Effects of Dexamethasone on Lymphocyte Proliferation and Cytokine Production in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** We evaluated the pattern of dexamethasone mediated inhibition of concanavalin-A (Con-A) stimulated peripheral blood mononuclear cell (PBMC) proliferation to classify patients with rheumatoid arthritis (RA) as corticosteroid resistant (CR) or sensitive (CS). We also studied the role of T helper 1, (Th1) and Th2 cytokines in the mechanism of glucocorticoid resistance in RA.

Methods. PBMC from 21 healthy controls and 15 patients with RA were isolated and cultured for the *in vitro* glucocorticoid sensitivity assay. Basal and Con-A stimulated PBMC proliferation levels and the inhibitory effect of different doses (10^{-8}, 10^{-6}, 10^{-4} M) of dexamethasone (Dex) were evaluated. The IC_{50} was defined as the concentration of Dex that caused 50% inhibition of cell proliferation and subjects with an $IC_{50} > 10^{-6}$ M were considered to be CR. The supernatants were collected for cytokine [interleukin 4 (IL-4), IL-6, IL-10, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ)] measurement by ELISA.

Results. We observed lymphocyte proliferation after Con-A stimulation, which was inhibited by Dex in a dose-dependent manner in both groups. Two of 21 controls (9.5%) and 7/15 RA patients (53.3%) were CR ($p < 0.01$). Basal IL-4, IL-6, IL-10, and TNF-α levels were similar for both groups; however, basal IFN-γ levels were slightly higher in patients with RA compared to controls. Con-A stimulation did not increase IL-4 or IL-6 levels compared to basal production but significantly increased IL-10 levels. IL-6 and IL-10 levels were significantly inhibited by Dex 10^{-6} M in both the control and RA groups. Con-A stimulation significantly increased TNF-α and IFN-γ levels compared to the basal condition in the control and RA groups, and both cytokines were inhibited only by higher doses of Dex in the RA group.

Conclusion. These findings might reflect a predominance of Th1 cells in RA that might contribute to corticosteroid resistance in patients in RA. (J Rheumatol 2002:29:46–51)

Key Indexing Terms:
RHEUMATOID ARTHRITIS CYTOKINES Th1/Th2
DEXAMETHASONE LYMPHOCYTE PROLIFERATION

Glucocorticoids (GC) have been used therapeutically as potent immunosuppressive and antiinflammatory agents for a broad spectrum of diseases, including autoimmune and allergic inflammatory diseases and organ transplant rejection[1]. Although most patients respond to GC therapy, a few subpopulations of individuals fail to respond to the therapeutic effects of this class of medication, and can be classified as corticosteroid resistant (CR) or corticosteroid sensitive (CS)[2,3]. An understanding of the basis for corticosteroid resistance may provide insights into the molecular mechanisms involved in the mechanism of the antiinflammatory action of GC and in chronic inflammatory processes such as rheumatoid arthritis (RA)[4]. Studies have described diminished GC receptor numbers and binding affinity in peripheral blood mononuclear cells (PBMC) from patients with RA[5]. However, differences in either affinity or number of GC receptors seem insufficient to account for the observed steroid resistance. The response of the disease to GC therapy depends also on post-receptor mechanisms and interactions of the GC receptors with DNA hormone responsive elements[6] and other nuclear factors, such as AP-1[7] and nuclear factor-κB (NF-κB)[8], and these mechanisms play an important role in the immune response.

The etiology of RA remains elusive. The predominance of RA in women may be attributable to hormonal factors, and there is a clear genetic contribution to this disease within the HLA class II locus (DR1 and DR4 disease susceptible haplotypes). This epitope supports the concept that T lymphocyte recognition is important at some stage in the pathogenesis of RA, either in shaping the T cell receptor repertoire or in the presentation of an inducing microbial or autoantigenic pep-

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tide. Recently, other candidate genes including cytokine polymorphisms have been investigated[9]. Cytokines have been shown to play a key role in mediating communication between the endocrine and immune systems, modulating the sensitivity of immune cells to GC[10-12]. GC not only suppress cytokine production, but also induce the production and release of several cytokines[13-15], or enhance their actions, modulating the efficacy of immune reactions by upregulation of cytokine receptors[9].

