

# MECHANISMS OF STEROID ACTION AND RESISTANCE IN INFLAMMATION

## Glucocorticoid resistance in inflammatory bowel disease

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### Abstract

Glucocorticoids are potent inhibitors of T cell activation and proinflammatory cytokines and are highly effective treatment for active inflammatory bowel disease (IBD). However, failure to respond, acutely or chronically, to glucocorticoid therapy is a common indication for surgery in IBD, with as many as 50% of patients with Crohn's disease (CD) and approximately 20% of patients with ulcerative colitis (UC) requiring surgery in their lifetime as a result of poor response to glucocorticoids. Studies report that approximately one-third of patients with CD are steroid dependent and one-fifth are steroid resistant while approximately one-quarter of patients with UC are steroid dependent and one-sixth are steroid resistant. While the molecular basis of glucocorticoid resistance has been widely assessed in other inflammatory conditions, the pathophysiology of the glucocorticoid resistance in IBD is poorly understood. Research in IBD suggests that the

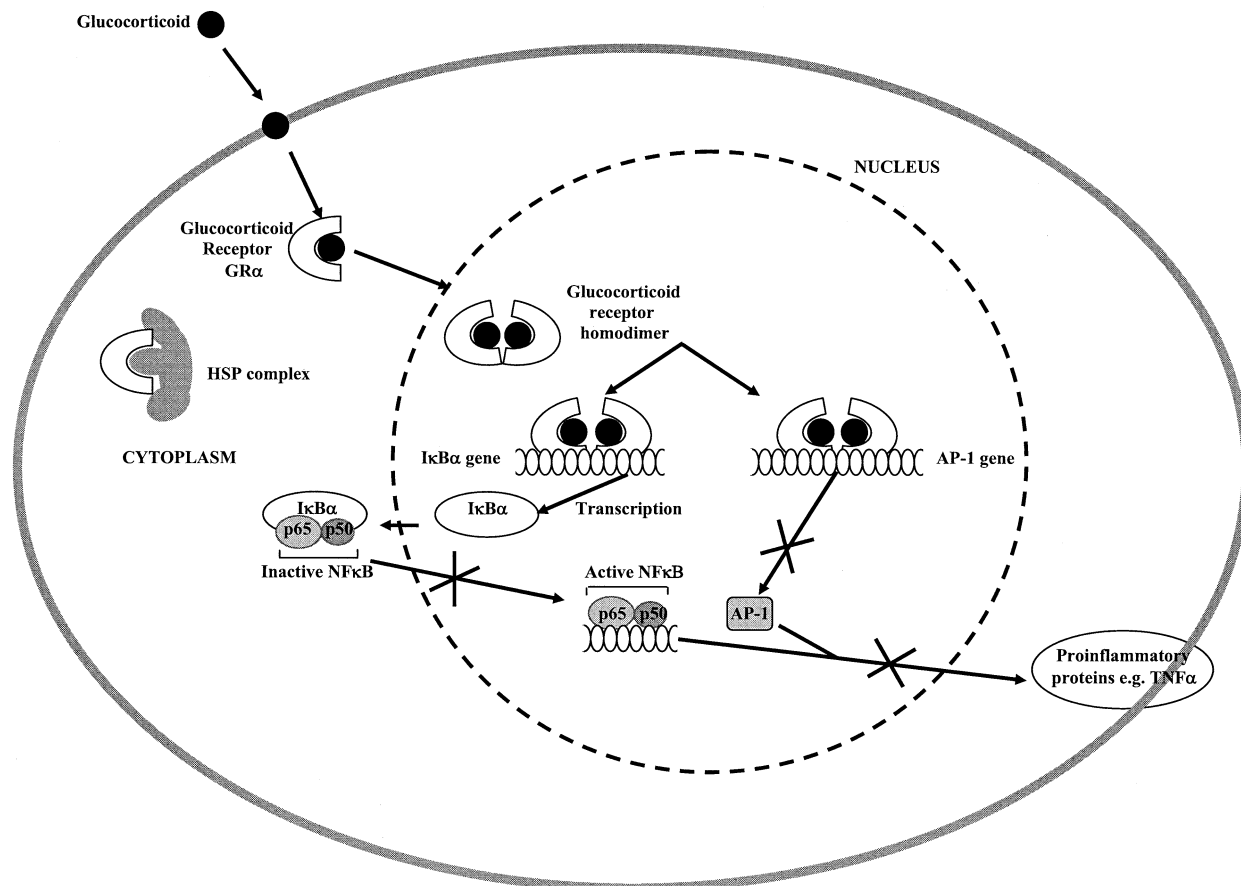
phenomenon of glucocorticoid resistance is compartmentalised to T-lymphocytes and possibly other target inflammatory cells. This review focuses on three key molecular mechanisms of glucocorticoid resistance in IBD: (i) decreased cytoplasmic glucocorticoid concentration secondary to increased P-glycoprotein-mediated efflux of glucocorticoid from target cells due to overexpression of the multidrug resistance gene (MDR1); (ii) impaired glucocorticoid signaling because of dysfunction at the level of the glucocorticoid receptor; and (iii) constitutive epithelial activation of proinflammatory mediators, including nuclear factor kappa B, resulting in inhibition of glucocorticoid receptor transcriptional activity. In addition, the impact of disease heterogeneity on glucocorticoid responsiveness and recent advances in IBD pharmacogenetics are discussed.

