Tissue production of pro-inflammatory cytokines (IL-1β, TNFα and IL-6) correlates with the intensity of the systemic inflammatory response and with corticosteroid requirements in giant-cell arteritis


Objectives. To investigate proinflammatory cytokine expression in temporal arteries from patients with giant-cell arteritis (GCA) and to analyse its relationship with the intensity of the initial systemic inflammatory reaction and response to corticosteroid therapy.

Methods. Quantification of interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and interleukin-6 (IL-6) mRNA by real-time quantitative PCR in temporal artery samples from 36 patients with biopsy-proven GCA and 11 controls. Immunohistochemical detection of IL-1β, TNFα, and IL-6 in temporal artery sections from 74 patients with GCA and 15 controls. Clinical and biochemical parameters of inflammation as well as the time (weeks) required to reach a maintenance prednisone dose < 10 mg/day were recorded.

Results. IL-1β (13.8 ± 2.5 vs 5.4 ± 1.3 relative units, P = 0.012) and IL-6 transcripts (34 ± 13.7 vs 7.8 ± 4.5 relative units, P = 0.034) were significantly more abundant in patients with a strong systemic inflammatory response compared with those with no inflammatory parameters. Immunohistochemical scores for IL-1β (2.7 ± 0.3 vs 1.9 ± 0.2, P = 0.018), TNFα (3.2 ± 0.2 vs 2.4 ± 0.3, P = 0.028) and IL-6 (3 ± 0.2 vs 2.1 ± 0.3, P = 0.023) were also significantly higher in patients with strong systemic inflammatory reaction. A significant correlation was found between the amount of tissue TNFα mRNA and the time required to reach a maintenance dose of prednisone < 10 mg/day (r = 0.586, P = 0.001).

Conclusion. GCA patients with a strong systemic inflammatory response, who have been previously shown to be more resistant to corticosteroid therapy, have elevated tissue expression of proinflammatory cytokines IL-1β, TNFα and IL-6. High production of TNFα is associated with longer corticosteroid requirements.

Key words: Giant-cell arteritis, Vasculitis, Inflammation, Cytokines, Acute phase response.

Giant-cell arteritis (GCA) is a large vessel granulomatous vasculitis preferentially involving large and medium-sized vessels. Although vascular inflammatory lesions can be widespread, most of the classical clinical manifestations of GCA are derived from symptomatic involvement of the carotid artery branches [1, 2]. In addition, GCA is a disease characterized by a strong acute-phase response. About 50% of patients experience fever and 60% weight loss [2]. Chronic anaemia is common and an elevated serum concentration of acute-phase proteins leading to an accelerated erythrocyte sedimentation rate (ESR) is so frequent that it has been considered one of the clinical criteria for the classification of GCA [3]. However, the intensity of the systemic inflammatory response is quite variable among patients. Patients with no constitutional symptoms and a normal or close to normal ESR have been repeatedly reported [4, 5].

We have previously shown that the intensity of the systemic inflammatory response defines clinically relevant subgroups of patients with different prognoses. For incompletely understood reasons, patients with a strong acute-phase response are at low risk of developing vascular occlusive events but are more refractory to therapy, requiring higher cumulative steroid doses and a longer duration of treatment [6, 7]. The mechanisms regulating the intensity of the systemic inflammatory response in GCA have not been investigated.

Accumulated experience in experimental settings and in human disease indicates that the systemic inflammatory response is triggered by pro-inflammatory cytokines, the best studied being interleukin 1 (IL-1), tumour necrosis factor (TNFα) and IL-6 [8, 9]. These cytokines have profound effects on numerous cell types that, in turn, secrete a variety of inflammatory mediators, creating complex networks of interactions and leading to multiple inflammatory cascades [10, 11]. In addition, IL-1 and TNFα have potent effects on vessel wall components, particularly endothelial cells, and may influence mechanisms involved in vessel occlusion and repair [12, 13]. Pro-inflammatory cytokines IL-1, TNFα and IL-6 are known to be produced in temporal artery lesions from patients with GCA [14–18], but the clinical impact of cytokine tissue expression has not been investigated.

The aim of our study was to measure IL-1β, TNFα and IL-6 production in temporal artery samples from a large series of
patients with biopsy-proven GCA in order to investigate the relationship between the magnitude of cytokine expression in lesions and clinically relevant findings such as the intensity of the systemic inflammatory reaction, which generates the general feeling of sickness, and response to corticosteroid therapy.

