

# Expression Analysis of the Glucocorticoid Receptor and the Nuclear Factor- $\kappa$ B Subunit p50 in Lymphocytes from Patients with Rheumatoid Arthritis

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**ABSTRACT.** *Objective.* To study a possible relationship between expression of the transcription factor glucocorticoid receptor (GR), which mediates antiinflammatory effects, and the transcription factor p50, which mediates proinflammatory effects, in peripheral blood mononuclear cells (PBMC) of patients with rheumatoid arthritis (RA).

*Methods.* Expression analysis of GR and nuclear factor- $\kappa$ B subunit p50 in PBMC was performed by semiquantitative immunoblotting.

*Results.* GR and p50 expression in PBMC were significantly increased in patients with RA who had never received corticosteroids. In contrast, GR density is decreased in glucocorticoid treated RA patients. In addition, a dependency between increased GR expression and increased p50 expression was found.

*Conclusion.* The pathogenesis of RA is not reflected in diminished GR expression but rather in an increased expression level of GR, as well as increased p50 expression in PBMC. Corticosteroids as the major therapeutic drugs result in a reduction of these increased GR and p50 expression levels. (J Rheumatol 2002;29:2500–6)

## Key Indexing Terms:

RHEUMATOID ARTHRITIS  
GLUCOCORTICOID RECEPTOR

NUCLEAR FACTOR- $\kappa$ B  
LYMPHOCYTES

Glucocorticoids are widely used in the therapy of chronic inflammatory diseases. They exert their activity after binding to the cytoplasmic glucocorticoid receptor (GR), resulting in a nuclear translocation of the receptor/hormone complex, which ultimately leads to modulation of gene transcription<sup>1-3</sup>. The molecular mechanisms underlying this modulation are based on either transactivation of glucocorticoid dependent genes or transrepression of genes induced by proinflammatory transcription factors<sup>3-6</sup> such as the nuclear factor- $\kappa$ B (NF- $\kappa$ B) or activating protein 1 (AP-1).

Transrepression of proinflammatory transcription factors seems to be one of the major mechanisms of how glucocorticoid induced antiinflammatory effects are achieved<sup>3-6</sup>.

In the last decade several investigations were performed to detect a correlation between the expression of GR and autoimmune diseases, but results are contradictory. In some cases a decrease in GR expression in lymphocytes of patients with rheumatoid arthritis (RA) or in mononuclear cells of patients with lupus nephritis who had not received corticosteroids for at least 6 months was reported<sup>7-9</sup>. Others detected decreased GR expression in lymphocytes from children with autoimmune disease independent of glucocorticoid treatment<sup>10</sup>. Another study confirmed a decrease in GR expression in mononuclear cells from patients with RA following glucocorticoid pulse therapy, that returned to pretreatment values shortly after the last treatment<sup>11</sup>. Moreover, an increase in receptor density was observed in interleukin 1 $\beta$  (IL-1 $\beta$ ) prestimulated rabbit synovial fibroblasts compared to unstimulated cells when dexamethasone was added<sup>12</sup>, suggesting regulation of GR expression by glucocorticoids and IL-1 $\beta$ . A higher expression of GR was detected in mononuclear cells of patients with inflammatory bowel diseases before steroid treatment, which decreased to normal levels when treated with glucocorticoids<sup>13</sup>.

In addition, there is evidence that the proinflammatory transcription factor NF- $\kappa$ B is also involved in the pathogen-

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esis of RA. NF- $\kappa$ B is known to play a pivotal role in the transcriptional regulation of proinflammatory factors<sup>3,14,15</sup>. In its active form this factor consists of 2 subunits, p65 and p50<sup>3,5,6</sup>, with the p50 subunit being reported to be important in joint inflammation and destruction<sup>16</sup>. This crucial role for p50 is also true for other autoimmune diseases, for example in experimental autoimmune encephalomyelitis<sup>17</sup>.

