THE PATHOLOGICAL MICROBIOTA

Gut microbiota and IBD

Microbiote intestinal et MICI

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Summary

Gut microbiota contains about $10^{14}$ bacterial cells classified within 4 bacterial phyla, namely Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Much of the information has been generated through the application of nucleic acid-based methodologies (16S rRNA) which provide a cornerstone of microbial taxonomy. Inflammatory bowel disease (IBD) involves a dysregulated immune response to the gut microbiota in genetically predisposed hosts. Experimental animal models of colitis provide the best evidence that bacteria present in the bowel of the animals have an essential role in the pathogenesis of colitis since in most models, germ-free animals do not develop disease. Moreover, in the immunodeficient mouse model of colitis called TRUC (T-bet-/- x RAG2-/-), a colitogenic gut microbiota is selected and can be transmitted to mice with intact immunity and induce colitis. Current interest therefore focuses on the bacterial community as the source of antigens that fuel the chronic inflammation seen in IBD. Dysbiosis, an imbalance between harmful and protective bacteria, has been evoked and investigated in IBD. Thus, besides the classical pathogens, gut microbiota can drive pathogenicity via two mechanisms: an expansion of ‘pro-inflammatory’ species or a restriction in the protective compounds of the microbiota. Complexity of the microbiota suggests that both mechanisms may contribute to chronic gut inflammation in IBD.

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Résumé

Le microbiote intestinal contient environ $10^{14}$ bactéries classées en 4 phyla bactériens: Firmicutes, Bacteroidetes, Actinobacteria, et Proteobacteria. Une grande partie des informations concernant cet écosystème a été générée par l’application de méthodes moléculaires visant la reconnaissance des acides nucléiques (16S ARNr) motifs moléculaires clefs de la taxonomie microbienne. Les maladies inflammatoires de l’intestin (MICI) résultent d’une réponse immunitaire dérégulée vis-à-vis du microbiote intestinal chez des sujets génétiquement prédisposés. Les modèles murins de colite expérimentale fournissent des arguments expérimentaux solides pour incriminer la microflore intestinale dans la pathogénèse d’une colite. En effet, dans la plupart des modèles, la colite ne se développe pas en absence de microbiote (situation axénique). Récemment, avec le

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modèle de souris immunodéfécientes de colite appelée TRUC (T-bet -/- x Rag2 -/- , ulcercative colitis), un nouveau paradigme a emergé puisque le microbiote intestinal semble pouvoir transmettre la colite suggérant ainsi l’existence d’un microbiote ‘colitogénique’. Un intérêt grandissant centré sur l’étude des communautés bactériennes comme source antigénique alimentant l’inflammation chronique au cours des MICI a vu le jour. Une dysbiose, i.e. un déséquilibre entre des bactéries ‘délétères’ et ‘bénéfiques’, a été évoquée et recherchée dans les MICI. A côté des agents pathogènes classiques, le microbiote intestinal pourrait porter une pathogénicité de deux façons: par l’expansion d’espèces «pro-inflammatoires» ou par la diminution de bactéries protectrices «anti-inflammatoires». La complexité du microbiote suggère que ces deux mécanismes pourraient contribuer à l’inflammation intestinale chronique rencontrée au cours des MICI.

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**Introduction**

Despite great advances in recent years, the pathogenesis of inflammatory bowel disease (IBD) has not been completely clarified. It now appears evident that Crohn’s disease (CD) and ulcerative colitis in genetically predisposed subjects are due to inappropriate activation of the gut’s mucosal immunity, with the stimulus coming from the bacterial contents of the intestine (microbiota). Trends in the incidence of IBD in different geographic locations highlight the role of environmental factors in its pathogenesis. One of the main candidates is the gut microbiota. In addition, most of the genes that are currently known to be involved in IBD pathogenesis [NOD2, IL-23R, ATG16L1 and IRGM (autophagy)] are related to interactions between the bacteria and the immune system. Finally, the hypothesis involving a deficit of defensin (antibacterial peptides synthesized in the intestine) in Crohn’s disease strengthens the predominant role of the gut microbiota in intestinal homeostasis. All of these elements place the focus on gut microbiota in IBD pathogenesis. Two complementary strategies have been used until now: 1) an investigation for a candidate ‘pathogen’ microorganism; and 2) an extensive analysis of the gut microbiota while investigating a compositional imbalance (dysbiosis). Many teams worldwide are therefore interested in describing the gut ecosystem. Indeed, the existence of a persistent pathogen or dysbiosis may contribute to the chronic and inappropriate activation of the gut immune system. The study of gut microbiota has advanced in recent years due to the development of molecular descriptive methods applied to bacteria.