Patients and methods

Patients

The entire study group consisted of 74 patients (25 men and 49 women) with an average age of 77 yr (range 58–91) with biopsy-proven GCA. These patients were consecutively selected from those who had fully developed inflammatory lesions involving the three arterial layers of their temporal arteries. Patients whose samples exhibited only adventitial involvement, even enough to establish the histological diagnosis of GCA, were excluded from the study in order to avoid variations in cytokine expression mostly determined by the density of the inflammatory infiltrates. Sixteen patients (22%) had received prednisone (1 mg/kg per day) for an average of 2 days (range 1–5) and the remaining 58 (78%) were untreated at the time of the temporal artery excision. As control samples we included 15 histologically normal temporal arteries from 15 patients (10 women and 5 men) with an average age of 76 yr (range 52–87) in whom a surrogate diagnosis was obtained. The ultimate diagnoses in these patients were isolated polymyalgia rheumatica (5 patients), non-vasculitic ischaemic optic neuropathy (2 patients), self-limited constitutional symptoms with anaemia (2 patients), temporomandibular osteoarthritis (1 patient), chronic otitis media (1 patient), slowly resolving symptoms with anaemia (2 patients), temporomandibular osteoarthritis (1 patient), headache associated with cutaneous lesions and clinically relevant findings such as the intensity of the systemic inflammatory reaction than any individual inflammatory marker [6, 7]. Patients with 0–2 inflammatory parameters were considered to have a weak systemic inflammatory reaction, whereas patients with 3–4 inflammatory markers were considered to have a strong systemic inflammatory response.

All patients were treated by the authors (JHR, MCC and JMG) according to uniform criteria. The initial prednisone dose was 1 mg/kg per day (up to 60 mg/day) for 1 month and was subsequently tapered at a rate of 5–10 mg/week. Reductions below 20 mg/day were slower and individualized. A rate of 2.5 mg every 3 months was attempted. When the ESR rose above 50 mm/h and clinical symptoms appeared or haemoglobin fell below 110 g/l, or when clear and worsening symptoms occurred with a normal or slightly elevated ESR, a disease flare was considered. When the ESR rose with no clinical symptoms or anaemia, the maintenance prednisone dose was held until the ESR returned to normal or a flare could be defined. When a disease flare occurred, prednisone was increased 10 mg above the previous effective dose. At the end of the study, 31 patients had a follow-up long enough to attempt a reduction of the prednisone dose below 10 mg/day. Time (weeks) required to reach a maintenance dose less than 10 mg/day, cumulative prednisone dose at that point, and the number of relapses during the first 18 months of follow-up were recorded.

This study was approved by the Internal Review Board of our institution (Hospital Clinic, University of Barcelona), and all patients signed informed consent.

Cytokine mRNA quantification

RNA isolation. Surgically excised temporal artery biopsies were embedded in optimal cutting temperature (OCT), quickly frozen in isopentane prechilled in liquid nitrogen and stored at –80°C until used. Total RNA was obtained from 100 serial sections (20 μm thick) per sample using the commercially available Micro RNA isolation kit (Stratagene, La Jolla, CA) following the instructions of the manufacturer. In order to construct the standard curves for real-time polymerase chain reaction (PCR), RNA was also isolated from different cell lines using TRIzol® (Gibco, Life Technologies, Gaithersburg, MD). Both procedures are based on the Chomczynski method [19].

cDNA synthesis. Total RNA (1 μg) was reverse transcribed to cDNA using the SuperScriptTM II First-Strand Synthesis kit (Gibco) employing random hexamers as the priming method. Reaction conditions were carried out according to the manufacturer’s recommendations. Samples were stored at –20°C until use.