We investigated the expression of the transcription factor GR, which mediates antiinflammatory effects, and compared it with expression of the proinflammatory NF- $\kappa$ B subunit p50 in patients with RA who had and had not been treated with glucocorticoids. Since we know from earlier studies<sup>18,19</sup> that untreated patients with RA showed levels of cortisol that positively correlated with variables of immunological activity, we were interested in whether this correlation might also be reflected in the expression level of the cortisol target, the GR, and its counterpart NF- $\kappa$ B. Since there is no p50-specific ligand, there was no possibility to measure both protein levels by ligand binding in parallel. Therefore the detection of GR and p50 expression in lymphocytes from patients with RA was performed in parallel using semiquantitative immunoblotting, which should result in the detection of the entire amount of both full length GR and p50 molecules present in these cells.

## MATERIALS AND METHODS

**Patients and control subjects.** We studied 37 patients who met the American College of Rheumatology (ACR) criteria for RA<sup>20</sup> and 14 healthy controls. The patients were divided into 2 groups: one group had never received corticosteroids (16 patients) and the other (21 patients) had received corticosteroid therapy continuously for at least one year (range 5–15 mg prednisolone). All 3 groups were matched for sex but not age, but there was no correlation between age and p50 expression ( $p = 0.52$ ) or GR expression ( $p = 0.12$ ), respectively, as determined by Spearman rank correlation. Blood samples were taken between 9:00 and 10:00 AM.

**Preparation of PBMC.** Immediately after blood was obtained peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque Plus (Amersham Pharmacia Biotech, Freiburg, Germany) and washed 3 times in phosphate buffered saline (PBS) solution. The separated cells consisted of about 90 to 95% PBMC as detected by microscopic analysis. Additional FACS analysis revealed about 85–88% lymphocytes and 7–10% monocytes when using this method (without any striking difference between the cell ratio for all 3 groups).

**Preparing the whole cell extracts.** Whole cell extracts (WCE) were prepared by incubating the PBMC in double the cell volume of NETN-buffer (100 mM NaCl; 1 mM EDTA, pH 8; 10 mM Tris, pH 8; 0.5% Nonidet P-40), which was supplemented with protease inhibitors aprotinin and leupeptin to a final concentration of 10  $\mu$ g/ml. Briefly, the cell pellet was resuspended in NETN buffer following 3 freeze (liquid nitrogen) and thaw (30°C) cycles and an incubation at 4°C for 15 min with gentle shaking. After centrifugation at 14,000 g at 4°C for 10 min the supernatant was carefully removed, the protein concentration was determined, and samples were frozen in aliquots. All chemicals were purchased from Sigma (Munich, Germany).

**SDS-PAGE and western blotting.** Protein samples (50 and 100  $\mu$ g per individual on the same gel, and 12.5, 25, 50, and 100  $\mu$ g for Hela WCE) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli<sup>21</sup>. The resulting gel was electroblotted for 1.75 h to a polyvinylidene difluoride membrane (Millipore,

Freiburg, Germany), using a semidry blotting system. The membrane was blocked overnight with PBS-T (PBS, 0.1% Tween 20) with 5% skim milk powder. Antibody incubation was carried out for 1.5 h with the respective primary antibody (monoclonal  $\alpha$ -GR antibody NCL-GCR, Novocastra, Balliol, United Kingdom; polyclonal  $\alpha$ -p50 antibody C19, Santa Cruz Biotechnology, Heidelberg, Germany) and with the respective peroxidase coupled secondary antibody (Amersham, Pharmacia Biotech) for 1 h. The membranes were processed by enhanced chemiluminescence according to the manufacturer's protocol (Amersham Pharmacia Biotech) and immune complexes visualized by exposing the membranes to radiographic film.

**Scanning of radiographic films and statistical methods.** The developed films were scanned with a Primax Colorado Direct Scanner (TaskBridge version 1.1) with a resolution of 300 dpi. Optical density (OD) was measured with the Tina program (version 2.07d). To ensure that the measured values fell within a linear range, each analysis included a standard curve consisting of 12.5, 25, 50, and 100  $\mu$ g of Hela WCE. The resulting OD/mm<sup>2</sup> value for each GR or p50 band of every individual was then normalized to the 12.5 or 100  $\mu$ g of HeLa-WCE on the same blot, respectively. The value expressed for each patient was calculated as the average of the ratio of OD/mm<sup>2</sup> per 50  $\mu$ g patient protein divided by OD/mm<sup>2</sup> of Hela WCE, and OD/mm<sup>2</sup> per 100  $\mu$ g patient protein divided by OD/mm<sup>2</sup> of Hela WCE. Data were then evaluated by SPSS for Windows (V.6.1.3) and described by median and range, the diagnostic groups were compared by Kruskal-Wallis procedure, correlation between age and proteins was calculated by Spearman rank correlation and graphical figures were depicted as box plots<sup>22</sup>.

