Pattern of Serum Cytokines in Patients With Rheumatoid Arthritis According to PPD Reactivity

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Introduction: We demonstrated, in a recently published study, far more PPD negative reactivity among patients who had RA (70%) than among controls (30%). To evaluate the hypothesis that different response to PPD in RA patients is associated with different profiles of serum cytokines, we compared the serum levels of IL-2, IL-4, IL-6, IL-10, TNF alpha, and IFN gamma from PPD negative and PPD positive RA patients. We also evaluated any correlations between serum cytokines and RA activity.

Material and methods: Forty RA patients and 21 controls were enrolled. Those with an induration <5mm were considered as negative and those with ≥5mm as positive PPD. Disease activity was calculated using DAS28. Plasma levels of cytokines were determined using the multiplex BD TM Cytometric Bead Array Kit Assay.

Results: Of the RA patients, 27 (67.5%) had negative reaction to PPD and 13 (32.5%) a positive reaction to PPD. There was no statistical difference in sex profile, age or activity index between both negative and positive PPD RA patients. There was no significant difference in the cytokines measured between PPD positive and PPD negative RA patients. Index activity showed a positive correlation with IFN gamma (r = -0.433; P = 0.005) and IL-6 (r = 0.325; P = 0.041) in RA patients.

Conclusions: Positive and negative tuberculin RA patients seem to show a similar cytokine serum profile.

Key words: Rheumatoid arthritis (RA). PPD. Cytokines.

Introduction: After the intradermic injection of purified protein derivative (PPD) also known as tuberculin, antigen-specific T cells are activated and secrete cytokines that mediate a
hypersensitivity reaction, including tumoral necrosis factor (TNF)–α, interferon (IFN)–γ, and lymphotixin (TNF–β), among others. Patients with rheumatoid arthritis (RA) are known to present attenuated delayed type hypersensitivity responses and a reduced lymphocyte proliferation to universal antigens. In a recent study we demonstrated that negative reactivity to PPD is much larger in patients with RA (70%), compared to controls (30%) and the general population (32%).

Serum Cytokine Determination

The blood sample of each participant was centrifuged during 10 minutes at 1000 g. Serum sample aliquots were frozen at –80°C immediately after collection of the sample. Afterwards, serum concentrations of IL–2, IL–4, IL–6, IL–10, IFN–γ, and TNF–α were measured using a flow cytometry technique that employed the Multiplex BD Cytometric Bead Array (CBA) kit.

Material and Methods

Patients and Controls

The study was carried out in the Department of Rheumatology and Molecular Biology of the Hospital Nacional Guillermo Almenara, Lima Peru. Peripheral blood samples of 40 patients with RA were studied, 27 of them with negative reactivity to PPD. As a reference group, 21 samples were obtained from healthy individuals, hospital workers, without any concomitant illness or immunosuppressive treatment. Patients with rheumatoid arthritis (RA) were included, with a mean age of 50.33±10.2 years, 92.5% were women, 70% with active disease according to DAS 28, and 92.5% were women. Of the 40 patients with RA, 27 (67.5%) presented negative reactivity to PPD and 13 (32.5%) positive reactivity. There was no significant difference between these 2 groups with respect to age (50.8±10.6 vs 49.3±9.15; P=.66), female gender (92.6 vs 92.3%; P=.97), or disease activity (66.6 vs 76.9%; P=.71). In patients with RA there were no significant differences in the serum concentrations of cytokines among the groups with positive and negative PPD reactive. The serum concentrations of IL–6 were significantly larger in patients with RA (P=.042) and negative PPD (P=.002) compared to controls (Table 1). In patients with RA, the group of active disease had larger serum concentrations of IFN–γ.