Real-time quantitative PCR. Cytokine mRNAs were measured by real-time PCR using specific Pre-Developed TaqManR Target kits from Applied Biosystems (Foster City, CA) [20]. The cytokine target probe and an 18S ribosomal RNA control probe, used as endogenous control, were covalently linked to a different reporter dye (FAM or VIC). A multiplex PCR reaction was carried out with 1 μl of the temporal artery cDNA sample, TaqManR Universal PCR Master Mix, and primers and probe from the target and the endogenous control in a final reaction volume of 25 μl, as recommended by the manufacturer. Each cDNA sample was tested twice. After an initial denaturation step at 95°C for 10 min, 40 cycles were performed as follows: 95°C for 15 s and 60°C for 1 min. The reaction was monitored by measuring the fluorescence signal after each cycle with ABI PrismTM 7700 Sequence Detection system (Applied Biosystems). For TNFα expression, a standard curve was constructed using serial dilutions of cDNA obtained from the Jurkat cell line; for IL-6, with cDNA obtained from the U-937 cell line; and for IL-1β, with cDNA obtained from peripheral blood mononuclear cells of a healthy human donor stimulated with phorbol myristate acetate and calcium ionophore (Calbiochem, CN Biosciences, Inc., Darmstadt, Germany). Standard curves were constructed by plotting the log of standard dilutions vs the threshold cycle (CT) values, CT being the fractional cycle number at which the fluorescence passes a fixed threshold. The cytokine mRNA concentration in arterial samples was calculated referring the sample CT to the standard curve, and normalized with the corresponding value of the endogenous control CT. Values were expressed as relative units.

Immunohistochemical study

Serial 4–6-μm cryostat sections were obtained from frozen temporal arteries of the 74 patients and 15 controls described above. In all cases, one section was stained with haematoxylin and eosin to ensure that all samples included had a similar degree of histopathological involvement. The remaining sections were immunostained with the following antibodies: polyclonal rabbit anti-human TNFα (Genzyme, Minneapolis, MN), monoclonal mouse anti-human IL-1β (clone B1 from Genzyme), and monoclonal mouse anti-human IL-6 (clone 6708.111) from R&D Systems (Minneapolis, MN). Preliminary studies were carried out to determine the optimal concentration of the antibodies
providing the strongest specific staining with the lowest background. Anti-TNFα antiserum was used at a 1/500 dilution and purified monoclonal antibodies anti-IL-1β and anti-IL-6 were used at 10 μg/ml.

Temporal artery sections were air-dried and fixed with cold acetone. After several blocking steps, sections were incubated with the primary antibodies diluted in phosphate-buffered saline for 30 min. Immunodetection was carried out with an avidin–biotin–peroxidase system with the EnVision kit from Dako (Carpinteria, CA), according to the instructions of the manufacturer. Peroxidase activity was visualized by 0.02% 3–3′ diaminobenzidine (Sigma, St Louis, MO) and 0.05% hydrogen peroxide. All sections were slightly counterstained with Harris’ haematoxylin. In each procedure, previously immunostained arteries were included to ensure consistency and additional sections were incubated with just the secondary antibody and detection system to exclude non-specific binding.

Quantification was performed according to the following semiquantitative scores based on the percentage of cells positively stained at the granulomatous area at the intima–media junction: 0, no staining; 1, ≤25%; 2, 26–50%; 3, 51–75%; and 4, 76–100% (Fig. 1). After confirming agreement in the scoring system, cytokine expression in two sections per condition was independently evaluated by three investigators (JHR, AGM and MCC) who were blinded to the patients’ clinical information. Slides without optimal immunostaining quality were discarded and the final quantification was performed on 67 cases for IL-1β, 59 cases for TNFα and 54 cases for IL-6.

**Statistical analysis**

The Mann–Whitney U-test was applied to quantitative data. The χ²-test for trend was used for contingency tables and Pearson coefficient for correlations.

---

**Table 1.** IL-1β, TNFα and IL-6 mRNA levels (relative units) in temporal artery samples from GCA patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 11)</th>
<th>Patients (n = 36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>7.6 ± 3.3</td>
<td>12 ± 2.1</td>
<td>0.04</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.9 ± 0.2</td>
<td>5.1 ± 0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>13.4 ± 6.2</td>
<td>20.6 ± 5.5</td>
<td>0.36</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Immunostaining scoring system. Pictures represent scores for IL-1β expression. (A) Score 1 (≤ 25% positive cells) in a patient with no inflammatory parameters. (B) Score 2 (26–50% positive cells) in a patient with one inflammatory parameter. (C) Score 3 (51–75% positive cells) in a patient with three inflammatory parameters. (D) Score 4 (76–100% positive cells) in a patient with four inflammatory parameters.
the main cell type expressing pro-inflammatory cytokines. In some cases, particularly in those displaying the highest scores, smooth muscle cells, which have been demonstrated to be able to produce pro-inflammatory cytokines [22], also stained positive (Figs 1 and 2).