## RESULTS

We investigated the expression of GR and NF- $\kappa$ B subunit p50 in 3 groups: healthy controls, RA patients untreated, and those treated with glucocorticoids. Patient characteristics are summarized in Table 1.

To examine the expression of GR and p50 we separated PBMC, extracted the cellular proteins, detected GR and p50 by Western blotting, and calculated the relative amount of each protein. Two representative Western blots are depicted in Figure 1, as well as standard curves generated by quantifying the 12.5, 25, 50, and 100 mg Hela WHC after probing for GR and p50. A representative blot, shown in Figure 1, reveals a signal for the GR in whole cell extract derived from PBMC from a healthy control (Figure 1A, lane 1) and an even stronger signal in an untreated RA patient (Figure 1A, lane 3), whereas GR expression in a patient receiving glucocorticoid was decreased (Figure 1A, lane 2).

Analysis of p50 expression revealed differences between untreated RA patients (Figure 1B, lane 3) and healthy controls, as well as with glucocorticoid treated RA patients (Figure 1B, lanes 1 and 2), but there was no striking difference between the amount of p50 expressed in PBMC of healthy controls compared to RA patients receiving glucocorticoid.

As shown in Figures 1C and 1D, a linear relationship is obtained between the amount of protein and the signal intensity in the Hela WCE standard curve when using either antibody. Statistical analysis resulted in significant differences between all 3 groups concerning the expression of GR in PBMC ( $p = 0.0001$ ). Since there was a wide range of GR expression in the groups (Table 2), the results of the statis-

Table 1. Characteristics of patients with RA treated and not treated with glucocorticoids.

	Patient	Age, yrs	Sex	Duration	Medication	CRP, mg/dl	ESR, mm/h
Treated	1	69	F	1982	7.5	1.3	17
	2	75	M	1970	15	1	39
	3	59	F	1999	12.5	3.9	30
	4	66	M	1992	15	3.4	19
	5	79	F	1999	12.5	8.5	56
	6	63	F	1993	7.5	1.8	17
	7	64	F	1996	15	4.9	17
	8	58	M	1998	15	1.1	15
	9	59	F	1990	15	5.6	45
	10	82	M	1996	10	2.5	13
	11	64	F	1980	5	0.8	29
	12	71	M	1994	15	5.3	60
	13	64	F	1961	12.5	1.5	23
	14	78	F	1996	7.5	1.3	25
	15	69	M	1973	10	2.4	43
	16	58	M	1994	7.5	1.4	22
	17	78	F	1997	7.5	0.9	20
	18	53	F	1994	10	2.4	15
	19	76	F	1977	15	1.5	13
	20	65	F	1990	10	2.3	41
	21	50	F	1998	10	1.2	12
Untreated	1	71	F	Oct 2000	0	1.4	77
	2	30	F	Aug 2000	0	1.1	40
	3	60	M	Sept 2000	0	5.3	51
	4	53	F	Sept 2000	0	2.7	46
	5	31	M	Sept 2000	0	12.8	104
	6	56	F	Dec 2000	0	7.4	107
	7	81	F	Sept 2000	0	0.8	15
	8	75	F	Dec 2000	0	1.9	60
	9	80	F	Aug 2000	0	5.7	74
	10	71	M	June 2000	0	3	5
	11	49	F	Jan 2001	0	4.5	45
	12	66	M	Aug 2000	0	0.6	12
	13	49	F	Oct 2000	0	1.5	34
	14	39	M	June 2000	0	0.8	15
	15	41	M	May 2001	0	1.1	7
	16	34	F	May 2001	0	2.9	66

tical analysis are presented as box plots (Figure 2). Statistical analysis of the p50 revealed a significantly higher expression in untreated RA patients versus healthy controls and RA patients receiving glucocorticoid ( $p = 0.0007$ ); in contrast, there was no striking difference between the latter 2 groups. The results are shown in Table 3 and in Figure 3.

