SYMMETRICAL SYNOVIAL FLUID CELL CYTOKINE MESSENGER RNA EXPRESSION IN RHEUMATOID ARTHRITIS: ANALYSIS BY REVERSE TRANSCRIPTION/POLYMERASE CHAIN REACTION

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SUMMARY
To investigate the complex intra-articular immune activity in rheumatoid arthritis (RA), we analysed the expression of a wide range of cytokine mRNAs in synovial fluid cells from patients with rheumatoid arthritis. To minimize in vitro artefact, mRNA was rapidly extracted from synovial fluid leucocytes taken from single joints of seven patients and simultaneously from both knee joints of four patients. Expression of interleukin (IL) 1β, IL-2, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, tumour necrosis factor-α and interferon-γ (IFN-γ) was detected using the reverse transcription/polymerase chain reaction. The expression of cytokines varied between patients. IFN-γ mRNA was detected in 60% of the patients and IL-4 mRNA in 10%. Cytokine expression in both knees was very similar. These results suggest that T-cell activity in RA is detectable using sensitive techniques and that the intra-articular immunopathology of RA is systemically very similar.

KEY WORDS: Rheumatoid arthritis, Cytokines, Polymerase chain reaction.

Although many cytokines have been detected in the synovial compartment of patients with rheumatoid arthritis (RA), most studies have investigated a very limited number of cytokines from each joint [1, 2]. Cytokines are known to interact and it has been suggested that they should be considered as a network [3]. The importance of simultaneously detecting multiple cytokines is illustrated by T-lymphocyte cytokines, where patterns of cytokine production have profound implications for the outcome of immune responses. Although recent in vivo evidence shows that few T cells secrete the very restricted cytokine patterns originally described as T helper 1 (Th1) and T helper 2 (Th2) subsets [4, 5], it is clear that cell-mediated immune responses are dominated by Th1 cytokines, e.g. interferon-γ (IFN-γ) and humoral responses by Th2 cytokines, e.g. interleukin 4 (IL-4) [6].

The reverse transcription/polymerase chain reaction (RT/PCR) technique provides a useful method for detecting the expression of a large number of cytokine mRNA species from sites of human disease which provide limited sample size. The technique is very sensitive and is useful for detecting mRNA expressed at low levels, such as T-lymphocyte mRNA. Cytokines such as IL-2 and IFN-γ are expressed at low levels compared to cytokines produced predominantly by monocyte/macrophage cells, even in models of T-lymphocyte-dependent processes such as allograft rejection and graft-versus-host reactions [7, 8]. In graft-versus-host lesions, only 3% of T cells produce IFN-γ, with an even lower proportion producing other cytokines [8]. In these models, T-lymphocyte cytokine proteins are rarely detectable at sites of disease which may explain the difficulties of detecting them in RA joints [9]. Studies of T-lymphocyte cytokine mRNA expression in RA synovial samples using RT/PCR have reported variable results [10, 11]. Although RA is considered a systemic disease, there are several studies comparing the immune response from different joints of the same individual. We analysed the mRNA expression of nine cytokines in SF cells from one or two joints of patients with RA.

METHODS

Subjects
Eleven patients with active RA, fulfilling at least four of the seven American College of Rheumatology criteria, had synovial fluid (SF) samples taken from large joints. Four of these patients had samples taken simultaneously from both knee joints. Patient details are given in Table I.

Sample preparation
SF samples were immediately placed into EDTA-containing tubes. Samples for RNA analysis were diluted 1:4 in cold phosphate-buffered saline (PBS) and filtered through a 0.45 μm filter. Total SF leucocytes were isolated by centrifugation, with an even lower proportion producing other cytokines [8]. In these models, T-lymphocyte cytokine proteins are rarely detectable at sites of disease which may explain the difficulties of detecting them in RA joints [9]. Studies of T-lymphocyte cytokine mRNA expression in RA synovial samples using RT/PCR have reported variable results [10, 11]. Although RA is considered a systemic disease, there are several studies comparing the immune response from different joints of the same individual. We analysed the mRNA expression of nine cytokines in SF cells from one or two joints of patients with RA.