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### Introduction

Glucocorticoids are potent inhibitors of T cell activation and cytokine secretion and are consequently an effective treatment for inflammatory bowel disease (IBD). However, failure to respond, acutely or chronically, to glucocorticoid therapy is a common indication for surgery in IBD, with as many as 20% of patients with ulcerative colitis (UC) and approximately 50% of patients with Crohn's disease (CD) requiring surgery in their lifetime as a result of poor response to medical therapy. Several studies have attempted to define the prevalence of glucocorticoid resistance in IBD. A landmark retrospective study, describing the long-term outcome at 1 year in patients with CD treated with their first course of oral prednisolone dosed at 40 to 60 mg/day and tapered within weeks to a maintenance dose of 10 to 15 mg (Munkholm *et al.* 1994),

found that 36% of patients were steroid dependent and 20% were steroid resistant. A high frequency of surgical intervention was reported in steroid dependent (26%) and steroid resistant (59%) patients within 1 month after glucocorticoid treatment. A recent retrospective American study reported steroid dependency in 28% and 22% of CD and UC patients respectively, and steroid resistance in 16% of CD and UC patients (Faubion *et al.* 2001). The 1-year operation rate was 38% in CD patients and 29% in UC patients. While poor response to glucocorticoids may be influenced by such factors as underdosing and abrupt tapering, a prospective study in 48 consecutive patients with active CD, many of whom had previously been treated with glucocorticoids, using a higher oral glucocorticoid dose and a slower tapering schedule than used in the previous studies reported steroid dependency and resistance rates of 63% and 13% respectively (Reinisch



**Figure 1** Mechanism of action of glucocorticoids. Glucocorticoids mediate their anti-inflammatory responses by passively transporting themselves into target cells and binding the intracellular glucocorticoid receptor (GR), also known as the classic GR or GR- $\alpha$  (GR $\alpha$ ). The unliganded receptor is sequestered in the cytoplasm, bound to the heat-shock protein (hsp) complex which comprises chaperone molecules hsp90 and hsp70 and immunophilin FKBP59, a 59-kDa protein. In the cytoplasm the glucocorticoid ligand binds to GR $\alpha$  which becomes activated. This allows the formation of a homodimer of two activated GRs which is transported into the nucleus of the target cell within less than a minute after GR binding. Glucocorticoid action is dependent on GR-mediated transcriptional regulation of specific target genes as a result of sequence-specific DNA binding which, in turn, inhibits the promoter regions of genes such as nuclear factor kappa B (NF- $\kappa$ B) and activator protein-1 (AP-1) which are potent transcription factors for many proinflammatory cytokines and adhesion genes. Central to the anti-inflammatory action of glucocorticoids is the induction of inhibitor kappa B alpha (I $\kappa$ B $\alpha$ ) which binds to and inhibits NF- $\kappa$ B by sequestering it in the cytoplasm. TNF $\alpha$ , tumor necrosis factor  $\alpha$ .

