The differential expression of corticosteroid receptor isoforms in corticosteroid-resistant and -sensitive patients with rheumatoid arthritis

D. L. Kozaci¹*, Y. Chernajovsky¹ and I. C. Chikanza¹,²

Objective. A proportion of patients with rheumatoid arthritis (RA) fail to respond adequately to corticosteroid (CS) therapy. Using an in vitro CS sensitivity bioassay, we have subdivided RA patients into steroid-sensitive (SS) and -resistant (SR) subgroups and this correlates with clinical responses to CS therapy. CSs exert their effects via the CS receptor (CR), which exists as two main isoforms, CRα and CRβ. CRβ can function as a negative inhibitor of CRα. We have hypothesized that steroid resistance in RA patients is due in part to a relative over-expression of the CRβ.

Methods. Peripheral blood mononuclear cells (PBMCs) were isolated from SS and SR RA patients. CRα and CRβ mRNA expression was determined by quantitative real time polymerase chain reaction (qRT–PCR). The ratio of CRβ/CRα mRNA expression was determined. CRα and CRβ protein expression by PBMCs was analysed by flow cytometry.

Results. qRT–PCR analysis showed a trend towards higher expression of both CRβ and basal CRβ/CRα ratio in SR RA patients. Stimulation of PBMCs in vitro with concanavalin-A induced a significantly higher CRβ mRNA expression, and CRβ/CRα ratio in SR RA patients compared with SS patients, which was not inhibited by hydrocortisone. Flow cytometry showed that the percentage of PBMCs staining for CRβ protein was significantly lower in the SS RA group (SS 43.3 ± 14.8% vs SR 88.6 ± 8.6%; P < 0.0010). The mean intensity of fluorescence CRβ staining was higher in the SR RA patients (P < 0.001).

Conclusion. We show for the first time that CRβ is over-expressed in SR RA patients and that hydrocortisone fails to inhibit concanavalin-A stimulated increase in CRβ mRNA in SR RA patients. This mechanism may contribute in part to the CS hyporesponsiveness seen in some RA patients.

Key words: Rheumatoid arthritis, Inflammation, Corticosteroid resistance, Corticosteroid receptor, Corticosteroids.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that is often treated with corticosteroids (CSs) [1]. A proportion of patients with RA fail to respond adequately to CS therapy [2]. RA patients can be subdivided into CS-sensitive (SS) and -resistant (SR) subgroups on the basis of the in vitro inhibition by CSs of T-cell proliferation induced by concanavalin-A (con-A), which correlates with the clinical observations [2]. The IC₅₀ of hydrocortisone on cell proliferation in SS subjects is ≤10⁻⁶ M, whilst the value is ≥10⁻⁵ M in the majority of SR patients [2]. Patients with asthma, an allergic condition associated with bronchial spasm and eosinophilic infiltration of the bronchial mucosa, have also been divided into similar subgroups that correlate with clinical responses to CS therapy [3, 4]. The underlying mechanisms involved in the SS and SR phenomenon in patients with RA remain unknown but may be related in part to the functional status of the CS receptor (CR).

We have gained more insight into the molecular basis of the anti-inflammatory actions of CS. CSs inhibit T-cell proliferation via the inhibition of interleukin (IL)-2 production and other cytokines via the CR [5]. The promoter region of the IL-2 gene contains cis-acting elements required for the maximal induction of IL-2 production that are recognized by the nuclear transcription factors AP-1, NF-κB, NF-AT and OCT-1 [6, 7]. When CS binds to CR, the CS/CR complex is released from the heat shock protein 90 containing multi-protein complex, it dimerizes, transactivates and translocates to the nucleus to bind to specific DNA motifs, the glucocorticoid response elements (GREs) [8]. The GREs can mediate both positive and negative effects [8, 9]. The CS/CR complex also interferes with the binding of AP-1 to its site on DNA (trans-repression) [6, 7, 10], and CSs up-regulate IκB production, which sequesters NF-κB in the cytoplasm [10,11]. NF-κB exists in the cytoplasm as a heterodimer (most commonly p50 and p65 subunits) bound to an inhibitor IκB that exists in a number of isoforms [12]. Activation of cells by a variety of stimuli, leads to the degradation of IκBs and the released NF-κB then translocates to the nucleus to initiate transcription of target genes [12–14]. The binding of AP-1 and NF-κB to DNA sites on target genes induces the production of pro-inflammatory cytokines such as IL-1β, IL-2, IL-3, IL-6, IL-8, tumour necrosis factor α (TNFα) and adhesion molecules [7, 15, 16].