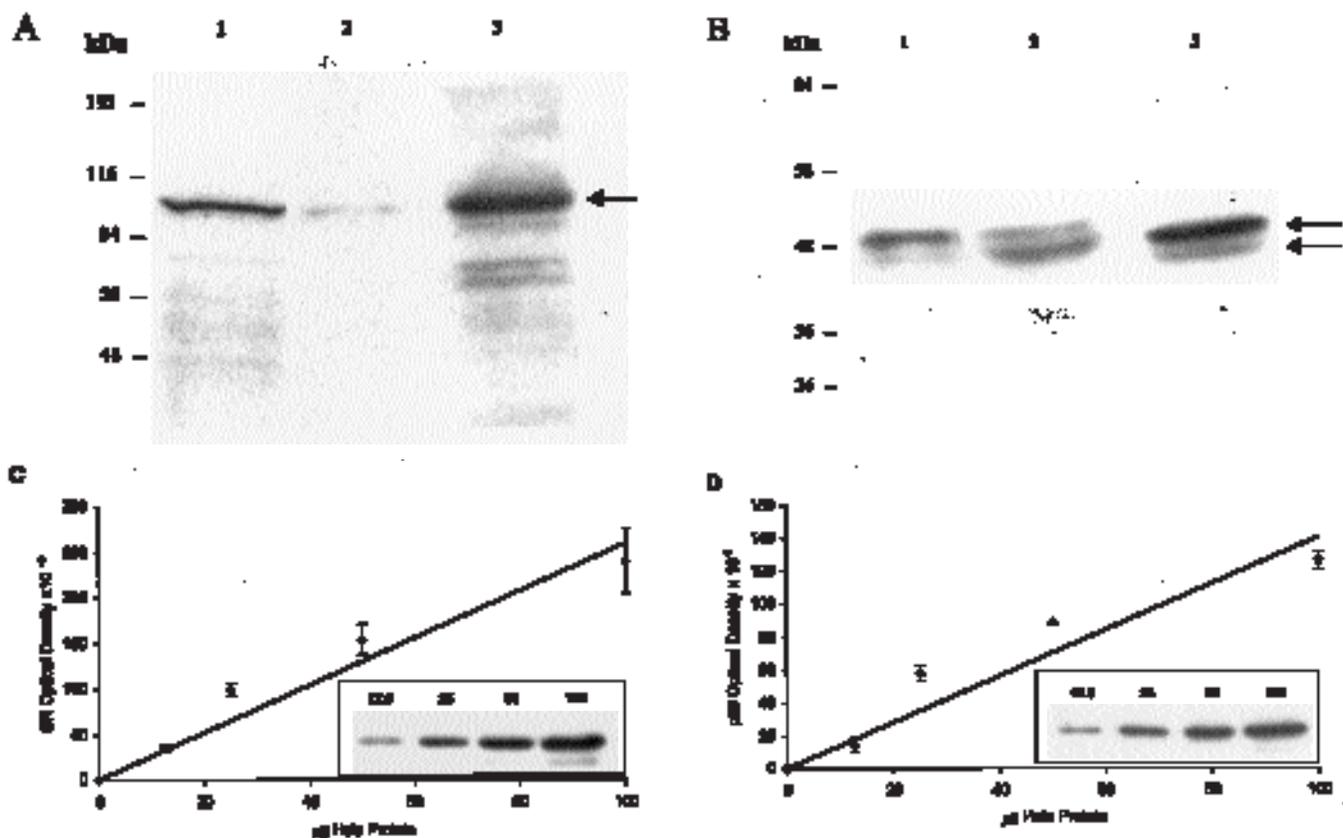
Moreover, we detected a relationship between expression of GR and expression of p50: higher GR levels tended to correspond to higher p50 levels in untreated RA patients. This tendency between GR and p50 expression in PBMC of RA patients untreated with GC is presented in Figure 4.

## DISCUSSION

We report on the expression of the GR and the NF- $\kappa$ B subunit p50 in PBMC of patients with RA. We determined the relative amounts of both proteins expressed in PBMC of

each individual using semiquantitative immunoblotting. This method should result in the detection of the entire amount of full length GR and p50 molecules present in these cells.

