Fecal Microbiota in Early Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To compare the composition of intestinal microbiota of patients with early rheumatoid arthritis (RA) or fibromyalgia (FM), fecal samples were collected from 51 patients with RA and 50 with FM.

Methods. RA patients fulfilled the RA criteria of the American College of Rheumatology, and duration of their disease was ≤ 6 months. Only nonhospitalized patients from outpatient care were included. Patients having extreme diets or previous disease modifying antirheumatic drug or glucocorticoid medication were excluded, as were those taking antibiotics or having gastroenteritis for at least 2 months prior to sampling. Fecal bacterial composition was analyzed with a method based on flow cytometry, 16S rRNA hybridization, and DNA-staining. A set of 8 oligonucleotide probes was used.

Results. In comparison to patients with FM, the RA patients had significantly less bifidobacteria and bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *Bacteroides fragilis* subgroup, and *Eubacterium rectale – Clostridium coccoides* group. Results from the 8 probes showed a significant overall difference between the 2 patient groups, indicating widespread microbial differences.

Conclusion. These findings support the hypothesis that intestinal microbes participate in the etiopathogenesis of RA. (First Release June 1 2008; J Rheumatol 2008;35:1500–5)

Key Indexing Terms:
MICROBIOTA RHEUMATOID ARTHRITIS FIBROMYALGIA
CYTOMETRY 16S rRNA HYBRIDIZATION

In recent years the treatment of rheumatoid arthritis (RA) has developed significantly. With a combination of disease modifying antirheumatic drugs (DMARD) used in the early phase of disease, and with new immunomodulatory antirheumatic drugs, the prognosis has evidently improved[1–3]. Despite the advances in treatment, RA is still one of the most disabling musculoskeletal diseases. Most important, its etiopathogenesis remains unknown, and even the new treatments do not act on the fundamental causative factors of the disease.

Factors causing inflammatory processes and leading to clinical symptoms of RA are unknown. It has been accepted that both genetic and environmental predisposing factors are involved in the etiopathogenesis[4-6]. The most important genes that influence the susceptibility and course of the disease are located within the human major histocompatibility complex (MHC) or the human leukocyte antigen (HLA) region. While the genotype is an important factor affecting the risk for RA, disease heritability is estimated to be 50%–60%, of which HLA genes account for less than half[5]. Among the potential pathogenic environmental factors, microbes and particularly the gastrointestinal (GI) bacteria have been suggested to play an essential role in the etiopathogenesis of RA[6-9].

Intestinal microbiota comprise a complex ecosystem with an exceptionally high bacterial density and diversity. The bacteria living in the human GI tract achieve the highest cell densities recorded for any ecosystem: the adult alimentary tract contains 1–2 kg of microbial cells of hundreds of bacterial species, of which over 80% have not been cultured[10-12]. Despite the vast microbial burden and the close contact between the microbes and the host, commensal intestinal microbiota are considered to be beneficial. Intestinal microbes are known to interact actively with the mucosa-associated immune cells and to promote immunologic maturation[13,14]. The nonpathogenic normal microbiota also protect the gut from the colonization of harmful microbes[13].

Several observations highlight the possible role of the normal intestinal microbiota in the etiopathogenesis of RA. In healthy adults, fragments of normal intestinal bacteria are found in circulating blood leukocytes and in the spleen. In patients with inflammatory arthritis, similar degradation

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products of bacterial cell walls and bacterial nucleic acids are detected in the inflamed joints[15-18]. In animal models, parenterally injected cell-wall fragments of several intestinal bacteria have been shown to be arthritogenic. The arthritogenicity of the bacterial structures depends on the bacterial species, and it is notable that even bacteria of normal intestinal microbiota cause experimental arthritis in animals[19-21]. Further support for the role of intestinal bacteria comes from a study showing that patients with early-phase RA had intestinal microbiota significantly different from those of control patients[22]. Interestingly, modulation of the RA patients' microbiota with a vegetarian diet alleviates the symptoms of the disease, and the most significant changes in the microbiota have been displayed by patients showing the greatest clinical improvement[23-25].

