Microbiome Composition by Pyrosequencing in Mesenteric Lymph Nodes of Rats with CCl₄-Induced Cirrhosis

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Key Words

Carbon tetrachloride · Cirrhosis · Microbiota · Mesenteric lymph node

Abstract

Background: The cross talk between the gut microbiota and the immune system, which is essential to maintain homeostasis, takes place at the intestinal lymphoid tissue such as the mesenteric lymph nodes (MLNs). Here, we investigated the presence of bacterial DNA in MLNs of control and cirrhotic rats and its relationship with inflammatory responses. **Methods:** The MLN microbiome of cirrhotic rats with ascites, which was induced by carbon tetrachloride (CCl₄), was compared to that of control rats using quantitative real-time PCR

and pyrosequencing of the 16S rRNA gene. Cytokines in blood samples were assessed by ELISA. **Results:** Unexpectedly, sequence analysis revealed a high microbial diversity in the MLNs of both control and cirrhotic rats with Proteobacteria as one of the most dominant phylum. CCl₄-induced liver injury was not associated with a change in bacterial load, but it was linked to a decrease in microbial diversity ($p < 0.05$) and alterations in the microbial community in MLNs. A high proportion of *Bifidobacterium animalis* was also positively correlated with elevated interleukin-10 expression ($p = 0.002$, false discovery rate = 0.03, $r = 0.94$). **Conclusions:** For the first time, the high microbial diversity observed in MLNs of both controls and CCl₄-induced cirrhotic rats provides evidence that bacterial translocation is more than a mere dichotomic phenomenon.

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Silvia Cuenca and Elisabet Sanchez are co-first authors. The genetic sequence data will be submitted to the GenBank database upon publication.

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Introduction

Cirrhosis is the final phase of chronic liver disease, in which inflammation is associated with dying hepatic cells and fibrosis, which lead to poor liver function and portal hypertension.

Bacterial translocation is defined as the migration of viable bacteria or their products from the gastrointestinal tract to mesenteric lymph nodes (MLNs) and other extra-intestinal sites, such as the systemic circulation or extra-intestinal organs [1]. MLNs are a vital part of the immune system. They carry specialized cells that trigger immune system responses and play a crucial role in the interplay with intestinal microbiota. Bacterial translocation has been postulated as a main mechanism in the pathogenesis of spontaneous infections in cirrhosis and also in the hyperdynamic circulatory state, a key factor in the pathogenesis of portal hypertension, ascites development and other cirrhosis complications [2–7].

Until recently, bacterial translocation in cirrhosis was considered a dichotomic and mostly monomicrobial phenomenon [8, 9]. However, recent studies using techniques of advanced molecular biology have questioned this interpretation [10, 11]. Bacterial DNA and also live bacteria have been detected in the MLNs of healthy mice, where they have been transported from the digestive tract via dendritic cells [12–14]. Using a 16S rRNA gene clone library analysis by conventional Sanger technique to analyze the bacterial composition, MLNs were found to be dominated mainly by *Pseudomonas* and *Alcaligenes* spp., which belong to the Proteobacteria phylum [14]. These results confirmed that bacterial translocation into the MLNs can be polymicrobial.

Dysbiosis (microbial community alteration) in fecal microbial communities has been evidenced in patients with liver cirrhosis, where Bacteroidetes phylum was significantly reduced while Proteobacteria and Fusobacteria phyla were highly enriched [15]. Members of the Proteobacteria and Bacteroidetes phyla have also been associated with inflammation in cirrhotic patients presenting a hepatic encephalopathy complication [16]. However, to our knowledge, no study using pyrosequencing has shown dysbiosis in MLNs in liver disorders, where the interplay between microbiota and the immune system could play a crucial role in the inflammation and therefore in the evolution of the disease.

Chronic liver damage induced by carbon tetrachloride (CCl₄) administration in rodents is an experimental model that has been widely used to study the pathogenesis of cirrhosis, ascites and bacterial translocation [17].

In the present pilot study, using a rat model of cirrhosis and a high-throughput sequencing technology, we evaluated the microbial composition of MLNs of cirrhotic and control rats, and its relationship with immune responses in the systemic circulation. Our findings disclose the dysbiosis down to the species level and link an anti-inflammatory cytokine to a specific bacterial taxon.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 35–49 g were purchased from Harlan Laboratories (Indianapolis, Ind., USA) and provided by Research Models and Services Production (Udine, Italy). Rats after weaning and their mothers were fed a rodent chow diet (2018S; Teklad, Madison, Wisc., USA). After a 1-week quarantine, all animals were placed in individual cages and kept at a constant room temperature of 21°C, exposed to a 12-hour light:12-hour dark cycle and allowed free access to water and rodent chow (A04; SAFE, Augy, France). There was no contact between rats neither through water, chow or feces of other animals. The study was approved by the Animal Research Committee at the Institut de Recerca of Hospital de la Santa Creu i Sant Pau (Barcelona) and by the Department of Agriculture, Livestock and Fisheries of the Generalitat de Catalunya (Departament d'Agricultura, Ramaderia i Pesca). Animal care complied with the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*.

