Cell specific effects of glucocorticoid treatment on the NF- κ Bp65/I κ B α system in patients with Crohn's disease

K Thiele*, A Bierhaus*, F Autschbach*, M Hofmann, W Stremmel, H Thiele, R Ziegler, P P Nawroth

*KT, AB, and FA contributed equally to this work.

Department of Medicine University of Heidelberg, Im Bergheimer Strasse 58, 69115 Heidelberg, Germany
K Thiele
W Stremmel
R Ziegler

Institute of Pathology, University of Heidelberg, Im Neuenheimer Feld 220/221, 69120 Heidelberg, Germany
F Autschbach

Section of Vascular Medicine, Department of Medicine IV, University of Tübingen, Otfried-Müller-Strasse 10, 72076 Tübingen, Germany
A Bierhaus
P P Nawroth

Columbia University, Department of Physiology, 630 West 168th Street, NY 10032, USA
M Hofmann

Department of Surgery, Hospital Bruchsal, Gutleutstrasse 9-14, 76646 Bruchsal, Germany
H Thiele

Correspondence to: Dr P P Nawroth, Section of Vascular Medicine, Department of Medicine IV, Otfried-Müller-Strasse 10, 72076 Tübingen, Germany.

Accepted for publication 27 April 1999

Abstract

Background/Aims/Patients—Glucocorticoid treatment is known to reduce nuclear factor-kappa B (NF- κ B)p65 binding activity and activation in lamina propria cells of patients with Crohn's disease. However, lamina propria cells of glucocorticoid treated patients did not show increased expression of I κ B α , and the hypothesised upregulation of I κ B α by glucocorticoid treatment has not yet been shown in vivo. To investigate whether cells other than lamina propria localised mononuclear cells contribute to increased I κ B α , resection gut specimens from patients matched for Crohn's disease activity index (CDAI) with or without glucocorticoid treatment were studied, and changes in the NF- κ B/I κ B α system were determined in the lamina propria as well as in underlying submucosal and endothelial cells.

Methods—Changes in the NF- κ B/I κ B α system were determined by immunohistochemistry, electrophoretic mobility shift assay, and western blot analysis in resected gut specimens from patients matched for CDAI and van Hees index with or without long term glucocorticoid treatment.

Results—Resection gut specimens from patients with Crohn's disease under glucocorticoid treatment had significantly lower nuclear NF- κ Bp65 levels in mononuclear, epithelial, and endothelial cells than samples from CDAI and van Hees index matched patients not having glucocorticoid treatment. Nuclear NF- κ Bp65 showed a strong positive correlation with both the CDAI ($r = 1$ for both groups) and the van Hees index ($r = 0.605$ for untreated and $r = 0.866$ for glucocorticoid treated specimens). Lower nuclear translocation of NF- κ Bp65 in the glucocorticoid treated group was paralleled by higher I κ B α levels in vascular endothelial cells, but not in infiltrating mononuclear cells.

Conclusion—A comparison of resection gut specimens from untreated and treated CDAI matched patients with Crohn's disease showed downregulation of NF- κ B binding activity and NF- κ Bp65 expression and cell specific induction of endothelial I κ B α expression in the glucocorticoid treated group. As the two groups showed similar disease activity (CDAI, van Hees index), the activation of the NF- κ Bp65/I κ B α system must be only part of the

inflammatory cascade leading to the clinical appearance of Crohn's disease.

(Gut 1999;45:693-704)

Keywords: Crohn's disease; CDAI; inflammation; transcription factor; NF- κ Bp65; I κ B α

Increased oxidative stress and activation of redox sensitive cellular signalling systems are well recognised key steps in the development of chronic inflammatory disease.¹ Several molecular mechanisms have been described that convert the transient appearance of oxygen free radicals into sustained cellular changes and altered gene expression. One ubiquitous mechanism is represented by the redox sensitive transcription factor NF- κ B, which has been shown to be activated in response to a number of immune and inflammatory stimuli.²⁻⁶ Recently, Crohn's disease has been shown to be a model system for understanding the biological implications of NF- κ B activation.⁷⁻¹³

The NF- κ B family of transcription factors consists of the members p105/p50, p100/p52, p65 (RelA), relB, c-Rel, the proto-oncogene v-rel, and the *Drosophila* morphogen.²⁻⁶ Characteristically, NF- κ B proteins are sequestered in the cytoplasm as the result of retention by a class of inhibitory proteins, referred to as the I κ B family.^{4-6 14 15} On stimulation, I κ B α is phosphorylated at two serine residues in its N-terminal portion by a large I κ B kinase (IKK) complex in which IKK α and IKK β are associated with the newly identified scaffold proteins NEMO/IKK γ and IKAP and thus physically linked to upstream activators.^{16 17} Phosphorylation targets I κ B α for ubiquitination and subsequent rapid proteolytic degradation,¹⁴⁻¹⁷ thereby releasing free NF- κ B (preferentially the NF- κ B heterodimer p50/p65) which translocates into the nucleus, where it binds to decameric DNA sequences and activates transcription of NF- κ B regulated target genes.²⁻⁶ As I κ B α expression itself is controlled by NF- κ Bp65, NF- κ Bp65 activation is shut down by an I κ B α dependent autoregulatory feedback loop.^{2-5 14 15} It has been shown in vitro that newly synthesised I κ B α not only retains NF- κ Bp65 in the cytoplasm but translocates to the nucleus, where it can

Abbreviations used in this paper: BSA, bovine serum albumin; CDAI, Crohn's disease activity index; EMSA, electrophoretic mobility shift assay; GR, glucocorticoid receptor; NF- κ B, nuclear factor-kappa B; rNF- κ Bp65, recombinant NF- κ Bp65; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; TBS, Tris buffered saline.

sequester free NF- κ Bp65 and promote translocation of NF- κ B/I κ Ba complexes from the nucleus back to the cytoplasm.¹⁵ However, recent studies show that this autoregulatory mechanism can be compensated by induction of perpetuated NF- κ Bp65 mRNA synthesis, which provides a continuously growing pool of free NF- κ Bp65 which can translocate into the nucleus and thereby overrides the I κ Ba dependent self regulation.¹⁸

Glucocorticoids are steroid hormones with potent immunosuppressive and anti-inflammatory action, frequently used in the treatment of a broad range of chronic diseases such as allergic reactions, asthma, rheumatoid arthritis, systemic lupus erythematosus, transplantation dependent tissue rejection, and inflammatory bowel disease.^{19–24} The molecular mechanisms underlying the therapeutic effects of glucocorticoids on chronic inflammation are

poorly understood. However, there is growing evidence that they exert part of their action by downregulating gene expression by interfering with the activity of transcription factors such as STAT-1,²⁵ NFAT-1,²⁶ AP-1,²⁷ and NF- κ B.^{2 6 28} The highly immunosuppressive and anti-inflammatory action of glucocorticoids is at least in part related to a glucocorticoid dependent repression of NF- κ B mediated gene expression.^{2 6 28}