We hypothesized that cytokines could differentially regulate the sensitivity of PBMC to GC in patients with RA. We evaluated the pattern of dexamethasone mediated inhibition of concanavalin-A (Con-A) stimulated PBMC proliferation to classify steroid sensitive or resistant patients with RA. Also, to evaluate the role of Th type 1 [tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)] and Th type 2 [interleukin 6 (IL-6), IL-10] cytokines in the mechanism of glucocorticoid resistance in patients with RA, we studied the effect of different dexamethasone doses on these cytokine secretions by Con-A stimulated PBMC in culture.

MATERIALS AND METHODS

Subjects. We studied 21 healthy volunteers (12 women and 9 men) ranging in age from 24 to 53 years, with no signs of acute or chronic illness and taking no drugs. We also studied 15 patients (11 women and 4 men) who fulfilled the American College of Rheumatology revised criteria for RA (Table 1). Mean [± standard deviation (SD)] age was 50.3 ± 14.6 years (range 32 to 79). Mean (± SD) disease duration was 6.8 ± 7.1 years (range 3 mo to 26 yrs). All patients had active disease and some were taking nonsteroidal antiinflammatory drugs, but no patient was or had been taking GC, chloroquine, or methotrexate for at least 18 months.

Cell preparation. Blood samples were obtained from healthy donors and patients with RA between 8:00 and 9:00 AM. PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (Histopaque, Sigma Chemical Co., St. Louis, MO, USA), washed 3 times in Hanks' buffered saline solution, and resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 2 mM HEPES buffer (Sigma), 10% fetal calf serum, 100 IU/ml penicillin, 100 g/ml streptomycin, and 10 mg/ml gentamicin.

Proliferation and in vitro corticosteroid sensitivity assay. To perform the *in vitro* CS sensitivity assay we measured the inhibitory effect of dexamethasone on Con-A stimulated PBMC proliferation. PBMC (2×10^{-6} cells/well) were plated onto 96 well flat bottom plates (Nunc, Kamstrup, Denmark) and cultured at 37°C in the presence of 5% CO_2. Con-A at the dose of 50 $\mu g/ml^{-1}$ (established in preliminary studies) was used to stimulate the cells in the presence or absence of different doses (10^{-8}, 10^{-6}, 10^{-4} M) of dexamethasone (Dex). Dex was dissolved in ethanol at an initial concentration of 10^{-3} M and serially diluted to the working concentrations with RPMI. After 48 h of culture, the cells were pulsed with 1 μCi/well tritiated thymidine (^{3}H-thymidine, Amersham, Pharmacia Biotech, Little Chalfont, UK) for 18 h prior to collecting the supernatants for cytokine quantification. The cells were harvested with a multiple automated sample harvester and radioactivity was counted in a liquid scintillation ß counter (Beckman, Fullerton, CA, USA).

Percentage inhibition of proliferation by corticosteroid was calculated using the formula:

$$\{1 - (x - n/y - n)\}\ 100$$

where: x = counts in Dex and Con-A, n = counts in RPMI alone, and y = counts in Con-A alone. The IC_{50} was defined as the concentration of Dex that caused a 50% inhibition of cell proliferation. By using this calculation, the variations in Con-A induced PBMC proliferation occurring between and within individuals at different times were compensated for. We also made a linear regression analysis that clearly correlated with the calculated IC_{50}. Subjects with an $IC_{50} > 10^{-6}$ M were considered to be corticosteroid resistant.

Cytokine quantification. The supernatants collected during cell culture were stored at –70°C for measurement of IL-4, IL-6, IL-10, TNF-α, and IFN-γ levels by ELISA. The antibodies used for coating 96 well plates were 8D4-8 (anti-IL-4, PharMingen, San Diego, CA, USA), 18891D (anti-IFN-γ, PharMingen), JES3-19F1 (anti-IL-10, PharMingen), MAB206 (anti-IL-6, R&D Systems, Minneapolis, MN, USA), and MAB610 (anti-TNF-α, R&D Systems). Second step biotinylated detection monoclonal antibodies were 25D2 (anti-IL-4, PharMingen), 19751N (anti-IFN-γ, PharMingen), JES3-12G8 (anti-IL-10, PharMingen), BAF206 (anti-IL-6, R&D Systems), BAF210 (anti-TNF-α, R&D Systems). The minimum detection limits for IL-4, IFN-γ, IL-10, IL-6, and TNF-α were 54, 78, 78, 3.9, 7.8 pg/ml, respectively.

Statistical analysis. All results are expressed as the mean ± SD. Data were compared using the Wilcoxon Mann-Whitney 2 tailed U test and 2 tailed Fisher exact test, with the level of significance set at $p < 0.05$.