Induction of Cirrhosis and Study Groups

Cirrhosis was induced as previously described [18]. When rats reached a weight of 200 g, they were administered weekly doses of CCl₄ (Sigma-Aldrich, St. Louis, Mo., USA) intragastrically using a sterile pyrogen-free syringe (ICO plus 3 Novico Médica, S.A., Barcelona, Spain) with an attached stainless-steel animal feeding tube (Popper and Sons, New Hyde Park, N.Y., USA) without anesthesia. The first dose of CCl₄ was 20 µl, and subsequent doses were adjusted on the basis of changes in weight 48 h after the previous dose, as reported previously [19]. When cirrhotic rats presented ascites, the dose of CCl₄ was maintained at 40 µl. Non cirrhotic rats receiving tap water were used as controls.

Laparotomy

When ascites was suspected in cirrhotic rats based on the increase in abdominal girth, a paracentesis was carried to confirm the presence of ascitic fluid. Paracenteses were performed under air anesthesia with isoflurane (Forane®; Abbott, Madrid, Spain) in sterile conditions, and approximately 0.1 ml of ascitic fluid were removed. One week later, a laparotomy was carried out on all cirrhotic rats, and in control rats, laparotomy was performed at the corresponding weeks.

For laparotomy, rats were anesthetized with 10 mg/kg xylazine (Rompun®; Bayer, Kiel, Germany) and 50 mg/kg ketamine (Ketolar®; Parke-Davis, Madrid, Spain) in sterile conditions. In brief, the abdominal fur was removed with a depilatory cream and the skin was sterilized with iodine. The abdomen was then opened via a 4-cm median incision, and the remaining fluid was removed. Samples of ascitic fluid (and pleural fluid if present) were collected

for bacterial culture. Ten MLNs were aseptically and randomly collected from the ileocecal area, weighed and homogenized in sterile saline solution for later analyses. Blood was collected from the vena cava into a sterile EDTA-containing BD Vacutainer® tube (BD Biosciences, San Jose, Calif., USA) without additives, centrifuged and stored at -80°C until later analysis. Liver and spleen tissue was sampled for histological evaluation and bacterial culture and also frozen at -80°C . Rats were then euthanized with intravenous sodium thiopentate (Pentothal®; Abbott Laboratories).

Bacterial Culture

We performed bacterial cultures of samples of MLN homogenate, spleen and liver of cirrhotic and control rats, and ascites and pleural samples were obtained from cirrhotic rats. All the samples were inoculated on Columbia blood agar, Columbia CNA agar and chromogenic medium (CPS ID2; BioMérieux, Marcy-l'Etoile, France). Isolates were identified on the basis of their growth and morphology. Gram stain, catalase, coagulase or oxidase assays were performed when required.

Biochemical Analysis

Serum supernatants were tested for interleukin (IL)-6 and IL-10 using an ELISA kit (PeproTech, London, UK) according to the manufacturer's instructions. Cytokines were quantified with standard curves provided by the kit. The detection limits were set at 30 pg/ml for both cytokines.

Genomic DNA Extraction

From each rat, a sample of the MLN homogenate was subjected to genomic DNA extraction using the DNeasy® blood and tissue kit (Qiagen, Madrid, Spain) according to the manufacturer's recommended protocol.

Assessment of Microbial Load

To assess microbial load, we used extracted DNA to amplify the V4 region of the 16S rRNA gene by quantitative real-time PCR (qPCR) using the following primers: V4F_517_17 (5'-GCCAGCAGCCGCGGTAA-3') and V4R_805_19 (5'-GACTACCAGGGTATCTAAT-3'). qPCR was performed with the 7500 fast real-time PCR system (Applied Biosystems, Foster City, Calif., USA) using optical-grade 96-well plates. PCR was carried out in a total volume of 25 μl using the power SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) containing 6.25 pmol/ μl of each of the above-cited universal forward and reverse primers. The reaction conditions for DNA amplification were as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate and mean values were calculated. Data were analyzed using Sequence Detection Software (version 1.4) supplied by Applied Biosystems. The target DNA copy number was determined by comparison with serially diluting standards (10^1 to 10^6 copies of plasmid DNA containing the amplicon) running on the same plate, as previously described [20]. Bacterial load was expressed as copy numbers of 16S rRNA gene per gram of MLN.

