Bacterial DNA activates cell mediated immune response and nitric oxide overproduction in peritoneal macrophages from patients with cirrhosis and ascites

R Francés, C Muñoz, P Zapater, F Uceda, I Gascón, S Pascual, M Pérez-Mateo, J Such

Background and aims: Translocation of intestinal bacteria to ascitic fluid is probably the first step in the development of episodes of spontaneous bacterial peritonitis in patients with cirrhosis. We have recently reported the detection of bacterial DNA in blood and ascitic fluid from patients with advanced cirrhosis, what we consider as molecular evidence of bacterial translocation. Several studies have shown the immunogenic role of bacterial DNA in vitro, and we hypothesised that the presence of bacterial DNA could activate the type I immune response in peritoneal macrophages from these patients, leading to greater cytokine synthesis (interleukin (IL)-2 and IL-12, tumour necrosis factor α, and interferon γ) and effector molecules such as nitric oxide.

Methods: Peritoneal macrophages obtained from patients with cirrhosis and culture negative non-neutrocytic ascitic fluid were collected and characterised by flow cytometry. Inducible nitric oxide synthase, nitric oxide levels, and cytokine production were measured by immunoenzymometric assays in basal and harvested conditions according to the presence/absence of bacterial DNA.

Results: The ability of peritoneal macrophages to synthesise nitric oxide and levels of all cytokines were significantly increased in patients with bacterial DNA. There was a positive correlation between inducible nitric oxide synthase and nitric oxide levels.

Conclusions: The presence of bacterial DNA in patients with decompensated cirrhosis is associated with marked activation of peritoneal macrophages, as evidenced by nitric oxide synthesising ability, together with enhanced cytokine production.

Patients and Methods

Patients

Since April 2000, all patients with cirrhosis and culture negative non-neutrocytic AF have been consecutively included in a prospective study in our Liver Unit to detect and identify the presence of bactDNA in blood and/ or AF, according to the methodology previously described. A series of 10 consecutively admitted patients with or without bactDNA in blood and AF were included in the study protocol.

Cirrhosis was diagnosed by histology or by clinical, laboratory, and/or ultrasonographic findings. Exclusion criteria were the presence of culture positive blood or AF, neutrocytic AF (>250 polymorphonuclear leucocytes (PMN)/μl), signs or symptoms of systemic inflammatory response syndrome according to previously published criteria, upper gastrointestinal bleeding or intake of antibiotics in the preceding two weeks, including selective intestinal decontamination with norfloxacin, hepatocellular carcinoma and/or portal thrombosis, alcoholic hepatitis, and refusal to participate in the study. The ethics committee of Hospital General Universitario approved the study protocol and all patients gave informed consent for inclusion in the study.

Abbreviations: SBP, spontaneous bacterial peritonitis; AF, ascitic fluid; BT, bacterial translocation; bactDNA, bacterial DNA; NK, natural killer; IFN-γ, interferon γ; NO, nitric oxide; iNOS, inducible NO synthase; PMN, polymorphonuclear leucocytes; LPS, lipopolysaccharide; MoAbs, monoclonal antibodies; TNF-α, tumour necrosis factor α; IL, interleukin
Blood was obtained for routine haematological, biochemical, and coagulation studies. Simultaneously, large volume paracentesis was performed in all patients on admission under aseptic conditions, following the usual procedures, and samples for routine biochemical study and PMN count were obtained. Total protein, albumin, leukocyte count, and PMN count were performed on all AF specimens. Both blood and AF were inoculated at the bedside in aerobic and anaerobic blood culture bottles, 10 ml each. Samples of blood and AF were inoculated in rubber sealed pyrogen free tubes (Endo Tube ET; Chromogenix AB, Vienna, Austria).

Peritoneal macrophages culture
A volume of 800–1500 ml of AF, collected after therapeutic paracentesis, was centrifuged at 1500 g for 10 minutes to obtain the cellular pellet. After washing with phosphate buffer saline (PBS) three times, more than 80% of the macrophage population was counted from a total number of 10–16 million cells, in all cases using Testisimplets prestained slides (Roche Diagnostics Ltd, Sussex, UK). Cell viability was also evaluated by trypan blue (Sigma, Madrid, Spain) and resulted in more than 95% viability. Cells (4 × 10⁶) were separated for macrophage characterisation by flow cytometry; 1 × 10⁶ cells were prepared to obtain the cell lysates for iNOS quantification according to the manufacturer’s instructions and 5 × 10⁶ macrophages were resuspended in 5 ml of phenol red free RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, UK) supplemented with 10% human serum AB (BioWhittaker, Walkersville, Maryland, USA), 100 IU/ml penicillin/streptomycin, and 2.5 µg/ml amphotericin B (Gibco BRL). Macrophages (2 × 10⁶) were incubated as controls, and 2 × 10⁶ macrophages were cultured adding 0.2 µg of LPS from E coli serotype 0111:B4 (Sigma) in a 24 well plate for 24 hours at 37°C in a humidified atmosphere with 5% CO₂. Supernatants were obtained by centrifugation at 2500 g for five minutes and stored at −80°C until assay. The maximum period of time between being frozen and thawing was three months and in the current study none of the specimens was thawed and refrozen prior to analysis.

