Concise report

Distinct bacterial colonization patterns of *Escherichia coli* subtypes associate with rheumatoid factor status in early inflammatory arthritis

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Abstract

Objectives. The aetiology of RA is unknown; however, bacterial exposure, particularly to *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae*, has been linked to disease pathogenesis. The strongest association was observed for RF⁺ RA. We compare colonization patterns of these bacteria, and the anti-bacterial antibody levels in early onset RF⁺ and RF⁻ inflammatory arthritis.

Methods. Bacteria isolated from stool and urine of early-stage RF⁺ and RF⁻ patients recruited to the Early Arthritis Registry were biochemically identified and genotyped. IgM and IgA anti-bacterial and RF antibodies were assessed by ELISA.

Results. Differences in the types of colonizing pathogenic *E. coli* were identified. RF⁺ patients were more commonly colonized with phylogenetic Group D *E. coli*, whereas RF⁻ patients were more commonly colonized with phylogenetic Group B2 *E. coli* and these individuals also had lower joint scores and inflammatory markers yet higher IgA anti-*E. coli* antibody responses.

Conclusions. These studies link the type of colonizing bacteria in the gut and urine with the immune response (anti-bacterial and RF) in early-onset inflammatory arthritis and provide evidence for a role of the host–pathogen response in the aetiology of RF.

Key words: Bacterial colonization, Anti-bacterial antibodies, Rheumatoid arthritis, Rheumatoid factor, Early inflammation.

Introduction

The aetiology of RA is thought to be multi-factorial. Studies of monozygotic twins report 15% concordance for RA [1]. This low correlation suggests that environmental factors may play a major role in disease development. In 1968, Hill [2] proposed that bacterial infection may be associated with RA, since some infections can stimulate tissue destruction. In 1985, Ebringer et al. [3] identified *Proteus mirabilis* as possibly being involved in RA pathogenesis. Elevated serum anti-*P. mirabilis* antibodies were found in RA patients and they suggested that such antibodies might contribute to joint damage and inflammation. Research has been conducted to determine the mechanism by which this occurs [4, 5]. However, what remains debatable is whether exposure to these bacteria contributes to the development of RA. There have been reports of elevated levels of anti-*P. mirabilis* antibodies in RA patients in some studies [6–15], but not in others [16, 17]. The elevated levels when identified appear to be primarily in RA patients who are RF⁺ [15, 18]. RFs are autoantibodies that bind the Fc of immunoglobulin G (IgG). Production of RF requires two signals to the B cell. IgG binding as part of an immune–pathogen
To date, no study has simultaneously evaluated *P. mirabilis* or other Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumonia* colonization in either the gastrointestinal tract (GIT) or the urinary tract (UT) and the immune responses to these bacteria in RA patients at disease onset. Wilson *et al.* [21] reported an increased frequency of *P. mirabilis* isolation from female RA patient’s urine compared with healthy controls and non-RA patients. Senior *et al.* [10] examined RA patients without clinical signs of UT infection (UTI) and found 33% of RA patient’s urine contained *P. mirabilis*. These bacterial exposures are considered ‘non-significant’, *P. mirabilis* bacteriuria (<10⁴ cfu/ml urine), being insufficient to induce clinical symptoms.

The role of *E. coli*, a common intestinal microorganism and cause of most UTIs in the general population, in RA pathogenesis is unknown. Several studies show no elevation of antibodies against *E. coli* [7, 11, 13, 14, 21], even during the early stages of RA [11]. Newkirk *et al.* [15], however, showed a slight elevation of IgA anti-*E. coli* antibodies in RA patients compared with patients with undifferentiated arthropathy. *Klebsiella pneumonia* has been linked to SpA [22] with very few studies conducted investigating the link between *K. pneumonia* and RA. Increased IgA anti-*Klebsiella* antibodies have been found in patients with RA [23, 24].

This study evaluated bacterial colonization patterns in GIT and UT and the serum anti-bacterial antibody levels in patients with early-onset inflammatory arthritis stratified according to RF status. We hypothesized that exposure to specific types or strains of *P. mirabilis*, *E. coli* and/or *K. pneumonia* could play a role in early stage seropositive RA pathogenesis.