Analysis of Microbial Community Composition

To analyze the microbial community composition of the MLNs, we performed a 16S rRNA gene survey. For this purpose, extracted genomic DNA was subjected to PCR amplification of the

V4 region of the bacterial and archaeal 16S rRNA gene. The V4 primer pairs used in this study are expected to amplify >95% of the archaeal and bacterial domains according to our analysis done using PrimerProspector software [21]. The 5' ends of the forward (V4F_517_17) and reverse (V4R_805_19) primers targeting the 16S gene were tagged with the following specific sequences for pyrosequencing: 5'-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-(multiplex identifier)-(GCCAGCAGCCGCGGTAA)-3' and 5' CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-(GAC-TACCAGGGTATCTAAT)-3'. Tag pyrosequencing was performed using multiplex identifiers of 10 bases, which were provided by Roche (Nutley, N.J., USA) and specified upstream of the forward primer sequence. Standard PCR [1 unit of Taq polymerase (Roche) and 20 pmol/ μl of the forward and reverse primers] was run in a Mastercycler gradient (Eppendorf, Hamburg, Germany) at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 56°C for 20 s, 72°C for 40 s and a final cycle of 72°C for 7 min. As a negative control for the PCR, H_2O was used instead of the template. The integrity and specificity of the 16S rRNA V4 amplicons were confirmed by microcapillary electrophoresis using an Agilent 2100 Bioanalyzer with the DNA 1000 kit. Subsequently, the amplicons were sequenced on a 454 Life Sciences (Roche) FLX system (Scientific and Technical Support Unit, Vall d'Hebron Research Institute, Barcelona, Spain) following standard 454 platform protocols. To rule out possible contamination, we also confirmed that no amplicon was produced after PCR of spleen samples of all rats for which we were not expecting bacterial DNA amplification.

The sequences were analyzed using the QIIME (Quantitative Insights into Microbial Ecology) pipeline [22]. From the pyrosequencing experiment, 25,579 high-quality sequences with an average of 290 bp were recovered from all the samples (with an average of 3,654 sequences per sample) after filtering high-quality readings, as previously described [23]. From all samples, using a 97% similarity cutoff, we obtained 412 taxa (or molecular species). After removing taxa with low abundance (i.e. we considered only taxa that were represented by at least 0.2% of the sequences in a sample), we recovered 143 microbial taxa.

Rarefaction analysis was done for all samples, with 10 repetitions using a step size of 100 (100–2,700 sequences per sample). For β diversity analyses, which examine changes between microbial communities, sequence data were normalized at 2,718 sequences per sample. This number was chosen to avoid the exclusion of samples with a lower number of sequence readings from further analysis. The UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis was performed on pairwise unweighted UniFrac distances [24].

Statistical Analysis

Differences in microbial load between groups were analyzed using the nonparametric Mann-Whitney U test, with values of $p < 0.05$ considered statistically significant for all tests. We used the `otu_category_significance.py` script from the QIIME pipeline to test the taxa associated with experimental cirrhosis status and cytokine concentrations. This analysis provided the false discovery rate (FDR) value, which is defined to be the false discovery rate of the p value (corrected p value) and is considered significant when <0.1 [18]. In order to determine the microbial taxon or group of taxa that is increased or decreased in the cirrhotic rats versus controls, we performed analysis of variance (ANOVA) to compare differences between group means.

Fig. 1. High microbial diversity in MLNs. The microbial diversity of MLNs of control rats (average proportion of sequences of the 4 healthy rats) is shown at several taxonomic levels based on the analysis of 16S RNA gene sequences. P = Proteobacteria; F = Firmicutes; B = Bacteroidetes; A = Actinobacteria.

Results

We included a total of 7 rats: 3 with CCl₄-induced cirrhosis and 4 control rats. In cirrhotic rats, to confirm the presence of ascites paracentesis was performed 14, 17 and 21 weeks after the first CCl₄ dose, respectively.

Bacterial Culture

Cultures of the spleen, liver and MLN homogenate were negative in the 4 control rats. Cultures of liver, spleen, ascites and pleural fluid tissue from the 3 cirrhotic rats were also negative. Cultures of homogenates from MLNs were positive only in 1 (rat G24) of the 3 cirrhotic rats. In this rat, *Escherichia coli* was isolated.