Glucocorticoids mediate their effects by binding to intracellular glucocorticoid receptors (GRs),²⁹ which function as ligand activated nuclear transcriptional regulators.^{29–31} Binding results in conformational changes in the ligand activated GR, which translocates into the nucleus and directly interferes with gene transcription.³¹ Two putative mechanisms have been proposed to explain GR mediated inhibition of NF- κ B: (a) in vitro studies indicate that ligand bound GR can physically interact with the NF- κ B subunit p65, thereby preventing transactivation of NF- κ B^{31–35}; (b) GR can upregulate I κ Ba gene transcription and expression possibly by yet undiscovered positive GR responsive elements in the I κ Ba promoter.^{36 37} The latter mechanism has been proposed to play a role in attenuating NF- κ Bp65 activity under chronic conditions.⁶ Only a few data are available that describe the relative contribution of each mechanism under physiological conditions.^{6 10}

It has recently been reported that I κ Ba was not upregulated in lamina propria biopsy specimens of patients with Crohn's disease receiving steroid treatment for seven days, although there was a significant decline in nuclear NF- κ Bp65.¹⁰ The authors suggest that clinical relapse of disease from remission may override an upregulation of I κ Ba¹⁰ and thereby contrasts with the in vitro situation.^{36 37} Another likely explanation may lie in the fact that the above study¹⁰ examined lamina propria specimens only, leaving the question open of what happens to the underlying submucosal and endothelial cells. Furthermore, because of the study design, the patients presumably had a different disease activity after glucocorticoid treatment, making it difficult to define whether changes in the NF- κ B/I κ Ba system are the cause or consequence of the glucocorticoid effect. The study presented here therefore evaluates I κ Ba expression and NF- κ B activation in resection gut specimens from patients with Crohn's disease with and without glucocorticoid treatment matched with respect to Crohn's disease activity index (CDAI) and van Hees index. As the two groups show the same disease activity and the same extent of inflammation, the study design allows us to define the effect of glucocorticoid treatment on NF- κ Bp65 activation independent of other factors that may modulate NF- κ Bp65 activity.

Methods

REAGENTS

[γ -³²P]ATP (3000 Ci/mmol at 10 Ci/ml), ECL-nitrocellulose membranes, ECL detection reagents, and Hyperfilm α ray films were obtained from Amersham, Braunschweig, Ger-

Table 1 Clinical characteristics of patients with Crohn's disease treated with and without glucocorticoids

	Without glucocorticoids	With glucocorticoids	p Value
Sex (M/F)	6/4	3/2	—
Age (years)	33.3 (7.2) (23–44)	39.0 (14.5) (21–57)	0.319
Duration of disease (years)	8.41 (7.5) (0.1–24)	8.7 (6.4) (1.5–15)	0.942
Haemoglobin (g/l)	119 (40) (14–149)	112 (53) (34–168)	0.780
Leucocytes ($\times 10^9$ /l)	10.9 (4.7) (6.2–18)	14.3 (8.9) (5.9–28)	0.336
Time of glucocorticoid therapy (months)	—	3.4 (2.3) (1–6)	—
CDAI	265 (110) (95–427)	289 (127) (170–443)	0.707
van Hees index	197 (21) (165–228)	187 (72) (107–289)	0.689

Values are mean (SD) (range).

Table 2 Treatment regimens

Patient no	Medication	Duration of treatment
<i>Without glucocorticoids</i>		
1	Mesalazine 250 mg	Twice a day for 6 weeks
2	—	—
5	—	—
6	—	—
7	—	—
8	—	—
10	—	—
11	Mesalazine 500 mg	Three times a day for 3 weeks
13	Mesalazine 500 mg	Three times a day for 2 years
14	—	—
<i>With glucocorticoids</i>		
3	Prednisolone 20 mg, Mesalazine 500 mg	Once a day for 1 month, Twice a day for 6 months
4	Methylprednisolone 8 mg	Once a day for 4 months
9	Prednisolone 20 mg	Once a day for 1 month
12	Prednisolone 10 mg	Once a day for 11 years
15	Methylprednisolone 8 mg, Mesalazine 500 mg	Once a day for 6 months, Three times a day for 1 week

Table 3 Evaluation score used for immunohistochemistry

Result of staining		
Score	Mononuclear cells	Endothelia
0	No positive cells	Negative
1	1–10% positive cells, weak staining	Weak staining, up to 10% of vessel circumference positive
2	1–10% positive cells, strong staining	Strong staining, up to 10% of vessel circumference positive
3	11–50% positive cells, weak staining	Weak staining, up to 11–50% of vessel circumference positive
4	11–50% positive cells, strong staining	Strong staining, up to 11–50% of vessel circumference positive
5	51–80% positive cells, weak staining	Weak staining, up to 51–80% of vessel circumference positive
6	51–80% positive cells, strong staining	Strong staining, up to 51–80% of vessel circumference positive
7	81–100% positive cells, weak staining	Weak staining, up to 81–100% of vessel circumference positive
8	81–100% positive cells, strong staining	Strong staining, up to 81–100% of vessel circumference positive

many. Monoclonal anti-p65 antibodies, specific for active NF- κ B, were obtained from Boehringer Mannheim, Mannheim, Germany. Anti-p65 (#sc-109), anti-c-Rel (N, sc-70), and anti-I κ B α (C-15, #sc-203) polyclonal antibodies were obtained from Santa Cruz Inc, Heidelberg, Germany. The anti-p105/p50 sera no 1157, 1140, 1613, and 1614 were generously given by Dr N Rice (NCI-Frederick Cancer Research and Development Centre, Frederick, Maryland, USA). Anti-CD3, anti-CD20, and anti-CD68 antibodies were from Dako, Hamburg, Germany. Anti-CD38 antibodies, Cy3 conjugated streptavidin, and Cy2 conjugated donkey anti-sheep antibodies were obtained from Dianova, Hamburg, Germany. Normal human immunoglobulins (gamma-venin) were

from Behring, Marburg, Germany. Bovine serum albumin (BSA) and Naphthol AS-biphosphate were obtained from Sigma, Deisenhofen, Germany. New Fuchsin was purchased from Merck, Darmstadt, Germany. Poly(dI/dC) was from Pharmacia, Freiburg, Germany. The NF- κ B consensus oligonucleotides and the rabbit reticulocyte lysate were purchased from Promega, Heidelberg, Germany.

PATIENTS

Table 1 gives a description of the patients, including sex, age, duration of disease, haemoglobin (g/dl), leucocyte concentration ($10^9/l$), CDAI, and van Hees index. Table 2 lists their treatments. The patients presented were under medical treatment in the Department of

Figure 1 Immunohistochemical staining of activated NF- κ Bp65 antigen in resection gut specimens from patients with Crohn's disease. (A) Representative staining in a resection gut specimen from a patient without glucocorticoid treatment (original magnification $\times 40$; representative positive stained cells are marked with an arrow). (B) Representative staining in a resection gut specimen from a patient with glucocorticoid treatment (original magnification $\times 40$; representative positive stained cells are marked with an arrow). (C) Representative staining in a resection gut specimen from a patient without glucocorticoid treatment; counterstaining with haematoxylin was omitted (original magnification $\times 63$). Many mononuclear cells in the lamina propria show nuclear staining. Some endothelial cells as well as crypt epithelial cells also express the molecule. (D) Representative staining in a resection gut specimen from a patient with glucocorticoid treatment; counterstaining with haematoxylin was omitted (original magnification $\times 100$). Few mononuclear cells in the lamina propria show positive nuclear staining (arrows). Some additional positive cells display weak cytoplasmic expression of the molecule (arrowheads). (E) Summary of the scoring and the statistical results.

