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

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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.1 4

At present evidence is obtained that the balance of produced cytokines will determine the disease course in rheumatic diseases.5

Cytokine production is usually studied in vitro by isolation of peripheral blood mononuclear cells (PBMCs) 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.6–10

This paper focuses on the measurement of a variety of cytokines produced by PBMCs 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 (Venoject, Ferumo, Belgium) containing preservative free sodium heparin (150 U5P 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.11 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 (MTX), 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.10
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 TNFα, 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 FACScan 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 age, 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>
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<tbody>
<tr>
<td>Age (y)</td>
<td>60–16*</td>
<td>44–11</td>
</tr>
<tr>
<td>Number of CD-14 positive cells</td>
<td>6961–3393</td>
<td>7235–2585</td>
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<tr>
<td>Number of CD-3 positive cells</td>
<td>12 070–4734</td>
<td>18 900–7500</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>I</td>
<td>II</td>
<td>I</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td>IL6</td>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
<td>389***</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></td>
<td></td>
<td>5</td>
<td>7</td>
<td>220</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></td>
<td></td>
<td>0</td>
<td>2</td>
<td>nt</td>
</tr>
<tr>
<td>Range</td>
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<td>8</td>
<td>nt</td>
<td>nt</td>
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<td>IL10</td>
<td></td>
<td></td>
<td>0</td>
<td>7</td>
<td>nt</td>
</tr>
<tr>
<td>Range</td>
<td>7</td>
<td>10</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
</tbody>
</table>

*** p < 0.001, † p < 0.005. nt = not tested.
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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 as 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 Neind JL, Griffiths JK, van der Meer JMM. Interleukin-1 (IL-1), IL-1 receptor antagonist and TNFα production in whole blood. J Leukoc Biol 1992;52:687–92.

Figure 1 Cytokine production measured in WBC cultures of patients with RA stimulated with anti-CD3 combined with anti-CD28.