We found a significant increase in GR as well as in p50 expression in PBMC of patients untreated with glucocorticoids when compared to healthy controls. Moreover we showed diminished receptor density in glucocorticoid treated RA patients when compared with the same healthy controls. These results support the model that during inflammation many proinflammatory cytokines (e.g., IL-1, IL-2, and tumor necrosis factor- $\alpha$ , TNF- $\alpha$ ) are produced, which are known to activate NF- $\kappa$ B<sup>15,23-25</sup>. This results in a positively regulated feedback loop due to NF- $\kappa$ B induction of several of these proinflammatory cytokine genes<sup>15,26-28</sup>. Increased NF- $\kappa$ B activity might lead to an increase in GR



**Figure 1.** Typical immunoblots of whole cell extracts (WCE) from patients and generation of a standard curve. **A.** Typical anti-GR immunoblot of WCE derived from PBMC of a healthy control (lane 1), a patient with RA treated with glucocorticoid (lane 2), and an untreated RA patient (lane 3). Glucocorticoid receptor (GR) is marked with an arrow. **B.** Typical anti-p50 immunoblot of WCE derived from PBMC of a healthy control (lane 1), a glucocorticoid treated (lane 2), and an untreated (lane 3) patient with RA. The 2 isoforms of p50 that are quantitated together as one band are marked with arrows. **C and D.** Typical immunoblot of 12.5, 25, 50, and 100  $\mu$ g HeLa WCE probed with either anti-GR antibody or anti-p50 antibody (see Materials and Methods). Arbitrary OD units of duplicate experiments were plotted against the amount of HeLa protein loaded in each lane. **C.** Typical curve generated from a blot probed with the anti-GR antibody; inset shows signals obtained from one such experiment (regression coefficient  $R^2 = 0.94$ ). **D.** Typical standard curve generated from a blot probed with the anti-p50 antibody; inset shows the signals obtained from one such experiment (regression coefficient  $R^2 = 0.90$ ).

expression, since NF- $\kappa$ B can induce GR transcription via a NF- $\kappa$ B binding site in the 5' region of the human GR gene<sup>29</sup>. This is in accord with a study that showed an increased GR expression after TNF- $\alpha$  or IL-1 treatment in HeLa S3 and COS1-cells, most likely mediated by NF- $\kappa$ B<sup>30</sup>. Increased GR expression would result in an accumulation of cytoplasmic GR that needs to be activated by glucocorticoids.

Endogenous glucocorticoids are the products of the hypothalamus-pituitary-adrenal (HPA) axis, and cytokines (e.g., TNF- $\alpha$ , IL-1) can activate the HPA axis at all 3 levels, forming a cytokine-HPA axis circuit. This should lead to an activated, nuclear localized GR, able to both transrepress transcriptional NF- $\kappa$ B activity<sup>31</sup> and transactivate the NF- $\kappa$ B specific inhibitor molecule I- $\kappa$ B<sup>32,33</sup>. This in turn may contribute to the disruption of this proinflammatory circuit, resulting in inactivation of the proinflammatory transcription factor NF- $\kappa$ B, leading to cessation of inflammation. In RA there is evidence of increased ACTH and cortisol driven

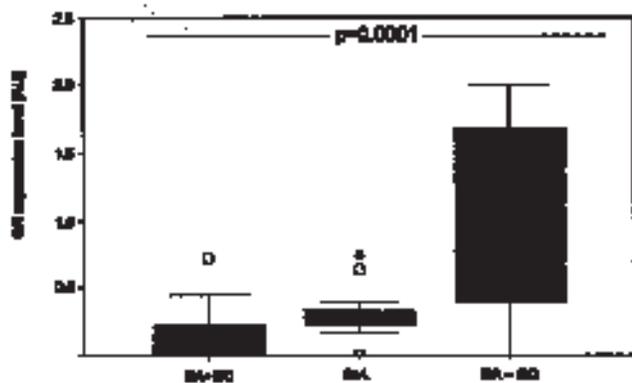
by elevated cytokines, but it appears that the elevations are inappropriately low with respect to inflammation<sup>34,35</sup>. This might explain the benefit of exogenously applied glucocorticoid in RA. In this hypothesis the pathogenesis of RA is reflected in increased GR density induced by increased NF- $\kappa$ B activity, together with an inappropriately low glucocorticoid level. This should be overcome by additional corticosteroids. Indeed exogenous treatment with glucocorticoid leads to both a reduced GR and p50 density (see Results). A simplified model describing the possible crosstalk between pro- and antiinflammatory circuits is depicted in Figure 5.