*et al.* 1995). Similarly, the bioavailability of oral glucocorticoid therapy can be significantly reduced in patients with active IBD, particularly those with severe diarrhea, and many patients who fail to respond to oral glucocorticoids achieve remission with intravenous glucocorticoids. However, a recent retrospective study of 97 hospitalized patients with severe UC showed that despite high dose intravenous glucocorticoid therapy, 34% required colectomy within 30 days of presentation (Lindgren *et al.* 1998).

### Normal physiology

Glucocorticoids mediate their anti-inflammatory responses by binding the intracellular glucocorticoid receptor (GR),

also known as the classic GR or GR $\alpha$ , a phosphorylated 92-kDa protein, which is a member of the nuclear receptor superfamily (Beato *et al.* 1995). The unliganded receptor is sequestered in the cytoplasm, bound to heat-shock protein chaperone molecules hsp90 and hsp70 and immunophilin FKBP59, a 59-kDa protein. In the cytoplasm the glucocorticoid ligand binds to the GR which becomes activated. This allows the formation of a homodimer of two activated GRs which is transported into the nucleus of the target cell within less than a minute after GR binding. As illustrated in Fig. 1, glucocorticoid action is dependent on GR-mediated transcriptional regulation of specific target genes as a result of sequence-specific DNA binding which, in turn, inhibits the promoter regions of genes such as nuclear factor kappa B

(NF- $\kappa$ B) and activator protein-1 (AP-1) which are potent transcription factors for many proinflammatory cytokines and adhesion genes. Central to the anti-inflammatory action of glucocorticoids is the induction of inhibitor kappa B alpha (I $\kappa$ B $\alpha$ ) which binds to and inhibits NF- $\kappa$ B by sequestering it in the cytoplasm (Scheinman *et al.* 1995).

