN-γ, leading to the suggestion that CCR5 might be part of a negative regulatory feedback loop on acute T cell activation.

In CCR5 deficient mice infected with mouse hepatitis virus there was reduced T cell infiltration at day 7, but by day 12 T cell infiltration was similar to wild-type and this study also suggested that IFN production may have been increased in the CCR5−/− group. Infection with leishmania donovani showed a shift from an initial low to an exaggerated antigen specific IFN response at eight weeks post infection in CCR5−/− mice, suggesting that perhaps the impact of CCR5 alters during the course of an infection. In contrast with the above, a study by Belnoue et al showed that CCR5−/− mice infected with cerebral malaria had significantly reduced T cell cerebral infiltration. These contrasting results may reflect CCR5 interaction with parasitic rather than viral infection.

This study also showed a trend towards less severe hepatic inflammatory scores in CCR5WT/WT versus 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 CCR5A32 in DRB1*03011 positive individuals, suggesting a dominant role for this HLA allele. However, we observed significantly lower hepatic inflammatory scores for the CCR5A32/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 necroinflammatory score, was found among CCR5A32 heterozygotes. CCR5A32 is associated with reduced migration of circulating lymphocytes in response to ligands such as MIP-1α and we also observed this in vitro in CCR5A32 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 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 showed increased expression of CCR5, which correlated with histological severity. Similarly, animal studies have shown a key role for CCR5 in hepatic lymphocyte migration.

Hence reduced expression of CCR5, associated with heterozygosity for CCR5A32, 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 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 of 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 coinfected with HIV, particularly as anti-CCR5 directed medications are already being investigated for the treatment of HIV.11,12

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Authors’ affiliations

C Goulding, R McManus, A Murphy, G MacDonald, S McKiernan, D Kelleher, Department of Clinical Medicine and the Dublin Molecular Medicine Centre, Trinity Centre for Health Sciences, St. James Hospital, Dublin, Ireland

S Barrett, J Crowe, Centre for Liver Diseases, Mater Misericordiae Hospital, Dublin, Ireland

J Hegarty, Liver Unit, St Vincent’s University Hospital, Dublin, Ireland

Conflict of interest: None declared.

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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. 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. We therefore observed that peritoneal 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. 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, suggesting that in this setting, as yet unknown, nitric oxide 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 hepatoportal syndrome. Ascitic fluid nitric oxide levels are independently related to the development of renal impairment in patients with spontaneous bacterial peritonitis.

Thus 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 demonstrated the presence of bacteria in patients with advanced cirrhosis.

J Such
Liver Unit, Hospital General Universitario, Pintor Baeza s/n, Alicante, Spain

C Muñoz
Department of Immunology, Hospital General Universitario, Pintor Baeza s/n, Alicante, Spain

P Zapater
Department of Clinical Pharmacology, Hospital General Universitario, Pintor Baeza s/n, Alicante, Spain

M Pérez-Mateo
Liver Unit, Hospital General Universitario, Pintor Baeza s/n, Alicante, Spain

Correspondence to: Dr J Such, Liver Unit, Hospital General Universitario, Pintor Baeza s/n, Alicante, Spain; such.js@hotmail.com

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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. 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 endotoxaemia, might be an additional step in the sequence of events outlined in fig 2 of our review, maybe even preliminary to endotoxaemia.

U Thalheimer, C K Triantos, D N Samonakis, D Patch, A K Burroughs
Liver Transplant and Hepatobiliary Medicine, Royal Free Hospital, London, UK

Correspondence to: Professor A K Burroughs, Liver Transplant and Hepatobiliary Medicine, Royal Free Hospital, Hampstead, London NW3 2QG, UK; andrew.burroughs@rfh.nhs.uk

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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;54:393–401).

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. 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 controls at 29% (p = 0.01; odds ratio (OR) 2.87 (95% confidence interval (CI) 1.23–6.61). 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 4.28 (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
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 9.25% of control mothers (odds ratio (OR) 0.95 (95% confidence interval (CI) 0.93–0.97); p = 0.87). Moreover, concerning passive smoking also during the perinatal period, 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 did not find any link between IB and passive smoking, including exposure during pregnancy and at birth.

