CONCISE REPORT

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

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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.[1 2] Increased permeability, raised numbers of inflammatory cells, and increased HLA-DR expression in the gut have been reported in patients with rheumatoid arthritis (RA).[3 4] 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,[5] but markers of gut inflammation are not restricted to the use of non-steroidal anti-inflammatory drugs.[4] Also, an increased expression of the gut associated surface molecule, $\alpha_E\beta_7$, 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.[6] This suggests an accumulation of lymphocytes derived from the gut in the inflamed joints.[7]

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 CCD3, CCR4, CCR8, and secretion of IL4, IL5, and IL13. In humans, this dichotomy between type 1 and 2 cells is, however, not so clear.[7]

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,[8] 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℃ 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℃, 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 $MgCl_2$, 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℃, followed by heat inactivation at 75℃ for 5 minutes and cooling to 25℃. 1.25 U/µl multiscribe reverse transcriptase enzyme was added and the mixture was subjected to 48℃ for 30 minutes and inactivated at 95℃ for 5 minutes. The cDNA was stored at −20℃ until use.

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

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

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

Patient	Age (years)	Duration of RA (years)	Treatment DMARDs/PRED	Gastroscopy Indication	Endoscopic findings	Histological findings Stomach	Duodenum
RA1	75	28	MTX+SSZ/−	Anaemia	Mild gastritis	Mild chronic inflammation (antrum, corpus)	Normal
RA2	80	25	MTX/+	GERD	Oesophagitis, gastric atrophy	Mild chronic inflammation (corpus), mild oesophagitis	Normal
RA3	83	50	−/+	Heartburn, abdominal pain	Gastric atrophy, antral intestinal metaplasia	Atrophy and intestinal metaplasia in antrum	Normal
RA4	63	17	LEF/−	Abdominal pain	Large prepyloric ulcer	Normal	Normal
RA5	43	2	MTX/−	Anaemia	Normal	Normal	Normal
RA6	56	15	MTX/+	Abdominal pain	Gastric atrophy	Mild chronic inflammation (antrum, corpus)	Mild chronic inflammation
RA7	55	7	MTX/+	Abdominal pain	Duodenogastric reflux	Mild chronic inflammation (corpus)	Normal
RA8	50	10	LEF/+	Anaemia	Hiatus hernia	Normal	Normal
RA9	56	14	MTX+SSZ/+	Abdominal pain, vomiting	Normal	Normal	Normal
RA10	47	24	−/−	Abdominal pain	Mild antrum gastritis	Mild chronic inflammation (corpus)	Normal
RA11	42	<1	−/−	Diarrhoea	Normal	Normal	Normal
RA12	39	4	SSZ+HCQ/−	Abdominal pain, anaemia	Mild corpus gastritis	Intestinal metaplasia, atrophy in corpus, mild chronic inflammation (corpus, antrum)	Normal
RA13	84	4	HCQ/+	Anaemia	Angular ulcer	Severe chronic inflammation (angulus), severe intestinal metaplasia and atrophy in antrum	Normal
CTRL1	39			Abdominal pain	Normal	Normal	Normal
CTRL2	53			Barrett's oesophagus (control)	Barrett's oesophagus	Mild chronic inflammation (antrum), severe intestinal metaplasia in oesophagus	Normal
CTRL3	43			Heartburn	Mild chronic gastritis	Mild chronic gastritis (antrum, corpus, angulus), Hp+, mild intestinal metaplasia and atrophy in corpus	Normal
CTRL4	70			Barrett's oesophagus (control)	Normal	Normal	Normal
CTRL5	65			Barrett's oesophagus (control)	Barrett's oesophagus	Mild chronic inflammation (corpus) with intestinal metaplasia in antrum and oesophagus	Not examined
CTRL6	39			Diarrhoea	Normal	Normal	Normal
CTRL7	53			Hypoalbuminaemia	Erosions in antrum, corpus	Moderate chronic inflammation (antrum, corpus), Hp+	Normal
CTRL8	56			Reflux (control)	Haematomatous cystic polyps in corpus	Normal	Normal
CTRL9	52			Barrett's oesophagus (control)	Barrett's oesophagus, hiatus hernia	Gastric biopsies not taken; severe intestinal metaplasia in oesophagus	Not examined
CTRL10	76			Pyloric ulcer (control)	Deformed pylorus, no ulcer	Mild chronic inflammation (corpus), with intestinal metaplasia	Not examined
CTRL11	36			Suspected submucous tumour (control)	Normal	Mild intestinal metaplasia (antrum)	Normal
CTRL12	30			Abdominal discomfort	Normal	Normal	Normal
CTRL13	75			Problems in swallowing	Hiatus hernia	Normal	Normal
CTRL14	30			Abdominal pain	Suspected antral polyps	Normal	Normal
CTRL15	38			Heartburn	Oesophagitis	Gastric histology normal, candida oesophagitis	Normal

