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 all the cytokines measured between PPD positive and PPD negative RA patients. Index activity show a positive correlation with IFN gamma (r =0.433; P=.005) and IL-6 (r=0.325; P=.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.

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Patrón de citocinas séricas en pacientes con artritis reumatoide de acuerdo a su reactividad al PPD

Introducción: En un reciente estudio, se demostró una alta reactividad negativa al PPD o tuberculina en pacientes con artritis reumatoide (AR) (70%) comparado con controles (30%). Para determinar si esta alta reactividad negativa al PPD está asociada con un determinado patrón de citocinas, se compararon las concentraciones séricas de interleucina (IL)-2, IL-4, IL-6, IL-10, factor de necrosis tumoral (TNF)-α e interferón (IFN)-γ en pacientes con AR con reactividad positiva y negativa al PPD. Se analizó también la correlación entre las citocinas y la actividad de la AR.
Material y métodos: Se estudiaron 40 pacientes con AR y 21 individuos sanos. Se consideró reactividad positiva al PPD a una induración ≥ 5mm y reactividad negativa cuando es < 5 mm. La actividad de la AR se determinó según el DAS28. Las citocinas se determinaron por citometría de flujo utilizando el Kit Multiplex Cytometric Bead Array.
Resultados: De los pacientes con AR, 27 (67,5%) presentaron reactividad negativa al PPD y 13 (32,5%) reactividad positiva al PPD, similares en edad, sexo femenino y enfermedad activa. No se encontraron diferencias significativas en las citocinas entre los grupos con PPD positivo y PPD negativo. El IFN-γ (r = 0,433; p = 0,005) y la IL-6 (r = 0,325; p = 0,041) fueron las únicas que mostraron correlación positiva con la actividad de la enfermedad.
Conclusiones: No parece que haya diferencias en el patrón de citocinas séricas en los pacientes con reactividad negativa y positiva al PPD.

Palabras claves: Artritis reumatoide (AR). PPD. Citocinas.

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 lymphotoxin (TNF-β), among others.[1] Patients with rheumatoid arthritis (RA) are known to present attenuated delayed type hypersensitivity responses and a reduced lymphocyte proliferation to universal antigens.[2-5] 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%),[6] responses that could be related to different patterns and serum concentrations of cytokines. The exact mechanism of this attenuated response to in vitro PPD by the mononuclear cells in peripheral blood of patients with RA is not completely defined, though some have been proposed, such as the participation of some cytokines, including interleukin 10 (IL)-107, IL-23, and the chronic exposure to TNF-α.[8] The fundamental objective of this study is to compare the pattern of serum cytokines in patients with RA and to stratify them according to their reactivity to PPD. Secondary objectives were to compare the serum concentrations of cytokines among patients with active and inactive RA and to determine if there was any correlation with the activity of the disease and the serum concentration of cytokines.

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.

Clinical and Laboratory Research

In patients with RA we collected the following information: duration of disease, number of painful and swollen joints (28 joint count), morning stiffness, global disease activity evaluation by the patient, and erythrocyte sedimentation rate (ESR). The activity of RA was evaluated according to the Disease Activity Score in 28 joints (DAS 28). Patients with a score of >2.6 were considered active. All patients received low dose prednisone (<7.5 mg/day) and methotrexate between 7.5 and 15 mg/weekly.

PPD Injection

Using the Mantoux technique, 5 units of tuberculin were applied to all of the participants and the skin reaction was measured at 72 hours. The cutpoint to determine positive reaction to PPD was base on the American Thorax Society's guidelines.[9] A positive reaction was considered as ≥5 mm induration for patients with RA and ≥10 mm in controls. A negative PPD reaction was considered when it was <5 mm, both in RA patients as in controls.

Serum Cytokine Determination

The blood simple 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 flux cytometry technique that employed the Multiplex BD Cytometric Bead Array (CBA) kit.