These observations led to the hypothesis that bacterial antigenic material from the GI tract leaks to the blood circulation, and in RA patients ends up in joints, causing prolonged inflammation. Differences in the composition of intestinal microbiota and in the functions of the immune system would determine which patients get RA. Of note, the host's genotype appears to affect the composition of the microbiota[26-29]. MHC genes responsible for modulation of the immune system and affecting the risk for RA seem also to guide the composition of intestinal microbiota, and consequently both the microbial antigen exposure from the gut and the immunological responses against microbial antigens could be genetically modulated. Individuals with a certain genotype could harbor microbiota consisting of arthritogenic bacteria, and the seepage of bacterial structures from intestines to circulation and further to joints would lead to chronic articular inflammation observed in RA[8,9].

The analytic method used in previous studies of the composition of intestinal microbiota in RA, gas-liquid chromatography of the bacterial structural fatty acids, has not enabled quantitative and bacterial group- or genera-specific analyses[22-24]. Our objective was to verify earlier observations of alterations in RA patients' intestinal microbiota with a genome-based quantitative analysis, and to determine what bacterial groups or genera are responsible for the potential changes. A method based on flow cytometry, 16S rRNA hybridization, and DNA-staining was used to analyze the compositions of fecal microbiota of nonhospitalized patients with early-phase RA and control patients with fibromyalgia (FM).

MATERIALS AND METHODS

Patients. Fifty-one consecutive patients with early RA and 50 consecutive patients with FM were included in the study. Inclusion criteria for patients were: no previous DMARD medication, no glucocorticoids, antibiotics, or gastroenteritis for at least 2 months prior to sampling, and the duration of disease for RA patients of not more than 6 months. Nonsteroidal antiinflammatory drugs (NSAID) were allowed. All RA patients fulfilled the RA criteria of the American College of Rheumatology[30]. Mean duration of RA was 3.8 (SD ± 1.7) months and the average number of swollen joints at the beginning of the disease was 8.0 (SD ± 4.9). Only nonhospitalized patients from outpatient care were included, and patients with extreme diets such as vegetarians were excluded. Similarly, patients working in institutional healthcare units or in seniors' homes were excluded. Laboratory measures and other characteristics, use of NSAID, for example, and cigarette smoking, a known risk factor for RA, are shown in Table 1[31,32].

The study was approved by the ethical committee of Satakunta Central Hospital. The study was explained to all patients prior to enrollment, and their oral consent was obtained.

Analysis of fecal samples. Fecal samples were collected at a specialist's practice and stored immediately at 4°C. Within 1 h, the sample was suspended in 5 ml of phosphate buffered saline (PBS). The suspension was vortexed, incubated at 4°C for 2 h, and centrifuged at 80 g for 1 min to remove debris as a pellet. One volume of the supernatant was transferred into 3 volumes of fresh 4% paraformaldehyde and fixed at 4°C overnight. After fixation, the bacteria were centrifuged at 22,000 g for 3 min and washed with PBS. The centrifuging and washing were repeated 3 times. The bacteria were stored in 50% ethanol-PBS at –20°C until hybridized.

The fixed bacteria were hybridized overnight in hybridization buffer [0.9 M sodium chloride, 20 mM Tris/HCl (pH 7.2), 0.1% sodium dodecyl sulfate] containing the probe (2.5–5.0 ng/µl). Eight 16S rRNA-targeted oligonucleotide probes labeled at the 5′-end with Cy5 indocarbocyanin (Ex/Em 646/662 nm; Molecular Probes, Eugene, OR, USA) were used (Table 2). Probes Bif662 and Bif164 are partially complementary in their target bacteria, Bif662 covering more *Bifidobacterium* species. Similarly, Clep866 and Fprau645 probes have partially the same target species, Clep866 having the wider target spectrum, and *Bacteroides fragilis* subgroup hybridized with Bfra602 probe is nested in *Bacteroides-Porphyromonas-Prevotella* group hybridized with Bacto1080.