Immunostaining of cell surface and characterisation of the monocyte/macrophage population
At the indicated time of culture, cells were harvested and washed once in ice cold PBS, suspended in 500 µl of PBS, and distributed (50 µl per tube) to polystyrene round bottom tubes (Becton Dickinson, San Diego, California, USA) for immunolabelling. Surface determinant immunostaining was performed with unfixed cells using fluorochrome conjugated monoclonal antibodies (MoAbs) against the surface determinants. The combinations CD45 PerCP/CD3 FITC, CD45 PerCP/CD33 PE, CD45 PerCP/CD14 FITC, and CD14 FITC/CD33 PE were added for better discrimination between monocytes and lymphocytes in AF mononuclear cells during flow cytometry analysis. Cells were incubated in the dark for 15 minutes, washed in cold PBS, fixed, and permeabilised with IntraPrep permeabilisation reagent ( Immunotech Beckman Coulter, Marseille, France). Then, cells were washed in PBS and stained (30 minutes in the dark) for intracellular cytokines using FITC conjugated MoAbs against tumour necrosis factor α (TNF-α) and interleukin (IL)-6, and PE conjugated MoAbs against IL-8 and IL-1-β. FITC and PE conjugated isotype controls were used in parallel. After cells were washed in PBS they were suspended with paraformaldehyde 1% in PBS for flow cytometry analysis. Samples were analysed in a FACS sort flow cytometer (Becton Dickinson, Immunocytochemistry Systems, Palo Alto, California, USA) using Cellquest (version 3.2) software. Typically, list mode data for 20 000 events for CD45+ cells in a “live gate” mode were acquired.

Measurement of NO production and human iNOS levels
The sum of the NO metabolites nitrite (NO 2⁻) and nitrate (NO 3⁻) is widely used as an index of NO generation and expressed as NOx levels per ml, which corresponds to 10⁶ cells in this study. NOx levels in basal AF, and in supernatants from cultured macrophages, were calculated by measuring conversion of NO 3⁻ to NO 2⁻ by the enzyme nitrate reductase using an ELISA assay (R&D Systems, Minneapolis, USA) based on the Griess reaction that absorbs visible light at 540 nm. All samples were tested in duplicate and values were corrected by running samples with culture media without macrophages to assess background NOx levels. Quantitative determination of iNOS concentrations was performed through the Quantikine Human iNOS Immunoassay (R&D Systems) in cell lysates, obtained according to the manufacturer’s instructions and expressed as U/ml.

Measurement of IFN-γ, TNF-α, IL-2, and IL-12
Immunoenzymometric assays for quantitative measurement of human IFN-γ, TNF-α, IL-2, and IL-2 in AF samples were performed by handling Biosource IFN-γ EASIA kit (Biosource Europe SA, Nivelles, Belgium), human TNF-α HS Quantikine, and IL-12 and IL-2 Quantikine (R&D Systems) according to the manufacturer’s instructions. All samples were tested in duplicate and read at 450 nm and 490 nm in a ThermoMax microplate reader ( Molecular Devices, Sunnyvale, California, USA).

Statistical analysis of experimental data
All observations are reported as mean (SD). Statistical differences in basal characteristics between groups were analysed using the χ² test for categorical data applying Yates’ correction when required, the Mann-Whitney U test for quantitative data without a normal distribution according to the Komolgorov-Smirnov test, and the Student’s t test for variables with a normal distribution. The ANOVA test with the post hoc Tukey test was used for multiple comparisons. Bivariate correlations were evaluated using the Pearson test. All p values were two tailed. A p value <0.05 indicated statistical significance. Analyses were performed with the SPSS statistical package (SPSS Inc. version 11.0, Chicago, Illinois, USA).

RESULTS
Characteristics of patients and laboratory data
All patients fulfilling the inclusion and exclusion criteria were considered for entry into the study. Ten consecutively admitted patients in whom we did not detect or detected the presence of bactDNA comprised groups I and II, respectively. Clinical and analytical characteristics of the patients in both groups at admission are shown in table 1. No significant differences were observed in any of the parameters between the two groups of patients.

BactDNA was always detected simultaneously in blood and AF. Similarity in sequences between blood and AF was more than 99.5% in all cases, and identifications obtained from the NCBI Database by the advanced BLAST search included E coli (n = 7), Klebsiella (n = 2), and S aureus (n = 1).

Upper gastrointestinal endoscopy was performed at the index admission or in the preceding three months in eight patients in group I and in seven patients in group II. No differences were observed in the presence or size of oesophageal varices or in portal hypertensive gastropathy between the two groups of patients.

Patients were followed up during admission from inclusion in this study. Two patients from group II died during admission; one from terminal liver failure and one from...
acute pancreatitis. One patient in group I developed culture negative spontaneous bacterial peritonitis and one patient in group II developed a vertebral abscess. Infections developed seven and 12 days, respectively, after the initial paracentesis.

Gating of monocyte population and production of intracellular cytokines
Monocyte populations were defined on the basis of cell granularity and fluorochrome conjugated MoAbs CD14-FITC and CD33-PE (fig 1). No differences between groups were found in an ANOVA test, with the stimuli and MoAbs as factors in the analysis. The average monocyte population activated was 76.1 (SD 14.7)%.