**Evidence involving gut microbiota in the pathogenesis of IBD**

There is increasing evidence that microbiota have a key role in the immunological homeostasis of the normal gut and in the pathogenesis of IBD.

**Clinical evidence**

The topography of the lesions is one of the first elements mentioned when implicating microbiota in the pathogenesis of IBD. The distal ileus and colon are the segments with the highest concentrations of bacteria (10^{11} to 10^{12} bacteria/g of material) [1]. Several studies have shown that the concentration of bacteria associated with the mucosa is significantly higher in patients with IBD than in control subjects [2]. In the 1980’s, the team at Oxford observed that diverting the luminal flux enabled healing of underlying lesions in patients with CD. More precisely, Harper et al. showed that elements greater than 0.2 microns in the chyme could exacerbate the inflammation of CD [3]. In patients with CD who were surgically treated with an ileostomy and colostomy, reintroduction of the effluent from the small intestine into the colon induced colonic inflammation. Conversely, the ultrafiltrate of this chyme, which did not contain bacteria, did not have this pro-inflammatory effect. Similarly, there is a strong suspicion that the microbiota plays a role in the recurrence of CD after ileal resection. P. Rutgeerts et al. showed that after terminal ileal resection for CD, recurrence occurred in 70% of patients within six months following restoration of gastrointestinal continuity and was not observed in patients that had an ileostomy [4].

**Experimental evidence**

The role of bacteria in the pathogenesis of IBD is also supported by work done in germ-free animals. In different models, the presence of microbiota is necessary for triggering any type of colitis. This was observed both in models of spontaneous colitis (IL-10 or IL-2 knockout mice) [1] and in those of chemically-induced colitis [5]. Experiments that sought to colonize germ-free mice with one bacterial species (thus creating monoxenic mice) showed that all the bacteria did not have the same pro-inflammatory effects and could also influence the phenotype of the enterocolitis. In IL-10 knockout mice for example, *Escherichia coli* induces proximal colitis, whereas *Enterococcus faecalis* induces distal colitis [6]. A recent study showed that microbiota selected by the immune state of TRUC mice (T-bet -/- x Rag2 -/- ulcerative colitis) could transmit colic inflammation to wild-type mice [7]. This vertical and horizontal transmission gave rise to a new paradigm, ‘colitogenic microbiota’. 
Genetic evidence

The human genome study and its polymorphisms (SNPs) demonstrated associations between IBD and some genes mainly involved in recognition and/or antibacterial response. The most clearly established genetic association is that of the Nod2 gene with ileal CD [8]. Nod2 is an intracellular sensor of bacterial peptidoglycan and can be activated by a minimally bioactive compound, muramyl dipeptide (MDP), which is present both in Gram-positive and Gram-negative bacteria and which activates the NF-kB and MAP kinase pathways [9]. A meta-analysis of 39 studies demonstrated an odds ratio of 2.4 (IC: 2.9-3.9) for simple heterozygotes, and 17.1 (IC: 10.7-27.2) for compound heterozygotes and homozygotes [10]. These functional polymorphisms endorse the already old hypothesis that CD results from an inadequate immune response to the gut microbiota in a genetically predisposed host. In a more recent study which tested over 300,000 SNPs, one SNP of the IL-23R gene located on the 1p31 chromosome was associated with CD. The Arg381Gln allele conferred protection against iBd [11].

The effect of this polymorphism should be integrated in relation to recent data that showed the role of IL-23 in the activation and perpetuation of the inflammatory and antifungal response, especially in Th17 polarization. This pathway was clearly identified as a major factor in the pathogenesis of iBd. Finally, the ATG16L1 gene was strongly associated with Cd. The ATG16L1 polymorphism is involved in the transport and elimination of intracellular bacteria. In addition, it is remarkable that the NOD2 and ATG16L1 polymorphisms are associated with defensin expression deficit [13].