M Baldé, C Gower-Rousseau
Department of Epidemiology and Public Health, CHRU de Lille and Registre Epimad, Lille, France

D Turk
Division of Gastroenterology, Hepatology, and Nutrition, Department of Paediatrics, CHRU de Lille and Registre Epimad, Lille, France

J F Colombel
Department of Hepato-Gastroenterology, CHRU de Lille and Registre Epimad, Lille, France

Correspondence to: Professor J-F Colombel, Department of Hepato-Gastroenterology and Registre Epimad, Hôpital Claude Huriez, CH et U de Lille, 59037 Lille Cedex, France; jfcolombel@chru-lille.fr

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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 deciding which patients are completely but also studying 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 not 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 for an extended resection was made in a patient before the pathological staging of the tumour is known, 45% of patients will sustain a substantial decrease in QOL with no counter-part in quantity (that is, LE). The committee referred to the survival (55%) 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 1–2.3 years. Fifthly, they stressed that different indications should be made in men and women because of their different risks for metachronous 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 females than males. 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.

H F A Vasen
The Netherlands Foundation for the Detection of Hereditary Tumours, Leiden, the Netherlands

W H de Vos tot Nederven Cappel
Department of Gastroenterology, Leiden University Medical Centre, Leiden, the Netherlands

Correspondence to: Dr H F A Vasen, The Netherlands Foundation for the Detection of Hereditary Tumours, Rijnboerweg 10, 2333 XX Leiden, the Netherlands; hfavasen@wxs.nl
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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.

R A Goodlad

Correspondence to: Dr R A Goodlad, Cancer Research UK, 44 Lincoln’s Inn Fields, London WC2A 3PX, UK; goodlad@cancer.org.uk

Conflict of interest: None declared.

References


Author’s reply

We thank Goodlad for his interest in our article. In our study (Gut 2004; 53: 1610–16), we assessed expression of these 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. 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, with the aim of providing 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. 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 anions in the colon of 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. The observed lack of effect of prebiotic carbohydrates on colonocyte 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).

J Cummings

Ninewells Hospital and Medical School, Dundee, UK

N Coleman

Hutchison/MRC Centre, Cambridge, UK

Correspondence to: Dr N Coleman, MRC Cancer Cell Unit, Hutchison/MRC Centre, Hills Rd, Cambridge CB2 2XZ, UK; nc109@cam.ac.uk

Conflict of interest: None declared.

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. 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, 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 women and ispaghula had a more general adverse effect on polyps.3

R A Goodlad, D Alvarez
Cancer Research UK, London, UK
Correspondence to: Dr R A Goodlad, Cancer Research UK, 44 Lincoln’s Inn Fields, London WC2A 3PX, UK; goodlad@cancer.org.uk
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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, 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 study used a fibre supplement, ispaghula, not found in most diets in the world, and at a very small dose of only about 3 g/day.

J Cummings
Ninewells Hospital and Medical School, Dundee, UK
Conflict of interest: None declared.

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 were not density and to assess individual cell cycle phases in our samples, either by immunostaining or by counting mitotic figures. While additional roles for minichromosome maintenance (MCM) proteins have been proposed, there is strong evidence that they function as essential replication factors. MCMs are displaced from chromatin following DNA replication, yet remain abundant in the nucleus throughout the cell cycle. Importantly, several groups have shown that MCMs are lost following cell cycle exit (into differentiation, quiescence, or senescence).1,6 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 and variability 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

Recurrence 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 chemotherapy. In the FOLFOX (leucovorin, 5-fluorouracil, oxaliplatin) regimen every 14 days for six months. During the second course of CT the patient experienced severe hiccup which was treated with metoclopramide without improvement. Hiccup was ascribed to the use of irinotecan and the patient subsequently rejoined at the CT programme. Discontinuing prophylactic oral chlorpromazine with significant reduction of the symptom. This approach yielded completion of the CT programme.