AF NOx and cytokine levels
Basal AF NOx levels in patients from group II were higher than those observed in patients from group I although values did not reach statistical significance, probably due to the reduced number of cases (table 2). NOx levels measured in the supernatant of non-stimulated cultures from patients in group II were significantly higher than those observed in patients in group I. Furthermore, addition of cellular stimulants to the culture media induced a marked increase in NOx levels in both groups of patients but values only reached significance in patients in group I (table 2).

Table 1  Basic clinical and analytical characteristics of the cohort distributed according to the absence (group I) or presence (group II) of bacterial DNA in serum and ascitic fluid

<table>
<thead>
<tr>
<th></th>
<th>Group I (n = 10)</th>
<th>Group II (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (y)</td>
<td>65 (10)</td>
<td>65 (12)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>9/1</td>
<td>8/2</td>
</tr>
<tr>
<td>Aetiology (n)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Alcohol</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>HCV</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Alcohol-HCV</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Diuretics (yes/no)</td>
<td>9/1</td>
<td>9/1</td>
</tr>
<tr>
<td>Child Pugh (mean score)</td>
<td>8.63 (1.27)</td>
<td>8.89 (1.19)</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>2.6 (1.6)</td>
<td>2.5 (1.2)</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>2.8 (0.8)</td>
<td>2.5 (0.5)</td>
</tr>
<tr>
<td>Quick (%)</td>
<td>64.1 (15.9)</td>
<td>59.4 (15.1)</td>
</tr>
<tr>
<td>WBC/mm³</td>
<td>6351 (2172)</td>
<td>6520 (3034)</td>
</tr>
<tr>
<td>AF total protein (g/dl)</td>
<td>1.9 (0.9)</td>
<td>1.6 (0.5)</td>
</tr>
<tr>
<td>AF WBC/mm³</td>
<td>248 (267)</td>
<td>106 (67)</td>
</tr>
<tr>
<td>% AF PMNs</td>
<td>14.9 (15.1)</td>
<td>15.2 (26.3)</td>
</tr>
</tbody>
</table>

Data are mean (SD). WBC, white blood cell; AF, ascitic fluid; HCV, hepatitis C virus; PMN, polymorphonuclear leucocytes.

No significant differences between the two groups.

Although the limited number of bacteria isolated (seven E. coli, two K. pneumoniae, one S. aureus) precludes any statistical analysis, we did not observe any difference in the immune response according to the type of bacteria detected in patients in group II.

Human iNOS concentrations in cell lysates
Relative mass values for naturally occurring iNOS under all three experimental conditions are detailed in table 2. Statistically significant differences were observed between values obtained at baseline and in non-stimulated cultures from patients in group II were significantly higher than those observed in patients in group I. Furthermore, addition of LPS to the culture medium induced a significant increase in iNOS concentration compared with samples obtained without stimulation in both groups of patients (table 2).

Figure 1  Gating of the monocyte population. Monocyte populations were defined on the basis of cell granularity (SSC-H) and the fluorochrome conjugated monoclonal antibodies anti-CD33-PE (FL2-H) and anti-CD14-FITC (FL1-H). Regions R1 and R2 correspond to monocytes and polymorphonuclear cells, respectively.

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A direct and significant correlation was found between basal iNOS and NOx levels in the overall group of patients ($r^2 = 0.72$, $p = 0.03$) (fig 2) and also between basal values of iNOS and all cytokines studied, with the exception of TNF-α ($r^2 = 0.77$, $p = 0.04$; TNF-α $r^2 = 0.65$, $p = 0.09$; IL-12 $r^2 = 0.79$, $p = 0.001$; IL-2 $r^2 = 0.84$, $p = 0.001$).

**DISCUSSION**

In this study, we have shown that peritoneal macrophages, obtained from patients with cirrhosis and AF, and the presence of bactDNA are primed to synthesise significantly higher amounts of NO than macrophages obtained from patients without bactDNA, and this is associated with marked activation of the cytokines implicated in the type 1 immune response.

Bacterial infections are common complications in patients with advanced cirrhosis, and SBP is the most frequent and severe complication of cirrhosis.36 SBP is the most frequent complication in cirrhotic patients and is associated with a significant increase in the incidence and mortality of these patients.37-39 Its origin is multifactorial, involving the interaction of several factors, such as bacterial translocation, gut dysmotility, and the gut-liver axis.40-42 The main pathogenic factor of SBP is bacteria, which can be blocked by the use of antibiotics.43,44 However, the use of antibiotics is not always effective, and the pathogenic role of bacteria is still a subject of debate.45-47

The presence of bacteria in the peritoneal cavity is a clinical hallmark of SBP.48-50 Moreover, the detection of bacteria in peritoneal fluid is a strong predictor of mortality in patients with SBP.51-54 The release of bacterial DNA (bactDNA) in peritoneal fluid is a late but sensitive marker of bacterial translocation and SBP.55,56 BactDNA is a nucleic acid fragment derived from bacterial cells, which can be detected in the peritoneal cavity of patients with SBP and is associated with an increased risk of mortality.57,58 The detection of bactDNA in peritoneal fluid is a sensitive and specific indicator of SBP, and its presence in the peritoneal cavity is associated with an increased risk of mortality.59,60