**Correlation between tissue production of pro-inflammatory cytokines and the intensity of the acute-phase response**

The amount of tissue cytokine mRNA was different in patients at the edges of the spectrum: patients with a strong systemic inflammatory reaction (3–4 inflammatory parameters) had more abundant IL-1β and IL-6 transcripts compared with those with no inflammatory parameters (Fig. 3). Differences in TNFα mRNA between both groups did not achieve statistical significance. Since cytokine mRNAs have instability sequences at the 3’ untranslated region and significant amounts of cytokine transcripts may not undergo translation into protein [10, 23], we subsequently evaluated protein expression by immunohistochemistry.

Patients with a strong systemic inflammatory response (3–4 inflammatory parameters) had significantly higher scores for all cytokines than patients with no inflammatory parameters (Figs 2 and 4A). When the whole series of patients including the complete spectrum of the intensity of the systemic inflammatory reaction was considered, patients with a strong acute-phase response (3–4 inflammatory parameters) had significantly higher scores for TNFα and for IL-6 than patients with a weak systemic inflammatory reaction (0–2 inflammatory parameters) (Fig. 4B). A similar
A trend was observed for IL-1β expression in the whole series but the difference was not significant. However, as shown in Fig. 4C, the percentage of patients displaying the maximum score for IL-1β increased significantly according to the number of inflammatory parameters.

**Correlation between cytokine expression and response to therapy**

Since we have previously shown that patients with an initial strong systemic inflammatory response have higher and longer
We investigated whether there was any correlation between cytokine expression in tissue at the moment of diagnosis and the subsequent duration of treatment. Thirty-one patients had a follow-up long enough to have attempted a prednisone dose reduction to less than 10 mg/day, according to current treatment schedules [7, 24]. In these patients, a significant correlation was found between TNFα transcripts and the time required to achieve a maintenance dose of prednisone less than 10 mg/day (Fig. 5). A similar trend was also observed for IL-1β, although it did not reach statistical significance (Fig. 5). A significant correlation was also found between TNFα mRNA and the cumulative prednisone dose ($r = 0.532$, $P = 0.002$). Among the 29 patients who completed a follow-up of 18 months, six patients experienced two or more relapses, whereas the remaining patients relapsed once or did not relapse at all. TNFα mRNA concentrations tended to be higher in the six patients with multiple relapses but the difference did not reach statistical significance (8.88 relative units, range 1.40–15.04, vs 4.27 relative units, range 0.60–11.37; $P = 0.095$), possibly owing to the low number of recurrent relapsers in this series.

**Discussion**

In this study we found that pro-inflammatory cytokines IL-1β, TNFα and IL-6 are remarkably expressed in temporal artery lesions from patients with GCA, particularly at the granulomatous areas. Although normal temporal arteries did not display significant immunostaining for pro-inflammatory cytokines, cytokine mRNA was detected in all specimens. Several reasons may account for the notable cytokine gene expression observed in histologically normal temporal arteries which, in contrast, did not contain significant amounts of cytokine protein, as assessed by immunohistochemistry. First, the surgical procedure itself is an injury and ligation of small tributaries or the main artery may convey ischaemia–reperfusion events. Both phenomena are well-known inducers of pro-inflammatory cytokine mRNAs [25, 26], but may not last long enough to allow their translation into proteins. Second, temporal artery specimens are bloody and contain vascularized surrounding connective tissue which may include activated peripheral blood mononuclear cells, given that normal temporal artery biopsies are often obtained from patients with inflammatory conditions in whom the diagnosis of GCA may be suspected [14, 27]. Cytokine production by activated circulating white blood cells may then be detected by highly sensitive PCR. Finally, pro-inflammatory cytokines are subjected to a tight post-transcriptional regulation [23]. Even if some baseline constitutive expression takes place in normal arteries, it may not necessarily be translated into a significant amount of protein. All these limitations must always be taken into account when evaluating cytokine mRNAs in temporal arteries.

Tissue expression of pro-inflammatory cytokines, both at the mRNA and at the protein level, were highly variable among patients, even those disclosing a similar degree of histopathological involvement, indicating variability among patients in the ability to produce cytokines. Previous studies addressing pro-inflammatory cytokine expression in GCA have studied only mRNA [14, 15] or have included a small number of cases focusing on the cellular and topographic distribution of cytokine expression with no attempts to correlate cytokine production with disease expression or outcome [16–18]. At the mRNA level, IL-1β and IL-6 were more abundant in patients with a strong systemic inflammatory response compared with those with no inflammatory parameters. At the protein level, IL-1β, TNFα and IL-6 expression was higher in patients with a strong systemic inflammatory reaction.