Defensin deficit

Several recent studies have demonstrated a malfunction of the antibacterial barrier, particularly a dysfunction of defensin in CD. Defensins are antibacterial peptides which are produced by the Paneth cells and play a major role in the innate immunity of the small intestine. Wehkamp et al. showed that the ileal expression of alpha defensins (HD-5 and HD-6) is decreased in patients with ileal CD, and even more so in patients that are carriers of a NOD2 mutation [13]. They also reported that the expression of beta defensins (HBD-2 and HBD-3) is slightly induced in patients with colic localized CD compared to patients with ulcerative colitis (UC), as if there were insufficient induction of clonic defensin synthesis in CD [14]. Nevertheless, a recent study called into question the hypothesis of a primary deficit of defensin in CD and reported that the deficit of alpha defensin in the mucosa was only found in the inflamed ileum and not in the healthy areas [15].

Serological evidence

Several publications have shown high immunoglobulin G concentrations in the digestive lumen, which are directed against the antigens of commensal bacteria in the gastrointestinal tract of patients with IBD [16]. In patients with UC, the presence of significantly high titers of antibodies directed against certain bacteria of the commensal microbiota (Bacteroides vulgatus) has also been described. The perinuclear antineutrophilic cytoplasmic antibodies (p-ANCA) recognize an epitope belonging to a colonic commensal bacteria (Bacteroides caccae and Escherichia coli), which are found in 60 to 90% of UC cases [17]. The presence of high levels of anti-Omp antibodies (external membrane protein of E. coli), anti-i2 (Pseudomonas aeruginosa antigen) and ASCA (Saccharomyces cerevisiae) in CD appears to be associated with particularly severe forms of the disease (stenosing, perforating or requiring intestinal surgery).

In conclusion, all of these observations support the key role of gut microbiota in IBD, as it carries the antigens which cause inflammation, and its compositional imbalance (dysbiosis) is highly suspected as an aggravating and/or causal factor of IBD.

Microorganism candidates and IBD

Several microorganism candidates have been studied with IBD; more specifically, two bacteria have to this date been the subject of intensive research concerning mainly CD.

Adherent-invasive E. coli (AIEC)

Some strains of Escherichia coli with special adhesive properties were isolated from ileal mucosa samples from patients with chronic active CD and from those with post-operative neontal ileum (with and without CD recurrence) [18]. These E. coli strains from ileal mucosa affected by CD did not have the virulent characteristics of other E. coli pathogens and were designated AIEC (adherent-invasive E. coli, reference strain LF82) due to their adhesive properties compared to Caco-2 cells. These strains were also able to survive and greatly multiply inside macrophages without inducing cellular death, with the infected macrophages secreting high levels of TNF-alpha. The prevalence of AIEC in the ileal lesions rose to 36.4% in patients with CD versus only 6.2% in the controls [19]. In addition, AIEC bacteria potentiate the colonic inflammation induced by the DSS (murine model) and increase the expression of TLR5 and IPAF, innate immune receptors which recognize flagellin [20]. Recent results show that the AIEC reference strain LF82 is able to adhere to the brush border of ileal enterocytes isolated from patients with CD; this follows the recognition of the CEACAM6 host receptor by the type 1 pili variants as expressed by the AIEC bacteria [21]. The CEACAM6 protein is a glycosyl phosphatidylinositol (GPI)-anchoring antigen which belongs to the immunoglobulin superfamily. This protein is normally expressed in the colon, lungs and spleen. The CEACAM6 receptor is overexpressed in the ileum in about 35% of patients with CD, which could explain the high prevalence of AIEC strains associated with the ileal mucosa in the patients [19, 21]. In addition, an inflammatory state and/or infection by the AIEC LF82 strain induces an increased expression of the CEACAM6 receptor in the cultured Caco-2 intestinal epithelial cells. These results suggest that in predisposed patients with an abnormal expression of the CEACAM6 receptor in the ileum, the AIEC bacteria would be able to promote and/or potentiate their own...
colonization of the intestinal epithelium. After colonizing the ileal mucosa, the AiEC bacteria could cross the intestinal barrier, be phagocytized and then multiply in the resident macrophages of the lamina propria by inducing a high secretion of TNF-alpha proinflammatory cytokine, which could result in the increased expression of the CEACAM6 receptor. There is therefore a colonization/inflammation sequence in patients with CD since the AiEC bacteria, as well as the TNF-alpha and IFN-gamma proinflammatory cytokines, induce an overexpression of the CEACAM6 receptor in the ileal mucosa.

