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Immune activation in the small intestine in patients with rheumatoid arthritis

R Nissinen, M Leirisalo-Repo, A M Nieminen, L Halme, M Färkkilä, T Palosuo, O Vaarala


Objectives: To determine whether inflammation in the gut associated immune system is activated in rheumatoid arthritis (RA). The expression of chemokine receptor- (CCR4, CCR5) and cytokine- (interleukin (IL)2, IL10, interferon γ (IFNγ), tumour necrosis factor α (TNFα), and transforming growth factor β (TGFβ)) specific mRNA in intestinal biopsy samples from patients with RA was examined.

Methods: Duodenal biopsy samples from 13 patients with RA and 15 control subjects were studied. The mRNA expression of CCR4, CCR5, IL2, IL10, IFNγ, TNFα, and TGFβ in intestinal biopsy samples was demonstrated by real time quantitative reverse transcriptase-polymerase chain reaction.

Results: The mRNA expression of CCR4, CCR5, and IL10 in intestinal biopsy samples was increased in patients with RA in comparison with control subjects (p = 0.001, p = 0.046, p = 0.019). No difference in the expression levels of IL2, IFNγ, TNFα, or TGFβ was seen between patients with RA and controls.

Conclusions: The increased intestinal mRNA expression of IL10, CCR5, and CCR4 suggests that gut associated immune cells are activated in patients with RA.

Gut lesions have been found in patients with rheumatic diseases, while peripheral arthritis is occasionally found in patients with gastrointestinal diseases. Increased permeability, raised numbers of inflammatory cells, and increased HLA-DR expression in the gut have been reported in patients with rheumatoid arthritis (RA). Occult intestinal inflammation, which may be related to non-steroidal anti-inflammatory drug treatment or may be associated with disease, occurs in about 67% of patients with RA, but markers of gut inflammation are not restricted to the use of non-steroidal anti-inflammatory drugs. Also, an increased expression of the gut associated surface molecule, α4β7, which is expressed on >95% of intestinal intraepithelial lymphocytes and on 40% of lamina propria lymphocytes, is found on synovial fluid derived T cells in comparison with peripheral blood lymphocytes in patients with RA. This suggests an accumulation of lymphocytes derived from the gut in the inflamed joints.

The expression profile of cytokines and chemokine receptors in tissue reflects the stage of inflammation and may also be an indicator of the functional phenotype of immune cells. T cells are divided into type 1 and type 2 cells supporting cytotoxic or humoral immune response, respectively. Type 1 response has been associated with interferon γ (IFNγ) and interleukin (IL)2 secretion and expression of CCR5 and CXCR3, whereas type 2 response has been associated with expression of CCR3, CCR4, CCR8, and secretion of IL4, IL5, and IL13. In humans, this dichotomy between type 1 and 2 cells is, however, not so clear.

To date only a few reports have been published dealing with the profile of cytokines and chemokine receptors expressed in the gut of patients with rheumatic diseases and, as far as we know, no such studies exist on patients with RA. Therefore, we studied the mRNA expression of CCR4, CCR5, IL2, IL10, IFNγ, tumour necrosis factor α (TNFα), and transforming growth factor β (TGFβ) in duodenal biopsy samples from patients with RA.

PATIENTS AND METHODS

Patients

Duodenal gut biopsy samples were obtained from 13 patients with RA (12 women, 1 man, mean age 59, range 39–84 years) and 15 control subjects (10 women, 5 men, mean age 50, range 30–76 years) in whom gastroscopy was performed with clinical indications. In patients with RA the endoscopy was performed in the presence of abdominal pain or if bleeding was suspected. In the controls the endoscopy was performed in the presence of abdominal pain or symptoms of reflux. Table 1 presents the characteristics of patients and controls.

In addition to routine samples, three additional biopsy samples of duodenum were obtained in 0.9% NaCl. The biopsy samples were frozen at −70°C for later analysis. In all the cases, the duodenum was normal both by inspection and by histology. The patients and controls gave their written informed consent. The study protocol was approved by the ethics committee of Helsinki University Central Hospital.

Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA expression of cytokines and chemokine receptors was demonstrated by real time RT-PCR from mucosal samples. Total RNA (tRNA) was extracted from frozen biopsy samples, stored at −70°C, by RNA Total Gen Elute Mammalian RNA kit. Reverse transcription reaction was carried out in a final volume of 75 μl using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The reaction mix contained 10×RT buffer, 5.5 mg MgCl2, 500 μmol/l of each dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 10 ng/μl template RNA. The solution was treated with 0.01 U/μl DNAase (Boehringer Mannheim) for 30 minutes at 37°C, followed by heat inactivation at 75°C for 5 minutes and cooling to 25°C. 1.25 U/μl multiscrube reverse transcriptase enzyme was added and the mixture was subjected to 48°C for 30 minutes and inactivated at 95°C for 5 minutes. The cDNA was stored at −20°C until use.

Real time PCR was performed with an automated fluorometer, ABI Prism 7700 Sequence Detection System.

Abbreviations: IL, interleukin; IFN, interferon; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; TGFβ, transforming growth factor β; TNFα, tumour necrosis factor α.

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Table 1 Clinical features and endoscopic findings in patients with RA and in control subjects

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Duration of RA (years)</th>
<th>Treatment DMARDs/PRED</th>
<th>Gastroscopy Indication</th>
<th>Endoscopic findings</th>
<th>Histological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA1</td>
<td>75</td>
<td>28</td>
<td>MTX/SSZ/–</td>
<td>Anaemia</td>
<td>Mild gastritis</td>
<td>Mild chronic inflammation (antrum, corpus) Normal</td>
</tr>
<tr>
<td>RA2</td>
<td>80</td>
<td>25</td>
<td>MTX/–</td>
<td>GERD</td>
<td>Oesophagitis, gastric atrophy</td>
<td>Mild chronic inflammation (corpus) Normal</td>
</tr>
<tr>
<td>RA3</td>
<td>83</td>
<td>50</td>
<td>–/–</td>
<td>Heartburn, abdominal pain</td>
<td>Gastric atrophy, antral intestinal metaplasia</td>
<td>In antrum Normal</td>
</tr>
<tr>
<td>RA4</td>
<td>63</td>
<td>17</td>
<td>LIF/–</td>
<td>Abdominal pain</td>
<td>Large proplastic ulcer</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>RA5</td>
<td>43</td>
<td>2</td>
<td>MTX/–</td>
<td>Anaemia</td>
<td>Normal</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>RA6</td>
<td>56</td>
<td>15</td>
<td>MTX/–</td>
<td>Abdominal pain</td>
<td>Gastric atrophy</td>
<td>Mild chronic inflammation (antrum, corpus) Normal</td>
</tr>
<tr>
<td>RA7</td>
<td>55</td>
<td>7</td>
<td>MTX/–</td>
<td>Abdominal pain</td>
<td>Duodenogastric reflux</td>
<td>Mild chronic inflammation (corpus) Normal</td>
</tr>
<tr>
<td>RA8</td>
<td>50</td>
<td>10</td>
<td>LIF/–</td>
<td>Anaemia</td>
<td>Hatus hemis</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>RA9</td>
<td>56</td>
<td>14</td>
<td>MTX/SSZ/–</td>
<td>Abdominal pain, vomiting</td>
<td>Normal</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>RA10</td>
<td>47</td>
<td>24</td>
<td>–/–</td>
<td>Abdominal pain</td>
<td>Mild antrum gastritis</td>
<td>Mild chronic inflammation (corpus) Normal</td>
</tr>
<tr>
<td>RA11</td>
<td>42</td>
<td>&lt;1</td>
<td>–/–</td>
<td>Diarrhoea</td>
<td>Normal</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>RA12</td>
<td>39</td>
<td>4</td>
<td>SSZ/HCQ/–</td>
<td>Abdominal pain, anaemia</td>
<td>Mild corpus gastritis</td>
<td>Intestinal metaplasia, atrophy in corpus, mild chronic inflammation (corpus)</td>
</tr>
<tr>
