Int J Colorectal Dis (2002) 17:223–232
DOI 10.1007/s00384-001-0366-3

ORIGINAL ARTICLE

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Abrogated lymphocyte infiltration and lowered CD14 in dextran sulfate induced colitis in mice treated with p65 antisense oligonucleotides

Accepted: 19 September 2001
Published online: 20 February 2002
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Abstract *Background and aims:* Dextran sulfate sodium (DSS) induced colitis exhibits a predominantly NF-κB dependent proinflammatory cytokine profile and shares similarities with human inflammatory bowel disease (IBD). Lamina propria macrophages of IBD patients display elevated levels of NF-κB p65. Knowing the role of NF-κB in IBD, we investigated the beneficial cellular mechanisms underlying the lasting effect of a single p65 antisense treatment in DSS-colitis mice. *Methods*: One local dose of p65 antisense oligonucleotides was administered in DSS colitis mice. Ten days later the mice were killed and examined at cellular and biochemical levels. The level of p65 in lamina propria cells was determined by electrophoretic mobility shift assay and by intracellular immunofluorescent staining of nuclear p65 levels, using laser scanning cytometer. *Results:* FACS analysis demonstrated a considerable drop in infiltrating lymphocytes and a drastic reduction in $CD14^+$ cells in mice treated with p65 antisense oligonucleotides. Moreover, abrogation of inflammation extended all the way to the cecum in treated mice. Treatment was correlated with decreased DNA binding activity of NF-κB. *Conclusions:* Our data strongly support a model in which p65 antisense treatment possesses the capacity to disrupt the pathogenic autocrine loop propagated by NF-κB at the chronic phase of IBD.

Keywords Inflammatory bowel disease · Lamina propria mononuclear cells · Antisense oligonucleotides · Nuclear factor κB · CD14

Introduction

The transcription factor NF-κB plays a key role in chronic inflammatory diseases such as IBD [1] (as reviewed in [2, 3]) and rheumatoid arthritis [4, 5, 6]. Proinflammatory cytokines including interleukins 1 and 6 and tumor necrosis factor α are induced in inflammatory conditions of the intestine, both in mice and humans [1, 7]. All these proinflammatory cytokines are known to be regulated by nuclear factor (NF) κB [8, 9, 10], indicating its pivotal role. Macrophages are one of the major producers of these cytokines and have been implicated in the pathogenesis of IBD [11, 12]. Recently NF-κB was shown to be activated both in macrophages and epithelial cells of inflamed intestinal mucosa [13], substantiating the connection between NF-κB, innate immunity and IBD in vivo. In addition, nuclear levels of NF-κB p65 were shown to be higher in lamina propria biopsy specimens from IBD patients than in controls [14]. NF-κB is an inducible transcription factor consisting of a heterodimeric complex containing a 50-kDa subunit (p50 or NF-κB1) and a 65-kDa subunit (p65 or RelA). The functional importance of NF-κB in inflammation is due to its ability to regulate promoters critical for inflammatory processes. NF-κB is normally bound in the cytoplasm to its inhibitory protein IκB, until activated by a multitude of stimuli such as bacteria, oxidants, stress, UV radiation, and viruses. Activation induces disintegration of

IκB through proteolytic degradation, resulting in NF-κB translocation into the nucleus where it binds to and activates its target genes [8, 15, 16]. Inhibition of NF-κB-mediated signaling, by gene disruption of NF-κB-encoding genes [17], or by exposure to glucocorticoids [18] results, however, in a decreased inflammatory response.

To study normal and pathophysiological conditions in the gut and the effect of immunomodulating substances, relevant animal models of intestinal inflammation are imperative [19]. In 1996 it was reported that a single local administration of antisense phosphorothioate oligonucleotides targeting the p65 subunit of NF-κB blocks the inflammatory state both in 2,4,6-trinitrobenzene sulfonic acid treated and in interleukin-10 deficient mice. Although the effects were dramatic, no experiments have addressed the underlying cellular effects of p65 antisense oligonucleotides of the innate and the adaptive immune system. Another well characterized model is that using dextran sulfate sodium (DSS) to induce intestinal inflammation in mice. This model has been reported to respond to classical treatment with sulfasalazine and related substances [20, 21], all being anticolitis drugs that are used in human ulcerative colitis and that possess several immunomodulating functions [22]. Histopathological examination of mice given DSS has shown that an induction phase of about 5 days precedes the stage of histopathologically evident intestinal inflammation with infiltration of inflammatory cells [23, 24]. In analogy with the findings in human IBD [25], the presence of the inflammatory cells in the inflamed mucosa and in the spleen following the early noninflammatory phase suggests that cellular immunological reactions are involved in the pathogenesis of DSS-induced colitis. The characteristics of the DSS substance, its ability to induce inflammation in colon and cecum, and its stability in solution have been assessed previously [21].