Visceral Surgery in Bruchsal, Germany (H T), which is an academic teaching hospital associated with the University of Heidelberg. The study was approved by the ethics committee of the University of Heidelberg, according to the guidelines of the Helsinki Declaration.

PREPARATION OF TISSUES AND IMMUNOHISTOCHEMISTRY

Resection gut specimens of patients with Crohn's disease were taken during operation and snap frozen in liquid nitrogen. Depending on the conditions during surgery, time between

Figure 2 Double immunofluorescence staining of activated NF- κ Bp65 in combination with other cell markers in a resection gut specimen of a patient with Crohn's disease without glucocorticoid treatment. As leucocytes in the inflamed lamina propria show positive nuclear as well as cytoplasmic staining of NF- κ Bp65 (fig 1), simultaneous detection of CD3, CD20, CD68, or CD38 (column A; Cy2, green fluorescence) and NF- κ Bp65 (column B; Cy3, red fluorescence) was performed. This showed that NF- κ Bp65 is expressed by cells of all these leucocyte subsets. The double staining is shown in column C (Cy3, red; Cy2, green). Some vascular endothelial cells also express NF- κ Bp65 (arrowheads) (original magnifications: CD3, $\times 600$; CD20, $\times 900$; CD68, $\times 600$; CD38, $\times 900$).

sampling and freezing in liquid nitrogen varied slightly but never exceeded 20 minutes. Under these conditions, however, it could not be excluded that some intestinal tissue underwent ischaemia before it was frozen. Cryostat sections (5 µm) were mounted and fixed in cold acetone or 2% paraformaldehyde in phosphate buffered saline (pH 7.4) for 10 minutes at room temperature, followed by a wash in Tris buffered saline (TBS, pH 7.4). Primary antibodies (mouse anti-human NF-κBp65, IgG3 isotype) and rabbit anti-human IκBα were diluted 1:10 and 1:20 in TBS, pH 7.4, containing 0.2% BSA and 2.5 mg/ml normal human immunoglobulins (gamma-venin) and applied to the sections for 90 minutes at room temperature. After being washed in TBS/0.2% BSA, sections were incubated with 5% inactivated normal sheep (NF-κBp65) or goat (IκBα) serum to reduce non-specific background. Acetone treated slides were post-fixed with 2% paraformaldehyde before this step. After removal of the blocking solution, a biotinylated secondary antibody, adsorbed against human immunoglobulin (biotin-sheep anti-mouse (1:100; Amersham) or biotin-goat anti-rabbit (1:20; Dako) respectively was added for 30 minutes, followed by a 30 minute incubation with streptavidin complexed with

alkaline phosphatase (Dako). After subsequent washes with TBS and a final wash in distilled water, the colour reaction was developed using Naphthol AS-biphosphate and New Fuchsin as chromogen.³⁸ After counterstaining with haematoxylin, sections were mounted in glycerol/gelatin.

The immunohistochemical results were evaluated according to a score depicted in table 3. Coded slides were analysed by one investigator (K T) blinded to the clinical diagnosis and medication. Expression of NF-κBp65 and IκBα was determined on consecutive serial sections in each case. In each slide, the number of positive mononuclear cells was determined in at least five visual fields (magnification × 160) in five different representative areas of the lamina propria as well as in the submucosa. For determination of the endothelial expression, 40 vessels in the lamina propria and the submucosa were analysed in each case. Median values of the respective results were obtained for statistical evaluation.

IN SITU DOUBLE IMMUNOFLOUORESCENCE AND LASER SCAN MICROSCOPY

To determine which subsets of mononuclear cells express NF-κBp65 in Crohn's disease, in situ double immunofluorescence for NF-κBp65 in combination with other cell markers was performed. Sections were incubated for one hour with a mixture of monoclonal mouse anti-NF-κBp65 (IgG3 isotype) and monoclonal mouse anti-CD3 (clone OKT-3, IgG2a isotype; 10 µg/ml), mouse anti-CD20 (clone L-26, IgG2a isotype; dilution 1:50), mouse anti-CD68 (clone EBM11, IgG1 isotype dilution; 1:200), or mouse anti-CD38 (clone CBL 167, IgG2b isotype; dilution 1:50). Incubations with the primary reagents omitted and with the use of a mixture of non-binding monoclonal mouse antibodies of different isotypes served as negative controls. The antibodies were diluted in TBS/0.2% BSA/2.5 mg/ml gamma-venin. TBS/0.2% BSA was used for all washing steps and to dilute the secondary and tertiary reagents. As secondary antibodies, a combination of biotinylated goat anti-mouse IgG3 (dilution 1:50; Southern Biotech, distributed by Bibby Dunn, Asbach, Germany) and sheep anti-mouse IgG1, IgG2a, or IgG2b (dilutions 1:50, 1:100 and 1:50, respectively; Serotec Ltd, Kidlington, UK) plus gamma-venin was used, followed by simultaneous incubation with Cy3 conjugated streptavidin (dilution 1:1000; red fluorescence) and Cy2 conjugated donkey anti-sheep antibodies (dilution 1:100; green fluorescence) for 30 minutes respectively. After the final washes, the sections were mounted with histogel mounting medium (Cameron, Wiesbaden, Germany). Slides were viewed with a Laserscan microscope (Ernst Leitz GmbH, Wetzlar, Germany) using 570 nm (red emission) and 508 nm (green emission) filters.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSA was performed as described previously in detail.^{39, 40} In brief, frozen tissue (0.1 cm × 0.2 cm) was broken down mechanically, trans-

Figure 3 Immunohistochemical staining of activated NF-κBp65 antigen in resection gut specimens from patients with Crohn's disease (original magnification × 40); representative positive stained cells are marked with an arrow. (A) Representative staining of vascular endothelial cells in a resection gut specimen from a patient without glucocorticoid treatment. (B) Representative staining of vascular endothelial cells in a resection gut specimen from a patient with glucocorticoid treatment. (C) Summary of the scoring and the statistical results.

ferred to a 50 ml Falcon tube containing 5 ml cold buffer A (10 mM Hepes/KOH, pH 7.9 at 4°C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethanesulphonyl fluoride, 0.6% Nonidet P40) and homogenised with an Ultraturrax homogeniser (Wheaton, Milleville, New Jersey, USA) for one minute. Insoluble material was removed by centrifugation (30 seconds at 400 g (2000 rpm), 4°C) and the supernatant was incubated on ice for 10 minutes before being centrifuged for five minutes at 6000 g (8000 rpm), 4°C. The supernatant was discarded and the nuclear pellet was resuspended in 100 µl buffer B (25% glycerol, 20 mM Hepes/KOH, pH 7.9 at 4°C, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethanesulphonyl fluoride, 2 mM benzamidine, 5 mg/ml leupeptin) and incubated on ice for 20 minutes. Cellular debris was removed by two minutes centrifugation at 18000 g (14000 rpm) at 4°C and the supernatant was quick frozen at -80°C. Protein concentrations were determined by the Bradford assay.^{40a} A 10 µg portion of nuclear extract was included in the binding reaction.