High Microbial Diversity in the MLNs of All Control Rats

From the sequence data analysis, we unexpectedly detected an interestingly high microbial diversity in all MLNs of control rats. The microbial community was dominated by 4 bacterial phyla: Proteobacteria (61.5%, SD = 9.7%), Firmicutes (28.2%, SD = 10%), Actinobacteria (5.5%, SD = 0.8%) and Bacteroidetes (4.4%, SD = 1.3%). At a lower taxonomic level, we identified an average of 10 microbial classes, 28 orders, 63 families, 82 gen-

era and 88 bacterial taxa. No Archaea were found in our control rats (fig. 1). Among the Proteobacteria phylum, an unknown species from the Enterobacteriaceae family was the most abundant taxon (36%, SD = 8%).

Dysbiotic Microbiome in Cirrhotic Rats

Microbial DNA was detected in MLN homogenate of all cirrhotic rats. The community diversity was lower in cirrhotic than in control rats, which was estimated by 3 indexes: Chao1, phylogenetic diversity and the number of taxa observed ($p < 0.05$; Mann-Whitney test; fig. 2). Control rats presented individually higher numbers of bacterial taxa: 75 (G31), 74 (G32), 78 (G35) and 76 (G37) than cirrhotic rats: 55 (G24), 51 (G25) and 45 (G28). However, this finding was not associated with a difference in the microbial load between the two groups, as evaluated by qPCR (fig. 3).

We used the UPGMA method based on the unweighted UniFrac metric to compare samples. This analysis measures the phylogenetic distance between bacterial communities in a phylogenetic tree, thereby providing a measure of similarity among microbial communities present in distinct samples [24]. As shown in the figure 4, control rats presented more homogeneous microbial communities than cirrhotic rats.

In order to determine the microbial taxon or group of taxa that is increased or decreased in the cirrhotic rats versus controls, we performed ANOVA. Clostridiales, a group of bacteria belonging to the Firmicutes phylum, were found to be 6.6-fold more abundant in controls than in cirrhotic rats ($p = 0.006$; FDR = 0.087; fig. 5a). Furthermore, an unknown species belonging to the *Janibacter* genus (Actinobacteria phylum) was found to be 2.5-fold more abundant in rats with cirrhosis (2.9% of the sequences) than in controls (1.2% of the sequences; $p = 0.019$; FDR = 0.37; fig. 5b).

Correlation with Immune Responses

In order to identify the possible association between microbial taxa and inflammation, we measured cytokines such as IL-6 and IL-10 in blood samples in all the 7 study rats. The concentration of these two cytokines, although higher in cirrhotic rats [492 (SD = 293) vs. 179 pg/ml (SD = 213), respectively] than in control rats [388 (SD = 100) vs. 47 pg/ml (SD = 35 pg/ml), respectively], was not significantly different between the two groups. However, using the Pearson correlation, we found that MLN bacteria belonging to the Actinobacteria phylum, and in particular *Bifidobacterium animalis*, were significantly and positively correlated with high IL-10 expression ($p =$

Fig. 2. Higher microbial richness in control rats. Control rats ($n = 4$) showed a higher microbial diversity ($p < 0.05$) than cirrhotic rats ($n = 3$) based on the 16S rRNA gene sequence analysis, as assessed by three richness metrics: number of observed species, Chao1 and phylogenetic diversity (PD).

Fig. 3. Microbial load in MLNs of control and cirrhotic rats. Concentration of microbial DNA is similar in control ($n = 4$) and cirrhotic ($n = 3$) rats assessed by qPCR of the 16S rRNA gene. All reactions were performed in triplicate. The standard curve obtained from a 10-fold serial dilution of the template produced an R^2 value of 0.993 and a slope of -3.1 .

Fig. 4. Control and cirrhotic rats presented a distinct microbial composition. Two distinct clusters were identified using the UPGMA tree based on unweighted UniFrac analysis of 16S rRNA gene sequences. Scale bar represents 3% sequence divergence.

Fig. 5. Dysbiosis in MLNs of cirrhotic rats compared to control rats. **a** Control rats presented a higher proportion of Clostridiales (arrow, order level) than cirrhotic rats ($p = 0.006$; FDR = 0.087). **b** Cirrhotic rats showed a higher proportion of a taxon from the *Janibacter* genus (arrow, species level) than cirrhotic rats ($p = 0.014$; FDR = 0.27). Only taxa or order of taxa accounting for >1% of the sequences are represented in the legends.

0.002; FDR = 0.03; $r = 0.94$; fig. 6). However, we did not observe a significant correlation between a bacterium or a group of bacteria and IL-6 expression.

