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 (TNF α), interleukin 6 (IL6) and IL12, while the anti-CD3 (1 μ g/ml) and anti-CD28 (5 μ g/ml) combination was used for T cell stimulation with the measuring of IL4 and interferon gamma (INF γ) production. Twenty seven patients with RA and 23 healthy controls were studied.

Results—The results showed a decreased IL6 (LPS stimulus 4–6 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 INF γ 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 INF γ production in WBCs are inhibited by eventual circulating serum factors.

(*Ann Rheum Dis* 1997;56:693–695)

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 (INF γ) 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 (PBMNs) 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 PBMNs 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 μ g/ml) and 2-mercaptoethanol (5×10^{-5} M).

One hundred and fifty μ l of the diluted blood was stimulated with lipopolysaccharide (LPS) *Escherichia coli*, Sigma, St Louis) at 4, 16, 62 or 250 pg/ml.¹⁰

Department of
Rheumatology, Zuider
Ziekenhuis,
Rotterdam, the
Netherlands
A J G Swaak

Department of Auto
Immune Diseases,
Central Laboratory
Red Cross
Bloodtransfusion
Service, Amsterdam,
the Netherlands
A J G Swaak
H G van den Brink
L A Aarden

Correspondence to:
Dr A J G Swaak,
Zuiderziekenhuis,
Department of
Rheumatology, Groene
Hilledijk 315, Rotterdam, the
Netherlands.

Accepted for publication
10 September 1997

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

	RA (median range)	Controls (median range)
Age (y)	60–16*	44–11
Number of CD-14 positive cells	6961–3393	7235–2585
Number of CD-3 positive cells	12 070–4734	18 900–7500

* $p < 0.01$.

To stimulate T lymphocytes a combination of anti-CD3 (CLB-T3/4E) 1 $\mu\text{g/ml}$ and anti-CD28 (CLB-CD 28/1) 5 $\mu\text{g/ml}$ ¹² 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 -70°C until cytokine analysis was performed.

CYTOKINE ASSAYS

Values of TNF α , IL4, IL6, IL10, and INF γ in supernatant were measured using a sandwich ELISA. These ELISAs were developed and used as previously described.¹³ 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 2 Cytokine production in whole blood cultures of patients with RA (group I) and normal controls (group II)

Cytokine (pg/ml)	Lipopolysaccharide stimulus (pg/ml)									
	0		4		16		62		250	
	I	II	I	II	I	II	I	II	I	II
IL6										
Median	5	10	389***	602	1250***	1920	3685	3261	3685	3547
Range	27	11	526	337	906	918	2383	1861	2385	3241
TNF α										
Median	5	7	220	290	530	770	1540	1210	2210	1190
Range	197	150	357	669	1085	1792	2652	2387	2935	2822
IL12 (P40)										
Median	0	2	nt		168†	183	260***	519	317	464
Range	10	8			135	147	169	373	220	386
IL10										
Median	0	7	nt		nt		nt		57	79
Range	7	10							177	92

*** $p < 0.001$, † $p < 0.005$. 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 significant older ($p < 0.01$) than the controls (table 1).

CYTOKINE PRODUCTION (IL6, TNF α , 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 TNF α .

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 (INF γ 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 INF γ 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

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

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 PBMNs 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 IFN γ 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 IFN γ production.¹³ These findings confirmed our results that in RA the production of IL12 as well as IFN γ 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 patients¹⁴ 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,¹⁰ 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.

- 1 Scott P. IL-2: Initiation cytokine for cell-mediated immunity. *Science* 1993;260:496–7.
- 2 Heinzel FP, Sadick MD, Haladay BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon gamma on interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T-cell subsets. *J Exp Med* 1989;169:59–71.
- 3 Yamamura MK, Uyenura RJ, Deans K, Weinberg TH, Rea BR, Madlin RL. Defecting protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* 1991;254: 277–9.
- 4 Barnes PF, Madlin RL, Ellmer JJ. T-cell responses and cytokines. In: Bloom BR, ed. *Tuberculosis: pathogenesis, protection and control*. Washington DC: American Society for Microbiology, 1994: 417–35.
- 5 Rooney M, Symons J, Di Giovine F, Varsani H, Woo P. Inflammatory cytokine responses in juvenile chronic arthritis. *Br J Rheumatol* 1995;34:454–60.
- 6 Malavé I, Searles RP, Montano J, Williams PC. Production of tumour necrosis factor (cachectin) by peripheral blood mononuclear cells in patients with systemic lupus erythematosus. *Int Arch Allergy Appl Immunol* 1989;89:355–61.
- 7 Nerad 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;52:687–92.
- 8 Bloemena E, Roos MTL, van Heyst JLAM, Vossen JMJJ, Schellekens PTA. Whole blood lymphocyte cultures. *J Immunol Methods* 1989;122:161–9.
- 9 De Forge IE, Kenney JS, Jones ML, Warren JS, Resnick DG. Biphasic production of IL-8 in lipopolysaccharide (LPS) stimulated whole blood. *J Immunol* 1992;148:2133–41.
- 10 Swaak AJG, van den Brink HG, Aarden LA. Cytokine production (IL-6 and TNF- α) in whole blood cell cultures of patients with systemic lupus erythematosus. *Scand J Rheumatol* 1996;25:233–8.
- 11 Arnett FC, Edworthy SM, Block DA, Mc Shane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association, 1987, revised criteria for classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315–24.
- 12 van der Pouw-Kraan TCTM, van Kooten C, Rensink J, Aarden LA. Interleukin (IL)-4 production by human T cells: differential regulation of IL-4 vs. IL-2 production. *Eur J Immunol* 1992;22:1237–41.
- 13 Schulze MA, Koops H, Lipsky PE, Karanagh AF, Davis LS. Elevated Th-1 or Th-0 like cytokine in mRNA in peripheral circulation of patients with rheumatoid arthritis. Modulation by treatment with anti-ICAM-1 correlates with clinical benefit. *J Immunol* 1995;155:5029–37.
- 14 Swaak AJG, van Rooyen A, Nieuwenhuis E, Aarden LA. Interleukin-6 (IL-6) in synovial fluid and serum of patients with rheumatic diseases. *Scand J Rheumatol* 1988;17: 469–74.