Binding to an NF-κB consensus oligonucleotide (5'-AGTTGAGGGGACTTTCCAGG C-3') was performed in 10 mM Hepes, pH 7.5, containing 0.5 mM EDTA, 100 mM KCl, 2 mM dithiothreitol, 2% glycerol, 4% Ficoll 400,

0.25% Nonidet P40, 1 mg/ml BSA (DNase free), and 0.1 µg/µl poly(dI/dC) in a total of 20 µl as described.⁴⁰ Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabelled consensus oligonucleotides and by characterisation with specific polyclonal antibodies (Santa Cruz).

PREPARATION OF RECOMBINANT

NF-κBp65-CONTAINING LYSATE

Recombinant NF-κBp65 (rNF-κBp65) was prepared as previously described in detail.^{41 42} In brief, rNF-κBp65 was synthesised using the T7 promoter of the plasmid Rc-CMV-p65, kindly provided by Dr P A Baeuerle (Tularik, San Francisco, California, USA). NF-κBp65 RNA (3 µg) was added to 70 µl of rabbit reticulocyte lysate to translate proteins in vitro. Efficient translation was monitored in a parallel reaction using [³⁵S]methionine as substrate. A 0.15–20 µl volume of lysate, containing about 0.15–10 µg of in vitro translated protein,⁴² was used in the EMSA. Unprogrammed lysate without NF-κBp65 served as control.

SEMIQUANTITATIVE DETERMINATION OF NF-κBp65

BINDING ACTIVITY IN EMSA

To quantify the NF-κB signal obtained in each resection gut specimen, rNF-κBp65 was included in each EMSA as previously described in detail.⁴¹ Binding reactions with 5, 7.5, and 10

Figure 4 Total gut nuclear extract (10 µg) was prepared as described in Methods and assayed for NF-κB binding activity, monitored by electrophoretic mobility shift assay (EMSA). Radioactively labelled oligonucleotides spanning the consensus NF-κB recognition motif were incubated with equal amounts of nuclear extracts, and complexes were separated by electrophoresis on non-denaturing 5% polyacrylamide gels. (A) EMSA of four representative patients without (patients 1 and 2, lanes 1 and 2) and with (patients 3 and 4, lanes 3 and 4) glucocorticoid treatment. Specificity of NF-κB binding was shown by including a 160-fold molar excess of unlabelled consensus NF-κB oligonucleotide in the reaction with the extract from patient 2 (last lane). The position of NF-κB is indicated by an arrow. (B) Nuclear extracts of all resection gut specimens (table 1) were assayed for NF-κB binding activity by EMSA. Intensity of signals was expressed as recombinant NF-κBp65 (rNF-κBp65) equivalents using an internal standard curve for rNF-κBp65 (data not shown⁴¹). (C,D) Characterisation of the NF-κB subunits contributing to the observed shift formed at the NF-κB consensus sequence was performed by including 2.5 µg of anti-p50 (lane 2), anti-p65 (lane 3), anti-p52 (lane 4), anti-cRel (lane 5), or anti-relB (lane 6) antibodies in the binding reactions with nuclear extracts of a Crohn's disease patient without (C) or with (D) glucocorticoid treatment. Specificity of NF-κB binding was shown by including a 160-fold molar excess of unlabelled consensus NF-κB oligonucleotide in the reaction with extract from patient 2 (lane 7). The position of NF-κB is indicated by arrows.

Figure 5 (A, B) Cytoplasmic (CP) and nuclear (NE) extracts were prepared from resection gut specimens of two patients without (patients 2 and 14; A) and two with (patients 3 and 15; B) glucocorticoid treatment as described in the Methods. Fractions were separated by SDS/PAGE (12.5% gel) and subjected to immunoblotting with an antibody for NF-κBp65. Horseradish peroxidase coupled antibodies were used as secondary antibodies, and signals were detected with the ECL-western blot detection system. The specific complexes are indicated by arrows. (C) Nuclear NF-κBp65 detected by western blot in samples without ($n = 10$) and with ($n = 5$) glucocorticoid treatment was evaluated by densitometry. The determination of the signal area to be measured and the quantitative evaluation was performed twice for two independent experiments. The mean of the two measurements was taken for statistical analysis. (D) To adjust for individual differences in NF-κB expression, the ratio of nuclear to cytoplasmic NF-κBp65 (table 4) determined in western blot analysis was calculated to give an NF-κB activity coefficient for resection gut specimens without and with glucocorticoid treatment.

μg rNF-κBp65-containing lysate were included in each gel. The resulting NF-κB signals were quantified by densitometry (Bio-Rad, Munich, Germany) and used to establish a standard curve for each gel. The densitometric value for the NF-κB signal of the resection gut specimens was converted into rNF-κBp65 equivalents by using the internal standard curve for rNF-κBp65.⁴¹

WESTERN BLOT ANALYSIS

Cytoplasmic and nuclear fractions were prepared as previously described in detail.⁴³ Cytoplasmic extract (20 μg) or nuclear extract (10 μg) was subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE; 12.5% gel), followed by electroblotting on ECL-nitrocellulose membranes. Equal loading was confirmed by Ponceau Red staining. Filters were probed with the respective antisera for p65 (1:100), c-Rel (1:100), p50/p105 (1:2000), and IκBα (1:100) as described previously.^{40, 43} Membranes were washed (2×7 min; TBS/0.05% Tween) before the secondary antibody (horseradish peroxidase coupled rabbit IgG, diluted 1:2000 in Blotto A) was added and incubation was continued for 30 minutes at room temperature. Membranes were washed (3×15 minutes) as above followed by a last five minute wash in TBS. Immunoreactive proteins were detected with the ECL-western blot system and subsequent autoradiography for two minutes (p65,

p50), four minutes (IκBα), or five minutes (c-Rel).

DENSITOMETRIC QUANTIFICATION OF WESTERN BLOT AUTORADIOGRAMS

Signals obtained in western blot analysis were quantified using a GS-700 imaging densitometer (Bio-Rad). Determination of the signal

Table 4 Densitometric evaluation of NF-κBp65 signals obtained on western blot analysis of resection specimens from patients with Crohn's disease treated with and without glucocorticoids

Patient	Cytoplasmic NF-κBp65 (arbitrary units)	Nuclear NF-κBp65 (arbitrary units)	NF-κBp65 activity coefficient
<i>Without glucocorticoids</i>			
1	5.825	10.603	1.821
2	7.117	14.637	2.056
5	5.805	9.561	1.866
6	9.328	11.911	1.277
7	5.437	5.337	0.981
8	14.73	8.7	0.591
10	2.855	2.896	1.014
11	5.948	5.879	0.983
13	7.252	7.58	1.045
14	10.941	23.08	2.109
<i>With glucocorticoids</i>			
3	10.618	5.919	0.557
4	3.899	1.788	0.459
9	16.656	6.473	0.388
12	3.347	1.757	0.524
15	6.463	3.606	0.557

The determination of the signal area to be measured and the quantitative evaluation was performed twice in two independent experiments. The mean of the two measurements is given. To adjust for individual differences in NF-κB expression, the ratio of nuclear NF-κBp65 to cytoplasmic NF-κBp65 was calculated to establish an NF-κBp65 activity coefficient.