Discussion

To our knowledge, although it has been shown that more than 1 bacterial species is translocated into MLNs [14], our study is the first to report a high microbial diversity of bacterial DNA.

Our previous studies demonstrated that fecal microbiota of healthy rats is dominated by Firmicutes (74%) and Bacteroidetes (23%), whereas Actinobacteria and Proteobacteria are minor constituents [20]. However, in this study, our observation showed that MLNs from control and cirrhotic rats harbored a majority of Proteobacteria. This would suggest that bacteria from this phylum more than other phyla have a greater ability to attach and thus penetrate the mucosal barrier. Indeed, Clegg et al. [25] showed that Gram-negative bacteria such as *Escherichia coli* (Proteobacteria) produce functional adhesins

Fig. 6. Correlation between microbial taxa present in all MLNs and serum IL-10 concentrations using ANOVA. The analysis provided an r and a p value for each microbial taxon in the 7 rats. The plot illustrates the r value against the p value for each taxon (represented by a dot).

that play a role in bacterial-host cell interactions. Thus, more antigens from these bacteria would be presented to the immune cells in MLNs and would therefore play an important role in homeostasis. This hypothesis would explain cases where lower abundant intestinal phyla, such as Proteobacteria, have been found to be associated with conditions like irritable bowel syndrome, a low-grade inflammatory disorder [26, 27], inflammatory bowel disease [28] and cirrhosis [29, 30].

Previous studies using microbiological culture and conventional PCR to detect bacterial DNA have shown that bacterial translocation to MLNs is rare in control rats and occurs between 37 and 87% of the cirrhotic rats with ascites [9, 30–33]. Moreover, the bacterial translocation in these studies was mostly monomicrobial and less frequently bimicrobial. However, using pyrosequencing, our study suggests that the presence of polymicrobial DNA in MLNs is a physiological feature in control rats and that bacterial translocation should no longer be considered a dichotomic and monomicrobial event but instead as a constant and polymicrobial phenomenon. This notion is in agreement with a recent study, also using pyrosequencing, that found polymicrobial DNA in most sterile ascitic fluid samples of patients with cirrhosis [11]. In this setting, what would be relevant is not that bacterial DNA was detected in the MLNs but the quantitative and qualitative characteristics of such polymicrobial

DNA. Moreover, we observed a decrease in Clostridiales in MLNs of cirrhotic rats. This is in line with the study of Gomez-Hurtado et al. [34] who reported lower *Clostridia* spp. in fecal samples in mice treated with CCl_4 compared to control animals, and this was associated with a proinflammatory scenario.

Our findings in MLNs reveal differences in the microbial community composition between cirrhotic and control rats and a lower microbial diversity without a decrease in microbial load in the former. The observation of lower diversity is a common trait associated with human disorders such as obesity, inflammatory bowel disease and colic in infants [35–37]. These observations support the notion of missing bacteria replaced by a few pathogenic ones in disease conditions.

Commensal bacteria provide defense against pathogenic ones, not simply by competing for nutrients and physical niches but also by inducing specific immune responses. Indeed, the development of the mucosal immune system is dependent on the type of bacteria that are present in the lumen and that can be captured by immune cells such as dendritic cells. Previous studies have reported that fecal *Bifidobacterium* is reduced in patients with cystic fibrosis or with hepatitis B virus-induced chronic liver disease [38, 39].

Investigating translocation of commensal bacteria using qPCR in a mouse model of type 2 diabetes, which is associated with systemic low-grade inflammation, Amar et al. [40] found high numbers of intestinal bacteria in the adipose tissue and blood. They demonstrated that the translocation event could be reverted by 6 weeks of treatment with the probiotic strain *B. animalis* subsp. *lactis* 420. Our finding, which showed a positive correlation between *B. animalis* and high anti-inflammatory cytokine levels in MLNs, is in line with this previous work.

Our study, although using a very high-throughput technique to study the MLN microbiome, presents several limitations. Indeed, considering that this work is a pilot and laborious study, we used a low number of rats. Furthermore, our study does not allow understanding whether the difference in MLNs is a consequence of the difference in the gut microbiome or a difference in selective translocation. In future studies, in order to be able to distinguish between these two possible causes, we could also consider analyzing the gut microbiome. Finally, our study does not distinguish the presence of viable from nonviable bacteria, which could be solved, in the future, using a PCR-based method using propidium monoazide [41].

In conclusion, this pilot study revealed the presence of high 16S rDNA diversity in MLNs of both control and

cirrhotic rats. The latter presented significantly lower bacterial diversity and a dysbiosis of the microbial community.

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Disclosure Statement

There is no conflict of interest.

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