In January 2005, relapse of disease occurred in the liver that was not surgically manageable and the patient was started on the FOL-FOX (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 regimes, 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,1,8 during the following cycles of CT this drug was omitted. This approach allowed the patient to continue CT without recurrence of hiccup.

The 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.1,9 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.1,8

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 oxaplatin, have been implicated as a cause of hiccup.8 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).3

D Errante, D Bernardi, A Bianco
Division of Medical Oncology, Ospedale Civile, Vittorio Veneto (TV), Italy
N Zanatta
Division of Internal Medicine, Ospedale Civile, Vittorio Veneto (TV), Italy
L Salvagno
Division of Medical Oncology, Ospedale Civile, Vittorio Veneto (TV), Italy

Correspondence to: Dr D Errante, UO Oncologia, Ospedale Civile, Via Forloni 71, 31029 Vittorio Veneto (TV), Italy; domenico.errante@hsus.it
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Laterally spreading tumour in which interstitial deletion of \(\beta\)-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.\(^1\) 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.\(^2\) Despite distinctive biological behaviours of LSTs, only a few genetic alterations have been reported, such as K-ras and p53 mutations\(^3\) and cyclooxygenase 2 overexpression.\(^4\)

A 62 year old Japanese woman was referred to our hospital for treatment of a colonic 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 of gene expression, we analysed the gene expression profiles by a cDNA array.\(^5\) Among 350 cancer related genes, bone morphogenetic protein 4 (BMP4) was one of the most differentially expressed genes in tumour tissues and matched normal tissues (fig 1B). BMP4 is one of the most differentially expressed genes in the tumour tissues and matched normal tissues. (C) Intense nuclear expression of \(\beta\)-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.

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 \(\beta\)-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.

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genic \(\beta\)-catenin in human colon cancer cells,\(^6\) we analysed alterations in \(\beta\)-catenin in tumour tissues. Intense nuclear expression of \(\beta\)-catenin was immunohistochemically seen within the nuclei of tumour cells (fig 1C).\(^7\) No point mutations of \(\beta\)-catenin were detected. Interstitial deletion was then examined by polymerase chain reaction. A shorter band was detected in tumor tissues compared with the normal size of 931 base pairs (bp) (fig 1D). DNA sequencing showed an interstitial deletion of 394 bp in tumor tissues (fig 2). Three base inserted repeats, AGC and GCT, were found in sequences flanking the interstitial deletion. The deletion included the part of exon 3 containing critical serine and threonine codons for GSK-3\(\beta\) phosphorylation.

Figure 2 DNA sequencing showing interstitial deletion of the 394 bp region in tumor tissue. Three base inserted repeats, AGC and GCT, were found in sequences flanking the interstitial deletion. The deletion included the part of exon 3 containing critical serine and threonine codons for GSK-3\(\beta\) phosphorylation.

References

Recent 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.

K Nosho, H Yamamoto, M Mikami, T Takahashi, Y Adachi, T Endo
First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan

K Hirota
First Department of Surgery, Sapporo Medical University, Sapporo, Japan

K Imai, Y Shinomura
First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan

Correspondence to: Dr K Nosho, First Department of Internal Medicine, Sapporo Medical University, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan; nosho@sapmed.ac.jp
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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). Currently, 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-mercaptophistidine trimethylbe- taine), a naturally occurring water soluble thiol compound of dietary origin, in 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 three-fold 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) of 503L (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 independent.

Figure 1 Ergothioneine and OCTN1. Concentration dependence, K, and V of specific ergothioneine (ET) uptake in HEK293 cells constitutively expressing the 503L variant or the 503F variant after one minute of loading with 10 μmol/l ET (A); 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 of both OCTN1 mRNAs was controlled by quantitative real time polymerase chain reaction (TaqMan assay). Linear correlation of ET concentrations in CD14+ monocytes (fractionated from peripheral blood mononuclear cells) with OCTN1 mRNA expression (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 assay for 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).