Results

Forty patients with RA were included, with a mean age of 50.33±10.2 years, 92.5% were women, 70% with active disease according to DAS 28, and 21 control individuals with a mean age of 44.62±9.68 years, 92.6 vs 92.3%; P=.97), or disease activity (66.6 vs 76.9%; P=.71). In patients with RA there were no significant differences in the serum concentrations of cytokines among the groups with positive and negative PPD reactive. The serum concentrations of IL–6 were significantly larger in patients with RA (P=.042) and negative PPD (P=.002) compared to controls (Table 1). In patients with RA, the group of active disease had larger serum concentrations of IFN–γ.
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(7.375±3.78 vs 3.263±3.02; \(P=0.05\)) and IL-6 (18.106±22.96 vs 9.513±10.86; \(P=0.09\)) compared to the control group, though this last difference was not statistically significant. There were no differences found in the serum concentrations of TNF-\(\alpha\), IL-2, IL-4, and IL10 between these 2 groups.

When compared to the control group, the group with active RA had larger concentrations of IL-6 (\(P=0.001\)) and lesser of IL-4 (\(P=0.03\)) compared to the control group; nonetheless, there were no differences in the concentrations of IL-6 and IL-4 in the group with inactive RA compared to the control group (Table 2). In the correlation studies between serum cytokines and disease activity according to DAS 28 (Table 3), only one positive correlation was observed, that between IFN-\(\gamma\) (\(r=0.433; \(P=0.005\)) and IL-6 (\(r=0.325; \(P=0.041\))) in patients with RA (see figure).

**Discussion**

In a previous study,\(^6\) we showed a high rate of negative reactivity to PPD in patients with RA (70%) compared to controls (30%). This high rate of negativity to PPD cannot be explained by a particular serum cytokine profile because in this study the serum concentrations of the studied cytokines were similar in patients with RA, both in the group with negative reactivity and in the group with a positive PPD. Although no previous studies have analyzed the profile of serum cytokines in patients with RA related to the PPD reactivity in vivo, some in vitro studies have pretended to study the mechanisms involved in the deficient proliferative response. It is a known that delayed type skin hypersensitivity in vivo\(^5,6\) and T cell proliferation to memorized antigens,\(^10\) by T lymphocytes of the rheumatoid synovial membrane is

<table>
<thead>
<tr>
<th>TABLE 1. Serum Concentrations of Cytokines in Patients With Rheumatoid Arthritis (RA) According to Their PPD Reactivity*</th>
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<tbody>
<tr>
<td>RA PPD (+), N=13</td>
</tr>
<tr>
<td>Mean±SD</td>
</tr>
<tr>
<td>INF-(\gamma)</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
</tr>
<tr>
<td>IL-2</td>
</tr>
<tr>
<td>IL-4</td>
</tr>
<tr>
<td>IL-6</td>
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<tr>
<td>IL-10</td>
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*SD indicates standard deviation; IFN-\(\gamma\), interferon-gamma; IL, interleukin; N, number of test sera; TNF-\(\alpha\), tumor necrosis factor-alpha.

†Calculated using Mann-Whitney test.

Serum concentrations are expressed in pg/mL.

<table>
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<tr>
<th>TABLE 2. Serum Concentrations of Cytokines in Patients With Rheumatoid Arthritis (RA) According to Disease Activity*</th>
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<tbody>
<tr>
<td>Active RA, N=28</td>
</tr>
<tr>
<td>Media±SD</td>
</tr>
<tr>
<td>INF-(\gamma)</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
</tr>
<tr>
<td>IL-2</td>
</tr>
<tr>
<td>IL-4</td>
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<tr>
<td>IL-10</td>
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</tbody>
</table>

*SD indicates standard deviation; IFN-\(\gamma\), interferon-gamma; IL, interleukin; N, number of test sera; TNF-\(\alpha\), tumor necrosis factor-alpha.

†Calculated using Mann-Whitney test.