CR exists in two main isoforms, CRα and CRβ, produced by alternative splicing [17–18]. Only CRβ binds to CS [18]. Other receptor isoforms such as CR-γ, CR-A and CR-P have been described but their functions are not fully characterized. Transfection experiments in COS-1, CV-1, HeLa S₃ and COS-7.
cells by two groups of investigators showed that the CRβ isoform can act as a negative inhibitor of CRα-mediated effects [18–20]. Bamberger et al. [19] showed that the CRβ isoform was able to inhibit the effects of hormone-activated human CRα (CS/CRα complex) on GREs in a dose dependent manner, [3H]-dexamethasone binding studies indicated that the CRβ isoform did not alter the binding affinity of CRα for CS [19]. The presence of the CRβ isoform in nuclear extracts and its ability to bind to radio-labelled GRE oligonucleotides suggested that its inhibitory effects were due to competition for the GRE target site [19]. Similar observations were made by Oakley et al. [20]. However, Brogan et al. using COS-7 and HEK-293 cells, showed that over-expression of the CRβ isoform did not interfere in a specific way with the interaction of CRα with AP-1 and NF-κB (transrepression) and they suggested that the effects in COS-7 cells could be due to non-specific transcriptional squelching [21]. Transfection studies in Jurkat cells showed that co-expression of CRα and CRβ did not interfere with CRα-mediated trans-activation or transrepression [22]. In contrast, CRβ from the same plasmid preparation caused a significant inhibition of transcriptional activity in COS-7 cells, indicating a cell type-specific pattern of CRβ-mediated inhibition of CRα-mediated CS effects [22].

Immuno histochemistry studies in asthma patients by Leung et al. [23] have recently shown that the CRβ isoform is over-expressed by a significantly higher percentage of broncho-alveolar lavage airway cells from SR asthmatic patients than in SS asthmatics. Furthermore, treatment of the broncho-alveolar lavage airway cells from SR asthmatics with a combination of IL-2 and IL-4 induced a further increase in the over-expression of the CRβ isoform above baseline, suggesting that cytokines could induce its expression [23, 24]. This observation in SR asthmatic patients suggests a potential role of CRβ in the SR phenomenon seen in asthma patients. Whether a similar situation exists in RA patients who are SR has not been determined.

We have previously shown that CSs fail to inhibit IL-2 and IL-4 production by peripheral blood mononuclear cells (PBMCs) from SR RA patients [2]. This failure to inhibit IL-2 and IL-4 production in SR could potentially result in CRβ over-expression. It can thus be hypothesized that the over-expression of the CRβ isoform relative to CRα could potentially contribute to a state of reduced responsiveness to CS therapy in patients with RA. We have investigated the hypothesis that the differences in the sensitivity to CS seen in patients with RA, may be a consequence, in part, of differences in the differential expression of the CRα and CRβ. Thus, we assessed the levels of both CRα and CRβ isoforms at mRNA and protein levels using quantitative real time polymerase chain reaction (qRT–PCR) and by immunocytofluorographic analysis of the cellular protein expression in vitro-defined SS and SR RA patients.

Methods

Patient selection. We studied a group of patients (n = 22) who were subdivided into SS (n = 10) and SR (n = 12) subgroups using a previously described CS sensitivity in vitro bioassay [2]. In order to control for the potential confounding effects of inflammation on CS sensitivity, only patients with similar levels of disease activity as measured by erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and number of active joints (i.e. tender and/or swollen) were selected. The demographics of these patients were not significantly different and are shown in Table 1. All the patients fulfilled the 1987 American College of Rheumatology diagnostic criteria for RA [25], had erosive joint disease, and had not received CS therapy within the past 3 months. All patients were on methotrexate and similar disease duration. None of the patients had another chronic inflammatory disorder other than RA. Patients with hypothalamic pituitary adrenal disorders, chronic obstructive pulmonary disease or on psychotropic drugs were excluded. Cells were isolated from each RA patient studied and then cultured; mRNA isolated from a portion of cells and cells were also immunocytofluorographically labelled for analysis of CRα and CRβ isoforms expression. The study was approved by our local research ethics committee and all patients gave informed and written consent.