Table 1. Clinical and laboratory data.

Criterion	No. Patients (%)
Morning stiffness	11/15 (73)
Arthritis of 3 or more joint areas	15/15 (100)
Symmetric arthritis	15/15 (100)
Rheumatoid nodules	0/15 (0)
Serum rheumatoid factor	7/15 (46)
Radiographic changes	12/15 (80)

Definition of RA: ≥ 4 of above 6 criteria, Criteria 1–4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses were not excluded.

RESULTS

Clinical and laboratory findings. Table 1 presents the clinical and laboratory findings for RA patients according to the American College of Rheumatology revised criteria.

Proliferation assays and sensitive or resistant phenotypes. These experiments were performed to evaluate the effects of Con-A on lymphocyte proliferation and the effects of several doses of Dex on the inhibition of Con-A stimulated PBMC proliferation in healthy controls and patients with RA. There was lymphocyte proliferation after Con-A stimulation in both the control (788 ± 115 vs 23682 ± 3015 cpm) and RA (794 ± 147 vs 21640 ± 4049 cpm) groups. Dex (10^{-8}, 10^{-6}, 10^{-4} M) inhibited lymphocyte proliferation in a dose-dependent manner in the control (18197 ± 2572; 7905 ± l459; 7686 ± 1759 cpm) and RA (15581 ± 2840; 11876 ± 2666; 6630 ± 1675 cpm) groups (Figure 1). Using the IC_{50} to classify CR or CS patients, we observed that the control group presented an IC_{50} of 10^{-6} M, while RA patients presented an IC_{50} of 10^{-4} M. The individual analysis showed that 2/21 (9.5%) controls and 7/15 (46.7%) patients with RA were CR. There was a significant difference in corticosteroid resistance between the control and RA groups ($p < 0.01$).

We analyzed the Con-A response of PBMC in the 2 RA subpopulations: corticosteroid sensitive (CS) and corticosteroid resistant (CR). We observed that there was no differ-

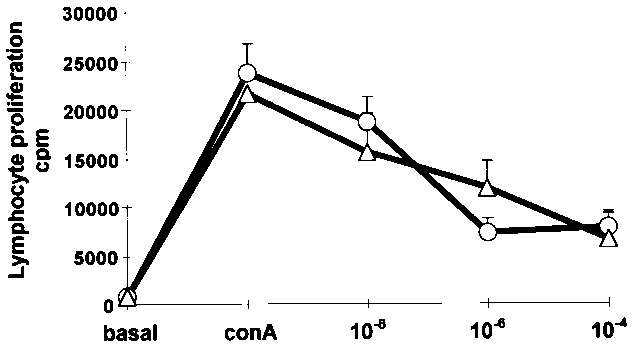

Figure 1. Lymphocyte proliferation after PBMC incubation in RPMI medium (basal), or RPMI with concanavalin-A (Con-A), or RPMI with Con-A plus different doses of dexamethasone (10^{-8}, 10^{-6}, and 10^{-4} M), in healthy controls (○) and patients with RA (△).

ence in the basal condition between CS and CR groups (758 ± 218 vs 836 ± 209 cpm; p = 0.61). However, after Con-A stimulation there was a significant increase in PBMC proliferation in CR compared to CS (29340 ± 7333 vs 14380 ± 2239 cpm; p = 0.04). The same pattern was also observed after treatment with Dex 10^{-8} or Dex 10^{-6} M (p = 0.005 and p = 0.003, respectively).

Cytokines. Cytokine production levels (pg/ml) were measured in the supernatant collected from fresh cultured PBMC in the basal condition and after stimulation with Con-A alone or Con-A plus different doses of Dex for control and CS and CR rheumatoid arthritis patient groups. The data are presented in Table 2. IL-4 production was undetectable in the basal condition and after Dex treatments in both groups (data not shown). There was no difference in IL-6, IL-10, or TNF-α production in the basal condition between the control and RA patient groups. However, RA patients had slightly higher basal IFN-γ levels than the control group (p = 0.07).