Flow cytometry. Flow cytometry of fecal bacteria was performed as described[39,40]. Briefly, 30 µl of hybridized bacteria was added to 4 ml PBS, vortexed, and sonicated. After sonication, 0.5 µl of 5 mM Sytox® DNA stain (Molecular Probes) was added per 100 µl of the sample. Samples were incubated in the dark at room temperature for 5 min and analyzed with a BD FACSCalibur® flow cytometer (Becton Dickinson, San Jose, CA, USA). Commercial FACSFlow (Becton Dickinson) was used as sheath fluid. The flow cytometry parameters were adjusted for bacterial counts. At least 20,000 bacteria per sample were counted. All samples were analyzed as triplicates. An example of flow cytometry analysis of fecal bacteria is shown in Figure 1. Data were analyzed using CellQuest™ software (Becton Dickinson).

Table 1. Clinical characteristics of patients.

	RA	FM	p
Patients (male)	51 (9)	50 (5)	
Mean age, yrs ± SD	56.7 ± 12.8	50.5 ± 12.6	0.016
RF-positive	41	0	
CCP antibody-positive	30	0	
Mean ESR, mm/h ± SD	21.2 ± 15.2	9.8 ± 8.3	< 0.001
Mean CRP, mg/l ± SD	9.1 ± 9.6	3.7 ± 5.9	0.001
Mean hemoglobin, g/l ± SD	132.5 ± 9.2	136.2 ± 10.0	0.058
NSAID medication			
None	10	23	
COXIB	14	4	
Non-COXIB	27	23	
Amitriptyline medication	0	5	
Cigarette smokers	14	17	

CCP antibody was determined from 50 RA patients and 48 FM patients. RF: rheumatoid factor (isotype IgM); CCP: anti-cyclic citrullinated peptide antibody; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; NSAID: nonsteroidal antiinflammatory drug. COXIB: celecoxib, etoricoxib, rofecoxib, or valdecoxib. Non-COXIB: ibuprofen, ketoprofen, naproxen, aceclofenac, diclofenac, or meloxicam.

Table 2. Oligonucleotide probes used in the study.

Phylogenetic Target	Probe	Sequence (5′–3′)	Reference
Atopobium cluster	Ato291	GGT CGG TCT CTC AAC CC	33
Bacteroides-Porphyromonas-Prevotella group	Bacto1080	GCA CTT AAG CCG ACA CCT	34
Bacteroides fragilis subgroup	Bfra602	GAG CCG CAA ACT TTC ACA A	35
Genus *Bifidobacterium*	Bifl64	CAT CCG GCA TTA CCA CCC	36
Genus *Bifidobacterium*	Bif662	CCA CCG TTA CAC CGG GAA	36
Clostridium leptum subgroup	Clep866	GGT GGA T(A/T) ACT TAT TGT G	37
Eubacterium rectale - Clostridium coccoides group	Erec482	GCT TCT TAG TCA(A/G) GTA CCG	35
Faecalibacterium prausnitzii group	Fprau645	CCT CTG CAC TAC TCA AGA AAA AC	38

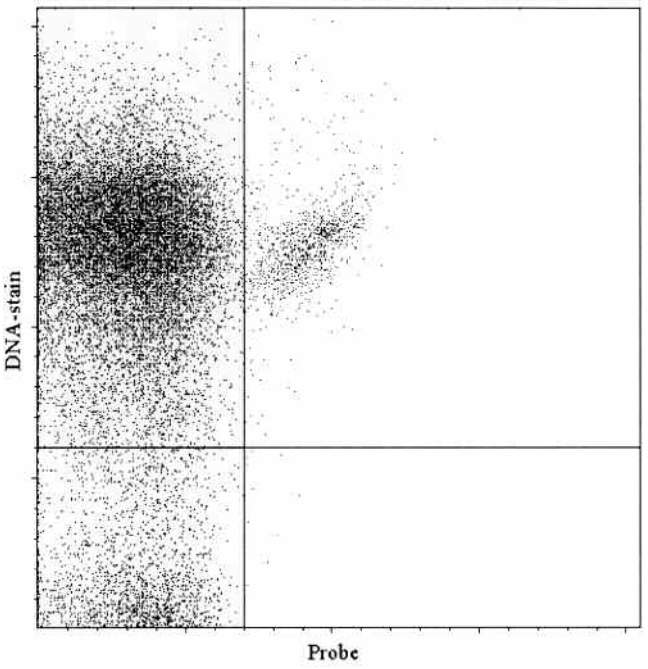

Figure 1. A flow cytometry analysis of hybridized and DNA-stained fecal bacteria. X-axis: fluorescence of the probe hybridizing target bacteria; Y-axis: fluorescence of DNA stain. Upper right quadrant: hybridized target bacteria; upper left quadrant: unhybridized bacteria. Lower left quadrant: DNA stain-negative and probe-negative particles; lower right quadrant: DNA stain-negative, probe-positive particles (i.e., false-positives). The bacteria are separated from the non-DNA-stained material, and the hybridized target bacteria are discriminated from other bacteria in the sample.