We have recently described the presence of bactDNA in the peritoneal cavity of patients with cirrhosis and culture-negative neutrocytic AF, and a previous episode of SBP.61,62 We have also shown that the presence of bactDNA is associated with an increased risk of mortality in patients with SBP.63,64

The presence of bactDNA in the peritoneal cavity is associated with an increased risk of mortality in patients with SBP.63,64 The detection of bactDNA in the peritoneal cavity is a sensitive and specific marker of SBP, and its presence is associated with an increased risk of mortality.65,66

**Table 2**

<table>
<thead>
<tr>
<th>NOx (nmol/10⁶ cells)</th>
<th>iNOS (U/10⁶ cells)</th>
<th>TNF-α (pg/10⁶ cells)</th>
<th>IFN-γ (nmol/10⁶ cells)</th>
<th>IL-2 (pg/10⁶ cells)</th>
<th>IL-12 (pg/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>21.2 (13.0)</td>
<td>4.5 (3.1)</td>
<td>15.6 (4.4)</td>
<td>26.9 (5.8)</td>
<td>22.1 (6.0)</td>
</tr>
<tr>
<td>24 h</td>
<td>57.5 (22.4)</td>
<td>7.5 (3.9)</td>
<td>21.9 (9.3)</td>
<td>31.0 (6.1)</td>
<td>29.3 (7.1)</td>
</tr>
<tr>
<td>LPS</td>
<td>85.6 (26.9)†</td>
<td>24.2 (10.3)†</td>
<td>57.6 (5.6)†</td>
<td>52.7 (5.7)†</td>
<td>61.0 (10.3)†</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>35.6 (20.8)</td>
<td>13.2 (4.5)†</td>
<td>35.3 (17.7)†</td>
<td>40.2 (8.0)&quot;</td>
<td>62.1 (10.3)&quot;</td>
</tr>
<tr>
<td>24 h</td>
<td>93.2 (35.6)&quot;</td>
<td>17.1 (5.1)&quot;</td>
<td>46.0 (9.0)&quot;</td>
<td>46.1 (8.8)&quot;</td>
<td>68.0 (10.3)&quot;</td>
</tr>
<tr>
<td>LPS</td>
<td>122.3 (44.5)&quot;</td>
<td>28.6 (7.7)&quot;</td>
<td>63.2 (10.2)&quot;</td>
<td>55.4 (8.2)&quot;</td>
<td>74.1 (9.0)&quot;</td>
</tr>
</tbody>
</table>

Data are mean (SD).

NOx, sum of nitric oxide metabolites, nitrate and nitrite; TNF-α, tumour necrosis factor α; IFN-γ, interferon γ; IL-2 and IL-12, interleukin 2 and 12, respectively; iNOS, inducible form of nitric oxide synthase.

* $p < 0.05$ versus the corresponding value in group I; † $p < 0.05$ versus the corresponding value after 24 hours without stimulation.

**Figure 2** Correlation between basal inducible NO synthase (iNOS) and NOx (sum of nitric oxide metabolites, nitrate and nitrite) levels in peritoneal macrophages obtained from ascitic fluid in patients with decompensated cirrhosis.
induction of iNOS has been reported previously. Elevated production of IFN-γ by CpG DNA activated NK cells is mediated by IL-12 and, in fact, we observed a significant correlation between basal levels of these two cytokines, which clearly suggests activation of a type I immune response in these patients. Supporting this assumption, TNF-α, which is directly synthesised by macrophages and also enhanced by IFN-γ, was significantly higher in patients in group II compared with group I.

In summary, peritoneal macrophages from patients with cirrhosis and ascites and the presence of bactDNA are primed for higher cytokine and NO synthesis. Our data suggest identification of a new subset of patients, with a marked preactivation of the cellular component of the immune system, probably in response to the immunogenicity of CpG motifs present in bactDNA.

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REFERENCE


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Genetic association between EPHX1 and Crohn’s disease: population stratification, genotyping error, or random chance?

We read with interest the article by de Jong and colleagues (Gut 2003;52:547–51) reporting studies of case control associations between DNA polymorphisms in xenobiotic metabolising genes and Crohn’s disease (CD). The authors employed a case control study design to test seven polymorphisms in five candidate genes in a control population (reviewed in de Jong et al.).

We noted for a significant association of a single nucleotide polymorphism (SNP), Tyr113His (348T>C), in the microsomal epoxide hydrolase 1 gene (EPHX1), with CD. Homozygosity for the T (Tyr 113) allele was significantly higher in cases than in healthy controls ($\chi^2 = 23.7$, p < 0.0001, odds ratio 2.9). The observed frequency of the T allele in controls was 41%, which is outside the range of 27–37% reported in other control populations (reviewed in de Jong et al.). Its frequency in CD cases was 67%. In view of the strength of reported association, we sought to replicate this observation. We genotyped the Tyr113His SNP (ref SNP ID rs1051740) in 307 independent sporadically ascertained cases of CD and 344 ethnically matched healthy control subjects. This compared with 151 cases and 149 controls typed in the published study. Our study design provided 80% power to detect a significant difference (p < 0.05) in allele frequency of $>7.5\%$ between cases and controls compared with the difference of 26% observed in the published study. Our power calculations were based on an observed minor (C) allele frequency of 30.2% in our control cohort, the common (T) allele frequency being 69.8%.