<td>RA13</td>
<td>84</td>
<td>4</td>
<td>HCQ/–</td>
<td>Anaemia</td>
<td>Angular ulcer</td>
<td>Normal</td>
</tr>
<tr>
<td>CTRL1</td>
<td>39</td>
<td></td>
<td></td>
<td>Abdominal pain</td>
<td>Normal</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>CTRL2</td>
<td>53</td>
<td></td>
<td></td>
<td>Barrett's oesophagus (control)</td>
<td>Barrett's oesophagus</td>
<td>Mild chronic inflammation (antrum) Normal</td>
</tr>
<tr>
<td>CTRL3</td>
<td>43</td>
<td></td>
<td></td>
<td>Heartburn</td>
<td>Mild chronic gastritis</td>
<td>MILD CHRONIC INFLAMMATION (ANTRUM, CORPUS) Normal</td>
</tr>
<tr>
<td>CTRL4</td>
<td>70</td>
<td></td>
<td></td>
<td>Barrett's oesophagus (control)</td>
<td>Barrett's oesophagus</td>
<td>Mild chronic inflammation (corpus) with intestinal metaplasia in antrum and oesophagus Not examined</td>
</tr>
<tr>
<td>CTRL5</td>
<td>65</td>
<td></td>
<td></td>
<td>Barrett's oesophagus (control)</td>
<td>Barrett's oesophagus</td>
<td>Mild chronic inflammation (corpus) with intestinal metaplasia in antrum and oesophagus Not examined</td>
</tr>
<tr>
<td>CTRL6</td>
<td>39</td>
<td></td>
<td></td>
<td>Diarrhoea</td>
<td>Normal</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>CTRL7</td>
<td>53</td>
<td></td>
<td></td>
<td>Hypealuminaemia</td>
<td>Erosions in antrum, corpus</td>
<td>Moderate chronic inflammation (antrum, corpus)</td>
</tr>
<tr>
<td>CTRL8</td>
<td>56</td>
<td></td>
<td></td>
<td>Reflux (control)</td>
<td>Haematematosus cystic polyps in corpus</td>
<td>Gastric biopsy not taken; severe intestinal metaplasia in oesophagus Not examined</td>
</tr>
<tr>
<td>CTRL9</td>
<td>52</td>
<td></td>
<td></td>
<td>Barrett's oesophagus (control)</td>
<td>Barrett's oesophagus, Hatus hemis</td>
<td>Gastric biopsy not taken; severe intestinal metaplasia in oesophagus Not examined</td>
</tr>
<tr>
<td>CTRL10</td>
<td>76</td>
<td></td>
<td></td>
<td>Pyloric ulcer (control)</td>
<td>Deformed pylorus, no ulcer</td>
<td>Mild chronic inflammation (corpus), with intestinal metaplasia Not examined</td>
</tr>
<tr>
<td>CTRL11</td>
<td>36</td>
<td></td>
<td></td>
<td>Suspected submucous tumour (control)</td>
<td>Normal</td>
<td>Mild intestinal metaplasia (antrum) Normal</td>
</tr>
<tr>
<td>CTRL12</td>
<td>30</td>
<td></td>
<td></td>
<td>Abdominal discomfort</td>
<td>Normal</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>CTRL13</td>
<td>75</td>
<td></td>
<td></td>
<td>Problems in swallowing</td>
<td>Hatus hemis</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>CTRL14</td>
<td>30</td>
<td></td>
<td></td>
<td>Abdominal pain</td>
<td>Suspected antral polyps</td>
<td>Normal Normal</td>
</tr>
<tr>
<td>CTRL15</td>
<td>38</td>
<td></td>
<td></td>
<td>Heartburn</td>
<td>Oesophagitis</td>
<td>Gastric histology normal, candida oesophagitis Normal</td>
</tr>
</tbody>
</table>

RA, rheumatoid arthritis; CTRL, control subject; DMARD, disease modifying antirheumatic drug; PRED, prednisone; MTX, methotrexate; SSZ, sulfasalazine; HCQ, hydroxychloroquine; LIF, leflunomide; Hpv, Helicobacter pylori positive at histology.

We used the comparative Ct method to measure the gene transcription in samples. The Ct of 18S was subtracted from the Ct of the cytokine to give the ΔCt value. The ΔCt of the analysed sample was then subtracted from the ΔCt of the calibrator. This difference is called the ΔΔCt value. The results are expressed as relative units based on calculation of 2−ΔΔCt, which gives the relative amount of cytokine normalised to endogenous control (18S) and compared with calibrator.