Statistical analysis. Statistical comparisons between the clinical characteristics (Table 1) and between the hybridization percentages (Table 3) in the RA and FM groups were by Student's t test. To analyze difference between patient groups in the hybridization percentages of all 8 oligonucleotide probes, Wilks' likelihood ratio statistic using 8 responses was used. The result was calculated with the R package. In all analyses, a p value ≤ 0.05 was considered to denote a significant difference.

RESULTS

Figure 1 shows an example of the flow cytometry analysis of the fecal sample. The bacteria are separated from the non-DNA-stained material, and the hybridized target bacteria are discriminated from other bacteria in the sample.

Table 3. Hybridization percentages in RA and FM patients (mean ± SD).

Probe	Hybridization Percentage RA	FM	p
Ato291	3.4 ± 4.0	3.2 ± 2.7	0.710
Bacto1080	6.9 ± 5.1	9.8 ± 7.2	0.021
Bfra602	4.4 ± 3.7	6.2 ± 5.2	0.044
Bifl64	3.8 ± 2.9	4.9 ± 5.4	0.232
Bif662	7.5 ± 4.0	10.0 ± 6.5	0.025
Clep866	14.8 ± 6.0	15.9 ± 6.4	0.363
Erec482	10.4 ± 6.6	13.7 ± 8.3	0.026
Fprau645	7.5 ± 3.6	7.8 ± 4.0	0.703
Probe additivity	58.7	71.5	

Altogether, the results revealed significant differences between fecal microbiota of the RA and FM patients (Table 3). Four of 8 oligonucleotide probes indicated proportionally less bacteria in RA than in FM. The RA samples contained less bifidobacteria than the FM samples. With the probe Bif662 hybridizing more comprehensively bacteria of genus *Bifidobacterium*, the percentages were 7.5% for RA patients and 10.0% for FM patients ($p = 0.025$). Correspondingly, with the Bif164 probe the hybridization percentages were 3.8% and 4.9%, respectively ($p = 0.232$), which was to be expected, considering the smaller coverage of this probe. RA patients also had significantly less bacteria of the *Bacteroides-Porphyromonas-Prevotella* group (6.9% and 9.8%; $p = 0.021$) and the *B. fragilis* subgroup (4.4% and 6.2%; $p = 0.044$). A significant difference was also observed in the *Eubacterium rectale – Clostridium coccoides* group (10.4% and 13.7%; $p = 0.026$). Of the bacterial groups studied, *C. leptum* subgroup bacteria were the most common in both patient groups, and the bacteria of *E. rectale – C. coccoides* group were the second most common. Comparison of the RA and FM groups using summarized results of all 8 probes also yielded a significant difference between the groups ($p = 0.039$).

DISCUSSION

Our study demonstrates that the fecal microbiota of patients with RA contain significantly less bifidobacteria and bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *B.*

fragilis subgroup, and the *E. rectale – C. coccoides* group than the fecal microbiota of patients with FM. These bacterial species are known to belong to the most common genera and groups in the human fecal microbiota[36,41]. Four of the 8 oligonucleotide probes indicated statistically significant differences between the groups. In addition, the combined results with the 8 probes showed a significant overall difference between the patient groups.