**Patients and methods**

**Patients**

Participants recruited to the McGill Early Arthritis Registry participated in this study [25]. The inclusion criteria were as previously described and included one or more joints inflamed >6 weeks, but <12 months. Approximately 50% met the ACR classification criteria for RA [26]. Patients were divided into groups based on RF status. Participants completed a short questionnaire about UTI history, recent infections, medications and exposures that could impact the intestinal flora. All subjects provided stool and mid-stream urine samples. Sera were available from the initial recruitment. Informed consent was obtained from all participants and this study was approved by the McGill University Faculty of Medicine Institutional Review Board (Subject #A00-M48-05B).

Biochemical identification of bacterial isolates from stool and urine

Urine and stool were cultured on commercially prepared plates containing a non-selective differentiated medium for the isolation, differentiation and enumeration of UT pathogens (Becton Dickinson, NJ, USA); indole, lysine and ornithine decarboxylase tests were also performed for bacterial identification. *Escherichia cloacae* (ATCC 13047) and *K. pneumonia* (ATCC 13883) were used as positive controls.

Genotype characterization of isolates via enterobacterial repeat intergenic consensus PCR

Bacterial isolates were evaluated by the enterobacterial repeat intergenic consenus (ERIC2 primer) PCR genotyping method as previously described [27]. The number of unique strains for each patient was recorded. These fingerprints were compared via product-moment Pearson’s correlation using GelCompar version 3.5 (Applied Maths, Sint-Martens-hatem, Belgium). PI 102 (CgA, ATCC BAA-457) served as the positive control.

Phylogenetic characterization of colonizing *E. coli*

*Escherichia coli* isolates were tested for the *E. coli* phylogenetic Groups A, B1, B2 and D using established PCR-based assays [28].

Measurement of anti-bacterial antibodies

Sera were assayed via ELISA for IgA and IgM antibodies against *P. mirabilis* (ATCC 4675), *E. coli* (ATCC 35218) and *K. pneumonia* (laboratory isolate) as previously described [15].

Measurement of IgM and IgA RF

Sera were assayed for IgM and IgA RF by ELISA as previously described [15] as well as by turbidity in the routine serological screen. There was good agreement whereby of 26 found negative by ELISA, 24 were negative by turbidity and of the positive RF sera by ELISA, 1 was negative for RF by turbidity.

Statistical analysis

Data analyses were conducted using GraphPad InStat version 3.00 (GraphPad Software, San Diego, CA, USA) and stata version 9.0 (StataCorp, College Station, TX, USA). RF⁺ RA and RF⁻ RA subjects were compared using Fisher’s exact test. For continuous measurement, the t-test was used to compare mean values between RF⁺ and RF⁻ groups.

**Results**

**Patient characteristics**

Forty subjects with inflammatory arthritis, 23 of whom were classified as RA by ACR criteria, were determined to be RF⁺ (*n* = 19) or RF⁻ (*n* = 21) according to ELISA results. Thirty-nine patients returned questionnaires. The study groups did not differ with respect to age, gender, education, income or marital status. The mean age (range) for the RF⁺ patients was 52 years (28–74 years) and for RF⁻ patients 56 years (30–87 years). Of the RF⁺ and RF⁻ patients, 68 and 71% were female, respectively.

No significant differences were found in DMARD or NSAID use between the groups. No patients were taking biological DMARDs. The mean tender and swollen joint
scores tended to be higher in the RF+ patient group compared with RF− [16.7 (8.4) and 10.9 (5.9), respectively] although the differences were not significant (P = 0.15 and 0.28, respectively). The mean ESR (Westergren method) was also higher in the RF+ patients than in the RF− (28.9 compared with 19.4; P = 0.09). Almost none of the patients had asymptomatic bacteriuria or experienced UTIs within 12 months prior to entering the study. However, 57% of RF− and 37% of RF+ patients had experienced one or more UTIs within their lifetimes.

*Escherichia coli* were the predominant bacteria isolated from the stool (~90%). *Klebsiella pneumonia* was isolated from the stool of 32 and 24% of RF+ and RF− patients, whereas *P. mirabilis* was found in the stool of one individual only. No significant differences in bacterial colonization patterns were found between RF+ and RF− patients in either the stool or urine specimens. According to ERIC2 PCR results, no particular strain was over-represented among the *E. coli, K. pneumoniae* or *P. mirabilis* isolates.