We used TaqMan chemistry (Applied Biosystems) to genotype DNA from cases and controls with an Applied Biosystems 7700 Sequence Detection System. Pre-optimised primers and fluorescent probes were obtained from Applied Biosystems (SNP assay ID C_14938_1). All cases and controls were previously genotyped for three CARD15 mutations: CD870 to G908R, L1007fs in CARD15, permitting stratification of data by CARD15 mutational status to identify potential gene–gene interactions.1 Allele and genotype frequencies were compared between cases and controls using a $\chi^2$ test for difference in proportions. Likewise, a $\chi^2$ test was used to assess Hardy-Weinberg equilibrium (HWE) across genotypes. We found no significant differences in allele or genotype frequencies between cases and controls (table 1). Stratification of the data by CARD15 mutation status showed no significant differences in Tyr113His allele frequencies in CD cases with none, one, or two CARD15 mutations. Genotypes in our cases and controls were in HWE (p > 0.5).

Case control based studies of genetic association assume that differences in allele frequencies relate directly to the phenotype under investigation, and that no unobserved confounding factors exist which may be attributable to the associated allele. While having greater power than family based studies to detect associations through linkage disequilibrium mapping, case control analysis is susceptible to type I errors (false positives). Thus, a number of commonly cited explanations for non-replication of genetic associations is stratification, through population admixture (mapping results present, and the difference in SNP frequencies and disease prevalence in the normal and affected populations. We noted that in de Jong et al. the distribution of genotypes in controls for SNP Tyr113His was not in HWE ($\chi^2 = 5.67$, p = 0.017). It is possible that this may have generated a type I error in their analysis. A degree of population admixture in their control cohort could account for the deviation from HWE and give rise to the observed association between the normally common T allele (as we observed) and Crohn’s disease. Alternative explanations are genotyping error and random chance. We examined the genotype distribution for the seven SNPs tested by de Jong et al. and found that in addition to Tyr113His, the Ille62Val (1506A>G) SNP in CYPIA1 was not in HWE ($\chi^2 = 7.87$, p = 0.005). A recent review of published association studies by Xu and colleagues found that 12% of SNPs tested were inconsistent with HWE in control subjects.

Our findings highlight the value of testing genetic association data for normal genotype distribution, and for rigorous replication of genetic associations with adequate statistical power.

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References

**Table 1** Allele and genotype frequencies between cases and controls

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>T/T</th>
<th>T/C</th>
<th>T/C</th>
<th>Tyr113His genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>344</td>
<td>167</td>
<td>146</td>
<td>31</td>
<td>69.8%</td>
</tr>
<tr>
<td>CD ALL</td>
<td>307</td>
<td>155</td>
<td>127</td>
<td>25</td>
<td>71.2%</td>
</tr>
<tr>
<td>CD 0 CARD15 DSAs 202</td>
<td>99</td>
<td>83</td>
<td>20</td>
<td>59.6%</td>
<td></td>
</tr>
<tr>
<td>CD 1 CARD15 DSA 69</td>
<td>33</td>
<td>33</td>
<td>3</td>
<td>71.7%</td>
<td></td>
</tr>
<tr>
<td>CD 2 CARD15 DSAs 20</td>
<td>12</td>
<td>7</td>
<td>1</td>
<td>77.5%</td>
<td></td>
</tr>
</tbody>
</table>

DSAs, disease susceptibility alleles; CD, Crohn’s disease.
A 36 year old woman presented for the first time with a five week history of bloody diarrhoea and mucus discharge in the 12th week of her first pregnancy. Ulcerative colitis was confirmed on flexible sigmoidoscopy and histology. She was started on mesalazine (Pentasa) 1 g twice daily orally and Pentasa enema was added subsequently. She failed to respond well to oral mesalazine (40–60 mg daily) for five weeks or to subsequent intravenous mesalazine given for a further two and a half weeks. Azathioprine (oral 150 mg daily) was also added. Repeat sigmoidoscopy confirmed severe distal colitis with ulceration. At the 23rd week of pregnancy, she was started on intravenous cyclosporin (2 mg/kg) with careful monitoring of serum levels. Significant improvement was noted in two weeks, after which cyclosporin was changed to the oral route. Steroids were gradually tapered to 2.5 mg daily. At 34 weeks she underwent an emergency Caesarean section because of antepartum haemorrhage and a healthy baby girl (birth weight 2.07 kg) was delivered. Two weeks later, cyclosporin was weaned off after minimal rise of serum creatinine that coincided with high serum cyclosporin levels. Her serum creatinine normalised four weeks later. She and baby remained well on azathioprine and mesalazine 14 weeks after delivery.

Intravenous cyclosporin induced remission in our pregnant patient who had failed to respond to high dose oral and intravenous mesalazine. Colectomy and the associated potential complications in pregnancy were avoided. There is only one other case report in the literature where cyclosporin was used in similar circumstances. While we would agree that cyclosporin should be used cautiously in pregnancy, our positive experience, and that of Bertschinger and colleagues, suggests that cyclosporin may induce remission and avoid colectomy during pregnancy.

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Reference


Is symptom control the correct end point for proton pump inhibitor treatment in Barrett’s oesophagus?