### Molecular mechanisms of glucocorticoid resistance

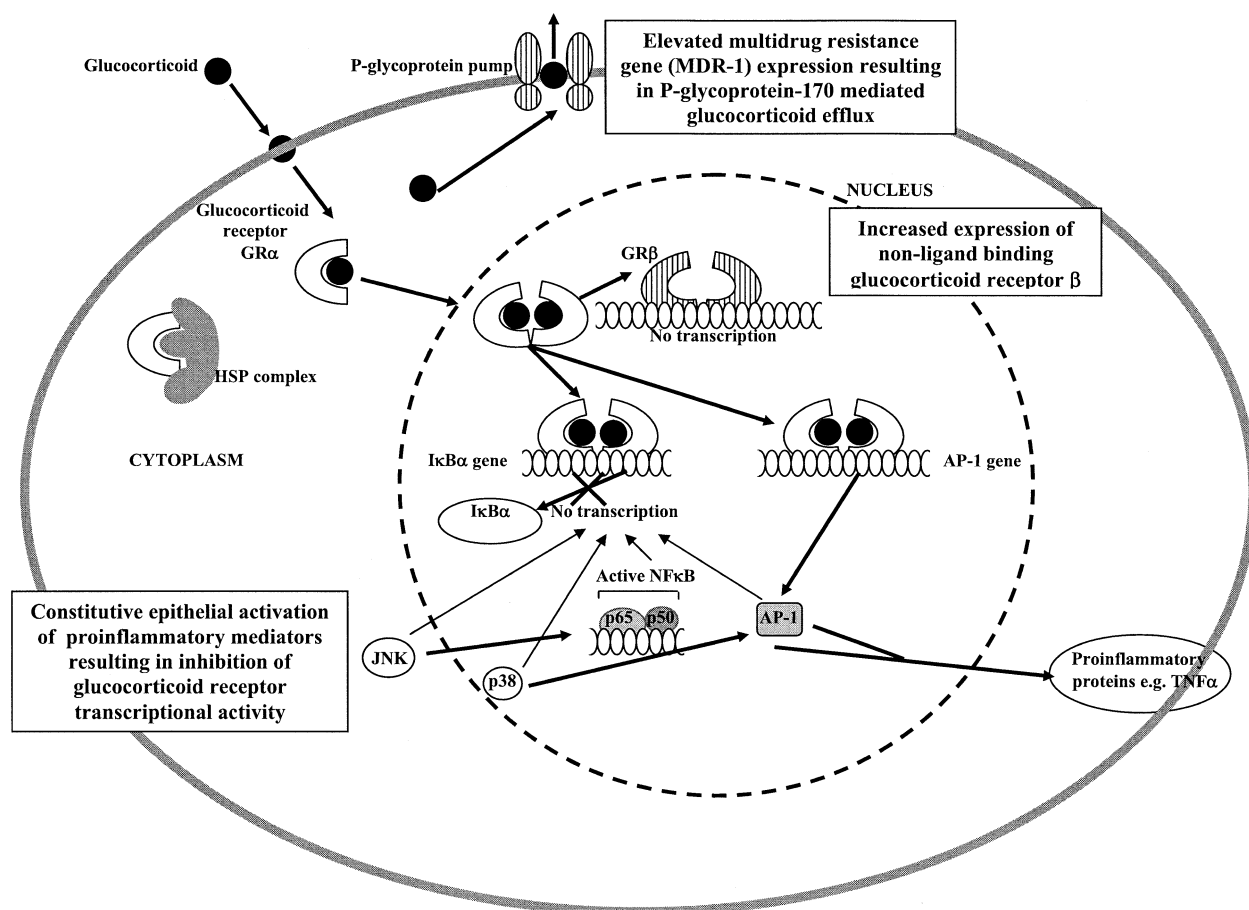
While the molecular basis of glucocorticoid resistance has been widely assessed in other inflammatory conditions, particularly asthma and rheumatoid arthritis, the pathophysiology of the glucocorticoid resistance in IBD has not been as well studied. Research in IBD suggests that similar to other inflammatory conditions the phenomenon of glucocorticoid resistance is not generalized but somehow compartmentalised to T-lymphocytes and possibly other target inflammatory cells. In a prospective study of 18 patients with acute severe UC, T-lymphocyte glucocorticoid resistance was found to be an important factor in determining response to glucocorticoid treatment (Hearing *et al.* 1999). Patients with acute severe UC were stratified, according to their clinical response 7 days after admission to a standard course of high dose intravenous glucocorticoid therapy, as complete responders ( $\leq 3$  stools daily with no visible blood), partial responders ( $\geq 4$  stools daily or visible blood with no indication for colectomy), or treatment failures (colectomy indicated). Concentration response curves were constructed for the antiproliferative effect of glucocorticoids on lectin-stimulated peripheral blood T-lymphocytes from these patients obtained within 48 h of admission. Proliferation of peripheral blood T-lymphocytes from five of seven patients classified as either partial responders or treatment failures was inhibited by less than 60% even at supra-physiological glucocorticoid concentrations. In contrast, proliferation of T-lymphocytes from all 11 complete responders was inhibited by at least 60% at glucocorticoid concentrations that might be expected to be achieved in the peripheral blood in the course of high dose intravenous glucocorticoid therapy. From a practical point of view, Hearing and colleagues (1999) demonstrated that a relatively fast, inexpensive *in vitro* T-lymphocyte proliferation assay may help predict which IBD patients will respond to glucocorticoid therapy and, more importantly, which patients will not respond to glucocorticoids, avoiding unnecessary and dangerous prolonged exposure to these drugs.

Similar correlations between *in vitro* T-lymphocyte sensitivity to glucocorticoid inhibition and the clinical response to glucocorticoid therapy have been reported in other inflammatory diseases such as asthma (Corrigan 1996), rheumatoid arthritis (Kirkham *et al.* 1991) and renal allograft rejection (Langhoff *et al.* 1986). These observations underscore the pivotal role of activated T-lymphocytes in the pathogenesis of these diseases and also

emphasise that, in a subset of IBD patients, glucocorticoid therapy is not viable because systemic concentrations achieved even at high dosage are insufficient to inhibit T cell function significantly and, by inference, disease progression. Research in impaired sensitivity to glucocorticoid inhibition in IBD has highlighted three potential molecular mechanisms, illustrated in Fig. 2. (i) Decreased cytoplasmic glucocorticoid concentration secondary to increased P-glycoprotein-mediated efflux of glucocorticoid from target cells due to overexpression of the multidrug resistance gene (MDR1); (ii) impaired glucocorticoid signaling because of dysfunction at the level of the glucocorticoid receptor; and (iii) constitutive epithelial activation of proinflammatory mediators, including NF- $\kappa$ B, resulting in inhibition of glucocorticoid receptor transcriptional activity.