**Mycobacterium avium paratuberculosis**

*Mycobacterium avium paratuberculosis* (MAP) is also suspected of playing a role in the pathogenesis of CD. MAP has been isolated in several types of samples, usually from patients with CD. These have been obtained from intestinal biopsies in culture and by PCR [22], within granulomas [23], and most recently in peripheral blood using specific culture methods [24]. Several therapeutic trials have tested the efficacy of antimycobacterial treatments for CD, but most of them are questionable due to the inappropriate use of antibiotics (use of ethambutol or isoniazid, which are not effective with complex MAP infections; use of single antibiotic therapy rather than combination) or concomitant use of corticosteroids. A recent Australian study partially avoided this bias [25]. This large randomized, double-blind, placebo-controlled study evaluated the efficacy of a combination antibiotic treatment over two years (including clarithromycin, rifabutin and clofazimine) in the maintenance of clinical remission after corticosteroid weaning. The proportion of patients experiencing relapse at one, two and three years was not significantly different between the two groups. At one year, however, the authors observed a trend of improvement in the antibiotic group (39% relapse vs. 56% in the placebo group, p=0.054). The authors interpreted this effect as being a non-specific effect of antibiotics on the gut microbiota. They concluded that their results did not support the role of MAP in CD. Nevertheless, this conclusion is debatable since the patients’ MAP status before inclusion in this trial was unknown. The existence of a subgroup of patients with a Crohn’s disease ‘phenotype’ induced by MAP has not been ruled out to date. As a result, it is possible that the benefit of anti-MAP treatment only concerns a subgroup of patient carriers of this mycobacterium.

**Dysbiosis and IBD**

The gut microbiota was studied until recently by using techniques based on bacterial culture, and as 70% of this microbiota was cultivable, it remained unexplored [26]. Today molecular tools independent of culture have provided an overall assessment of the diversity of gut microbiota. In order to highlight alterations in the composition of gut microbiota from patients with IBD, researchers compare the results to those of healthy subjects or those between the active (flare-up) and inactive phase (remission) of IBD. The sampling of the microbiota (fecal or mucosa-associated) is very important to the organization of a study. Although the study of fecal microflora may seem less relevant than that of mucosa-associated microbiota (MAM), it is still informative. Colon biopsies which are required for the analysis of MAM composition are usually obtained during a coloscopy, and the colonic preparation can distort the microbiota composition. In addition to difficulties related to obtaining the necessary material for exploring this compartment of gastrointestinal microbiota, the sampling methods also have their intrinsic difficulties. The study of fecal microbiota is therefore still relevant today. We provide a summary below of the current data by separating the diseases (CD, UC) and the explored compartments (fecal microbiota and MAM). These results are presented schematically in Tables 1, 2 and 3.

These results show an overall quantitative and qualitative (biodiversity) reduction of the Firmicutes phylum in patients with CD, especially from the *C. leptum* group, in the fecal microbiota of these patients. This phylogenetic group includes several butyrate-producing bacteria, notably *Faecalibacterium prausnitzii*, which is one of the main members of the *C. leptum* group [26]. Other bacteria that were considered ‘beneficial’ for the host have been shown to be quantitatively reduced in the fecal microbiota of patients with CD. Some studies using culture methods [27], in addition to recent molecular methods [28], have demonstrated a reduction of *Lactobacillus* and *Bifidobacteria* in the fecal microbiota of patients with CD, although others have not yet confirmed these results [29, 30]. An increased concentration of *Enterobacteria* was described in the fecal flora of patients with CD [27, 28]. The data concerning the Bacteroidetes phylum are more discordant. Some studies have reported a reduction in the *Bacteroides* group [28] or the *Bacteroides fragilis* sub-group [29], although other studies have reported an increase in the *Bacteroides fragilis* sub-group, especially the *B. vulgatus* species [31]. There appears to be a reduction in the relative biodiversity of the *Bacteroides fragilis* sub-group [29, 32], while this Bacteroidetes phylum seems to be preserved. There are still fairly few studies on the luminal gut microbiota of patients with UC. As with CD, they strongly suggest restricted biodiversity [33] and an increased proportion of unusual bacteria [30].