We have recently reported that abnormal acid reflux persists in up to 50% of patients with long segment Barrett’s oesophagus, despite good control of symptoms of gastro-oesophageal reflux disease (GORD) with proton pump inhibitor (PPI) therapy. The critical question is whether such persistence of abnormal acid reflux alters the risk of progression to adenocarcinoma. We investigated this issue by studying cellular proliferation and expression of cyclin D1, which is an important marker of neoplastic progression, in patients with Barrett’s oesophagus on PPI therapy.

A prospective cross-sectional survey of 20 patients with long segment Barrett’s oesophagus (defined as a length ≥3 cm and presence of specialised intestinal epithelium containing alician blue staining goblet cells) was conducted. In all cases, GORD symptoms had been well controlled with PPI therapy (omeprazole n = 13 patients, median dose 20 mg (range 10–40); lansoprazole n = 5, 30 mg; or rabeprazole n = 2, 20 mg). Patients had received PPI therapy for a median duration of 30 months (12–66). Oesophageal manometry, 24 hour ambulatory pHmetry, and Bilitec 2000 monitoring were conducted on all patients, without interruption of their usual PPI therapy. Representative endoscopic biopsy specimens of Barrett’s oesophagus from each patient were studied for expression of cyclin D1 protein (primary antibody 1:50 dilution; Novocastra Lab) and Ki-67 protein (primary antibody 1:75; Dako Lab), by standard immunohistochemistry. The histopathologist was blinded to clinical information. A proliferative index was computed for each patient by scoring the percentage of Ki-67 labelled specialised columnar epithelial cells, as previously described. Cyclin D1 expression was semi quantitatively assessed. The mean percentage of positive cells in areas of intestinal-type specialised columnar epithelium was assigned to one of three categories: 0, <5%; 1, 5–50%; or 2, >50%. The intensity of cyclin D1 immunostaining was scored as: weak = 1, moderate = 2, or intense = 3. The percentage category of positive cells and staining intensity were multiplied to produce a weighted score for each patient. All cases with weighted scores ≥1 were designated positive.

Despite PPI therapy and absence of GORD symptoms, pHmetry detected abnormal acid reflux in nine (45%) patients (pH <4 for (median) 19.2% (range 4.6–32.1) of 24 hours; DeMeester score 49.5 (20.2–109.8)). The remaining 11 patients had acid reflux within the normal range (pH <4 for ≤4.5% of 24 hours). Proliferative indices (mean (SD)) for patients with abnormal acid reflux and those with normal acid reflux were similar (56.5 (8.7) vs 37.4 (5.3), respectively; p = 0.3). Cyclin D1 expression was positive in seven (78%) patients with abnormal acid reflux and in seven (64%) patients with normal acid reflux (p = 0.4) (fig 1). The weighted score of cyclin D1 expression was identical (median 2 (range 2–6)) for patients with abnormal acid reflux and those with normal acid reflux.

These data imply that the risk of neoplastic progression was independent of the status of control of acid reflux by PPI therapy. We also examined the association between acid reflux and bile reflux. Absorbance ≥0.14 for ≤1.8% of the 24 hour monitoring period was considered the normal range for bile reflux in this study. Despite PPI therapy, abnormal bile reflux was detected in 12 (60%) patients, including six (55%) with normal acid reflux (absorbance ≥0.14 for 13.0% (2.5–46.5) and six (66%) with abnormal acid reflux (absorbance ≥0.14 for 17.4% (3.5–63.7)). Such persistent bile reflux may explain the similarity in expression of Ki-67 or cyclin D1 in the two groups with different control of acid reflux.

In contrast with PPI therapy, antireflux surgery that is successful in controlling acid reflux also controls bile reflux. Following successful antireflux surgery, proliferative indices in surface epithelial cells and crypts of Barrett’s oesophagus are significantly lower compared with a failed procedure. In the light of the present data, we propose the need for a novel clinical trial of PPI therapy versus antireflux surgery. Patients who are randomised to PPI therapy should undergo...
Twenty-four hours later.

Next day and patients were informed in function tests. Results were available the testing (hepatitis C antibody test and liver London. The clinic was manned between Bartholomew's Hospital in the City of clinic in the Minor Injuries Unit at St July 2003) and was widely publicised in the public information about HCV by organising the government identified need for more walk-in HCV testing service Improving hepatitis C services across the UK: response to a walk-in HCV testing service The Department of Health (DH) estimates that approximately 0.4% of the UK population are chronically infected with hepatitis C virus (HCV) (that is, 200,000 people). As few as 10% of these individuals, who are at risk of end stage liver disease, are thought to be aware of their infection. Clearly action is required to identify and treat these patients with current drugs (pegylated interferon and ribavirin) that can cure over 50% of infected patients. The UK voluntary sector has responded to the government identified need for more public information about HCV by organising a hepatitis C awareness day. We took advantage of the publicity around hepatitis C awareness days to assess the value of a walk-in HCV testing clinic. Our clinic was held over four days (4–7 July 2003) and was widely publicised in the local press and television. Patients who wished to be tested were invited to attend a clinic in the Minor Injuries Unit at St Bartholomew's Hospital in the City of London. The clinic was manned between 8am until 11am for counselling and informed testing (hepatitis C antibody test and over function tests). Results were available the next day and patients were informed in person 24 hours later. Nineteen people attended and two were infected. One of these patients had been lost to follow up due to non-attendance at a local liver clinic 12 years ago.