In agreement with the current study an increase in GR expression in PBMC in inflammatory bowel disease was shown in patients untreated with glucocorticoid using the [<sup>3</sup>H]dexamethasone binding assay<sup>13</sup>. Most of the studies investigating GR expression in patients with RA and other autoimmune diseases found a decreased density of GR<sup>7-10</sup>.

**Table 2.** GR expression in PBMC in 51 individuals (determined by semi-quantitative Western blotting and calculated as OD/mm<sup>2</sup>). All values were calculated and normalized against the GR amount in Hela whole cell extract used as standard control.

Patient	Patients with RA		
	Healthy Controls, n = 14	GC Treated, n = 21	GC Untreated, n = 16
1	0.63	0.08	1.12
2	0.22	0.00	0.46
3	0.17	0.00	0.94
4	0.00	0.00	1.70
5	0.74	0.00	0.00
6	0.30	0.00	0.00
7	0.21	0.00	0.73
8	0.28	0.00	0.82
9	0.34	0.45	0.41
10	0.39	0.42	1.89
11	0.28	0.23	0.36
12	0.24	0.72	1.75
13	0.30	0.00	0.63
14	0.20	0.00	2.00
15		0.00	0.13
16		0.00	1.67
17		0.00	
18		0.00	
19		0.00	
20		0.26	
21		0.38	

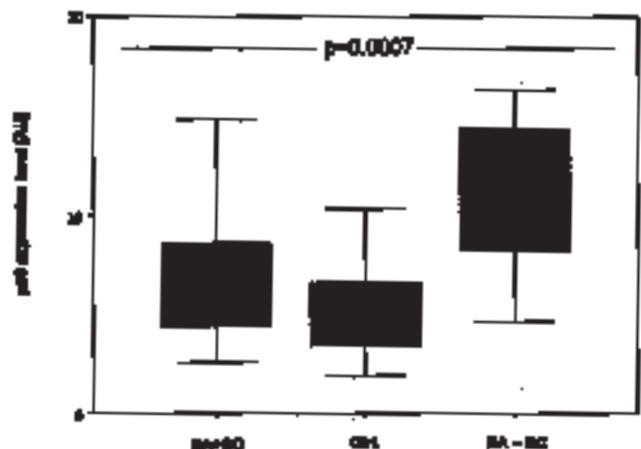
GC: glucocorticoids.



**Figure 2.** Box plot illustrating GR expression levels in the 3 groups investigated: 21 RA patients treated with glucocorticoid (RA+GC), 16 untreated RA patients (RA-GC), and 14 healthy controls (Ctrl). Expression levels were calculated and normalized using a Hela standard protein mixture (See Materials and Methods). Data are expressed as box plots, where the boxes represent the 25th to 75th percentiles, the lines outside the boxes represent the 10th and 90th percentiles, and the lines within the boxes represent the median (median for RA+GC = 0). Extreme values are marked with open circles and one outlier is marked with an asterisk.

**Table 3.** p50 expression in PBMC in 51 individuals (determined by semi-quantitative Western blotting and calculated as OD/mm<sup>2</sup>). All values were calculated and normalized against the p50 amount in Hela whole cell extract used as standard control.

Patient	Patients with RA		
	Healthy Controls, n = 14	GC Treated, n = 21	GC Untreated, n = 16
1	6.88	5.15	13.26
2	3.50	2.57	8.46
3	1.93	2.78	7.81
4	3.32	7.20	16.31
5	3.41	4.42	14.56
6	6.67	4.37	10.10
7	6.11	3.52	12.03
8	4.33	4.08	10.16
9	6.48	6.21	4.66
10	5.49	6.36	13.19
11	6.13	4.07	4.76
12	2.85	5.17	14.28
13	10.30	9.21	15.85
14	10.07	12.71	15.29
15		8.45	6.21
16		14.82	8.44
17		7.81	
18		9.37	
19		8.66	
20		7.47	
21		11.05	



**Figure 3.** Box plot illustrating the p50 expression levels in the 3 groups investigated: 21 patients with RA treated with glucocorticoid (RA+GC), 16 untreated RA patients (RA-GC), and 14 healthy controls (Ctrl). Expression levels were calculated and normalized by Hela standard protein mixture (See Materials and Methods). Data are expressed as box plots, where the boxes represent the 25th to 75th percentiles, the lines outside the boxes represent the 10th and 90th percentiles, and the lines within the boxes represent the median.