Statistical Analysis

A univariate analysis was done initially with a central tendency measurement calculation and dispersion for the quantitative variables, and frequency distribution for the qualitative variables. Comparisons between groups were done using Mann-Whitney's U test, and the relationship between the cytokine concentrations through the Spearman correlation coefficient. For the comparison of qualitative variables an exact Fishers test or χ^2 was done. A value of $P<.05$ was considered significant. The analysis was done using the SPSS version 11.0 software package and the serum concentration of cytokines were expressed as their mean values (pg/mL ± standard deviation [SD]).

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 and 90.5% 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±410.8 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

TABLE 1. Serum Concentrations of Cytokines in Patients With Rheumatoid Arthritis (RA) According to Their PPD Reactivity*

	RA PPD (+), N=13	RA PPD (–), N=27	Control, N=21	Statistical Difference†		
	Mean±SD	Mean±SD	Mean±SD	RA PPD (+) Versus RA PPD (–)	RA PPD (+) Versus Control	RA PPD (–) Versus Control
INF-γ	5.04±3.6893	5.49±8.9112	4.30±3.47	0.385	0.218	0.755
TNF-α	0.73±1.0349	1.31±1.4575	1.14±1.19	0.322	0.658	0.489
IL-2	1.19±1.4534	2.22±1.6312	2.27±1.27	0.071	0.129	1.000
IL-4	2.50±1.8921	2.72±2.3310	3.74±2.23	0.794	0.209	0.306
IL-6	11.1±10.4219	12.90±18.1146	11.04±35.5	0.891	0.042	0.002
IL-10	2.17±2.6960	1.97±2.2394	2.25±1.72	0.943	0.828	0.763

*SD indicates standard deviation; IFN-γ, interferon-gamma; IL, interleukin; N, number of test sera; TNF-α, tumor necrosis factor-alpha.
†Calculated using Mann-Whitney test.
Serum concentrations are expressed in pg/mL.

TABLE 2. Serum Concentrations of Cytokines in Patients With Rheumatoid Arthritis (RA) According to Disease Activity*

	Active RA, N=28	Inactive RA, N=12	Control, N=21	Statistical Differences*		
	Media±SD	Media±SD	Media±SD	Active RA Versus Inactive RA	Active RA Versus Control	Inactive RA Versus Control
INF-γ	7.375±3.78	3.263±3.02	4.30±3.47	0.050	0.393	0.197
TNF-α	1.425±1.604	1.438±0.94	1.14±1.19	0.673	0.659	0.969
IL-2	2.906±1.43	1.588±1.33	2.27±1.27	0.600	0.470	0.181
IL-4	3.063±2.28	3.725±2.48	3.74±2.23	0.295	0.036	0.586
IL-6	18.106±22.96	9.513±10.86	11.04±35.5	0.092	0.001	0.129
IL-10	3.631±2.331	3.35±1.72	2.25±1.72	0.562	0.409	0.835

*SD indicates standard deviation; IFN-γ, interferon-gamma; IL, interleukin; N, number of test sera; TNF-α, tumor necrosis factor-alpha.
†Calculated using Mann-Whitney test.
Serum concentrations are expressed in pg/mL.
Activity measured using DAS 28.

(7.375±3.78 vs 3.263±3.02; *P*=.05) and IL-6 (18.106±22.96 vs 9.513±10.86; *P*=.09) compared to the group of inactive patients, though this last difference was not statistically significant. There were no differences found in the serum concentrations of TNF-α, 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*=.001) and lesser of IL-4 (*P*=.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-γ (*r*=0.433; *P*=.005) and IL-6 (*r*=0.325; *P*=.041) in patients with RA (see figure).