**Cell preparation.** Peripheral blood (20 ml) was collected into heparinized tubes for all the studies between 10:00 and 11:00 a.m. in all the subjects to avoid variations due to diurnal rhythm of hypothalamic pituitary adrenal axis activity, which could potentially affect CR expression. PBMCs were isolated by density gradient centrifugation using Lymphoprep™ (Nycomed, Oslo, Norway), washed three times in Hank’s buffered saline solution and resuspended in tissue culture medium (TCM) as previously described [2]. The TCM consisted of RPMI 1640 (Life Technologies, UK) containing 10% fetal bovine serum, 100 000 IU/l penicillin and 100 mg/l streptomycin (GIBCO, Paisley, UK). Some of the resuspended cells were aliquotted and distributed equally into 1.8 ml sterile tubes, mixed with freezing medium [20% DMSO in fetal bovine serum (FBS)] 1:1 immediately frozen and stored in liquid nitrogen.

**In vitro CS sensitivity assay.** The in vitro CS sensitivity assay measured the inhibitory effect of hydrocortisone on con-A-stimulated PBMCs proliferation [2]. PBMCs (1 × 10⁶ cells in a final volume of 100 μl/well) were plated in triplicate in flat-bottomed Nunclon® 96-Well MicroWell® plates (Nunclon, Life Technologies, UK) and cultured at 37°C, 5% CO2 and 95% air in a humidified atmosphere. Con-A 10 μg/ml (dose established by preliminary experiments) was used to stimulate the cells in the presence of different concentrations of hydrocortisone (10⁻⁸ to 10⁻⁶, 10⁻⁵, 10⁻⁴ M). Hydrocortisone was always prepared fresh at the start of each experiment.

After 72 h of culture, 100 μl of Cell Titer-Glo reagent was added to each well according to manufacturer’s instructions, and inhibitory effects of hydrocortisone were determined on cell proliferation/viability (Promega G7570, Southampton, UK) by measuring the relative light units (RLU) using a MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA). The mean RLU of triplicate of each sample was calculated. The percentage inhibition of cell proliferation by hydrocortisone was calculated using the following formula:

\[
\left(1 - \frac{x - n}{y - n}\right) \times 100
\]

where, \(x\) RLU in hydrocortisone and Con-A; \(n\) RLU in TCM alone; \(y\) RLU in con-A alone.

The IC₅₀ of hydrocortisone on cell proliferation was determined from the dose-response curve. The IC₅₀ was defined as the concentration of hydrocortisone that caused a 50% inhibition of cell proliferation; by this calculation the variations in con-A-induced proliferation that occur between individuals and in the same individual were compensated for. Subjects with an

**TABLE 1. Demographics of patients studied for their CS responsiveness (mean±SE)**

<table>
<thead>
<tr>
<th></th>
<th>SR RA</th>
<th>SS RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Sex</td>
<td>F:M</td>
<td>9:3</td>
</tr>
<tr>
<td>Mean age (yrs)</td>
<td>53±7</td>
<td>52±5</td>
</tr>
<tr>
<td>Duration of RA (yrs)</td>
<td>8.3±4</td>
<td>8.1±3</td>
</tr>
<tr>
<td>Mean ESR (mm/h)</td>
<td>47±13</td>
<td>40±10</td>
</tr>
<tr>
<td>Mean CRP (mg/l)</td>
<td>17.2±6</td>
<td>16.8±5</td>
</tr>
<tr>
<td>Number of active joints</td>
<td>6.5±1.2</td>
<td>6.0±3</td>
</tr>
<tr>
<td>RF (positive:negative)</td>
<td>10:2</td>
<td>8:2</td>
</tr>
</tbody>
</table>
and CR sequences were selected to differentiate amplification from designed using Primer Express software (PE-Applied housekeeping gene. Sequences for forward and reverse oligo-

for 10 min at 70°C. MgCl2, 1 mM each dGTP, dATP, dTTP, dCTP, 0.5 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 5 mM 100 000 IU/l penicillin, and 100 g/ml streptomycin. Cells were