Figure 6 Correlation of nuclear NF-κBp65 (table 4) with (A) Crohn's disease activity index and (B) van Hees index in patients without and with glucocorticoid treatment.

area to be measured and the quantitative evaluation were performed twice for two independent experiments. The mean of the two measurements was taken for statistical analysis.

STATISTICAL ANALYSIS

Values are given as median for evaluation of immunohistochemistry and as mean (SD) for densitometric evaluation of EMSA results and

Figure 7 (A) Cytoplasmic extracts were prepared from resection gut specimens from four patients without (patient 5, 14, 2, 1; left) and four patients with (patients 15, 9, 3, 12; right) glucocorticoid treatment as described in the Methods. Fractions were separated by SDS/PAGE (12.5% gel) and subjected to immunoblotting with an antibody for full length IκBα. Horseradish peroxidase coupled antibodies were used as secondary antibodies and signals were detected with the ECL-western blot detection system. The specific complexes are indicated by arrows. (B) Cytoplasmic IκBα detected by western blot analysis in samples without ($n = 10$) and with ($n = 5$) glucocorticoid treatment was evaluated by densitometry. Determination of the signal area to be measured and the quantitative evaluation was performed twice for two independent experiments. The mean of the two measurements was taken for statistical analysis.

western blot signals. Student's two tailed t test was used to determine significance. $p < 0.05$ was considered to be statistically significant.

Results

NF-κBp65 BINDING ACTIVITY AND ACTIVATION IN RESECTION GUT SPECIMENS

To determine the influence of glucocorticoid treatment on NF-κB activation and IκBα expression in inflammatory bowel disease, we studied resection gut specimens, derived from 15 patients with Crohn's disease. Ten patients had never been treated with glucocorticoids, while five had taken glucocorticoids for at least four weeks (tables 1 and 2). Five patients (three without and two with glucocorticoid treatment) were taking sulfasalazine derivatives (table 2). Patients with and without treatment were matched for CDAl (196.8 v 187, $p = 0.69$) and van Hees index (265.1 v 289.4, $p = 0.707$).

Consistent with previous studies,^{10 11 13} immunohistochemical staining with an NF-κBp65 antibody that exclusively recognises activated NF-κBp65⁴⁴ showed positive red staining of numerous infiltrating mononuclear cells in sections obtained from patients without glucocorticoid treatment (fig 1A), and staining for activated NF-κBp65 was significantly lower in mononuclear cells in sections from glucocorticoid treated patients (fig 1B). When staining was repeated in the absence of haematoxylin, it became evident—that is, in sections from patients without glucocorticoid treatment, almost all NF-κBp65 positive cells displayed nuclear staining, and additional cytoplasmic staining was also noted (fig 1C). Activated NF-κB was present in mononuclear cells of the lamina propria and the submucosa as well as in endothelial and epithelial cells (fig 1C). In contrast, in sections from glucocorticoid treated patients, only a few mononuclear cells in the lamina propria showed positive nuclear staining (fig 1D, arrows). Some additional positive cells displayed weak cytoplasmic expression of NF-κBp65 (fig 1D, arrowheads). The lower level of activated NF-κBp65 observed in mononuclear cells of glucocorticoid treated resection gut specimens was confirmed by statistical evaluation (fig 1E).

To identify the mononuclear cells that express activated NF-κBp65 in Crohn's disease, double immunofluorescence staining for activated NF-κBp65 and CD3, CD20, CD38, and CD68 positive cells was performed on sections from untreated patients (fig 2). Activated NF-κBp65 was detected in CD3 positive T lymphocytes (first row), in CD20 positive B lymphocytes (second row), and CD68 positive mononuclear phagocytes/macrophages (third row) of the lamina propria, indicating that, in the inflamed mucosa, NF-κBp65 is activated in all major subsets of mononuclear cells. In addition, positive staining for activated NF-κBp65 was also observed in CD38 positive cells, presumably representing plasma cells.

Vascular endothelial cells of resection gut specimens without treatment also stained for activated NF-κBp65 (fig 3A), confirming that Crohn's disease also activates cells other than

mononuclear cells to participate in the inflammatory response. Glucocorticoid treatment was paralleled by a decrease in activation of NF- κ Bp65 in the vessel wall (fig 3B,C).

This prompted us to investigate the NF- κ B activation pattern in total resection gut specimens in order to define whether (a) it differed from the NF- κ B activation previously described for mononuclear cells in lamina propria biopsy specimens^{10,13} and (b) whether there were differences in nuclear NF- κ Bp65 in patients with and without glucocorticoid treatment. When EMSA was performed with nuclear extracts of resection gut specimens, three different concentrations (5, 7.5, and 10 μ g) of rNF- κ Bp65 programmed rabbit reticulocyte lysate were tested in parallel binding reactions and included on each gel as internal standard.⁴¹ This allowed us to normalise for intra-assay variation and to perform a semi-quantitative determination of the NF- κ B binding activity expressing NF- κ B binding activity as recombinant NF- κ B equivalents (rNF- κ B equivalents⁴¹). The results for four representative patients without (patient 1 and 2) and with (patient 3 and 4) glucocorticoid treatment are shown in fig 4A. Despite being normalised with respect to CDAI and van Hees index, NF- κ B binding activity was higher in untreated

patients (122.9 rNF- κ B equivalents) than in patients taking glucocorticoids (68.7 rNF- κ B equivalents; $p = 0.051$) (fig 4B). Characterisation of proteins contributing to the signal observed in EMSA by supershift experiments showed that in untreated samples the NF- κ Bp50/NF- κ Bp65 heterodimer preferentially bound to the NF- κ B binding motif, as evidenced by supershifts and reduction in NF- κ B binding activity in the presence of anti-p50 and anti-p65 antibodies (patient 2; fig 4C, lanes 2 and 3). Compared with samples of untreated patients, the contribution of NF- κ Bp65 and NF- κ Bp50 to the observed shift did not significantly differ in samples of glucocorticoid treated patients (fig 4D, lanes 2 and 3), although NF- κ B binding activity was lower in all samples tested (fig 4A,B). However, there was a tendency towards a slight increase in NF- κ Bp50 in samples from glucocorticoid treated patients as reflected by an increase in supershifted material in the presence of anti-p50 antibodies (patient 3; fig 4D, lane 2). In contrast with recently published results for epithelial and mononuclear cells isolated from inflamed mucosa,¹³ incubation with c-Rel antibodies did not result in supershifting or significant weakening of the NF- κ B complexes (fig 4C,D, lane 5). Only in one sample from a patient receiving glucocorticoid treatment did c-Rel antibodies result in a pronounced reduction in NF- κ B binding activity (data not shown). Although we cannot exclude a serological bias of detection, these findings imply that NF- κ B-cRel is not a major component of the NF- κ B complex detected in resection gut specimens from untreated and glucocorticoid treated patients with Crohn's disease.