### The multidrug resistance gene and glucocorticoid resistance

Glucocorticoid access to the cytoplasm is thought to be a passive process, permitted by the hydrophobicity of steroids. There is, however, an active process mediated by the MDR1 whereby cells can expel such ligands. The MDR1 gene codes for a drug efflux pump P-glycoprotein-170, which is expressed on the apical surface of lymphocytes and intestinal epithelial cells and actively transports glucocorticoids and other drugs out of target cells, thereby reducing their efficacy. We have demonstrated elevated peripheral T-lymphocyte and intestinal epithelial cell MDR expression in CD patients who require bowel resection and UC patients who require proctocolectomies for failed medical therapy (Farrell *et al.* 2000). This suggests that a subset of refractory IBD patients might escape effective immunosuppression by steroids and other immunosuppressive agents including cyclosporin, because these drugs are MDR substrates and are effectively 'pumped out' of target cells. T-lymphocyte MDR expression appeared to be constitutive as levels were similar when active IBD patients were followed up 3 months later, suggesting that genetic factors may play an important role in determining glucocorticoid resistance mediated through increased MDR1 expression. More recently, we have shown that specific MDR pump inhibitors (e.g. PSC 833) can significantly increase intracellular human intestinal epithelial and T-lymphocyte levels of cortisol and cyclosporin (Farrell *et al.* 2002): Demonstration of significant cortisol and cyclosporin efflux from the apical surface of human intestinal epithelial Caco-2 cells corroborates previous work which demonstrated an eightfold and twofold increase in small bowel and large bowel tissue cyclosporin levels respectively, and a threefold and twofold increase in small bowel and large bowel dexamethasone levels respectively in *mdr-1a*<sup>-/-</sup> mice (Schinkel *et al.* 1995, 1997), as well as *in vitro* data



**Figure 2** Mechanisms of glucocorticoid resistance in inflammatory bowel disease. Research in impaired sensitivity to glucocorticoid inhibition in inflammatory bowel disease has focused on three potential molecular mechanisms: (i) decreased cytoplasmic glucocorticoid concentration secondary to increased P-glycoprotein-mediated efflux of glucocorticoid from target cells due to overexpression of the multidrug resistance gene (MDR1); (ii) increased expression of glucocorticoid receptor  $\beta$  (GR $\beta$ ), a truncated splice variant of the normal isoform GR $\alpha$ , that does not bind glucocorticoid ligands, and is therefore unable to transactivate glucocorticoid-responsive genes; and (iii) functional interference with the glucocorticoid response by constitutive epithelial activation of proinflammatory mediators, NF- $\kappa$ B, AP-1 and upstream protein kinases p38 and c-Jun N-terminal kinase (JNK) which can directly inhibit (thin arrows) the anti-inflammatory action of a limited number of GR $\alpha$  molecules by preventing GR transcriptional activity.

demonstrating that complete blockade of intestinal MDR by PSC 833 significantly reduces cyclosporin efflux in mice (Mayer *et al.* 1997) and increases serum cortisol levels in rabbits (Cufer *et al.* 1998).

Significant progress has been made on the determination of the physiological role of MDR1 and related proteins. MDR1 expression is greatest in tissues where there is heavy exposure to toxic material – liver, lungs, intestine and kidneys – suggesting that it functions in normal tissues to actively export toxic compounds. The location of the MDR pump on the apical surface of bowel epithelium supports a defence mechanism in excreting toxic metabolites into the intestinal lumen. Of particular interest to refractory IBD, human studies demonstrate regional variation in MDR expression with moderate expression in the duodenum and jejunum and high levels

of expression in the ileum, with the highest levels found in the distal colon and rectum (Fojo *et al.* 1987). The very high level of MDR expression in the adrenal glands, the site of glucocorticoid synthesis, and studies on normal T-lymphocytes which show that MDR can act to protect lymphoid cells from glucocorticoid-induced apoptosis support a physiological role for P-glycoprotein-170 in glucocorticoid transport.