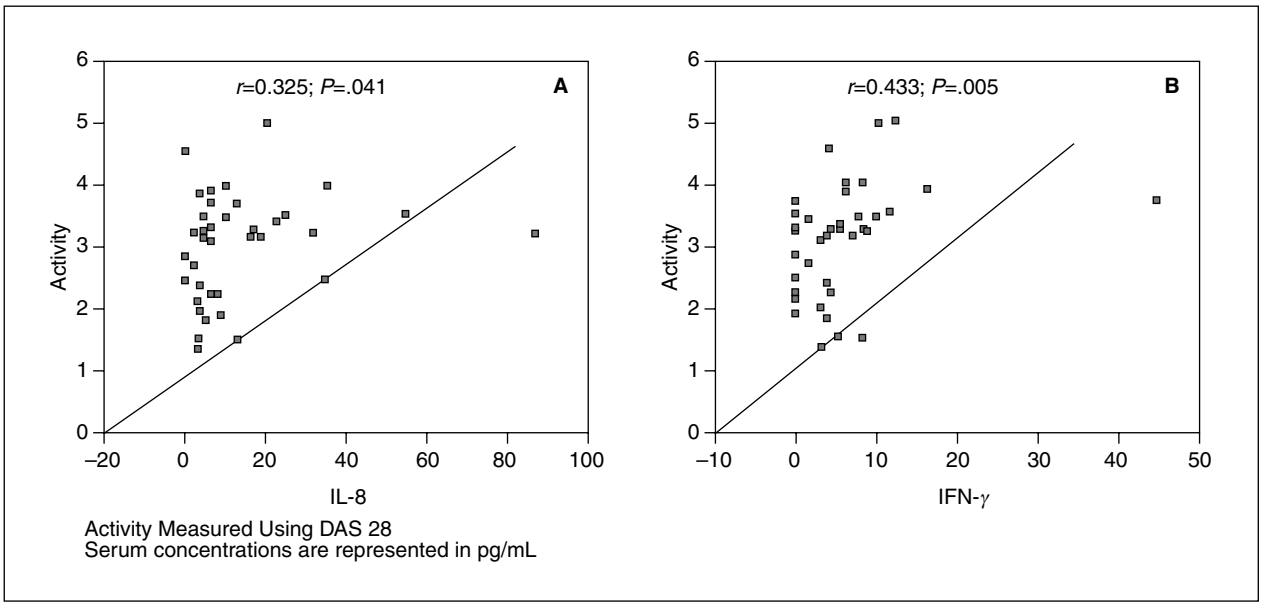
Discussion

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 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 hipersensitivity in vivo[5,6] and T cell proliferation to memorized antigens,[10] by T lymphocytes of the rheumatoid synovial membrane is

TABLE 3. Correlation Between Serum Cytokines Concentrations and Rheumatoid Arthritis Activity (RA) (N=40)*

	INF-γ	TNF-α	IL-2	IL-4	IL-6	IL-10	
Activity	*r* (Spearman)	0.433†	0.003	0.047	0.325‡	0.038	0.138
	P	.005	.987	.774	.041	.818	.397

*IFN-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 IFN-γ (B) were the only cytokines that showed a positive correlation with RA activity. RA indicates rheumatoid arthritis; IFN, interferon-gamma; IL, interleukin.

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 al[11] 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 al[12] were able to demonstrate that IL-10 had a negative regulatory effect, because the addition of neutralizing anti-Il-10 antibodies to rheumatoid synovial membrana 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 al[13] concluded that in RA, the reduced presence of the CD4+ T cell subgroup that produces IL-10 could be responsible for the predominant 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[8] or the production of type 2 cytokines such as IL-10. Contrary to the results of studies done in vitro, in many of them using mytogens 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 necessarily 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 mytogens as activators of mononuclear cells, in patients with a diminished response to PPD. This study shows that there is no predominance of TNF-α, IL-1, 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.

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.[14] 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=.09) and control subjects (P=.001). In a study done by Gratacós et al,[15] increased serum concentrations of IL-6 and TNF-α in patients with RA were found, compared to patients with ankylosing spondilytis 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.[16] 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.[17] IL-6 serum concentrations in our patients is highly correlated with the level of disease activity (P=.041), as it is in other studies where there is a correlation of Il-6 with the levels of C reactive protein, an indicator of activity in RA.[18] The lack of an increase of TNF-α in our patients with RA, especially if they are active with respect to controls is puzzling. It could be due to differences in medication or even to genetic polymorphisms that control TNF-α production. Nor should the fact that it might be a characteristic of this cytokine in its mechanism of action or an error due to sample size be dismissed. In conclusion, in light of the findings, there does not seem to be a difference in the pattern of serum cytokines in patients with RA according to their PPD reactivity.

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