CORRELATION OF DOWNREGULATION OF NF- κ Bp65 WITH DISEASE ACTIVITY

To extend further the observation of a glucocorticoid dependent switch in the extent of NF- κ B activation in CDAI and van Hees index matched Crohn's disease resection gut specimens, western blot analysis was performed. All probes from untreated patients showed a prominent translocation of NF- κ B from the cytoplasm to the nucleus. Although there was a signal for NF- κ Bp65 in the cytoplasm, stronger NF- κ B banding was detected in nuclear extracts of resection gut specimens as shown for two representative patients (patients 2 and 14; fig 5A, table 4). In contrast, a strong cytoplasmic NF- κ Bp65 signal was present in specimens from patients receiving glucocorticoid treatment, while nuclear NF- κ Bp65 staining was hardly detected. Two representative patients are shown (3 and 15; fig 5B, table 4). When cytoplasmic and nuclear signals of all patients were evaluated by densitometry, the signals obtained for nuclear NF- κ Bp65 confirmed a decrease in nuclear NF- κ B from 10.1 arbitrary NF- κ B units in samples from untreated patients to 3.9 arbitrary NF- κ B units in samples from glucocorticoid treated patients (fig 5C; table 4; $p = 0.0407$). To adjust for individual differences in NF- κ B expression, we calculated the ratio of nuclear to cytoplasmic NF- κ Bp65 (table 4),

Figure 8 Immunohistochemical staining of I κ B α antigen in resection gut specimens from patients with Crohn's disease (original magnification $\times 40$); representative positive stained cells are marked with an arrow. (A) Representative staining of mononuclear cells in a resection gut specimen from a patient without glucocorticoid treatment. (B) Representative staining of mononuclear cells in a resection gut specimen from a patient with glucocorticoid treatment. (C) Summary of the scoring and the statistical results.

thereby establishing an NF- κ B activity coefficient which allowed definition of the actual extent of NF- κ B translocation. This coefficient was 1.37 in untreated and 0.497 in glucocorticoid treated patients (fig 5D, table 4; $p = 0.0037$) and thus confirmed that NF- κ Bp65 nuclear translocation was lower in patients having glucocorticoid treatment.

The amount of nuclear NF- κ Bp65 in both groups correlated exactly with the given CDAI ($r = 1$ for both; fig 6A) and sufficiently with the van Hees inflammation index ($r = 0.605$ for untreated and 0.866 for treated patients; fig 6B), suggesting a relation between nuclear NF- κ Bp65 activation and the severity of the disease.

EMSA as well as a previous study¹³ showed a contribution of other members of the NF- κ B family to the observed NF- κ B binding activity. The above extracts were therefore tested for NF- κ Bp50 and NF- κ B-cRel. Samples from all patients without glucocorticoid treatment showed only moderate translocation of NF- κ Bp50 from the cytoplasm to the nucleus (data not shown). In contrast, samples from patients under treatment showed greater nuclear translocation of NF- κ Bp50 (data not shown), which is consistent with the increased contribution of NF- κ Bp50 to NF- κ B binding activity observed

by EMSA (fig 4D). However, nuclear NF- κ Bp50 showed only a weak negative correlation with the CDAI (data not shown) and therefore may be influenced by additional individual factors not necessarily determined by the outcome of disease or treatment.

Although the above supershifting experiments failed to detect NF- κ B-cRel in the NF- κ B binding complex (fig 4C,D), western blot analysis showed a slight increase in NF- κ B-cRel nuclear translocation in all patients with Crohn's disease when compared with disease specific controls (data not shown). As the data are in line with a previous report that describes the involvement of NF- κ B-cRel in NF- κ B complexes of lamina propria mononuclear cells from inflamed mucosa,¹³ the failure to detect NF- κ B-cRel by EMSA may be related to the fact that the antibody used is unable to react with traces of NF- κ B-cRel in an NF- κ B binding complex that is mainly formed by NF- κ B(p50/p65). In western blots, however, no difference was observed in the extent of nuclear translocation of NF- κ B-cRel in untreated and glucocorticoid treated samples (data not shown). Consistently, there was no correlation between nuclear NF- κ B-cRel and the CDAI (data not shown).

Figure 9 Immunohistochemical staining of I κ B α antigen in resection gut specimens of patients with Crohn's disease (original magnification $\times 40$); representative positive stained cells are marked with an arrow. (A) Representative staining of vascular endothelial cells in a resection gut specimen from a patient without glucocorticoid treatment. (B) Representative staining of vascular endothelial cells in a resection gut specimen from a patient with glucocorticoid treatment. (C) Summary of the scoring and the statistical results.

GLUCOCORTICOIDS UPREGULATE I κ B α IN VASCULAR ENDOTHELIAL CELLS BUT NOT IN MONONUCLEAR CELLS

Western blot analysis of cytoplasmic extracts of the above resection gut specimens showed that I κ B α expression was weak in samples from patients without treatment (fig 7A), while it was stronger in all patients having glucocorticoid treatment (fig 7A). Densitometric evaluation of the signal showed an increase from 7.13 relative I κ B α units in untreated samples to 13.07 relative I κ B α units in glucocorticoid treated samples ($p = 0.022$) (fig 7B).

These findings are in contrast with previous reports, in which steroid treatment for seven days did not change I κ B α expression levels in lamina propria biopsy specimens.¹⁰ Therefore the cells contributing to I κ B α expression were determined by immunohistochemistry in consecutive sections of the above tested resection gut specimens. Consistent with the previous observation,¹⁰ there was no significant difference in the extent of I κ B α expression in infiltrating mononuclear cells of patients with Crohn's disease without (fig 8A) or with (fig 8B) glucocorticoid treatment. Figure 8C summarises the quantitative results and the statistical analysis.

Vascular endothelial cells, however, which stained only weakly positive for I κ B α in sections from patients without treatment (fig 9A), showed stronger immunohistochemical expression of I κ B α in the glucocorticoid treated group (fig 9B). Statistical analysis confirmed a higher I κ B α expression in vascular endothelial cells in the glucocorticoid treated group (fig 9C), which may, at least in part, explain the increase in I κ B α observed in western blots of total gut extracts (fig 7A).

Discussion

Chronic NF- κ B activation in Crohn's disease seems to depend on NF- κ Bp65, as (a) local application of NF- κ Bp65 antisense oligonucleotides abrogated clinical and histological signs of mucosal inflammation in mice with experimental colitis,⁷ (b) NF- κ Bp65 antisense oligonucleotides reduced production of proinflammatory cytokines in cultured lamina propria macrophages of patients with Crohn's disease,^{7,8} and (c) increased expression of NF- κ Bp65 has been shown in lamina propria biopsy specimens^{10,13} and full thickness specimens of bowel¹¹ of patients with Crohn's disease. Glucocorticoids, frequently used in the treatment of Crohn's disease, have been shown to be effective inhibitors of NF- κ Bp65 and therefore seem to directly inhibit amplification and perpetuation of the inflammatory response.^{2,6,10,28,31-37} Several models have been proposed to explain the molecular mechanisms that underlie the inhibitory action of glucocorticoids on NF- κ B activation. Recent *in vitro* studies have reported direct protein-protein interactions between NF- κ Bp65 and GR, which result in mutual transrepression.³¹⁻³⁵ Other investigators suggest that expression of the NF- κ B specific inhibitor I κ B α is upregulated via a GR responsive element.^{36,37} At present, however, the relative contribution of each mechanism has not been defined under physiological conditions in human disease.