Using antisense oligonucleotides directed against the translation start site of NF-κB p65 we treated DSS-induced colitis mice to determine the beneficial mechanisms of the lasting effect when blocking NF-κB in this type of inflammation. In order to monitor NF-κB production and activation at the cellular level NF-κB activity was monitored by intracellular immunofluorescent staining in lamina propria macrophages from DSS-treated mice, both with and without antisense administration. In parallel we took advantage of a recently described method that combined the intracellular immunofluorescent staining in combination with laser scanning cytometry (LSC). The LSC is a microscope-based instrument that automatically measures multiple wavelength fluorescence and light scattering of cells on a microscope slide and generates a list of cytochemical and morphological features for each cell in the sample [26]. Previous reports have demonstrated that the nuclear vs. cytoplasmic levels of NF-κB p65 by using LSC can be determined [27]. We used this technique to evaluate the level of activated p65 in the nuclei of macrophages from colons of DSS colitis mice. This experimental setup is novel for this kind of application and could be instrumental for examining NF-κB activation at the cellular level in various inflammatory conditions. For comparison, NF-κB p65 levels were also determined by electrophoretic mobility shift assay (EMSA), a method previously established in similar systems. While EMSA requires substantial amounts of lamina propria cells to generate suitable quantities of protein, thus creating practical problems in material sampling, the advantage provided by LSC is the possibility of studying a few thousand cells both on cytoplasmic and nuclear levels. Moreover, LSC provides a computerized counting system enabling a rapid and quick read out. In addition, flow cytometry analysis added further information about how cells were affected by the antisense treatment.

Materials and methods

Animals

Conventional NMRI/KI mice were bred at the Laboratory of Medical Microbial Ecology, Karolinska Institutet, Sweden. Female and male mice 5–7 months old and weighing approx. 22–30 g at the start of the experiment were used. The animals were fed autoclaved animal chow (R3, Lactamin, Vadstena, Sweden) and water ad libitum. The experimental design was approved by the local animal ethics committee.

Induction of colitis

Inflammation in the large intestine was induced by administration of 2.0% DSS (molecular weight 41 kDa, sulfur content approx. 15%) in distilled water (final pH was adjusted to 8.5) as drinking water for up to 18 days. DSS was obtained from TdB Consultancy (Uppsala, Sweden). The drinking water bottles were replenished every day with fresh solutions. Animals were exposed to the DSS solution ad libitum. Water intake was not affected by addition of DSS.

In vivo administration of oligonucleotides

For therapeutic treatment 150 μg oligonucleotides directed against the 5′ end of NF-κB p65 were administered once locally in the rectum on day 8 of drinking 2.0% DSS. All DSS-drinking mice, including those treated with antisense oligonucleotides on day 8, had 2.0% DSS in the drinking water throughout the test period. Weight changes were monitored and organs were prepared and fixed in 4% formaldehyde for histological examinations.

Phosphorothioate oligonucleotides

Murine antisense phosphorothioate oligonucleotides consisted of 19-mer analogues to the 5′ end (translation initiation site) of the NF-κB p65 transcript and were synthesized by CyberGene (Huddinge, Sweden). The sequences of phosphorothioate oligonucleotides were as follows: murine p65 antisense: 5′- GAA ACA GAT CGT CCA TGG T -3′ compared to the murine p65 sense sequence: 5′- ACC ATG GAC GAT CTG TTT C -3′.

Evaluation of colonic inflammation

The animals were inspected daily for signs of colitis, i.e., diarrhea and rectal bleeding. Clinical parameters recorded were death and body weight. The histopathological examination of ileal, cecal, and colonic tissues were performed by an experimented mouse pathologist. Apart from a general anatomical evaluation, the tissues were scored in a blinded fashion on a scale from 0 to 4. Intensity of lesions: 0 normal, 1 mild, 2 moderate, 3 severe, and 4 very severe including edema, ulceration, leukocytic infiltration, transmural inflammation and mitoses/hyperplasia in the epithelium.

Cell isolation of murine lamina propria mononuclear cells

Mice were anesthesized with methophane and killed by cervical dislocation, and the intestines were quickly excised. Colon was excised out approximately between the cecal junction and the proximal rectum, close to its passage under the pelvisternum. The ileum and cecum with an adjoining 1 cm of ileum was collected separately. Following the removal of small specimens for histopathological examination, the remaining colon was cut longitudinally and washed in sterile phosphate-buffered solution (PBS), cut in small pieces and shaken in a waterbath at 37°C for 20 min in PBS containing 94 mM dithiothreitol and 1 mM ethylene diaminetetraacetate (EDTA). Lamina propria mononuclear cells were isolated as previously described [11]. Briefly, cells were collected by centrifugation and epithelial cells, as well as adipose tissue were removed. The pellet was resuspended in complete medium containing RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS), 25 mM hydroxyethylpiperazine ethane sulfonic acid, 0.15 mg/ml collagenase, 0.1 mg/ml DNAse I and penicillin/streptamycin, and incubated in a shaking waterbath at 37°C for 90 min. Cells were passaged through a cell strainer (100 µM, Falcon) and washed with PBS. Lamina propria mononuclear cells were isolated by Percoll gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) using 40% Percoll.