Open access confidential hepatitis C testing clinics may play an important role in encouraging people to come forward for HCV testing and may facilitate public education about this important treatable infection. However, these clinics are labour intensive and, in our experience, unlikely to provide a cost effective solution to the identification of people with this treatable, sometimes fatal, infection.

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R CF D’Souza, M J Glynn, E Alstead, G R Foster Hepatobiliary Group, Barts and The London, Queen Mary’s School of Medicine and Dentistry, London, UK Correspondence to: Professor G R Foster, Hepatobiliary Group, Department of Gastroenterology, DDRC, Turner St, London E1 2AD, UK; g.r.foster@qmul.ac.uk Conflict of interest: Dr Foster acts as a consultant to companies who sell drugs for the treatment of viral hepatitis and has received research funding from such companies. He has received fees from companies who market antiviral therapeutics.

Influence of mode of delivery on gut microbiota composition in seven year old children Intestinal microbiota development begins immediately following birth. The composition of the infant’s evolving microbiota is initially defined by the mother, the source of the newborn’s first microbial inoculum. Colonising bacteria rapidly adapt to breast milk and epithelial mucins as sources of nutrients. The prevalence of caesarean section delivery in Western countries is increasing. Caesarean born babies are deprived of contact with the maternal/vagal microbiota and the first exposure is characterised by a lack of strict anaerobes and the presence of facultative anaerobes such as Clostridium species. Caesarean born infants have a more slowly diversifying microbiota, with differences reported from normally born infants, even after six months of age. Aerceranics in early microbiota acquisition can affect immunophysiologival development with a heightened disease risk. This study assessed microbiota composition in seven year old children and compared the respective effects of normal delivery and caesarean section.

In all, 60 seven year old children were randomly selected from Southwestern Finland, representing caesarean and vaginal deliveries. The children were invited to attend a clinical examination, including skin prick testing and determination of serum total and antigen specific IgE antibodies. Perinatal data were derived from hospital medical records. Questionnaires were completed by the parents to verify a history of allergic symptoms. Faecal samples were produced at clinical examination and frozen at −70°C for microbioa assessment. Faecal microbiota profiles were determined using the culture independent fluorescent in situ hybridisation method. Probes specific for bifidobacteria, lactobacilli/enterococci, bacteroides, clostridia, and total bacterial numbers were applied. Written informed consent was obtained from parents and the study was approved by the ethics committee of the university.

Of the study population, 31 children had been delivered by caesarean section and 29 by vaginal delivery. At seven years of age, significantly higher numbers of clostridia were found in children delivered vaginally compared with caesarean born children (p = 0.0055) (table 1). No differences were observed in other faecal bacteria or total numbers of bacteria (table 1). Children with asthma diagnosed by a physician (n = 6) had lower numbers of clostridia in their faecal specimens while healthy children (n = 54) had higher clostridial numbers.

Early colonisation guides subsequent microbiota development which may later impact on health, to the extent of predisposing some infants towards specific diseases. Bifidobacteria are considered useful for health promotion. Reported effects are related to the individual “balance” of the gut microbiota and prevention of anarcheries within the gastrointestinal tract. Clostridia are generally considered harmful toxin producing species causing diarrhoea and food poisoning.

Our results show that bifidobacterial levels in the faeces of cohort children were comparable at seven years of age, independent of the mode of delivery at birth, while numbers of clostridia were significantly higher in normally born children seven years after birth.

Differences in neonatal gut microbiota, in particular the balance between Bifidobacterium species and Clostridium species, have been reported to precede heightened production of antigen specific IgE antibodies, a hallmark of the atopic responder type. Such differences may be related to external environmental

<table>
<thead>
<tr>
<th>Parameter (conc of specific microbe or total IgE)</th>
<th>Normally delivered</th>
<th>Caesarean born</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridia</td>
<td>9.29 (9.06–9.51)</td>
<td>8.83 (8.6–9.06)</td>
<td>0.0055</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>10.32 (10.13–10.5)</td>
<td>10.29 (9.99–10.59)</td>
<td>0.87</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>11.56 (11.46–11.68)</td>
<td>11.59 (11.5–11.68)</td>
<td>0.61</td>
</tr>
<tr>
<td>Lactobacilli/enterococci</td>
<td>9.07 (8.85–9.3)</td>
<td>9.05 (8.86–9.2)</td>
<td>0.85</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>9.95 (9.67–10.24)</td>
<td>9.84 (9.52–10.17)</td>
<td>0.63</td>
</tr>
<tr>
<td>Total IgE</td>
<td>79 (71–255)</td>
<td>65 (25–160)</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Values are median (interquartile range).
PostScript

et al

Sonwalkar

We read with interest the case described by a disease-related donor with Crohn’s ileitis after liver delivery may continue even beyond infancy. These findings call for further assessment of microbiota composition throughout childhood when dietary interventions may still offer a rational means of health improvement. It is of importance to characterise the optimal clostridial numbers and species composition at different ages following normal and caesarean delivery.