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RNasin (Promega), 1 mg random hexamers (Boehringer Mannheim), 2 mM deoxynucleotide triphosphate (dATP, dGTP, dCTP, dTTP) in equal concentrations (Boehringer Mannheim), 64 U of MMLV reverse transcriptase (Promega) and 500 ng of total RNA, incubated at 42°C for 60 min and stored at −20°C. Five microlitres of cDNA were amplified in 50 µl reactions containing PCR buffer [50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂] with 1.25 U of Taq polymerase (Perkin Elmer), and 0.2 mM of the appropriate sense and antisense primers. Samples were denatured at 94°C (1 min), annealed at 60°C (1 min) and extended at 72°C (2 min). Primer pairs were synthesized for IL-2, IFN-γ, IL-4, IL-10, tumour necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Beta-actin (β-actin) and CD3 delta chain (CD3δ) were used as a guide to total RNA and T-lymphocyte RNA, respectively. Negative control reactions for every PCR experiment were tubes without Taq polymerase, cDNA or primers.

To assess the reproducibility of RNA extraction and reverse transcription, aliquots of a conA PBMC culture were processed in parallel, from RNA extraction to PCR product analysis.

Sensitivity of the PCR reaction
We determined the lower detection limits of our PCR conditions using a plasmid containing artificial DNA complementary to the 5′ primers and copies of the 3′ primers similar to that described by Wang et al. [12]. Dilutions of plasmid amplified at optimal cycle numbers enabled us to determine the lower limit of molecules detected using the known molecular weight of the plasmid.

RESULTS
Reverse transcription/polymerase chain reaction performance
Messenger RNAs for all cytokines tested were detected at high levels in control 24 h conA PBMC cultures. Optimal PCR cycle numbers within the range reproducibly producing an exponential increase in PCR product are shown in Table II. A time course of conA PBMC reproduced cytokine mRNA changes previously detected by Northern blot analysis (results not shown).

The reproducibility of the RNA extraction and reverse transcription steps was demonstrated by the similar levels of PCR product obtained for the cytokines (all except IL-4 tested), β-actin and CD3δ, when three aliquots of a 24 h conA PBMC culture were analysed in parallel experiments. When the s.d. of the mean value of the PCR products from these triplicate PCR reactions was compared to the actual mean value, all s.d. values were <10% of the mean except for TNFα, s.d. 13.5% of the mean.

The lower limits of detection of our PCR process for IL-4, IFN-γ and TNF-α were: IL-4 500 molecules/PCR reaction; IFN-γ 600 molecules/reaction; TNF-α 1000 molecules/reaction.

### TABLE I
Clinical, treatment and synovial fluid leucocyte total and differential count details of 11 patients with rheumatoid arthritis analysed in this study

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Disease duration (yr)</th>
<th>ESR (mm/h)</th>
<th>RF*</th>
<th>Drugs†</th>
<th>SF, WCC‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>62</td>
<td>12</td>
<td>45</td>
<td>+</td>
<td>SSZ, HCQ</td>
<td>150 × 10⁹/l; 86% N.</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>66</td>
<td>14</td>
<td>36</td>
<td>+</td>
<td>AZA, NSAID</td>
<td>8.6 × 10⁹/l; 80% N.</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>71</td>
<td>55</td>
<td>52</td>
<td>−</td>
<td>MTX, PREV IA MPA</td>
<td>0.8 × 10⁹/l; 89% N.</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>71</td>
<td>6</td>
<td>66</td>
<td>+</td>
<td>GST, PREV IA MPA</td>
<td>22.5 × 10⁹/l; 77% N. 1 14% M 9%</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>59</td>
<td>2</td>
<td>100</td>
<td>+</td>
<td>GST, ORAL PRED</td>
<td>44.8 × 10⁹/l; 77% N. 1 14% M 9%</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>65</td>
<td>2</td>
<td>41</td>
<td>−</td>
<td>MTX, SSZ, NSAID</td>
<td>ND§</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>72</td>
<td>7</td>
<td>40</td>
<td>+</td>
<td>HCQ, GST</td>
<td>2 × 10⁹/l; 72% N. 1 24% M 3%</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>53</td>
<td>8</td>
<td>39</td>
<td>+</td>
<td>AZA, NSAID</td>
<td>R* 11.8 × 10⁹/l; 76% N. 1 18% M 16%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L* 11.7 × 10⁹/l; 85% N. 1 11% M 4%</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>35</td>
<td>0.3</td>
<td>48</td>
<td>−</td>
<td>NSAID</td>
<td>R) ND</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>60</td>
<td>2</td>
<td>108</td>
<td>+</td>
<td>MTX, PRED</td>
<td>R) 32.8 × 10⁹/l; 85% N. 1 4% M 13%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L) 35.1 × 10⁹/l; 77% N. 1 4% M 19%</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>73</td>
<td>5</td>
<td>8</td>
<td>−</td>
<td>GST, SSZ, NSAID</td>
<td>R) 1.3 × 10⁹/l; 5% N. 1 59% M 36%</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>L) 3.2 × 10⁹/l; 2% N. 1 87% M 10%</td>
</tr>
</tbody>
</table>