Regarding the potential role of the intestinal microbiota in the etiopathogenesis of RA, at least 2 alternatives exist to explain these results. First, it is possible that RA patients lack or have proportionally less of certain bacteria required for normal physiology or to prevent nonphysiological functions. For the latter, decreased frequency of the intestinal symbiont *B. thetaiotaomicron* resulting in leaking intestinal barrier would serve as an example[42]. As a second alternative, RA patients may have a more diverse microbiota containing bacteria that cannot be detected by the current set of oligonucleotide probes. That the probe additivity was nearly 13% less in the RA group could be an indication of this. For discussion of this second alternative, 2 facts must be taken into account: the limited scope of the probes and the enormous diversity of human GI microbiota. The probes we used cover about half of human intestinal bacteria. Further, of the estimated 800 bacterial species and over 7000 strains present in the human intestinal tract, roughly half are still unidentified[11,43]. Therefore, it is probable that the differences observed between the RA and FM patients extend to such bacteria that could not be detected by the probes used here. These findings leave open the possibility that individuals with genetic susceptibility for RA are determined to specifically harbor intestinal microbiota containing arthritogenic bacterial species or strains and leading to aberrant immune responses and chronic inflammation[9,26-29].

Seksik, *et al* have reported results parallel to ours in Crohn's disease (CD)[44]. In their study, counts for the *Bacteroides-Porphyromonas-Prevotella* bacteria were significantly lower in patients with CD than in healthy controls, and the overall proportion of the bacteria detected with a set of 6 oligonucleotide probes was lower in CD patients. About 30% of the dominant bacteria in the CD samples did not belong to the usual dominant phylogenetic groups, and species diversity remained high[44]. Further evidence for microbial changes in CD and ulcerative colitis (UC) was presented by Sokol, *et al*[45]. The proportions of bacteria detected with 6 oligonucleotide probes were lower in CD and UC patients, and the microbiota of the CD and UC patients were suggested to harbor uncharacterized discrepancies compared to healthy subjects[45]. All these findings indicate an aberration in the microbiota of the patients having microbe-associated autoimmune disease such as RA and inflammatory bowel disease.

Our observations are based on analysis of fecal samples. However, feces contain bacterial colonies from both the lumen and the mucosa of the upper GI tract, and consequently the differences in fecal microbiota can indicate differences in the microbiota in the upper gut regions[11]. Our results from patients with RA represent the state of intestinal microbiota before the start of intensive and potentially microbiota-modulating DMARD treatments. FM patients were chosen as controls because FM is a noninflammatory condition, while both RA and FM patients usually receive NSAID medication, and their age and sex distributions are similar[4,46]. All fecal samples were collected at the same specialist's practice and individuals of both patient groups were living in the same geographic region. Other variations between the patient groups were also minimized. For example, the smoking habits of RA and control patients were remarkably similar and only 5 FM patients used amitriptyline, a medication commonly used in FM but not in RA, with a very low dose of 10 mg/day. FM is not known to be a risk factor for RA and the control patients' risk to harbor RA can be estimated to be at the same level as in healthy individuals[46]. No participating FM patient was rheumatoid factor- or anti-cyclic citrullinated peptide antibody-positive. Although the RA patients' mean age was somewhat higher than that in the control group, and the age of the individual may affect intestinal microbiota, we believe it unlikely that this difference could explain the results[47,48]. Microbiota are known to evolve rapidly after birth and in infancy due to the radical changes in diet[12]. However, in adults the composition of the microbiota has been considered to remain roughly the same, and from the microbe ecological point of view the age difference of 6.2 years in our middle-aged patient groups is not remarkable. Similarly, while there were some differences in use of NSAID between the patient groups, this is not a probable cause of the microbial findings[49]. It is notable that if healthy individuals had been used as controls, a similar high-quality sampling protocol would have been impossible to arrange and the differences in the use of NSAID would have been even more pronounced.

Our findings support the hypothesis that intestinal microbes may participate in the etiopathogenesis of RA. Host genotype would guide both the composition of intestinal microbiota and immune responses against microbes, and in individuals susceptible for RA the arthritogenic bacterial antigens would pass from intestines to the joints, causing prolonged immunological response and articular inflammation. To elucidate the role of intestinal microbiota in the etiopathogenesis of RA and other autoimmune diseases, further studies with healthy control individuals having disease-associated genotypes are needed. They should undertake longterm monitoring of intestinal microbiota, immunological variables, lifestyle factors known to predispose to RA, and the development of disease symptoms. Determining the relationships among all these etiopathogenetic factors would help us to understand the pathogenesis of these diseases and

to develop new treatments or preventive methods based on the modulation of intestinal microbiota.

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