Figure 2 illustrates the T helper 1 (Th1) cytokine (TNF-α and IFN-γ) pattern, and Figure 3 the T helper 2 (Th2) cytokine (IL-6 and IL-10) pattern evaluated in the supernatant of the fresh cultured PBMC in the basal condition and after stimulation with Con-A alone or with Con-A plus different doses of Dex for control and RA groups. After Con-A stimulation,

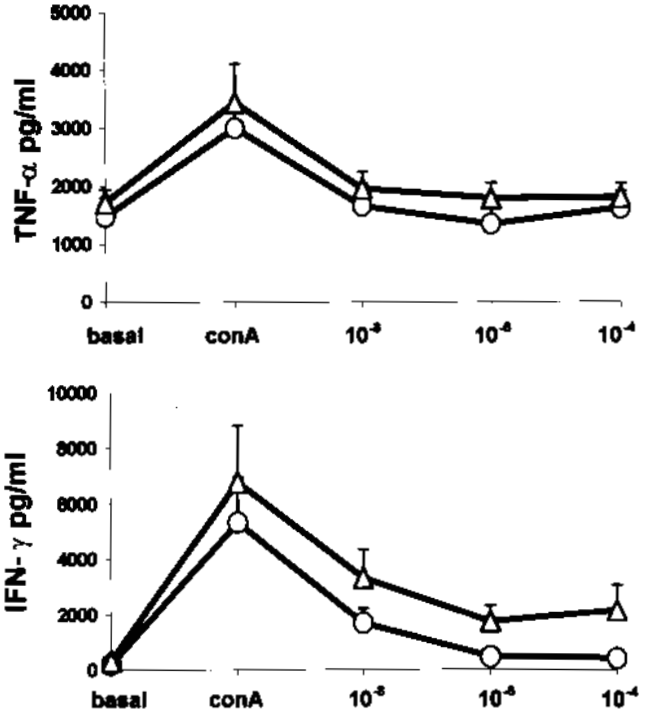

Figure 2. Th1 pattern of cytokine production (TNF-α and IFN-γ) after PBMC incubation in RPMI medium (basal), RPMI with concanavalin-A (Con-A), or RPMI with Con-A plus different doses of dexamethasone (10^{-8}, 10^{-6}, and 10^{-4} M) in healthy controls (○) and patients with RA (△).

TNF-α levels were significantly higher than in the basal condition in both the control (p = 0.01) and RA groups (p = 0.02). After Con-A stimulation, TNF-α was 50% inhibited by a low Dex dose (10^{-8} M) in the control group (p = 0.01) but only by a higher Dex dose (10^{-6} M) in the RA group (p = 0.04). Con-A stimulation increased IFN-γ secretion in both the control (p = 0.001) and RA (p < 0.0001) groups. IFN-γ was 50% inhibited by 10^{-6} M Dex after Con-A stimulation in the control group (p = 0.03) but only by 10^{-4} M Dex in the RA group (p = 0.01). IL-6 was not increased after Con-A stimulation compared to basal production; however, basal IL-6 levels were significantly inhibited by 10^{-6} M Dex in the control (p = 0.04)

Table 2. Cytokine levels (pg/ml) in the supernatant of lymphocyte cultures after 48 h incubation in RPMI (basal) or RPMI with concanavalin-A (Con-A) or Con-A plus different doses of dexamethasone (Dex 10^{-8}, Dex 10^{-6}, Dex 10^{-4} M) in healthy controls (C) and patients with corticosteroid sensitive (CS) and corticosteroid resistant (CR) rheumatoid arthritis.

	Basal			Con-A			Dex 10^{-8} M			Dex 10^{-6} M			Dex 10^{-4} M		
	C	CS	CR	C	CS	CR	C	CS	CR	C	CS	CR	C	CS	CR
IL-6	3584	2705	3707	4135	2797	3961	3290	1967	3372	2178	866	2477	1855	466	1957
	±453	±125	±284	±511	±133	±233	±396	±534	±298	±417	±325	±321	±331	±152	±446
IL-10	1036	1663	1207	10859	9858	13630	7715	7886	7872	4292	6010	7164	2929	2984	3215
	±279	±709	±485	±1243	±3139	±813	±1023	±2269	±955	±801	±2429	±1615	±415	±1059	±735
TNF-α	1473	1497	1808	3011	2226	3610	1666	1739	2061	1355	1441	1945	1629	1416	1955
	±330	±325	±285	±293	±1110	±777	±378	±530	±364	±326	±394	±328	±283	±255	±302
IFN-γ	203	376	250	5314	4903	8916	1664	1542	5312	469	1014	2534	402	586	3904
	±33	±65	±69	±1625	±2689	±3112	±543	±593	±1905	±117	±402	±1096	±111	±129	±1833