*RF, rheumatoid factor.
†SSZ, sulphasalazine; HCQ, hydroxychloroquine; AZA, azathioprine; MTX, methotrexate; PREV IA MPA, intra-articular methylprednisolone acetate 8–12 weeks previously in that joint; PRED, oral prednisolone; GST, gold sodium thiomolate.
‡SF, WCC, total and differential synovial leucocytes analysed in this study; N, neutrophils; L, lymphocytes; M, monocytes.
§ND, not determined.
*R, right knee; L, left knee
TABLE II
Synovial fluid cell cytokine mRNA expression, expressed as a percentage of the positive control for that cytokine (positive control arbitrarily equals 100)

<table>
<thead>
<tr>
<th>Patient</th>
<th>IL-1β</th>
<th>TNF-α</th>
<th>IL-6</th>
<th>GM-CSF</th>
<th>CD3β</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-10</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25 CYC*</td>
<td>30 CYC</td>
<td>30 CYC</td>
<td>35 CYC</td>
<td>40 CYC</td>
<td>35 CYC</td>
<td>40 CYC</td>
<td>35 CYC</td>
<td>40 CYC</td>
<td>35 CYC</td>
</tr>
<tr>
<td>2</td>
<td>66.7</td>
<td>42.4</td>
<td>151.9</td>
<td>58.3</td>
<td>103.0</td>
<td>120.1</td>
<td>65.1</td>
<td>63.3</td>
<td>70.1</td>
<td>92.8</td>
</tr>
<tr>
<td>3</td>
<td>9/11 (81.8%)</td>
<td>7/11 (63.6%)</td>
<td>8/11 (72.7%)</td>
<td>6/11 (54.5%)</td>
<td>5/11 (45.5%)</td>
<td>4/10 (40%)</td>
<td>3/10 (30%)</td>
<td>2/10 (20%)</td>
<td>1/10 (10%)</td>
<td>0/10 (0%)</td>
</tr>
<tr>
<td>4</td>
<td>7.2</td>
<td>68.7</td>
<td>112.9</td>
<td>1.7</td>
<td>55.8</td>
<td>67.1</td>
<td>0</td>
<td>31.9</td>
<td>68.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>R†</td>
<td>35.9</td>
<td>5.2</td>
<td>115.3</td>
<td>0</td>
<td>49.3</td>
<td>49.8</td>
<td>31.1</td>
<td>0</td>
<td>13.4</td>
</tr>
<tr>
<td>9</td>
<td>30.3</td>
<td>0</td>
<td>145.5</td>
<td>0</td>
<td>45.6</td>
<td>40.9</td>
<td>24.0</td>
<td>0</td>
<td>0</td>
<td>102.9</td>
</tr>
<tr>
<td>10</td>
<td>26.3</td>
<td>17.9</td>
<td>52.3</td>
<td>8.5</td>
<td>71.6</td>
<td>167.8</td>
<td>42.2</td>
<td>0</td>
<td>52.0</td>
<td>100.3</td>
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<tr>
<td>11</td>
<td>17.9</td>
<td>20.8</td>
<td>39.6</td>
<td>26.2</td>
<td>83.5</td>
<td>126.5</td>
<td>59.7</td>
<td>0</td>
<td>0</td>
<td>100.9</td>
</tr>
<tr>
<td>R</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>L</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Cycle no., number of cycles used in the PCR reaction for that cytokine.
†R, right knee; L, left knee.
‡Not determined.