RF+ and RF− groups were colonized with similar proportions of phylogenetic Groups A and B1, which are associated with commensal *E. coli* (Table 1). RF+ patients were colonized with the Group D phylogenetic strains, whereas the RF− patients were more commonly colonized with B2 E. coli strains. Group D and B2 E. coli are typically associated with extraintestinal infections. Those with B2 colonizing *E. coli* had lower tender and swollen joints compared with those lacking B2 (Table 2) suggesting that a component from these bacteria might be anti-inflammatory. Similarly, there was a trend for lower ESR in the subgroup of patients with B2 *E. coli* present. When the patients were grouped according to the presence (n = 10) or absence (n = 25) of the D phylogenetic type of bacteria, no differences in any of these inflammatory markers were observed (data not shown). The IgA anti-*E. coli* responses were significantly elevated in the individuals colonized with B2 *E. coli* compared with those lacking B2 *E. coli* (Table 2). There was little difference in the IgM anti-*E. coli* responses in these two subgroups. The anti-*E. coli* antibody responses in the patient groups colonized with or without type D *E. coli* were not different (data not shown).

Although the mean serum IgM and IgA antibody levels that recognized *P. mirabilis, K. pneumoniae* or *E. coli* tended to be slightly higher in the RF+ group, the differences when compared with the RF− group were not significant (data not shown), except for IgM anti-*E. coli*, which was 0.666 (0.298) and 0.465 (0.269) for the RF+ and RF− groups, respectively (P = 0.025). It is not possible to distinguish between the *E. coli* phylogenetic lineages using serological assays. Circulating antibodies to *E. coli* reflect not only current strains that are present, but also past exposure to different colonizing strains.

Serological analyses revealed that the IgM RF response correlated with the IgM anti-*E. coli* response (r = 0.44; P < 0.01) as predicted from the analyses of the means, but there was no correlation between the IgM RF response and the other anti-bacterial responses, indicating that IgM RF was not causing false positives in these assays. The IgA RF response correlated with the

**Table 1** *Escherichia coli* phylogenetic lineage and RF status in patients with early arthritis

<table>
<thead>
<tr>
<th>Phylogenetic type/group</th>
<th>RF+ (n = 17, n (%)</th>
<th>RF− (n = 19, n (%))</th>
<th>Total (n = 36, n (%))</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commensal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>7 (41)</td>
<td>6 (32)</td>
<td>13 (36)</td>
<td>0.73</td>
</tr>
<tr>
<td>Group B1</td>
<td>2 (12)</td>
<td>3 (16)</td>
<td>5 (14)</td>
<td>1.00</td>
</tr>
<tr>
<td>Pathogenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B2</td>
<td>4 (24)</td>
<td>10 (53)</td>
<td>14 (39)</td>
<td>0.09</td>
</tr>
<tr>
<td>Group D</td>
<td>7 (41)</td>
<td>3 (16)</td>
<td>10 (28)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

†In some cases, multiple unique *E. coli* isolates were recovered from study subjects; therefore, the total for each phylogenetic group can exceed the number of study subjects. *Escherichia coli* was not recovered from four study subjects. *Fisher’s exact, two-sided P-values are reported for each phylogenetic group.

**Table 2** Lower inflammation scores observed in arthritis patients with the B2 phylogenetic type of *E. coli*

<table>
<thead>
<tr>
<th>B2 Phylogenetic type of <em>E. coli</em></th>
<th>Mean inflammation score (s.d.)</th>
<th>Mean OD levels (s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tender joints</td>
<td>Swollen joints</td>
</tr>
<tr>
<td>B2 present, n = 14</td>
<td>8.54 (7.29)‡</td>
<td>4.38 (3.57)‡</td>
</tr>
<tr>
<td>B2 absent, n = 28</td>
<td>15.5 (14.39)‡</td>
<td>8.41 (8.39)‡</td>
</tr>
<tr>
<td>P-value</td>
<td>0.06*</td>
<td>0.06*</td>
</tr>
</tbody>
</table>

‡n = 13, °n = 12, §n = 23, £n = 16, *Fisher’s exact, one-tailed P-values. **Mann-Whitney, two-tailed P-value reported.
IgA anti-bacteria responses with the correlations for anti-K. pneumoniae > anti-P. mirabilis > anti-E. coli (r-values were 0.69, 0.60 and 0.38, respectively), likely reflecting mucosal colonization. The linear correlations for the IgM anti-bacterial responses correlated strongly (all with an r > 0.9; P < 0.05) suggesting that there was recognition of a similar structural component. The IgA anti-bacterial responses also showed significant, but weaker correlations (r-values ranged from 0.57 to 0.86; P < 0.05). All three bacteria are Gram negative and do have some common components.