There is also evidence that MDR inhibition may have therapeutic applications in overcoming glucocorticoid resistance in other inflammatory conditions. Significantly elevated T-lymphocyte MDR1 expression has been shown in patients with rheumatoid arthritis who require glucocorticoids (Maillefert *et al.* 1996), renal graft recipients who undergo graft rejection on cyclosporin therapy (Zanker *et al.* 1995) and, more recently, in patients

with systemic lupus erythematosus (Diaz-Borjon *et al.* 2000), compared with healthy controls. While MDR1 in circulating lymphocytes does not appear to be involved in steroid-resistant asthma (Montano *et al.* 1996), elevated MDR1 gene expression has also been implicated in patients with drug-resistant epilepsy (Tishler *et al.* 1995, Lazarowski *et al.* 1999).

To date, 15 MDR1 polymorphisms have been identified and a polymorphism in exon 26 (C3435T) of the MDR1 gene has been shown to be significantly correlated with levels of expression and function of P-glycoprotein-170 in healthy individuals. Healthy individuals homozygous for this polymorphism (C/C or 'resistant' genotype) have significantly higher duodenal and natural killer T-cell MDR1 expression, 38% lower digoxin plasma levels and 17% more efflux of rhodamine dye from CD56+ natural killer cells than volunteers with the T/T or 'responsive' genotype (Hoffmeyer *et al.* 2000, Hitzl *et al.* 2001). Substantial differences in the frequency of C3435T polymorphism have been reported between racial groups with a significantly higher frequency of the C/C genotype in West Africans (83%) and African Americans (61%) compared with 26% and 34% in caucasians and Japanese populations respectively (Cascorbi *et al.* 2001, Schaeffeler *et al.* 2001). As noted earlier, the prevalence of glucocorticoid-unresponsiveness in several population-based cohorts of caucasian IBD patients is approximately 20% to 40% which, while speculative, suggests that MDR1 pharmacogenetics may play an important role in these important IBD subgroups. Studies are currently underway assessing the frequency of MDR1 polymorphisms in IBD populations and, ultimately, these findings may prove useful in predicting glucocorticoid responsiveness among IBD patients and also help devise novel strategies using MDR inhibitors in the management of steroid-dependent and resistant IBD patients (Lum & Gosland 1995).

### Glucocorticoid receptor abnormalities and glucocorticoid resistance

Primary glucocorticoid resistance due to inherited abnormalities of the glucocorticoid receptor (GR) have been described (Iida *et al.* 1985) but very few cases have been reported worldwide and there are no reports in the literature of IBD patients with primary glucocorticoid resistance or grossly elevated cortisol levels characteristic of this condition. However, most glucocorticoid resistance research on T-lymphocytes and other target inflammatory cells have demonstrated several GR abnormalities as potential mechanisms influencing response to glucocorticoid treatment in several inflammatory conditions. Of these inflammatory conditions, steroid-resistant asthma has been the best studied to date. Steroid-resistant asthmatics have a disease which fails to respond to high dose steroid

therapy despite the fact that the obstruction of their airways is clearly reversible in response to inhaled beta-2-agonists. Typically, they show no abnormalities of glucocorticoid absorption and clearance, and no innate abnormalities of the hypothalamus/pituitary/adrenal axis (Loke *et al.* 2002). Furthermore, they are not immune to developing the unwanted Cushingoid effects of prolonged glucocorticoid therapy. Several investigators have demonstrated reduced peripheral T-lymphocyte GR binding affinity (Kam *et al.* 1993), abnormalities of GR-AP-1 binding in glucocorticoid resistant asthma, suggesting a post-receptor mechanism (Adcock *et al.* 1995), and increased expression of glucocorticoid receptor  $\beta$  (GR $\beta$ ), a truncated splice variant of the normal isoform GR $\alpha$  that does not bind glucocorticoid ligands, is unable to trans-activate glucocorticoid-responsive genes, and has therefore been suggested to act as a dominant-negative inhibitor of glucocorticoid action (Leung *et al.* 1997).