Depending on the way results were analysed, differences in cytokine expression were more significant for one cytokine with respect to the others. This is not surprising given that the final intensity of the systemic inflammatory response is determined by many factors. In addition to cytokine transcriptional and post-transcriptional regulation, proteasome-mediated cytokine

---

**Fig. 5.** Correlation between tissue mRNA levels of TNFα (A), IL-1β (B) and IL-6 (C), and the time required to reach a maintenance prednisone dose <10 mg/day.
turnover, cytokine processing by converting enzymes, cytokine receptor regulation, soluble cytokine receptors, decoy receptors and natural cytokine antagonists play in concert to determine the ultimate functional availability of a given cytokine [28, 29]. Moreover, pro-inflammatory cytokines produced in inflammatory lesions act in complex networks involving many cell types which, in turn, produce a second wave of inflammatory mediators [8, 9]. The responsiveness of target tissues, such as liver, bone marrow, hypothalamus and adipose tissue, to pro-inflammatory cytokines and to second-wave mediators may also determine the intensity of the acute-phase response [8, 9]. Finally the anatomical extent of inflammatory lesions in a typically segmental disease might also determine the net amount of pro-inflammatory cytokine production. In spite of such complexity, and the fact that we correlated a global response with data obtained from just a few millimetres of tissue, our findings suggest that the amount of locally produced pro-inflammatory cytokines in GCA lesions is a significant determinant of the intensity of the systemic inflammatory reaction in this disease.

In a previous study, we showed that patients with a strong systemic inflammatory response who had, indeed, elevated levels of circulating TNFα and IL-6 required higher corticosteroid doses and a longer duration of treatment [7]. In the present study we also found a correlation between tissue production of TNFα and, to a lesser extent, IL-1β and the time required to achieve a maintenance prednisone dose less than 10 mg/day, indicating that patients who are able to produce larger amounts of TNFα and IL-1β in lesions develop a long-lasting disease, more refractory to therapy. Corticosteroids are highly effective in controlling GCA clinical manifestations and in preventing ischaemic complications [1, 2]. However, corticosteroid requirements are highly variable among individuals. While some patients do remarkably well and easily achieve sustained remissions, other patients frequently relapse and require unacceptably high corticosteroid doses to control disease activity [7, 24, 30]. Our findings suggest that elevated tissue production of pro-inflammatory cytokines define a group of patients with an intense systemic inflammatory response who are more refractory to therapy. Whether persistent disease is determined by elevated levels of these cytokines or both phenomena are impelled by other factors remains to be elucidated, but an intense production of pro-inflammatory cytokines is likely to maintain inflammatory cascades leading to persistent clinical manifestations. The observation that IL-1 receptor antagonist knock-out mice develop large vessel vasculitis suggests that pro-inflammatory cytokines have a significant role in maintaining vessel inflammation [31].

Previous attempts to identify agents with corticosteroid-sparing effects on patients with GCA have led to conflicting results [32, 33]. Although not devoid of serious side-effects, anti-cytokine therapies have successfully expanded the therapeutic scope for several chronic inflammatory diseases. Our findings suggest that patients with GCA might potentially benefit from anti-cytokine therapies, particularly those with a strong systemic inflammatory reaction who, according to our results, produce higher levels of pro-inflammatory cytokines and are more refractory to conventional therapies.

Acknowledgements

We wish to thank I. Nayach, E. Gonzalvo and M. Maimar for technical support and Dr J. Casademont for advice on statistical analysis.

This work was supported by a grant from Fondo de Investigación Sanitaria (FIS 00/0689), Generalitat de Catalunya (2001/SGR/ 00379), ministerio de Ciencia y Tecnologia y Fondo Europeo de Desarrollo Regional (FEDER) (SAF 02-03307). J. Hernández-Rodriguez and M. J. Esteban were supported by a research award from Hospital Clinic and from Fundació Pedro M. C. Cid was a research award recipient from IDIBAPS. M. Sánchez was supported by Dako.

The results were partially presented at the 65th American College of Rheumatology Meeting, San Francisco, CA, November 2001, at the 10th ANCA and Vasculitis Workshop, Cleveland, OH, April 2002 and at the 66th American College of Rheumatology Meeting, New Orleans LA, October 2002.

References