In order to determine the effect of glucocorticoids on the NF- κ Bp65/I κ B α system, we studied whole gut segments (rather than biopsy specimens) of disease activity matched patients with and without glucocorticoid treatment. As presented here, the mode of glucocorticoid action seems to depend on the underlying cell type. Downregulation of NF- κ Bp65 was evident in both infiltrating mononuclear cells of the lamina propria and submucosa as well as in vascular endothelial cells. The fate of I κ B α , however, was different in different cell types: mononuclear cells located in the lamina propria and submucosa did not show higher levels of I κ B α in the glucocorticoid treated group (fig 8), which is in line with previous observations in sigmoid biopsy samples of patients with Crohn's disease.¹⁰ In contrast, vascular endothelial cells showed significantly higher I κ B α expression in resection gut specimens obtained from patients under glucocorticoid treatment (fig 9). These *in vivo* results contrast with the *in vitro* situation: mononuclear cells have been shown to respond to glucocorticoids *in vitro* with increased I κ B α expression while studies in cultured endothelial cells failed to detect glucocorticoid dependent upregulation of I κ B α .⁴⁶ This discrepancy points to an important, but mostly neglected, drawback of *in vitro* models of human disease: *in vitro* experiments on cell activation at the transcriptional level are performed over minutes to several hours, while human disease lasts much longer. *In vitro* experiments to identify the mechanism of glucocorticoid dependent NF- κ B downregulation have been performed only for hours and only with single cell types.^{31-37,46} They may therefore not be suffi-

cient to completely explain the complex physiological situation involving much longer time frames and cell-cell interactions.

It has been proposed that the glucocorticoid mediated increase in I κ B α synthesis is long lived³⁴ and thus plays a crucial role in attenuating NF- κ Bp65 activity under chronic conditions.⁶ This view is emphasised by the study presented here, as the prominent endothelial I κ B α upregulation shown was detected in samples of long term glucocorticoid treated patients, while other studies have so far only looked at the transient effects of glucocorticoid treatment on the NF- κ B/I κ B κ system *in vivo*.¹⁰ Recent *in vitro* studies that dissociated glucocorticoid dependent transrepression of NF- κ B and glucocorticoid mediated transactivation of I κ B α synthesis indicate that increased I κ B α synthesis is neither required nor sufficient for glucocorticoid mediated downmodulation of NF- κ B activity.³⁴ The authors postulate that the transrepressing function of glucocorticoids is the decisive factor in anti-inflammatory action while the transactivating capacity may account for unwarranted side effects.³⁴ Also the latter cannot be excluded, the study presented here demonstrating that upregulation of I κ B α may significantly contribute to the attenuation of NF- κ B activation in Crohn's disease. As depicted in table 2, some of the patients included in this study received the drug sulfasalazine which has been shown to inhibit NF- κ B activation by preventing I κ B α phosphorylation and subsequent degradation.⁴⁵ However, the NF- κ B activity score in patients that did not receive glucocorticoids but had sulfasalazine treatment was significantly higher (table 4) than the NF- κ B activity score in patients under glucocorticoid treatment. This implies that additional mechanisms such as increased I κ B α synthesis are present in glucocorticoid treated patients and thus allow further reduction in NF- κ B activation. It has recently been reported that induction of NF- κ Bp65 mRNA perpetuates NF- κ B activation under chronic inflammatory conditions.¹⁸ Although an excess of newly synthesised NF- κ Bp65 would activate I κ B α synthesis, the cellular defence would be much stronger if large amounts of I κ B α were already available as the result of glucocorticoid induced I κ B α synthesis. As the untreated and glucocorticoid treated groups investigated here were matched with respect to disease activity (CDAI and van Hees index), we conclude that activation of the NF- κ Bp65/I κ B α system is only part of the inflammatory cascade leading to the clinical appearance of Crohn's disease. As the patient groups presented here are too small to perform a careful multivariate analysis, larger studies will now be required to define further the effects of glucocorticoid treatment on the activation of the NF- κ Bp65/I κ B α system in patients with Crohn's disease.

To date, most of the research on inflammatory bowel disease has focused on the role of lamina propria infiltrating inflammatory mononuclear cells.¹⁰⁻¹³ The above data, however, show that vascular endothelial cells also participate in the inflammatory response by

activating the NF- κ B/I κ B α expression system. It will be important to define whether NF- κ B induced endothelial gene expression such as upregulation of the adhesion molecules VCAM-1 and ICAM-1 or the monocyte chemotactic protein-1 is particularly involved in the recruitment of inflammatory cells to the mucosa^{3,6,47} and therefore may actively participate in the maintenance of chronic inflammation in Crohn's disease. Part of the therapeutic effect of glucocorticoids may therefore be explained by downregulation of endothelial NF- κ Bp65 and the subsequent prevention of leucocyte recruitment to the inflamed mucosa. To elucidate the role of endothelial cells in Crohn's disease further, it will be necessary to define ligands and cellular receptors that mediate sustained endothelial NF- κ B activation. The ligand-receptor interactions involved would be attractive targets for the development of novel anti-inflammatory drugs and are likely to become a prime focus of future investigations.

The authors wish to thank Dr Nancy Rice (Fredericks, USA) for the gift of the NF- κ Bp50/p105 antibodies and Dr P A Baeuerle (Tularik Inc, San Francisco, California, USA) for providing the NF- κ Bp65 plasmid Re-CMV-p65. The excellent technical assistance of Mrs Christina Koch (immunohistology, reproduction of microphotographs) is acknowledged. This work was supported in part by Deutsche Forschungsgemeinschaft grants SFB no 601 (to PPN) and no 405/B9 (to FA) and a grant from the Deutsche Crohn/Colitis ulceroosa Vereinigung (to PPN). PPN performed this work during the tenure of a Schilling professorship.