RNA isolation and cDNA synthesis. Total RNA from PBMCs were isolated using RNA STAT-60 Reagent (Biogenesis, Poole, UK) according to manufacturer’s instructions. After washing, the mRNA was air dried and suspended in DEPC-treated water after which the optical density (OD) was determined (>1.7), and used for calculation of the concentration. cDNA was prepared using PROMEGA A3500 kit (Southampton, UK). 1.0 g of sample RNA was denatured for 10 min at 70°C, and then mixed with a buffer containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 5 mM MgCl2, 1 mM each dGTP, dATP, dTTP, dCTP, 0.5 g oligo dT primer, 20 U RNase inhibitor, 15 U AMV reverse transcriptase, in a final volume of 20 ml. This mixture was incubated for 15 min at 42°C, heated at 99°C for 5 min, and then incubated at 0–5°C for 5 min.

Quantitative real time PCR (qRT–PCR). For qRT–PCR analysis, TaqMan technology was applied, according to the manufacturer’s instructions. Taqman qRT–PCR involves the continuous detection of newly formed amplicons during PCR cycling, using a forward primer, a reverse primer and a FAM–TAMRA probe, which anneals between the two other primers. qRT–PCR was performed in triplicate for each sample using the ABI PRISM 7900HT Sequence Detector (Perkin–Elmer Applied Biosystems) and the TaqMan EZ RT–PCR Core Reagents Kit (Perkin–Elmer Applied Biosystems). The qRT–PCR was performed for CRα, CRβ and β-actin as a control housekeeping gene. Sequences for forward and reverse oligonucleotide primers as well as the TaqMan probes used in this study are detailed in Table 2. These oligonucleotides were designed using Primer Express software (PE-Applied Biosystems, Warrington, UK) and primers spanning intron sequences were selected to differentiate amplification from genomic DNA. All primers and probes were synthesized by Sigma (UK). The qRT–PCR primers and probes are shown in Table 2.

Before application of Taqman qRT–PCR to determine CRα and CRβ ratio, the capacity of the technique to detect differences in ratios of synthesized RNAs was validated. Amplicons presenting parts of exon 9α or exon 9β were generated from HeLaS3 cDNA, mixed in different ratios and analysed by Taqman qRT–PCR. Cycle conditions were as follows: initial denaturing at 95°C for 10 min, 50 cycles of 95°C for 15 s, 60°C for 60 s, elongating at 72°C for 60 s and one final elongation step of 5 min at 72°C. Tenfold serial dilutions (spanning four orders of magnitude) of HeLa S3 cDNA of known concentrations were used to construct standard curves of cycle threshold (Ct) values for CRα, CRβ and β-actin.

Analysis of cellular expression of CR protein isoforms by flow cytometry

PBMCs were isolated from blood collected between 10:00 and 11:00 A.M. from RA patients as described above. Cells were stained for CRα and CRβ using a modification of the flow-cytometry method for intracellular proteins as previously described [26] and analysed by fluorescent activated cell sorters (FACS). HeLaS3 cells, which over-express CRβ, were used as a positive test control.

Antibodies for immunofluorescence staining. The irrelevant non-specific Simulset™ IgG control was a mixture of FITC- and PE-conjugated mouse antibody isotype IgG2a (Becton Dickinson, San Jose, California). The first layer antibodies were an affinity purified protein rabbit polyclonal antibody GR(P-20) (Autogen Bioclear UK Ltd, Devizes, UK) and/or GCR-βPA3-514 (Affinity Bioreagents, Golden, USA) directed against CRα and CRβ, respectively, as indicated. The affinity purified protein antibodies only bind to their target protein molecules. The second antibody was FITC-conjugated polyclonal goat anti-rabbit (GAR) (BD Biosciences, San Diego, CA, USA). In order to reduce non-specific binding to other intracellular proteins, GAR-FITC was serially pre-adsorbed as previously described [25].