Serum concentrations are expressed in pg/mL. Activity measured using DAS 28.
diminished when compared to control subjects. The mechanisms implied in this reduced response is unknown, though several have been proposed, including the participation of TNF-α and IL-10. Corrigal et al11 proposed that the deficient proliferative response to PPD by peripheral blood T cells in RA was the result of a relatively high proportion of secreted IL-10 versus IL-2, more so that the absolute quantity of IL-2 produced. Katsikis et al12 were able to demonstrate that IL-10 had a negative regulatory effect, because the addition of neutralizing anti-IL-10 antibodies to rheumatoid synovial membrane explants in vitro led to an increase in the production of cytokines as well as an increase in the proliferation of T cells. Yudoh et al13 concluded that in RA, the reduced presence of the CD4+ T cell subgroup that produces IL-10 could be responsible for the predominance of Th1 cells over Th2 in sites of synovial inflammation and in peripheral blood. Additional mechanisms that result in a deficient proliferation of T cells after exposure to antigen include the chronic exposure to TNF-α and the production of type 2 cytokines such as IL-10. Contrary to the results of studies done in vitro, in many of them using myotogens as T cell activity triggers, we did not find increased serum concentrations of IL-2, IL-10, TNF-α or IFN-γ in our patients with RA with negative PPD reactivity. Though the explanation for these apparent discrepancies is not clear, it is probable that they are owed to differences in culture and isolation techniques, as well as different stimuli used. Resides, it must be taken into account that the stimulated production of cytokines does not necessary concur with the status of cytokines in vivo. It is important to remember that it is the first study in the literature to determine the serum cytokine concentration in vivo in a spontaneous state, without employing myotogens as activators of mononuclear cells, in a patient with a diminished response to PPD. This study shows that there is no predominance of TNF-α, IL-10, and IFN-γ in peripheral blood of patients with RA compared to controls. These findings call attention because we would expect that the high Th1 cell activity in synovium, which leads to macrophage activations and subsequent inflammation, should be also found in the periphery.

Table 3. Correlation Between Serum Cytokines Concentrations and Rheumatoid Arthritis Activity (RA) (%292)*

<table>
<thead>
<tr>
<th>Activity</th>
<th>INF-γ</th>
<th>TNF-α</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
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<tbody>
<tr>
<td>r (Spearman)</td>
<td>0.325</td>
<td>0.433</td>
<td>0.003</td>
<td>0.047</td>
<td>0.303</td>
<td>0.038</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*INF-γ indicates interferon-gamma; IL, interleukin; N, number of test sera; TNF-α, tumor necrosis factor-alpha; Activity measured using DAS 28.
†The significant correlation for alpha =0.01 (bilateral).
‡The significant correlation for alpha =0.05 (bilateral).

Serum concentrations of IL-6 (A) and INF-γ (B) were the only cytokines that showed a positive correlation with RA activity. RA indicates rheumatoid arthritis; INF, interferon-gamma; IL, interleukin.
These differences can be explained by the selective migration of Th1 cells from peripheral blood to the swollen joint and, as a consequence, a reduction in the cells that produce this cytokine in peripheral blood. In this study, findings of previous studies were the role of IL-6 in RA is proven are reinforced, because increased serum concentrations were found in patients with active disease compared to inactive RA (P<0.09) and control subjects (P<0.001). In a study done by Gratacos et al., increased serum concentrations of IL-6 and TNF-α in patients with RA were found, compared to patients with ankylosing spondylitis and non-inflammatory back pain. The production of IL-6 promotes differentiation of B cells and their development into antibody secreting cells. In fact, high concentrations of IL-6 correlate with high concentrations of rheumatoid factor. Moreover, IL-6 promotes bone resorption and can play an important role in periarticular osteoporosis characteristic of early RA. Apart from that, it induces the differentiation of B cells, activates T cells and induces the synthesis of acute phase reactant proteins in liver cells. IL-6 serum concentrations in our patients is highly correlated with the level of disease activity (P<0.041), as it is in other studies where there is a correlation of Il-6 with the levels of C reactive protein, reactant proteins in liver cells.

References