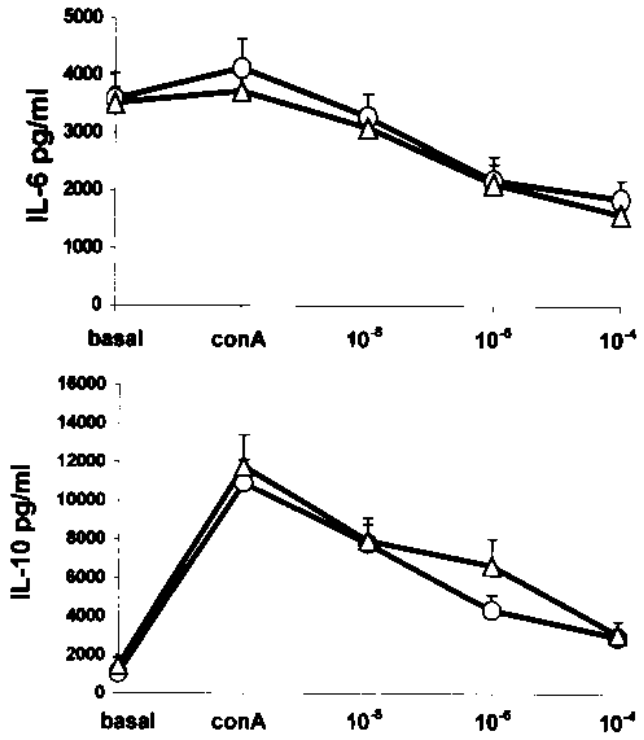

Figure 3. Th2 pattern of cytokine production (IL-6 and IL-10) after PBMC incubation in RPMI medium (basal), RPMI with concanavalin-A (Con-A), or RPMI with Con-A plus different doses of dexamethasone (10^{-8}, 10^{-6}, and 10^{-4} M) in healthy controls (○) and patients with RA (△).

and RA (p = 0.007) groups. After Con-A stimulation, IL-10 levels were much higher than basal levels in both groups (p = 0.0001). Dex (10^{-6} M) significantly inhibited IL-10 levels in both the control (p = 0.0002) and RA groups (p = 0.03). Therefore, Th1 cytokines were inhibited only in the RA group using higher doses of Dex (10^{-4} M) compared to control group (10^{-6} M). However, Th2 cytokines were inhibited in both groups using 10^{-6} M Dex.

In addition, we evaluated cytokine secretion in CS and CR RA patients. Cytokine levels did not differ between CS and CR patients, although it is important to point out that the number of samples was small (7 CR vs 8 CS patients with RA). Finally, we also analyzed the balance of TNF-α/IL-10 secretion during *in vitro* experiments, since patients with a high or low TNF-α/IL-10 ratio would be, respectively, less or more sensitive to endogenous and exogenous GC and more or less susceptible to the development of corticosteroid resistant or corticosteroid sensitive inflammatory disease[16]. However, there was no significant difference in TNF-α/IL-10 ratio between groups.

DISCUSSION

Corticosteroid resistance is a condition of clinical relevance to the steroid treatment of many human diseases, including RA. There is evidence that renal allograph recipients or individuals with asthma or RA can be divided into CS and CR subgroups on a clinical and *in vitro* basis using the ability of glucocorticoid to inhibit phytohemagglutinin and Con-A induced cell proliferation[3,17-19]. Glucocorticoids act by binding to a cytoplasmic glucocorticoid receptor, then translocate to the nucleus to act as a transcription factor[20,21]. Using a Con-A *in vitro* system similar to that described by Chikanza, *et al*[2], we divided RA patients and healthy controls into CS and CR phenotypes. Our study showed that the CR phenomenon is more frequently seen in RA patients than in healthy individuals (46.7% vs 9.5%; $p < 0.01$). CR could be an intrinsic property of each individual, probably having a genetic basis. However, the effects of GC on the immune system should also be considered. GC can both alter the circulating population of white cells and inhibit the production of IL-2, which is an important T cell growth factor influencing mitogen induced immunoglobulin synthesis in human lymphocytes *in vivo* and *in vitro*[13]. In addition, as previously observed, the majority of patients with GC resistant asthma presented a reversible cytokine induced reduction in GC receptor binding affinity, which could be sustained *in vitro* by the addition of IL-2 and IL-4[22]. Thus, we compared basal and Con-A stimulated cytokine production and the effects of different Dex doses on T cell cytokine production. The cell culture system used in this study has at least 2 advantages. First, maximal levels of some cytokines were obtained by using Con-A stimulation, and second, the secreted cytokines permitted the accurate determination of glucocorticoid dose-response curves over a 3 log range of concentrations.