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References


Crohn’s ileitis after liver transplantation from a living related donor with Crohn’s disease

We read with interest the case described by Sonwalkar et al of a patient who developed fulminant Crohn’s colitis after allogeneic stem cell transplantation (ASCT) (Gut 2003;52:1518–21). Although the donor had no known history of Crohn’s disease (CD) and did not carry the IBD3 or IBD5 haplotypes associated with CD, HLA class III mismatches at IBD3 and a CD associated polymorphism of the 5’UTR of NOD2/CARD15 were present in the donor and in the reconstituted immune cell population of the recipient post ASCT. The authors hypothesised that adoptive transfer of CD susceptibility may have occurred between ACST donor and recipient.

Herein, we report a case of a patient who developed CD after receiving a liver transplant from a donor with known CD. A 24-year-old female received a liver transplant for a living related donor with decompensated cirrhosis secondary to vertically transmitted chronic hepatitis C infection. The family history was significant for a maternal aunt diagnosed with CD, who served as the liver donor, and a maternal uncle and grandfather with colon cancer. Following liver transplantation, the patient was maintained on an immunosuppressive regimen consisting of tacrolimus 3 mg twice daily, sirolimus 5 mg daily, as well as TMP-SMZ prophylaxis. Her initial post-transplant course was uneventful but she later developed recurrent hepatitis C infection, treated with pegylated interferon and ribavirin. She presented with symptoms consistent with intermittent small bowel obstruction 11 months post-transplant. She was also receiving prednisone 15 mg daily at that time. A computed tomography scan of the abdomen and pelvis (see fig 1A on the Gut website: www.gutjnl.com) and an upper gastrointestinal with small bowel follow through study (see fig 1B on the Gut website: www.gutjnl.com) demonstrated marked fold thickening of the distal ileum. An enteroscopy demonstrated patchy ulcerations in the jejunum and Roux-en-Y limb of the small bowel. Biopsies showed focal ulceration and mild active inflammation without evidence of granuloma or viral inclusions. Wireless capsule endoscopy demonstrated multiple erosive and ulcerative changes in the distal small intestine (see fig 1C, 1D on the Gut website: www.gutjnl.com).

Because of persistent symptoms and concern for possible lymphoproliferative disorder, the patient underwent an open laparatomy which revealed nodularity of the terminal ileum. Intraoperative colonoscopy demonstrated nodularity and three ulcers in the distal ileum. Histopathological examination of the resected ileal specimen demonstrated focal villous blunting, expansion of the lamina propria with acute and chronic inflammatory cells, reactive crypt changes, and occasional crypt abscesses and focal gastric metaplasia (arrow and insert). SM, submucosa.

Figure 1 Histopathological examination of a resected ileal specimen demonstrated focal villous blunting, expansion of the lamina propria with acute and chronic inflammatory cells, reactive crypt changes, and occasional crypt abscesses and focal gastric metaplasia (arrow and insert). SM, submucosa.

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www.gutjnl.com
Enteric glia

von Boyen et al recently reported a study of glial fibrillary acidic protein (GFAP) expression in enteric glia (Gut 2004;53:222–8). Their new data are very interesting and add to our understanding of the possible role of enteric glia in gastrointestinal pathophysiology. However, we must take issue with some of the data presented that show extensive nuclear labelling with S-100 and with the description of the distribution of GFAP in the colon. Figure 1 of their paper shows labelling of enteric glia in the rat colon. The role of enteric glia in inflammation raised in the paper are very interesting, and of considerable importance in understanding the GFAP expression patterns in enteric glia (17 μm z stack of 1 μm optical sections; scale bar 50 μm) (fig 1). S-100 is also found in the cytoplasm of the glial perikarya; there is virtually no nuclear labelling, which was the most obvious element of the staining demonstrated by von Boyen et al.

In fig 1 of the paper of von Boyen et al, the nature of the GFAP immunoreactivity is not fibrous, but granular, while the predominant labelling of S-100 is nuclear. In our hands this is not the case (see our fig 1) and so we feel this calls into question whether the extensive nuclear labelling observed in both fig 1 and fig 2 is really reflective of the distribution of S-100. Moreover, in the paper cited by the authors in support of nuclear localisation, Ferri et al state that “only cytoplasmic localisation (of S-100) was consistently demonstrated in enteric glia,” contrary to von Boyen et al’s assertion that S-100 labelling is largely nuclear.

Finally, it should also be noted that GFAP expression in culture may reflect an altered state of differentiation as an adaptation to in vitro conditions rather than a pathophysiological response to cytokines. The issues of glial heterogeneity and the role of enteric glia in inflammation raised in the paper are very interesting, and of considerable importance in understanding the physiology and pathophysiology of the gastrointestinal tract. By analogy with the brain, it is likely that enteric glia play an important role in the function of the gut. However, we feel that the extensive glial heterogeneity suggested in the paper by von Boyen et al may be overestimated and we urge caution in extrapolation of these data based on the immunohistochemistry presented in this manuscript.

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References


Correction

In the paper by Wang et al Gastroenterol 2004;53:1096–1101), the acknowledgement and correct email address were not presented. The acknowledgement should have read as follows: "The authors thank senior technician Shu-Hao Wen for her assistance in processing the tissue slides, and Drs Jian-Ming Qian, Gang Sun, and Xiao-Hong Liu for their help in collecting the biopsy samples for the study project." In addition, the correct email address for Professor G-Z Pan is pgz@public3.bta.net.cn.