**Statistics**

A comparison of variables between the groups was carried out with the Mann-Whitney test. A p value <0.05 was considered significant.

**RESULTS**

**Real time quantitative RT-PCR**

Figure 1 shows the expression of chemokine receptors and cytokine IL10 mRNA in duodenal biopsy samples. The levels of CCR4, CCR5, and IL10 mRNA in intestinal biopsy samples were higher in patients with RA than in control subjects (median relative units being 1 v 0.2 and p = 0.001 for CCR4, median 1 v 15, p = 0.046 for CCR5, and median 1 v 0.3, p = 0.019 for IL10). No differences were seen in the IFNγ, IL2,
TGFβ, or TNFα mRNA levels in patients with RA when compared with controls.5

DISCUSSION

We found increased expression of IL10, CCR4, and CCR5 mRNA in duodenal biopsy samples from patients with RA. Normal human small intestinal lymphocytes express CCR5 and CXCR3, so called type 1 immune response associated chemokine receptors, and lack the expression of CXCR1, CXCR2,CCR1, CCR3, CCR4, and CCR7.9

CCR5 is expressed on a greater proportion of gut-homing peripheral blood lymphocytes than on those thought to home to extraintestinal sites in normal intestine,9 which may suggest that CCR5 is an important receptor for selective recruitment of lymphocytes to the intestine. Furthermore, the expression of RANTES, a ligand of CCR5, is increased in the inflamed intestine.10 The expression of CCR5 has been associated with type 1 immune response and with secretion of IFNγ, but despite findings related to CCR5 expression, we did not find increased expression of IFNγ or IL2 mRNA levels in our patients with RA. We observed highest levels of IFNγ mRNA expression in RA, but there was great variation in both patient and control groups.

CCR4 has been associated with type 2 immune response, but CCR4 positive cells are not exclusively type 2 restricted and are also expressed at target tissues in immunological disease with a type 1 deviation.7 Expression of CCR4 is found in the inflamed intestine, but it is not expressed on intestinal T cells of healthy subjects.9 In an animal model of chronic intestinal inflammation, IL10 deficient mice expressed CCR4, CCR2, and CCR6 mRNA locally in the inflamed mucosa.11 Another study showed that CCR4 was absent from the lung and skin of normal subjects, but was present in the lung of atopic patients.12 Supporting the induction of CCR4 in inflamed mucosa. The increased CCR4 mRNA expression in the intestine of patients with RA can be considered as a marker of inflammation, while it is too speculative to draw conclusions of the T1/T2 polarisation of the mucosal immune response in RA. IL10 has a dual role in the inflammatory process as it has both anti-inflammatory and proinflammatory potential.

Both CCR4 and CCR5 are also expressed on monocyte lineage cells. Thus, possibly, our findings reflect activation of the innate immune system. Interestingly, IL10, which is secreted by monocytes and T cells, was also found to be increased in the gut of patients with RA. The role of IL10 in the intestinal homeostasis is shown by studies on IL10 deficient mice, which develop chronic inflammation in the intestine with no obvious inflammatory lesions elsewhere.13 However, the anti-inflammatory role of IL10 has recently been questioned in RA. In patients with RA high IL10 concentrations have been detected in the serum and synovial fluid.14 15 B lymphocytes are also potent producers of IL10. IL10 has been shown to correlate with serum rheumatoid factor titre and in vitro levels of spontaneous IgM RF production,14 suggesting that activation of IL10 secretion is linked to inflammatory activity in RA. Also, an increased number of IL10 expressing CD3+ CD8+ T cells in the ileal lamina propria lymphocytes have been reported in patients with spondyloarthropathy.3

In conclusion, patients with RA have evidence of inflammatory activation in the gut, which may play a role in the pathogenesis of the disease.

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Figure 1 Chemokine receptor CCR4 (A) and CCR5 (B) and cytokine IL10 (C) specific mRNA detected by quantitative real time RT-PCR in duodenal biopsy samples from patients with RA and from healthy controls. Individual results are shown as relative amount (2^(-ΔΔCt)) of target gene compared with calibrator, both normalised to an endogenous reference (18S). The median values are indicated by horizontal lines and p values of the Mann-Whitney test are shown.