Although not as well studied as steroid-resistant asthma, a number of investigators have turned their attention to GR dysfunction in steroid-resistant IBD. While the basal density of glucocorticoid receptor (GR mRNA) in peripheral leukocytes has been shown to be higher in UC patients whose disease is in remission compared with controls, no significant differences have been shown in GR mRNA levels between glucocorticoid responders and glucocorticoid nonresponders who had required colectomy (Flood *et al.* 2001). Therefore, differences in GR density or GR mRNA levels do not appear to be important in determining glucocorticoid resistance in patients with UC. One Japanese group recently reported increased GR $\beta$ -specific messenger RNA (mRNA) expression in peripheral lymphocytes from 83% of patients with steroid-resistant UC, whereas transcripts were detectable in only 9% of the steroid-responsive patients, 10% of healthy volunteers and 10% of chronic active CD patients (Honda *et al.* 2000). These interesting observations mirror similar studies which have demonstrated a putative role for GR $\beta$  in steroid resistant asthma (Leung *et al.* 1997) and rheumatoid arthritis (Chikanza 2002). While GR $\beta$  expression deserves attention by further prospective and longitudinal studies, there is evidence that argues against the assumption that GR $\beta$  expression plays a major role in glucocorticoid resistance. First, the expression of GR $\beta$  in all the tissues and cells examined is far less than the amount of GR $\alpha$  transcripts, in fact Honda and colleagues (2000) found that the relative level of GR $\beta$  expression in UC was only 0.165% of the expression of GR $\alpha$  and transfection experiments have demonstrated that the GR $\beta$  isoform has to be expressed in an at least 5- to 10-fold excess relative to GR $\alpha$  to significantly inhibit glucocorticoid-mediated gene expression (Bamberger *et al.* 1995). Still other studies found no evidence at all for a repressing effect of GR $\beta$  (Oakley *et al.* 1999). Thus, it is unlikely that the very low amounts of GR $\beta$  found in UC patients can exert a transdominant-negative effect.

Furthermore, *in vitro* data suggest that splicing and generation of the GR $\beta$  isoform is inducible by cytokines such as interleukin (IL)-2 and IL-4 (Bamberger *et al.* 1997) as well as glucocorticoid administration (Bantel *et al.* 2000a). Therefore, studies will have to evaluate the pathogenic role of GR $\beta$  in IBD and exclude the possibility that GR $\beta$  expression is a mere secondary result of a systemic inflammatory reaction.

### Inflammation, NF- $\kappa$ B and glucocorticoid resistance

Because glucocorticoid resistance is most frequently observed in those patients with severe disease, it remains unclear whether glucocorticoid resistance is a primary phenomenon or whether the anti-inflammatory capacity of glucocorticoids is simply overwhelmed by an excessive synthesis of proinflammatory cytokines, owing to an excessive activity of various intracellular transcription factors which, in turn, may reduce the affinity of GR for its intracellular ligand. Both mechanisms favor an excess expression of inflammatory molecules and a positive feedback system with perpetuation of the inflammatory response. It has been suggested that relative glucocorticoid resistance may be induced in T-lymphocytes by the local inflammatory environment, as exposure of T-lymphocytes to inflammatory cytokines *in vitro* reversibly increases their resistance to glucocorticoid inhibition, possibly by reducing the binding affinity of their intracellular GR for ligand (Kam *et al.* 1993, Corrigan 1996). This is not, however, an entirely satisfactory explanation as most patients with severe IBD respond to glucocorticoid therapy, despite the fact that, presumably, their T-lymphocytes are in a similar inflammatory environment to those who do not. These phenomenon may be superimposed on a background of genetically determined T-lymphocyte glucocorticoid responsiveness mediated by MDR1 or even variable, innate T-lymphocyte glucocorticoid responsiveness which is known to be highly variable even in normal people (Walker *et al.* 1987).

