Cytokine production in whole blood cell cultures of patients with rheumatoid arthritis

A J G Swaak, H G van den Brink, L A Aarden

Abstract
Objective—The measurement of cytokine production of activated lymphocytes and monocytes in the whole blood cell (WBC) culture system may provide a sensitive tool for evaluating the actual ongoing immune response of patients with rheumatoid arthritis (RA).
Methods—Lipopolysaccharide (LPS) up to 250 pg/ml was used for the stimulation of monocytes for measuring the production of tumour necrosis factor α (TNFα), interleukin 6 (IL6) and IL12, while the anti-CD3 (1 µg/ml) and anti-CD28 (5 µg/ml) combination was used for T cell stimulation with the measuring of IL4 and interferon gamma (INFγ) production. Twenty seven patients with RA and 23 healthy controls were studied.
Results—The results showed a decreased IL6 (LPS stimulus 4–62 pg/ml) and IL12 (LPS stimulus 16–62 pg/ml) production in the RA patients. The maximal production of both cytokines was comparable with the normal controls. T cell stimulation showed a significant decreased INFγ production in the RA patients.

Conclusions—These findings obtained in the WBC culture system are highly suggestive for a decreased TH-1 derived cytokine production by a diminished IL12 production in RA patients. Another possibility is that both IL12 and INFγ production in WBCs are inhibited by eventual circulating serum factors.

The immune response can broadly be divided into two types, cell mediated and humoral. It has become clear that these two arms of the immune response are regulated by distinct subsets of CD4+ helper T cells, termed TH-1 and TH-2 cells, which secrete different patterns of cytokines. TH-1 cells produce interferon gamma (INFγ) and interleukin 2 (IL2) are responsible for cell mediated immunity, whereas TH-2 cells will produce IL4 and IL10 and mediate humoral immune responses. In different studies it could be shown that the immunological resistance and susceptibility to infectious diseases were mediated by the relative amount of these cytokines secreted. At present evidence is obtained that the balance of produced cytokines will determine the disease course in rheumatic diseases.

Cytokine production is usually studied in vitro by isolation of peripheral blood mononuclear cells (PBMs) separated by Ficoll-Hypaque gradient centrifugation. One of the main drawbacks of this method is the possible concomitant contamination by bacterial endotoxins and possible effect of the whole separation procedure. Recently a simple technique has been described using whole blood. Only small aliquots of blood are needed and separation procedures are unnecessary. Also the whole blood cell culture technique proved to be very reproducible.

This paper focuses on the measurement of a variety of cytokines produced by PBMs after an initial triggering with an antigenic or mitogenic stimulus as parameters for the actual ongoing immune response of patients with rheumatoid arthritis (RA) compared with healthy control persons.

Methods
BLOOD SAMPLES
Ten ml venous blood samples were collected in evacuated blood collection tubes (Venoeject, Ferumo, Belgium) containing preservative free sodium heparin (150 U/3P units). The samples were kept at room temperature and used within two hours.

PATIENTS AND CONTROLS
All 27 RA patients (11 male and 16 female) fulfilled the American College of Rheumatology (ACR) criteria for RA. Healthy male (7) and female (16) volunteers served as controls. To exclude variation caused by time and sex, experiments were done by matching the RA patients with the normal controls. Patients treated with cyclophosphamide or azathioprine, or both, were excluded. Treatment with a prednisolone dose of < 7.5 mg/day or methotrexate, or both, was permitted. Blood samples of the RA patients treated with MTX were taken at the day preceding the intake of MTX (six days after the intake). All patients received non-steroidal anti-inflammatory drugs (NSAIDs).

WHOLE BLOOD CELL CULTURE
Whole blood cell (WBC) cultures were performed in flat bottom microtitre plate (Nunc, Kamstrup, Denmark). Heparinised venous blood was diluted 1/10 with Iscove’s modified Dulbecco’s medium supplemented with penicillin (100 µg/ml) and 2-mercaptoethanol (5 × 10−5 M).

One hundred and fifty µl of the diluted blood was stimulated with lipopolysaccharide (LPS) Escherichia coli, Sigma, St Louis) at 4, 16, 62 or 250 pg/ml.
To stimulate T lymphocytes a combination of anti-CD3 (CLB-T3/4E) 1 µg/ml and anti-CD28 (CLB-CD 28/1) 5 µg/ml monoclonal antibodies was added to the cultures. After 16 and 72 hours of incubation with LPS and anti-CD3/anti-CD28 respectively, the supernatant was harvested and stored at −70ºC until cytokine analysis was performed.

CYTOKINE ASSAYS
Values of TNFά, IL4, IL6, IL10, and INFγ in supernatant were measured using a sandwich ELISA. These ELISAs were developed and used as previously described. Results were calibrated with serial dilutions of known quantities of recombinant cytokines.

For the detection of IL12, the IL12 P40 ELISA was used. Overall the detection limit was 10 pg.

MONOCYTE AND T LYMPHOCYTE DISTRIBUTION
EDTA blood was stained with conjugated monoclonal antibodies: phycoerythrin (PE) labelled anti-CD14 and fluorescein isothiocyanate labelled anti-CD3 (Becton Dickinson) to detect monocytes and T lymphocytes respectively. At the same time erythrocytes were removed by lysis through addition of FACS lysing solution (Becton Dickinson) to tubes. After incubation for 30 minutes at 20° C and washing twice with phosphate buffer (PBS with 0.1% azide), the samples were analysed with a FACSscan flow cytometer (Becton Dickinson). The percentage of CD14+ (monocytes) and CD3+ (lymphocytes) cells was calculated out of 5000 counted cells.

