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\textsubscript{50} was defined as the concentration of Dex that caused 50\% inhibition of cell proliferation and subjects with an IC\textsubscript{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-\alpha (TNF-\alpha), interferon-\gamma (IFN-\gamma)] 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-\alpha levels were similar for both groups; however, basal IFN-\gamma 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-\alpha and IFN-\gamma 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
DEXAMETHASONE
CYTOKINES
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\textsuperscript{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)\textsuperscript{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)\textsuperscript{4}. Studies have described diminished GC receptor numbers and binding affinity in peripheral blood mononuclear cells (PBMC) from patients with RA\textsuperscript{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\textsuperscript{6} and other nuclear factors, such as AP-1\textsuperscript{7} and nuclear factor-kB (NF-kB)\textsuperscript{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-
MATERIALS AND METHODS

Con-A stimulated PBMC in culture. To evaluate 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 T helper type 1 [tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)] and T helper 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 mMHEPES 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 (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 (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 β counter (Beckman, Fullerton, CA, USA).

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

\[
\frac{1 - (x - n y / n)}{100}
\]

where: x = counts in Dex and Con-A, n = counts in RPMI alone, and y = counts in Con-A alone. The IC50 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 IC50. Subjects with an IC50 > 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), BAF206 (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, 39, 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.

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 (10−4, 10−6, 10−8 M) inhibited lymphocyte proliferation in a dose-dependent manner in the control (18197 ± 2572; 7905 ± 1459; 7686 ± 1759 cpm) and RA (15581 ± 2840; 11876 ± 2666; 6630 ± 1675 cpm) groups (Figure 1). Using the IC50 to classify CR or CS patients, we observed that the control group presented an IC50 of 10−6 M, while RA patients presented an IC50 of 10−8 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-
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, 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 10⁻⁶, 10⁻⁴, 10⁻² M) in healthy controls (C) and patients with corticosteroid sensitive (CS) and corticosteroid resistant (CR) rheumatoid arthritis.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Con-A</th>
<th>Dex 10⁻⁶ M</th>
<th>Dex 10⁻⁴ M</th>
<th>Dex 10⁻² M</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>3584±453</td>
<td>4135±125</td>
<td>3961±396</td>
<td>3290±1967</td>
<td>2178±866</td>
</tr>
<tr>
<td>IL-10</td>
<td>10136±511</td>
<td>10859±133</td>
<td>813±1023</td>
<td>3139±2269</td>
<td>1666±325</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1473±485</td>
<td>1243±123</td>
<td>813±1023</td>
<td>3139±2269</td>
<td>1666±325</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>203±33</td>
<td>5314±65</td>
<td>1625±543</td>
<td>4903±593</td>
<td>4903±1905</td>
</tr>
</tbody>
</table>

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 (10⁻⁶, 10⁻⁴, and 10⁻² M), in healthy controls (C) and patients with RA (△).
There is evidence that renal allograft 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. Glucocorticoids act by binding to a cytoplasmic glucocorticoid receptor, then translocate to the nucleus to act as a transcription factor. Using a Con-A in vitro system similar to that described by Chikanza, et al., 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.

DISCUSSION
Corticosteroid resistance is a condition of clinical relevance to the steroid treatment of many human diseases, including RA. There is evidence that renal allograft 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. Glucocorticoids act by binding to a cytoplasmic glucocorticoid receptor, then translocate to the nucleus to act as a transcription factor. Using a Con-A in vitro system similar to that described by Chikanza, et al., 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. 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. 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. Thus, direct action on the cytokine balance might represent a way to bypass the initiating event. 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 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-

![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 (△).](image-url)
dominant with our findings concerning basal IL-4 cytokine super-

nant 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 asso-
ciated 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, simi-
larly 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/plas-
ma from patients with RA present 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 con-
centrations 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 proin-
flammatory 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 affect-
ed.31-33 Our study revealed that IL-4 and IL-6 levels pro-
duced 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 produc-
tion 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 phenome-
non in patients with RA.

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