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and pH dependent; only at unphysiologically low Na⁺ and pH values were the differences in transport activity between both variants lost (IC50). 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 CD4⁺ 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 CD4⁺ monocytes homozygous for the 503L variant, expression levels of SLC22A4 mRNA showed high interindividual heterogeneity and were directly proportional to cellular ET content 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 homogenous 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 control value. Hence rather than antioxidant typical inhibition of proliferation we noted. de Jong et al recently reported a fatal bird flu infection: a summary on bird flu infection: a summary on bird flu infected case in Vietnam with a significant correlation between presentation of diarrhoea and development of ARDS (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%). I therefore conclude that diarrhoeal presentation had a poorer outcome with infection among our subjects.

Diarrhoea as a presentation of bird flu infection: a summary on its correlation to outcome in Thai cases

Bird flu or avian flu, caused by H5N1 virus, is a new emerging infectious disease. There has been worldwide avian influenza infections in poultry since 1997. Recently, H5N1 caused severe disease with high mortality in humans in Vietnam and Thailand.1 Most infected cases usually developed progressive pneumonia with acute respiratory distress syndrome and consequently died. Atypical presentations of patients with bird flu were also noted. de Jong et al recently reported a fatal bird flu infected case in Vietnam with a presentation of diarrhoea, without respiratory symptoms.1 I performed a mini-study in order to document the magnitude of diarrhoeal presentation among reported Thai patients and the correlation with outcome. A literature review on papers concerning human bird flu 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 2–6 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 diarrhoea (CD) 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 fatality (p > 0.05) but there was a significant correlation between the development of ARDS and fatality (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%). I therefore conclude that diarrhoeal presentation had a poorer outcome with infection among our subjects.
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 di-, mono-, and non-glycosylated PrP in terminal ileum appeared to be closely similar to the type 41 PrPSc 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 endoscopy, biopsy forceps, or surgical instruments. These findings should assist policy makers in the UK and elsewhere in risk assessments about the use of disposable forceps 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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S Joiner, J M Linehan, S Brandner, J D F Wadsworth, J Collinge MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, London, UK

Correspondence to: Professor J Collinge, MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, UK; j.collinge@prion.ucl.ac.uk

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Figure 1 (A, B) High sensitivity immunoblots using anti-prion protein (PrP) monoclonal antibody 3F4. (A) Proteinase K digested sodium phosphotungstic acid pellets from 0.5 ml of 10% terminal ileum homogenates from variant Creutzfeldt-Jakob disease (vCJD) patients 1–4 or sporadic CJD (sCJD) patients 1 and 2. (B) Proteinase K digested sodium phosphotungstic acid pellets from 0.5 ml of 10% normal human tonsil homogenate (normal tonsil) or 0.5 ml of 10% normal human tonsil homogenate spiked with 2.5 μl of 10% brain homogenate from vCJD patient No 4 (spiked tonsil) were compared with a proteinase K digested sodium phosphotungstic acid pellet from 0.5 ml of 10% terminal ileum homogenate from the same vCJD patient. (C) Photomicrograph showing abnormal PrP immunoreactivity in a lymphoid follicle in vCJD terminal ileum (anti-PrP monoclonal antibody 3CSM 35). Scale bar, 100 μm. Inset, high power magnification of PrP deposits.

10% terminal ileum homogenate from the same vCJD patient. (C) Photomicrograph showing abnormal PrP deposits in vCJD terminal ileum (anti-PrP monoclonal antibody 3CSM 35). Scale bar, 100 μm. Inset, high power magnification of PrP deposits.

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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 2 The article of Moschen et al and a previous one of our group on bone loss in coeliac disease,1 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 colleagues 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 understand, at least in part, the relationship between inflammation and 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”.

M T Bardella
Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, IRCCS, Milan, Italy

M L Bianchi
Bone Metabolism Unit, Istituto Auxologico Italiano, IRCCS, Milan, Italy

Correspondence to: Dr M T Bardella, University of Milan, Mangiagalli e Regina Elena, IRCCS, Via Francesco Sforza, 33, Milan Italy; mariateresa.bardella@unimi.it

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