MAM is usually studied on biopsies taken during a coloscopy, but some studies also use operative samples. There seems to be a consensus among different authors using molecular methods independent of culture affirming that the MAM of patients with CD includes a higher bacterial concentration compared to healthy subjects [2]. This also concerns the anaerobes, as well as the facultative anaerobes, and could be related to a deficiency of the intestinal barrier. One hypothesis would be that a relative lack of defensins could enable intestinal bacteria to invade the mucosa, as observed in some studies [34], or to colonize the intestinal crypts [34]. The side-by-side coexistence of normal mucosa areas and ulcerated mucosa areas in Crohn’s disease led some authors to investigate a localized dysbiosis which could promote CD lesions. Several independent studies [35] did not demonstrate a significant difference between MAM in the normal and inflamed areas of patients with CD. Similar to the results from fecal microbiota studies, a significant decrease of bacteria from the phylum Firmicutes was demonstrated in the MAM of patients with CD. Two independent studies found a lower number of sequences of the

Gut microbiota and IBD
**Table 1** Characteristics of fecal microbiota and mucosa-associated microbiota in CD

<table>
<thead>
<tr>
<th>Fecal microbiota</th>
<th>Mucosa-associated microbiota</th>
<th>§</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbiota unstable over time</td>
<td>Increase in total concentration of bacteria</td>
<td>[28, 29]</td>
<td>[2]</td>
</tr>
<tr>
<td>Presence of unusual bacteria</td>
<td>Increase in total concentration of anaerobes and facultative anaerobes</td>
<td>[28, 30, 31]</td>
<td>[2, 35]</td>
</tr>
<tr>
<td>Bacterial invasion in the mucosa and presence of bacteria in the crypts</td>
<td></td>
<td>[34]</td>
<td></td>
</tr>
<tr>
<td>No difference between ileocolic MAM (dominant) in damaged and healthy areas</td>
<td></td>
<td>[35, 37, 43]</td>
<td></td>
</tr>
<tr>
<td>Overall reduction in biodiversity</td>
<td>Reduced concentration of Faecalibacterium prausnitzii</td>
<td>[36, 37]</td>
<td></td>
</tr>
<tr>
<td>Reduction in concentration of Bifidobacteria and Lactobacilli</td>
<td></td>
<td>[27, 28]</td>
<td></td>
</tr>
<tr>
<td>Increased concentration of Enterobacteria</td>
<td>AIEC associated with ileal CD</td>
<td>[27, 28]</td>
<td>[2, 37, 38]</td>
</tr>
<tr>
<td>Enteroadherent E. coli associated with CD</td>
<td>Increased concentration of E. coli pathogens of groups B2+ D</td>
<td>[45]</td>
<td>[18, 19]</td>
</tr>
<tr>
<td>Contradictory data on Bacteroidetes</td>
<td>Frequent detection of MAP</td>
<td>[29, 31]</td>
<td>[2, 34-36]</td>
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</tbody>
</table>

§: References concerning fecal microbiota  #: References concerning mucosa-associated microbiota