A shift in the balance between the Th1 and Th2-like cytokine response has been related to the development of autoimmune disease or atopic reactions. Regarding the failure to detect a precise cause of RA, recent studies with a new transgenic model have indicated that destructive arthritis can be present in the absence of an intraarticular antigen[23]. Thus, direct action on the cytokine balance might represent a way to bypass the initiating event[24]. In addition, the appearance of proinflammatory cytokines in joint tissue or synovial fluid and serum or plasma of patients with arthritic conditions suggests that they play a role in the local and systemic inflammatory responses. Our study did not show differences in IL-4 (undetectable levels), IL-6, IL-10, or TNF-α production in lymphocytes incubated for 48 h in culture medium (basal condition) between controls and RA patients. However, IFN-γ production, a marker of Th type 1 cells, was slightly higher in RA patients, suggesting activation of the Th1 cytokine pattern. A recent study[25] using a sensitive and quantitative reverse transcriptase-polymerase chain reaction method measured spontaneous T cell production of IL-2, IL-4, IL-10, and IFN-γ mRNA in unstimulated PBMC from 25 patients with active RA and 19 healthy controls. The authors found that IL-2 and IL-4 mRNA in PBMC of active RA were produced at a low spontaneous level and the response to *in vitro* activation by the mitogen was weak. The IL-4 mRNA expression is concor-

dant with our findings concerning basal IL-4 cytokine supernatant production using our *in vitro* system. The authors also found similar basal levels of IL-10 mRNA expression in the 2 groups, again in agreement with our results.

There are some controversies regarding the expression of Th1/Th2 cytokines in the bloodstream and synovium[26]. Changes in Th1 and Th2-type cell migration patterns are associated with differences between systemic and synovial sites. The major pathology in RA occurs in the synovium, and since synovial fluid is readily accessible the production of cytokines was first documented in this compartment[27]. During chronic inflammation, migration of proinflammatory cells is increased, whereas migration of protective cells is defective, leading to their relative accumulation in the bloodstream[28]. There is a predominant Th1 pattern in the RA synovium, similarly to data obtained with peripheral lymphocytes using this *in vitro* system in patients with RA. However, in contrast to our results, defects in IFN-γ production have been reported in blood from patients with RA[29]. Synovial fluid and serum/plasma from patients with RA presented substantially increased IL-6 levels, and this variable has been correlated with disease activity in RA. This result has been denied by others, who found a poor or no association between the serum/plasma concentrations of this cytokine and acute phase proteins such as C-reactive protein[30].

GC inhibit the cellular cascade of the inflammatory and immune response at virtually all levels, including cytokine production. Thus, GC can modify the Th1-type and proinflammatory Th2-type and antiinflammatory cytokine profile, favoring a Th2 cytokine response, and may represent one of the most important determinants of the microenvironment that ultimately contributes to the development of a specific Th1/Th2 pattern[13]. This is because the former is strongly inhibited by GC, whereas the latter is not positively affected[15,31-33]. Our study revealed that IL-4 and IL-6 levels produced after Con-A stimulation were not increased compared to basal production, whereas IL-10, TNF-α, and IFN-γ levels after Con-A stimulation were much higher than basal production in both groups. Dex inhibited T cell Th2-type cytokine production after Con-A stimulation in the RA and control groups. Dex also inhibited Th1-type cytokine production after Con-A stimulation in both groups. However, while in healthy individuals the IFN-γ levels returned to almost basal levels after 10^{-6} M Dex, in RA patients they remained elevated even after a high Dex dose (10^{-4} M). The same was observed with respect to TNF-α, with a higher Dex dose being necessary to inhibit this proinflammatory cytokine in the RA group. Thus, proinflammatory cytokines were only partially inhibited in patients with RA even when higher GC doses were used. Again, these findings might reflect a predominance of Th1 cells, possibly contributing to corticosteroid resistance. Since changes in the GC receptor may be one of several mechanisms for altering tissue sensitivity to GC and some cytokines were shown to increase or decrease GC sensitivity by increasing the receptor concentration or decreasing its binding affinity[34-36], we cannot rule out that GC receptor expression or affinity or GC mediated gene activation/repression may also be altered in such patients[37]. In conclusion, the proinflammatory cytokines are unlikely to cause glucocorticoid resistance, but they might be involved in the maintenance of this phenomenon in patients with RA.

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