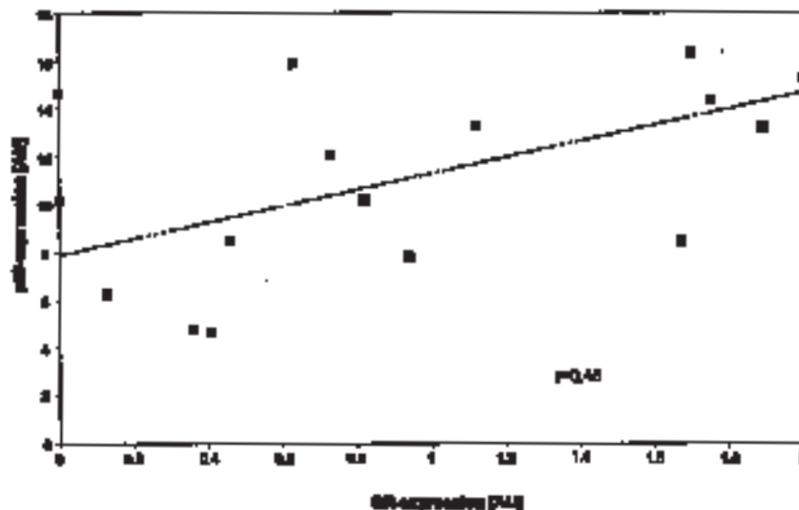


Figure 4. Relationship between expression levels of GR and p50 in whole cell extracts derived from PBMC of patients with RA who had never received glucocorticoids. Correlation coefficient is  $r = 0.45$  and indicates a tendency ( $p = 0.08$ ). The broken line indicates the trend. Values are given as arbitrary units (AU).

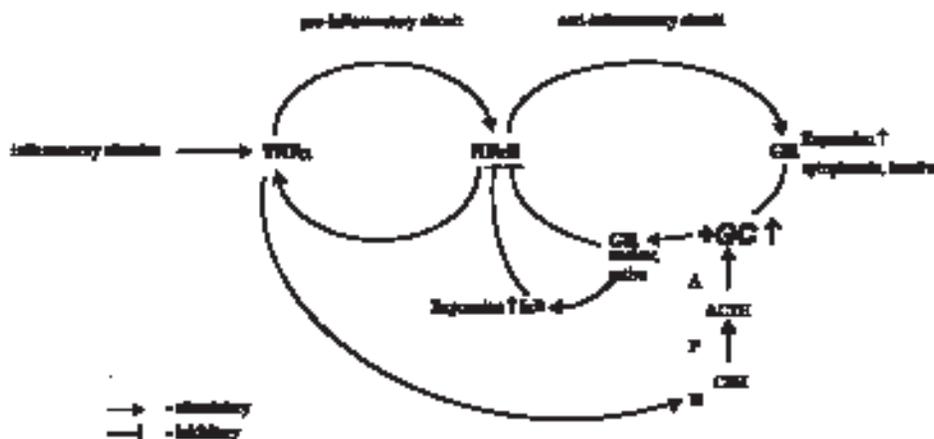


Figure 5. Simplified model of a possible crosstalk between pro- and antiinflammatory circuit: inflammation leads to induction of proinflammatory cytokines (e.g., TNF- $\alpha$ ) that activate NF- $\kappa$ B. NF- $\kappa$ B can enhance GR expression, resulting in an accumulation of inactive cytoplasmic located GR. GR can be activated by glucocorticoids, which are induced by cytokines. Once activated, GR translocates into the nucleus, where it can repress NF- $\kappa$ B activity (for details see text).

However, in contrast to our study, the patients analyzed in those studies had received corticosteroids at some time prior to or during measurement of GR.

Taken together our findings suggest that increased GR and p50 expression levels in PBMC reflect at least in part the pathogenesis of RA and the beneficial effect of glucocorticoids in RA can be monitored by a decrease in the expression of both these factors.

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