STATISTICS
Statistical evaluation of the results was undertaken with analysis of equality of median (matched sample sign test). p Values < 0.05 were considered significant.

Table 1 Differences between the patients with RA and the controls with regard to ages, number of circulation CD-14 and CD-3 positive cells

<table>
<thead>
<tr>
<th></th>
<th>RA (median range)</th>
<th>Controls (median range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>60–16*</td>
<td>44–11</td>
</tr>
<tr>
<td>Number of CD-14</td>
<td>6961–3393</td>
<td>7235–2585</td>
</tr>
<tr>
<td>positive cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of CD-3</td>
<td>12 070–4734</td>
<td>18 900–7500</td>
</tr>
<tr>
<td>positive cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.01.

Table 2 Cytokine production in whole blood cultures of patients with RA (group I) and normal controls (group II)

<table>
<thead>
<tr>
<th>Lipopolysaccharide stimulus (pg/ml)</th>
<th>0</th>
<th>4</th>
<th>16</th>
<th>62</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine (pg/ml)</td>
<td>II</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>IL6</td>
<td>56</td>
<td>10</td>
<td>389**</td>
<td>602</td>
<td>1250***</td>
</tr>
<tr>
<td>Range</td>
<td>27</td>
<td>11</td>
<td>526</td>
<td>337</td>
<td>906</td>
</tr>
<tr>
<td>TNFα</td>
<td>5</td>
<td>7</td>
<td>220</td>
<td>290</td>
<td>530</td>
</tr>
<tr>
<td>Range</td>
<td>197</td>
<td>150</td>
<td>357</td>
<td>669</td>
<td>1085</td>
</tr>
<tr>
<td>IL12 (P40)</td>
<td>0</td>
<td>2</td>
<td>nt</td>
<td>168†</td>
<td>183</td>
</tr>
<tr>
<td>Range</td>
<td>10</td>
<td>8</td>
<td>nt</td>
<td>135</td>
<td>147</td>
</tr>
<tr>
<td>IL10</td>
<td>0</td>
<td>7</td>
<td>nt</td>
<td>nt</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>7</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*** p < 0.001, † p < 0.005. nt = not tested.

Results
CELL SUBSETS, SEX RATIO, AND AGES IN RA PATIENTS AND HEALTHY CONTROLS
With regard to sex ratio, number of circulating monocytes (CD14 positive cells), and T cells (CD3 positive cells), no difference could be calculated in RA patients compared with controls. However, the RA patients were significantly older (p < 0.01) than the controls (table 1).

CYTOKINE PRODUCTION (IL6, TNFα, IL12 (P40), and IL10) IN WBC CULTURES STIMULATED WITH LPS
Table 2 summarises the results. The only differences found were in the IL6 and IL12 production compared with controls. When the WBC cultures were stimulated with a LPS stimulus between 4–16 pg/ml a decrease in IL6 production was seen, with a LPS stimulus between 16–62 pg/ml a decrease in IL12 production was found in RA patients. In the spontaneous cytokine production no differences were calculated and the values were under the detection level with exception of IL6 and TNFα.

No correlation could be found between ages and the production of separate cytokines and between the number of CD14 and CD3 positive cells with the separate cytokine production. In the individual patients and controls a linear correlation could be calculated between the stimulus with the cytokine production. Attention was also paid to possible treatment effects. Of the 27 RA patients only four had concomitant corticosteroid treatment. This group was too small for calculation. Between the 10 patients treated with MTX and the non-MTX treated RA patients (n = 17) no differences could be calculated.

CYTOKINE PRODUCTION (INFγ and IL4) IN WBC CULTURES STIMULATED WITH THE COMBINATION OF ANTI-CD3 AND ANTI-CD28
IL4 production in the RA patients was comparable with the normal controls, however, the INFγ production was strongly decreased in the RA patients (fig 1).

Discussion
Serum cytokine profiles may indicate the functional state of immune response with regard to the pathogenesis of the disease, but the serum
concentrations will strongly be affected by the consumption in the whole body. The ongoing immune response may remain localised and in this way the serum values will be influenced by the local consumption as well other factors, which will determine the diffusion into the circulation. By studying the PBMs information can be derived related to the potential responses. For this purpose the WBC system can be used to study the cytokine production with regard to the disease that is being studied.

Our study showed a decrease in IL12 production (with a LPS stimulus between 16–62 pg/ml). In close relation to this finding is the overall decreased INFγ production. In a previous study addition of inflammatory synovial fluids to isolated mononuclear cells of normal controls resulted also in an inhibition of both IL12 as well INFγ production. These findings confirmed our results that in RA the production of IL12 as well INFγ may be affected by circulating factors.

In the WBC cultures of RA patients IL4 production was comparable with the normal controls. Serum IL6 concentrations are increased in RA patients therefore we were surprised to find a somewhat decreased IL6 production when the WBC cultures were stimulated by LPS (4–16 pg/ml). The same finding was obtained in patients with systemic lupus erythematosus, but the decrease in IL6 production could be related with concomitant corticosteroid treatment. In our study treatment with corticosteroids (< 7.5 mg) or MTX had no influence on the cytokine production.

The interpretation of our results and the cited studies might be quite misleading. This is because the inflammatory process, which takes place in RA can cause a preferential margination of activated cells to the tissue leading to their depletion in the blood stream. In this case the opposite cell population is studied with the eventual contradictory results.

7 Neud JL, Griffiths JK, van der Meer JMM. Interleukin-1 (IL-1), IL-1 receptor antagonist and TNF-α production in whole blood. J Leukoc Biol 1992;52:867–92.
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