Discussion

This study evaluated comparable groups of early-stage inflammatory arthritis patients to determine whether or not colonization by E. coli, P. mirabilis or K. pneumoniae was associated with RF+ RA pathogenesis. Escherichia coli predominated in the intestine in both the RF+ and RF− patients. The genotypic profile of the bacterial strains was highly diverse. There was a significant difference in the phylogenetic types of E. coli identified, with the B2 type predominating in the RF− patients, whereas the D type predominated in the RF+ patients. The IgM RF response correlated with the IgM response to E. coli, whereas there was no correlation with IgM RF and the immune responses to the other bacteria. In contrast, the IgA RF response correlated with the IgA anti-K. pneumoniae and P. mirabilis better than it did with the IgA anti-E. coli response. Since the presence of the shared epitope of HLA-DR was not determined in this cohort, no comment can be made as to its influence on the RF and/or anti-bacterial antibody responses detected.

The phylogenetic studies of the E. coli isolates revealed surprising results. Bacteria from Groups B2 and D, which are more associated with pathology, were found to vary between the patient groups. The Group B2 isolates (53%) were predominantly found in RF− patients at similar levels to that previously reported by patients with UTI (54%) from a nearby hospital [29]. In contrast, in the RF+ patient group there was an over-representation of E. coli Group D (41%) vs B2 (24%). This contrasts with a study of IBD where 14% were found to be D positive, whereas 68% were B2 positive [30]. This level of D colonization in IBD is comparable with the RF− group (16%).

Groups B2 and D type E. coli produce virulence factors that can affect the immune response, and E. coli of the B2 group, such as CFT073, have recently been found to down-regulate signalling through TLR [31]. This is of interest, since TLR ligation is required for RF B-cell activation [19]. Thus, the presence of the B2 group of E. coli might suppress the RF response. Consistent with this were the lower inflammatory scores found in the patient groups with the B2 E. coli compared with those lacking them. An alternative explanation is that the RF− state is associated with less severe disease and is somehow permissive to B2 group E. coli colonization. More studies are needed to clarify this. It has been suggested that the TcpC protein from the B2 E. coli bacteria which is instrumental in the inhibition of MyD88 (and TLR signalling) might be a useful anti-inflammatory agent [32]. In the B2 E. coli colonized patients, it appeared that innate immunity was depressed, whereas their IgA responses to E. coli were significantly elevated suggesting that T-cell-dependent adaptive immune responses controlled these bacteria in these patients.

The correlations found between IgM RF and IgM anti-E. coli are consistent with a previous study of an early arthritis patient cohort. Similarly, the significant correlations observed between IgA RF responses and IgA anti-P. mirabilis antibodies, more so than with the anti-E. coli antibody response, are also consistent with that study [15]. However, the size of the study was limited and multiple statistical tests were performed, which increases the likelihood that some of the results may have been identified by chance.

The current study indicates that distinct bacterial colonization patterns and anti-bacterial antibody profiles can exist in inflammatory arthritis patients at disease onset in RF+ and RF− patients. It is also clear that no single unique strain of E. coli, P. mirabilis or K. pneumoniae was present. RFs were found to correlate with the anti-bacterial response. The IgM RF response was correlated with the IgM anti-E. coli antibody response and the IgA RF response correlated with the anti-P. mirabilis and K. pneumoniae responses. Unanswered questions remain concerning the role of different phylogenetic groups of pathogenic E. coli in the RF response and in inflammation which larger epidemiological studies could address.

Rheumatology key messages

- Early RF− but not RF+ inflammatory arthritis patients were commonly colonized with phylogenetic group B2 E. coli.
- B2 E. coli presence was associated with low inflammation, yet higher IgA anti-E. coli responses.

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Distinct bacterial colonization patterns of *E. coli* subtypes

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