PBMCs isolated by density gradient centrifugation were washed twice with a wash solution [WS; phosphate-buffered saline (PBS) containing 0.01% azide and 0.1% bovine serum albumin (BSA)] by resuspension of the cell pellet in the solution and centrifugation at 500 g. Cells were then fixed by incubation for 10 min on ice, in 1 ml of a 1% paraformaldehyde solution in PBS containing 0.1% BSA and then washed twice. Cells were then placed in each tube and treated as detailed below.

Permeabilization was performed with 1.2% n-octyl glucoside (NOG) for 7.5 min at 4°C. After permeabilization the cells were washed twice with WS and then stained. Cells were incubated with 20% normal human serum (NHS) for 10 min, washed twice. The first antibody was added (5 ml) and cells were incubated at 25°C for 20 min then washed twice. The second antibody pre-adsorbed GAR-FITC (5 ml) was added and cells were incubated at 25°C for 20 min then washed twice again with WS. Normal mouse serum (10 ml) was added, and cells were incubated at 25°C for 20 min then washed twice before adding the third final layer of antibodies. Finally, cells were washed twice and then resuspended in 500 ml of PBS/1% paraformaldehyde/0.1% BSA solution before FACS analysis.

### Table 2. The details of primers used for the quantitative real time PCR

| CRα probe: 5’-(FAM)-TGATAGAAGACCATGAGTAGGATTTCCCGCA-3’ (TAMRA). | Primers for CRα:
| i. forward: 5’-CTCTTTAACTGTTTCCCAAACATGTTTT-3’ [2326 (exon 8a)]; | Primers for CRβ:
| ii. reverse: 5’-TGATGTTTTACAGTATTTTAC-3’ [2387 (exon 9a)]. | i. forward: 5’-TAAATCTGTTTCTTCTCCCAAACATCT-3’ [2353 (exon 8)]; |
| CRβ probe: 5’-(FAM)-TGATAGAAGACCATGAGTAGGATTTCCCGCA-3’ (TAMRA). | ii. reverse: 5’-TGATGTTTTACAGTATTTTAC-3’ [2414 (exon 9b)]. |
| Primers for β-actin: | Primers for β-actin:
| i. forward: 5’-GGCCGGCTCAGCTTCTTCA-3’; | i. forward: 5’-GGCCGGCTCAGCTTCTTCA-3’; |
| ii. reverse: 5’-TTCCCTTATAGTGCACGCGAT-3’. | ii. reverse: 5’-TTCCCTTATAGTGCACGCGAT-3’. |
Flow-cytometric analysis was carried out using a Becton Dickinson FACScan Calibur and results were analysed using WinMDI V2.8 software. The cells were gated for forward and side scatter. The total number and percentage of positive PBMCs and the mean intensity of fluorescence (MIF) of CRβ and CRα staining over background were determined.

Statistical analysis
The results are expressed as mean±SEM. The results were analysed using SYSTAT 11 software. Non-parametric tests were used to analyse the differences between the observations seen in the SS and SR RA subjects with Wilcoxon matched-pair test for two-group comparisons with exact, two-tailed significance levels and analysis of variance. A value of \( P \leq 0.05 \) was considered to indicate statistical significance.

Results
Steroid sensitivity status of patients. There were no significant differences in the degree of inflammation between the two subgroups of patients as measured by the levels of ESR, CRP and number of active joints (Table 1). The distribution of RF seropositivity status, gender ratios and disease duration were similar between the SS and SR RA subgroups. Subjects were subdivided using the hydrocortisone IC\(_{50}\) of 10\(^{-8}\)M into SS and SR subgroups as previously described [2]. The mean dose–response curves of the subgroups are shown in Fig. 1. As can be seen, there are significant differences in the percentage inhibition of cell proliferation at each dose point between the SS and SR subjects.