- 1 Chapple IL. Reactive oxygen species and antioxidants in inflammatory diseases. *J Clin Periodontol* 1997;24:287-96.
- 2 Barnes PJ, Karin M. Nuclear Factor- κ B: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997;336:1066-71.
- 3 Baeuerle PA, Baltimore D. NF- κ B: ten years after. *Cell* 1996;87:13-20.
- 4 Verma I, Stevenson JK, Schwarz EM, et al. Rel/NF- κ B/I κ B family: intimate tales of association and dissociation. *Genes Dev* 1995;9:2723-35.
- 5 Collins T. Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. *Lab Invest* 1993;68:499-508.
- 6 Baeuerle PA, Baichval VR. NF- κ B as a frequent target for immunosuppressive and antiinflammatory molecules. *Adv Immunol* 1997;65:111-35.
- 7 Neurath MF, Pettersson S, Meyer zum Büschenfelde KH, et al. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF- κ B abrogates experimental colitis in mice. *Nat Med* 1998;2:998-1004.
- 8 Neurath MF, Pettersson S. Predominant role of NF- κ Bp65 in the pathogenesis of chronic intestinal inflammation. *Immunobiology* 1997;198:91-8.
- 9 Neurath MF. Pathogenesis of inflammatory bowel disease: transcription factors in the spotlight. *Gut* 1998;42:458-9.
- 10 Schreiber S, Nikolaus S, Hampe J. Activation of the nuclear factor kappa B in inflammatory bowel disease. *Gut* 1998;42:477-84.
- 11 Ellis RD, Limb GA, Thompson RPH. NF κ B in Crohn's disease. *Biochem Soc Trans* 1997;25:178S.
- 12 Ardite E, Panes J, Miranda M, et al. Effects of steroid treatment on activation of nuclear factor kappa B in patients with inflammatory bowel disease. *Br J Pharmacol* 1998;124:431-3.
- 13 Rogler G, Brand K, Vogl D, et al. Nuclear Factor κ B is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* 1998;115:357-69.
- 14 Beg AA, Baldwin AS. The I κ B proteins: multifunctional regulators of Rel/NF- κ B transcription factors. *Genes Dev* 1993;7:2064-70.
- 15 Zabel U, Baeuerle PA. Purified human I κ B can rapidly dissociate the complex of the NF- κ B transcription factor with its cognate DNA. *Cell* 1990;61:255-65.
- 16 Cohen L, Henzel WI, Baeuerle PA. IKAP is a scaffold protein of the I kappa B kinase complex. *Nature* 1998;395:292-6.
- 17 Rothwarf MK, Zandl E, Natoli G, et al. IKK-gamma is an essential regulatory subunit of the I kappa B kinase complex. *Nature* 1998;395:297-300.
- 18 Bierhaus A, Klevesath M, Illmer T, et al. Ligands of RAGE mediate continuous NF- κ B activation that results in persistent tissue factor induction and perpetuated endothelial dysfunction. *Thromb Haemost* 1997;77(suppl 1):595, OC-2429 [abstract].
- 19 Wilkens T, De Rijk R. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol Today* 1997;18:418-24.
- 20 Keatings VM, Jatakanon A, Worsdell YM, et al. Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and respiratory disease. *Am J Respir Crit Care Med* 1997;155:542-8.
- 21 Norbiato G, Bevilacqua M, Vago T, et al. Glucocorticoids and Th-1, Th-2 type cytokines in rheumatoid arthritis, osteoarthritis, asthma, atopic dermatitis and AIDS. *Clin Exp Rheumatol* 1997;15:315-23.
- 22 Andoh A, Fujiyama Y, Kitamura S, et al. Acute lupus peritonitis successfully treated with steroid pulse therapy. *J Gastroenterol* 1997;32:654-7.
- 23 Caesar I, Gross V, Roth M, et al. Treatment of active and postactive ileal and colonic Crohn's disease with oral pH-modified-release budesonide. German Budesonide Study Group. *Hepatology* 1997;44:445-51.
- 24 Geier DL, Miner PB Jr. New therapeutic agents in the treatment of inflammatory bowel disease. *Am J Med* 1992;93:199-208.
- 25 Lechner J, Welte T, Tomasi JK, et al. Promoter-dependent synergy between glucocorticoid receptor and Stat5 in the activation of beta-casein gene. *J Biol Chem* 1997;272:20954-60.
- 26 Wisniewska M, Stanczyk M, Grzelakowska-Sztartab B, et al. Nuclear factor of activated T cells (NFAT) is a possible target for dexamethasone in thymocyte apoptosis. *Cell Biol Int* 1997;21:127-32.
- 27 Vayssiere BM, Dupont S, Choquart A, et al. Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity in vivo. *Mol Endocrinol* 1997;11:1245-55.
- 28 van der Burg B, Liden J, Okret S, et al. Nuclear Factor- κ B repression in antiinflammation and immunosuppression by glucocorticoids. *Trends in Endocrinology and Metabolism* 1997;8:152-7.
- 29 Beato M. Gene regulation by steroid hormones. *Cell* 1989;56:335-44.
- 30 Evans RM. The steroid and thyroid hormone receptor family. *Science* 1988;240:889-95.
- 31 Caldenhoven E, Liden J, Wissink S, et al. Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol Endocrinol* 1995;9:401-12.
- 32 Scheinman RI, Gualberto A, Jewell CM, et al. Characterization of mechanisms involved in transrepression of NF- κ B by activated glucocorticoid receptors. *Mol Cell Biol* 1995;15:943-53.
- 33 De Bosscher K, Schmitz ML, Vanden Berghe W, et al. Glucocorticoid-mediated repression of nuclear factor-kappaB-dependent transcription involves direct interference with transactivation. *Proc Natl Acad Sci USA* 1997;94:13504-9.
- 34 Heck S, Bender K, Kullmann M, et al. I κ B α -independent downregulation of NF- κ B activity by glucocorticoid receptor. *EMBO J* 1997;16:4698-707.
- 35 McKay LJ, Cidlowski JA. Cross-talk between nuclear factor- κ B and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol Endocrinol* 1998;12:45-56.
- 36 Auphan N, DiDonato JA, Rosette C, et al. Immunosuppression of glucocorticoids: inhibition of NF- κ B activity through induction of I κ B synthesis. *Science* 1995;270:286-90.
- 37 Scheinman RI, Cogswell PC, Lofquist AK, et al. Role of transcriptional activation of I κ B- α in mediation of immunosuppression by glucocorticoids. *Science* 1995;270:283-6.
- 38 Cordell JJ, Falini B, Erber WN, et al. Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and anti-alkaline phosphatase (APAAP-complexes). *J Histochem Cytochem* 1984;32:219-24.
- 39 Böhler H, Qiu F, Zimmermann T, et al. Role of NF- κ B in the mortality of sepsis. *J Clin Invest* 1997;100:972-85.
- 40a Bradford MM. A rapid method for the quantitation of microgram quantities of protein utilizing the principles of protein dye binding. *Anal Biochem* 1976;72:248-54.
- 40 Bierhaus A, Illmer T, Kasper M, et al. Advanced glycation endproducts (AGEs) mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. *Circulation* 1997;96:2262-71.
- 41 Hofmann M, Schiekofer S, Kanitz M, et al. Insufficient glycaemic control increases NF- κ B binding activity in peripheral blood mononuclear cells isolated from patients with type 1 diabetes. *Diabetes Care* 1998;21:1-7.
- 42 Bierhaus A, Zhang Y, Deng Y, et al. Mechanism of the TNF α mediated induction of endothelial tissue factor. *J Biol Chem* 1995;270:26419-32.
- 43 Bierhaus A, Zhang Y, Quehenberger P, et al. The dietary pigment curcumin reduces endothelial tissue factor gene expression by inhibiting binding of AP-1 to the DNA and activation of NF- κ B. *Thromb Haemost* 1997;77:772-82.
- 44 Kaltschmidt C, Kaltschmidt VB, Henkel T, et al. Selective recognition of the activated form of transcription factor NF-kappa B by a monoclonal antibody. *Biol Chem Hoppe-Seyler* 1995;376:9-16.
- 45 Wahl C, Liptay S, Adler G, et al. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. *J Clin Invest* 1998;101:1163-74.
- 46 Brostjan C, Anrather J, Csizmadia V, et al. Glucocorticoid-mediated repression of NF-kappaB activity in endothelial cells does not involve induction of IkappaBalpha synthesis. *J Biol Chem* 1996;271:19612-16.
- 47 Vainer B, Nielsen OH. The influence of adhesion molecules in inflammatory bowel diseases. *Ugeskr Laeger* 1997;159:3767-71.