As described above, the anti-inflammatory activity of GR $\alpha$  is mainly mediated by its interference with potent transcription factors such as NF- $\kappa$ B, which results in inhibition of the synthesis of cytokines and other gene products of the inflammatory cascade. Conversely, NF- $\kappa$ B and GR $\alpha$  can mutually repress each other's transcriptional activity. Consequently, the debate as to whether inflammation drives glucocorticoid resistance or *vice versa* has refocused investigators' efforts into the critical role played by NF- $\kappa$ B (Dumont *et al.* 1998). Investigators assessed NF- $\kappa$ B activity in biopsy specimens from steroid-resistant and steroid-sensitive patients with CD and UC who all had severe disease activity (Bantel *et al.* 2000b). In sensitive patients, NF- $\kappa$ B activation was mainly found in lamina propria macrophages and in single scattered endothelial

cells. However, over 60% of steroid-resistant patients had a different staining pattern with active NF- $\kappa$ B predominantly localized in epithelial cells. The same investigators subsequently showed that while the activation of AP-1 and the upstream kinases p38 and c-Jun N-terminal kinase (JNK) in steroid-sensitive patients with CD was mainly found in lamina propria macrophages, steroid-resistant patients revealed activation of all these mediators mostly in epithelial cells (Bantel *et al.* 2002). The functional interference of these proinflammatory mediators with the glucocorticoid response was supported by reporter gene assays with expression of NF- $\kappa$ B, JNK and p38 all inhibiting the activity of GR $\alpha$ . This suggests that glucocorticoid resistance in CD is associated with constitutive activation of epithelial NF- $\kappa$ B and stress-activated protein kinases which, in turn, may inhibit the anti-inflammatory action of a limited number of GR $\alpha$  molecules by preventing GR transcriptional activity.

### Disease heterogeneity

While research into impaired target cell sensitivity to glucocorticoid inhibition in IBD has largely focused on potential molecular mechanisms, disease heterogeneity is another possible explanation as to why a subset of IBD patients are glucocorticoid resistant. Our increasing understanding of the pathogenesis of IBD has helped identify genetic factors that are associated with various disease phenotypes which, in turn, may influence the clinical response to glucocorticoid therapy. One study reported that patients with steroid refractory UC have a higher incidence of perinuclear anti-neutrophil cytoplasmic antibody than steroid responsive patients and normal controls (Sandborn *et al.* 1996). This suggests the possibility of heterogeneity in the pathogenesis of severe IBD which, in turn, might influence the clinical response to glucocorticoid therapy. Recently, multiple mutations have been identified in the NOD2 gene on chromosome 16 among CD patients, with many clustered in the leucine-rich repeat (LRR) domain (Ogura *et al.* 2001). Most significant among these is a frameshift variant, Leu1007 fsinsC, which truncates the last 3% of the protein. Two additional major variants, Arg702 Trp and Gly908 Arg have been identified, which confer similar genetic risks. Heterozygous carriage of any of the 3 major risk alleles increases susceptibility to CD 1.5- to 3-fold, whereas homozygotes or compound heterozygotes are at 18- to 44-fold increased risk. Taken together, these 3 major variants conservatively confer a 15% to 20% population attributable risk among familial CD, with a likely and lesser contribution among the more common, sporadic cases of CD. These frameshift mutations result in impaired binding to bacterial endotoxin and thus decreased activation of NF- $\kappa$ B, a process that is counterintuitive in a disease characterized by increased production of NF- $\kappa$ B

and tumor necrosis factor alpha (TNF $\alpha$ ). While this paradox remains to be explained, the discovery of the first gene for CD will have an important impact on future IBD research. It will help refocus research efforts in the rapidly expanding field of pharmacogenetics as clinical researchers can now incorporate knowledge about patients carrying NOD2 polymorphisms potentially to predict disease course and response to therapy. It is not known whether NOD2 mutations affect the response to glucocorticoids or immunomodulators in IBD, but in one study there was no relationship between infliximab responsiveness and NOD2 mutations (Vermeire *et al.* 2002). Ultimately, advances in pharmacogenetic research in NOD2, MDR1 and GR gene polymorphisms should help provide a unifying explanation linking disease heterogeneity, disease phenotype and glucocorticoid resistance in IBD.

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