Levels of CR isoform mRNA expression in PBMCs as determined by qRT–PCR. Taqman RT–PCR was used to quantitate levels of expression of the receptor isoforms. All PCR products were evaluated on the basis of the individual β-actin housekeeping gene expression. For CRα and CRβ isoform expression, CDNA samples from PBMCs from RA patients were analysed. The expression of mRNA levels of CRα and CRβ isoforms at basal and following stimulation with con-A, in the absence or presence of hydrocortisone were compared between the SS and SR subgroups (Fig. 2). The level of CRα mRNA expression was the same in both subgroups. A trend towards higher basal expression of CRβ mRNA in PBMCs was observed in SR RA patients than in the SS RA subgroup. Con-A stimulation without or with hydrocortisone caused a significant increase in the expression of the CRβ isoform in SR RA patients compared with the SS RA subjects (\( P < 0.05 \) and \( P < 0.02 \), respectively). Con-A stimulation did not cause an increase in CRβ mRNA in the SS RA subgroup and hydrocortisone caused a 14.5% downward trend inhibition in expression (Fig. 2B). In contrast, con-A enhanced CRβ isoform mRNA expression by 20%, in the SR RA patients, which was not inhibited at all by hydrocortisone (Fig. 2B). However, the differences in the relative over-expression of CRβ in SR RA patients become more highly significant when one looks at the CRβ/CRα ratio of mRNA expression (Fig. 2C). The basal CRβ/CRα ratio shows a trend towards higher levels in PBMCs from SR RA subjects than in SS RA patients. Significant changes are observed following stimulation of PBMCs in vitro with con-A alone and treatment of con-A-stimulated PBMCs with hydrocortisone with a significantly higher CRβ/CRα ratio in SR RA patients (\( P \leq 0.01 \) and \( P \leq 0.01 \), respectively). Thus, overall, the qRT–PCR data suggest that CRβ mRNA is over-expressed in SR RA patients.

Protein expression levels of the CR isoforms in PBMCs. Since overexpression of CRβ protein has been shown to reduce the effects of CS, we analysed the protein expression of the CR isoforms using flow cytometry. Fig. 3 (panels A–D) shows representative immunocytoluorographic data from an SR and SS RA patient. As can be seen, the percentage of PBMCs expressing CRβ protein is higher in SR RA subjects. The results from a group of SS and SR patients are summarized in Fig. 4 (panels A and B). For the CRα isoform, there were no significant differences between the SS and SR RA patients (Fig. 4, panels A and B). However, the percentage of PBMCs staining for CRβ was significantly lower in the SS RA group (\( P < 0.001 \)). The MIF of CRβ staining was also higher in the SR RA subgroup of patients than in the SS subjects (\( P < 0.001 \)). These data support the qRT–PCR observations in SR RA patients that CRβ is relatively over-expressed in PBMCs from RA patients who are SR as defined by the in vitro CS bioassay.

Discussion
A number of molecular mechanisms may be involved in SR and this may be related to perturbations of the cellular pathways used by CSs to exert their biological effects [27, 28]. One of these relates to perturbations of the CR isoform expression. CSs mediate their genomic effects via the CRα receptor. A number of cell line transfection experiments have shown that the relative over-expression of the CRβ isoform induces a state of reduced sensitivity to the inhibitory effects of CS on gene expression [18–20]. Using qRT–PCR analysis and flow cytometry, we show for the first time enhanced CRβ mRNA and protein expression in PBMCs from SR RA patients when compared with the SS RA patient subgroup. We also demonstrate that con-A stimulation of PBMCs from SR RA patients enhances overexpression of CRβ mRNA, which is not inhibited by hydrocortisone treatment. This observation is of pathophysiological relevance to the molecular basis of the SR phenomenon, since such a relative increase in CRβ expression during cell activation may occur during inflammation and could potentially contribute to a reduced state of responsiveness to the biological effects of CS therapy.