Patient sample results
These results (Table II) are given as the percentage of the conA PBMC positive control used in the same PCR reaction. There was wide variation in the expression of cytokine mRNA between patients. IL-8 and GM-CSF were the exceptions, being detected in 9/11 (81.8%) and 11/11 (100%) of the patients, respectively. TNF-α mRNA was detected in 7/11 (63.6%), IL-1β in 6/11 (54.5%) and IL-6 in 5/10 patients (50%). IL-10 mRNA was detected in 6/10 patients (60%). The T-cell cytokine IL-2 was detected in 4/10 (40%) and IFN-γ mRNA in 6/10 patients (60%). GM-CSF and IL-8 were expressed at high levels in 100% (GM-CSF) and 82% (IL-8) of the subjects, while TNF-α was expressed in 64% of the subjects. There was no association, either in a positive or negative way, between any of the cytokines. Slow-acting anti-rheumatic drug therapy was not associated with any definite pattern. Despite treatment, all patients had active disease, and no specific patterns were seen, even in patients with very active disease compared to those with moderate activity. IL-2 and IFN-γ mRNA were detected in 40 and 60% of the subjects, respectively. Our results are similar to those of Simon and colleagues [11] who detected IFN-γ and IL-2 mRNA in 8/10 RA SM samples, in contrast to IL-4 mRNA, detected in only 2/10 RA SM. In contrast, Chen and colleagues [10] rarely detected IFN-γ mRNA in separated RA SM and SF T lymphocytes. However, IFN-γ mRNA detection in their positive control samples was low,

CONCLUSIONS
This study, designed to process SF cells from patients with RA to investigate the intra-articular environment with minimal in vitro artefact, has demonstrated three important findings: (1) there is great concordance for the expression of multiple cytokine mRNA in different joints of the same individual with RA; (2) T-cell cytokine mRNA is found in RA SF leucocytes; (3) GM-CSF and IL-8 mRNA are expressed in most patients.

This study provides strong evidence supporting what has to date been largely an assumption: that individuals have the same immunopathology in each joint as part of a systemic disease. Further studies comparing different joints and SF and SM samples from the same joint are needed to confirm this evidence.

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compared to expected levels, suggesting that their T-cell separation techniques reduced IFN-γ mRNA levels.

Our results are similar to those obtained using immunohistological techniques to detect cytokine protein in RA SM, which also show varying patterns of cytokine staining [13, 14]. In contrast to the findings of one of those reports [13], our results show a low frequency of IL-4 expression, supporting findings that T lymphocytes isolated from RA SF mainly produce a Th1 pattern of cytokines [15]. GM-CSF is also produced by T lymphocytes and should not be assumed to be a non-T-lymphocyte cytokine [8]. Our detection of IL-10 mRNA correlates with studies using immunostaining techniques which detected IL-10 in RA SM macrophages and lymphocytes [13, 16].

Although the role of T lymphocytes in the pathogenesis of RA is unclear, T-lymphocyte and macrophage activity are interdependent. For example, culture supernatants of IFN-γ-secreting T lymphocytes from the central nervous system of animals with active experimental allergic encephalomyelitis stimulate both PB monocytes and epithelial microglial cells to produce TNF-α mRNA [17], suggesting that T lymphocytes produce stimulation of TNF-α production, an important effector cytokine in RA.

Cytokine mRNA expression may not always correlate with cytokine protein release, but it certainly reflects the ongoing response of SF leucocytes, and provides a guide for studies of cytokine peptides. With increasing interest in cytokine-directed therapy, knowledge of each individual’s cytokine ‘profile’ may contribute to the logical choice of these potentially highly effective, but very expensive, treatments.

ACKNOWLEDGEMENTS

Supported by the Clive and Vera Ramaciotti Research Foundation, and the Arthritis Foundation of Australia.

REFERENCES


APPENDIX

Sequences for oligonucleotide primers used in the RT/PCR reactions.

IL-2 sense: GAATGGAATTAATTACAAGAATCC
IL-2 antisense: TGTTCAGATCCCTTAGTCCAG
IFN-γ sense: ATATCTTGCCTTTICAGCTC
IFN-γ antisense: CTCCTTTTTCGCTTCTCCTG
IL-4 sense: GTCCTTCTTCTGCTAGTGTG
IL-4 antisense: ATTICTCTTCTCATGATGC
IL-10 sense: ATGCCTCAAGCTGAGACCC
IL-10 antisense: TCTCAAGGGGCGTTGCGTCATCC
TNF-α sense: GAAAGCATGATCCGGGACGT
TNF-α antisense: CTCCTTTTTCGCTTCTCCTGT
IL-6 sense: ATGCCTCAAGCTGAGACCC
IL-6 antisense: TCTCAAGGGGCGTTGCGTCATCC
GM-CSF sense: GCCCTGGGAGCATGTGAATG
GM-CSF antisense: ATAATCTGGGTTGCACAGGA