**Table 2** Characteristics of fecal microbiota and mucosa-associated microbiota in UC

<table>
<thead>
<tr>
<th>Fecal microbiota</th>
<th>Mucosa-associated microbiota</th>
<th>§</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of unusual bacteria</td>
<td>Increase in total concentration of bacteria</td>
<td>[30]</td>
<td>[2, 34]</td>
</tr>
<tr>
<td>Increase in total concentration of anaerobes</td>
<td>Increase in total concentration of anaerobes</td>
<td>[2]</td>
<td></td>
</tr>
<tr>
<td>Bacterial invasion in the mucosa and presence of bacteria in the crypts</td>
<td></td>
<td>[34]</td>
<td></td>
</tr>
<tr>
<td>No difference between colic MAM (dominant) in damaged and healthy areas</td>
<td></td>
<td>[39, 40]</td>
<td></td>
</tr>
<tr>
<td>Difference between colonic MAM (sub-dominant) in damaged and healthy areas</td>
<td></td>
<td>[40]</td>
<td></td>
</tr>
<tr>
<td>Overall reduction in biodiversity</td>
<td>Reduced concentration of Bifidobacteria</td>
<td>[30]</td>
<td>[34]</td>
</tr>
<tr>
<td>Reduction of Lactobacilli in active UC</td>
<td>Reduction of Bifidobacteria</td>
<td>[46]</td>
<td>[41]</td>
</tr>
<tr>
<td>Increase of Enterobacteria</td>
<td>Increase of Enterobacteria</td>
<td>[33]</td>
<td>[2, 38]</td>
</tr>
<tr>
<td>Enteroadherent E. coli associated with UC</td>
<td>Increased concentration of E. coli pathogens of groups B2+ D</td>
<td>[45]</td>
<td>[38]</td>
</tr>
<tr>
<td>Increased concentration of ‘active’ E. coli</td>
<td></td>
<td>[33]</td>
<td></td>
</tr>
<tr>
<td>Increased concentration of SRB</td>
<td>Contradictory data on Bacteroidetes</td>
<td>[47]</td>
<td>[2, 34, 36]</td>
</tr>
</tbody>
</table>

§: References concerning fecal microbiota  # References concerning mucosa-associated microbiota

SRB: sulfate-reducing bacteria

bacterial phylum Firmicutes [35, 36] in the ileocolic MAM of patients with CD, especially from the Lachnospiraceae family [36]. Within this phylum, *Fecalibacterium prausnitzii* seems to be particularly lacking in CD. Martinez-Medina et al. compared the ileocolic MAM of 19 patients with moderately active CD and 15 healthy subjects [37]. The analysis showed that *F. prausnitzii* was present in 13 of the 15 healthy subjects (87%), while its prevalence was only 53%
in the patients with CD (p= 0.035). In a recent study using molecular inventory, Frank et al. showed that F. prausnitzii was one of the most underrepresented species in the MAM of patients with iBd (compared to healthy subjects) [36]. In terms of Enterobacteriaceae family, the results are equally convincing. Several studies showed an increased concentration in the MAM of patients with Cd, whether using culture [2] or molecular methods [37, 38]. As with Cd, the MAM of patients with uC included an abnormally elevated overall concentration of bacteria [2, 34], especially anaerobes [2]. A restriction of MAM biodiversity similar to that observed in patients with CD has been found such as reduction of Firmicutes and overrepresentation of Enterobacteriaceae [2, 34, 36, 39-41].

**Conclusion**

There is converging evidence that places the role of gut microbiota at the center of iBd pathology. The study of this complex ecosystem has only been possible since the advent of molecular tools which have been able to overcome culture constraints. The data on gut microbiota has grown over recent years and knowledge of this entity, which is considered a hidden organ, is increasing exponentially. Two strategies, being that of the non-oriented description of the gut microbiota, and that of a candidate microorganism, have led to recent advances in the formulation of new pathophysiological hypotheses. This microbiota has direct and indirect interaction with the epithelial cells, the secreted components of the intestinal barrier (mucus, IgA, antimicrobial peptides, lysozymes) and the mucus immunity system (dendritic cells, lymphocytes). The most studied candidate microorganism is currently AiEC, which possesses characteristics that suggest a relevant colonization-inflammation ‘sequence’in CD. In addition, dysbiosis is an important element since it provides a therapeutic approach which aims to restore the normal microbial balance. Such an approach has been the subject of recent developments in CD based on the most concordant data, pointing to a deficit of Firmicutes (Faecalibacterium prausnitzii ) [42]. There is no doubt that in the coming years, dysbiosis of the microbiota functions will be the subject of intense research (transcriptomics, proteomics, bacterial metabolomics) and will open the way to new therapeutic possibilities.
Conflicts of interests

None.

References