Our observations in RA patients at the mRNA level were also reflected at the protein level in PBMCs. Our flow-cytometry data
are highly significant since the CRβ isoform does not undergo ligand-dependent down-regulation and consequently has a much longer half-life than that of CRα [29]. Thus, even low levels of CRβ protein may achieve a significant dominant negative effect on the activity of CRα isoform [29]. Hence, even the small increases in CRβ mRNA expression such as observed at the basal level in the SR RA patients, may translate into significant protein expression and the associated biological effects. Minimal perturbations of the CRβ/CRα mRNA ratio, may lead to prolonged and enhanced CRβ mediated inhibitory effects at the protein level. Our observations at the protein level in SR RA patients, are similar to the in vivo observations of Sousa et al. [30] and Hamid et al. [31] in SR asthmatic patients. Using fluorescence microscopy, Hamid et al. [31] examined broncho-alveolar lavage cell cytopsins from SS and SR asthmatic patients stained by immunohistochemistry for the expression of CRβ. Broncho-alveolar lavage cell preparations from SR asthmatic patients had a significantly higher expression of CRβ. Sousa et al. [30] measured the CRα and CRβ immunoreactive cells in skin
The mechanisms responsible for the enhanced expression of CRβ in SR RA patients are currently unknown. Alternative splicing of the CR pre-mRNA gives rise to the homologous CRβ mRNA. Knowing the exact mechanisms that regulate alternative splicing of CR pre-mRNA could be exploited as a therapeutic application. Such research is currently being done in Duchenne muscular dystrophy to produce a different isoform of dystrophin [33].

The physiological and pathophysiological role of the CRβ isoform is controversial. Co-transfection experiments with human CRβ and CRα expression vectors and GRE-containing reporter plasmid to COS cells by three independent groups have shown that CRβ over-expression inhibits GRE-containing gene expression [18–20]. Others have, however, provided conflicting data [21–22, 34]. The latter may represent a limitation of cell line co-transfection experiments. Human neutrophils are less sensitive than T cells to the apoptotic effects induced by CS and this may explain in part the observation that Cs are less effective at resolving neutrophil-associated inflammation [35]. This may be a consequence of the higher constitutive expression of CRβ by neutrophils in comparison with T cells [36]. Mouse neutrophils do not express CRβ and are very sensitive to CS-induced apoptosis [37]. Transfection of mouse neutrophils with a pRSh CRβ expression vector results in a significant reduction of dexamethasone-induced neutrophil apoptosis [36]. This is the closest indirect ex-vivo experimental evidence supporting the view that the over-expression of CRβ in SR RA patients we have observed in our studies has direct pathophysiological relevance to the phenomenon of SR. More recently, Charmandari et al. [37] have studied the mechanisms underlying the dominant negative effects of the CRβ isoform on steroid receptor coactivators [38]. They showed that CRβ suppressed the transcriptional activity of both activation of function (AF)-1 and AF-2 of CRα by binding to the p160 coactivators and the CR interacting protein 1 (CRIP1) [38]. This finding supports our hypothesis that in SR RA patients the enhanced CRβ expression is of pathophysiological relevance to the SR phenotype. RA patients have reduced numbers of CRs on their lymphocytes but this does not result in reduced biological effects of CS per se [39]. It therefore remains to be determined whether there are differences in post-receptor mechanisms in the SS and SR RA patients. We are currently investigating this possibility.

It has been suggested that methotrexate may increase CR expression and thus potentially increase susceptibility to CS inhibition. All the SS and SR RA patients were on methotrexate and thus the differences in the expression of CR isoforms are not secondary to methotrexate. Furthermore, the duration of RA disease, levels of inflammation as measured by the levels of ESR, CRP and number of active joints were not significantly different between the SS and SR subjects.

Conclusions

In conclusion, we show for the first time that the CRβ isoform is over-expressed in SR RA patients at both the mRNA and protein levels. Furthermore, hydrocortisone fails to inhibit the con-A induced increase in CRβ mRNA. This over-expression of CRβ by PBMCs may in part explain the reduced responsiveness to CS therapy observed in these patients.

Acknowledgements

The authors thank the Arthritis Research Campaign, UK for funding this research and C. Mein of the Genome Centre for technical support in the qRT-PCR studies and Rukshana Patel and Cherma St Claire Ali for logistical and administrative support. We are also most grateful to all the RA patients who volunteered to participate in these studies.
The authors have declared no conflicts of interest.

References