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

(Applied Biosystems), and TaqMan PDAR (predeveloped assay reagents) primers/probes. The PDAR primers/probes for TGFβ (catalogue No 4327054F), TNFα (catalogue No 4327055F), IL10 (catalogue No 4327043F), IFNγ (catalogue No 4327052F), IL2 (catalogue No 4327036F), CCR4 (catalogue No 4324583F), and CCR5 (catalogue No 4324596F) were used. Ribosomal 18S (catalogue No 4310893E) was used as endogenous control. The PCR reactions were run in triplicate wells with 50 ng (for target gene) or 5 ng (for endogenous control) of template cDNA in a final volume of 25 µl. Amplification conditions were 2 minutes at 50°C, 10 minutes at 95°C, and 50 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

The expression of each cytokine was measured also from a home-made calibrator sample, which was prepared by stimulating peripheral blood mononuclear cells from a healthy subject with phytohaemagglutinin for 48 hours.

We used the comparative Ct method to measure the gene transcription in samples. The Ct of 18S was subtracted from the cytokine Ct 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 $2^{-\Delta\Delta Ct}$, 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 61 *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,

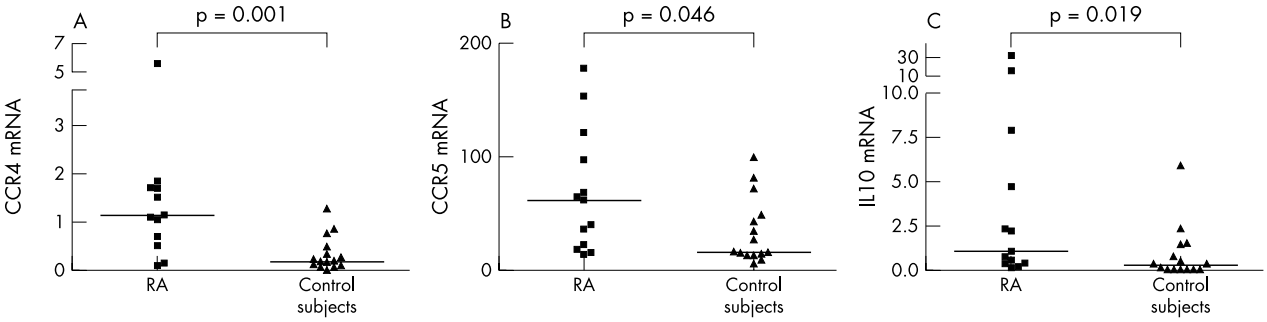

Figure 1 Chemokine receptor CCR4 (A) and CCR5 (B) and cytokine IL10 (C) specific mRNA detected by quantitative real time RT-PCR in duodenal biopsy samples from patients with RA and from healthy controls. Individual results are shown as relative amount ($2^{-\Delta\Delta Ct}$) of target gene compared 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.

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 homoeostasis 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 titres 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.[8]

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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Authors' affiliations

R Nissinen, A M Nieminen, O Vaarala, Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland
R Nissinen, T Palosuo, Department of Health and Functional Capacity, National Public Health Institute, Helsinki, Finland
M Leirisalo-Repo, Department of Medicine, Division of Rheumatology, Transplantation and Liver Surgery Unit, Helsinki University Central Hospital, Helsinki, Finland
L Halme, Department of Surgery, Transplantation and Liver Surgery Unit, Helsinki University Central Hospital, Helsinki, Finland
M Färkkilä, Department of Medicine, Division of Gastroenterology, Transplantation and Liver Surgery Unit, Helsinki University Central Hospital, Helsinki, Finland
O Vaarala, Division of Paediatrics, Department of Molecular and Clinical Medicine, Linköping University, Linköping, Sweden

Correspondence to: Dr R Nissinen, National Public Health Institute, Department of Molecular Medicine, Biomedicum, PO Box 104, 00251 Helsinki, Finland; riikka.nissinen@ktl.fi

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