Flow cytometry analysis of lamina propria mononuclear cells

Single cell suspensions were prepared as described above and cells from eight mice within the same test group were pooled. Immunofluorescent staining were carried out for 15 min on ice with a previously optimized dilution of antibody using PBS and 1% FCS as staining medium, in a total volume of 100 µl. The following conjugated antibodies were purchased from Pharmingen (San Diego, Calif.,, USA): RA3-6B2 (anti-B220/CD45R)-phycoerythrin, 30-H12 (anti-Thy-1.2) fluorescein, M1/70 (anti-CD11b/Mac-1α) fluorescein, and rmC5-3 (anti-CD14) fluorescein. After staining and one washing step, cells were finally resuspended in PBS containing propidium iodide (1 µg/ml) to exclude dead cells. Flow cytometry was performed on a fluorescence-activated cell sorter Calibur (Becton Dickinson, San Jose, Calif., USA) using the CellQuest software for analysis.

Intracellular immunofluorescent detection of NF-κB p65

Cells from eight mice within the same treatment group were pooled, washed and counted. The cell concentration was adjusted to 2–4×10^6/ml with Earle's buffered salt solution (EBSS) or PBS. Of the cell suspension 10 µl was transferred onto adhesion slides (Bio-Rad, Munich, Germany) and incubated for 20 min at room temperature. After a gentle wash with EBSS or PBS, the cells were fixed by adding ice-cold 4% formaldehyde for 5 min or methanol-free 1% formaldehyde in PBS for 15 min on ice, washed with EBSS or PBS and then with EBSS with 2.0% heat inactivated mouse serum or PBS plus 0.2% heat-inactivated FCS, transferred into ice-cold 70% ethanol and stored for up to 1 h at – 20°C. Before staining the cells were washed twice with PBS containing 0.1% saponin (Riedel de Haen, Seelze, Germany) and then incubated with PBS and 0.1% saponin for 2 min. Rabbit anti-mouse p65 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was diluted to optimal concentration determined previously, in PBS plus 0.1% saponin plus 2% FCS and incubated for 30 min at room temperature. After washing with PBS and 0.1% saponin, the second antibody, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit Ig (Vector, Burlingame, Calif., USA) was added, diluted in PBS and 0.1% saponin, and slides were incubated away from light for 30 min at room temperature. Cellular DNA was then counterstained by incubation with RNAse (200 µg/ml/) and 50 µg/ml propidium iodide in EBSS for 30 min. After washing with PBS, 10 µl glycerol:carbonate/bicarbonate (1:1; v/v) buffer plus 2% 1, 4-diazobicyclo-2, 2, 2-octane (Sigma, St Louis, Mo., USA) or SlowFade Light (Molecular Probes, Eugene, Ore., USA) was added as mounting medium to reduce UV-quenching of FITC, and a coverslip was carefully mounted to avoid air bubbles.

Laser scanning cytometry

Harvested cells on the adhesion slide were scanned in a LSC (Compucyte, Cambridge, Mass., USA). A LSC protocol for enumeration and subcellular fluorescence intensity analysis of NF-κB was setup with similar strategy to that previously described [27]. Briefly, using the propidium iodide signal as spatial marker for the nucleus, two thresholds were set to distinguish nuclear vs. cytoplasmic NF-κB associated immunoreactivity. The cytoplasmic signal was acquired by adding ten pixels of integration to the nuclear signal using the perimeter contouring feature of the LSC. Using either a reactive sensitive anti-p65 antibody or a nonreactive sensitive antibody (Santa Cruz Biotechnology), the nuclear presence of NF-κB was recorded.

Electrophoretic mobility shift assay

For EMSA the lamina propria mononuclear cells were isolated and resuspended in HB (10 mM Tris, pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 1.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1 µg/ml trasylol). Cells were centrifuged and resuspended in lysis buffer (HB + 0.4% NP40), and the nuclei were collected by centrifugation and resuspended in 20 mM hydroxyethylpiperazine ethane sulfonic acid (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 1 µg/ml trasylol. Supernatants were collected and snap frozen in liquid nitrogen. Binding reactions included 10 µg nuclear extract and were performed as described [18, 28] using 80,000 cpm end-labeled probe specific for NF-κB (top strand: GAT CCA GAG GGG ACT TTC CGA G).

Histology

Prior to fixation colon was opened longitudinally and feces were carefully removed. The tissues were fixed with 4% buffered formaldehyde, pH 7.0, and embedded in paraffin. Sections were made and stained with hematoxylin and eosin. The cecal specimens were embedded and sectioned in a manner showing the cecum and the ileocecal region in the same section. Specimens were taken in the midportion of colon. Cecal specimen was cut into four to six circular pieces and sectioned transversely. Colon biopsy specimens were collected in 10% formalin and analyzed histologically by Dr. R. Feinstein (Statens Veterinärmedicinska Anstalt, Uppsala, Sweden). Assessment of inflammation was made blindly and graded from mild to very severe by the same pathologist.

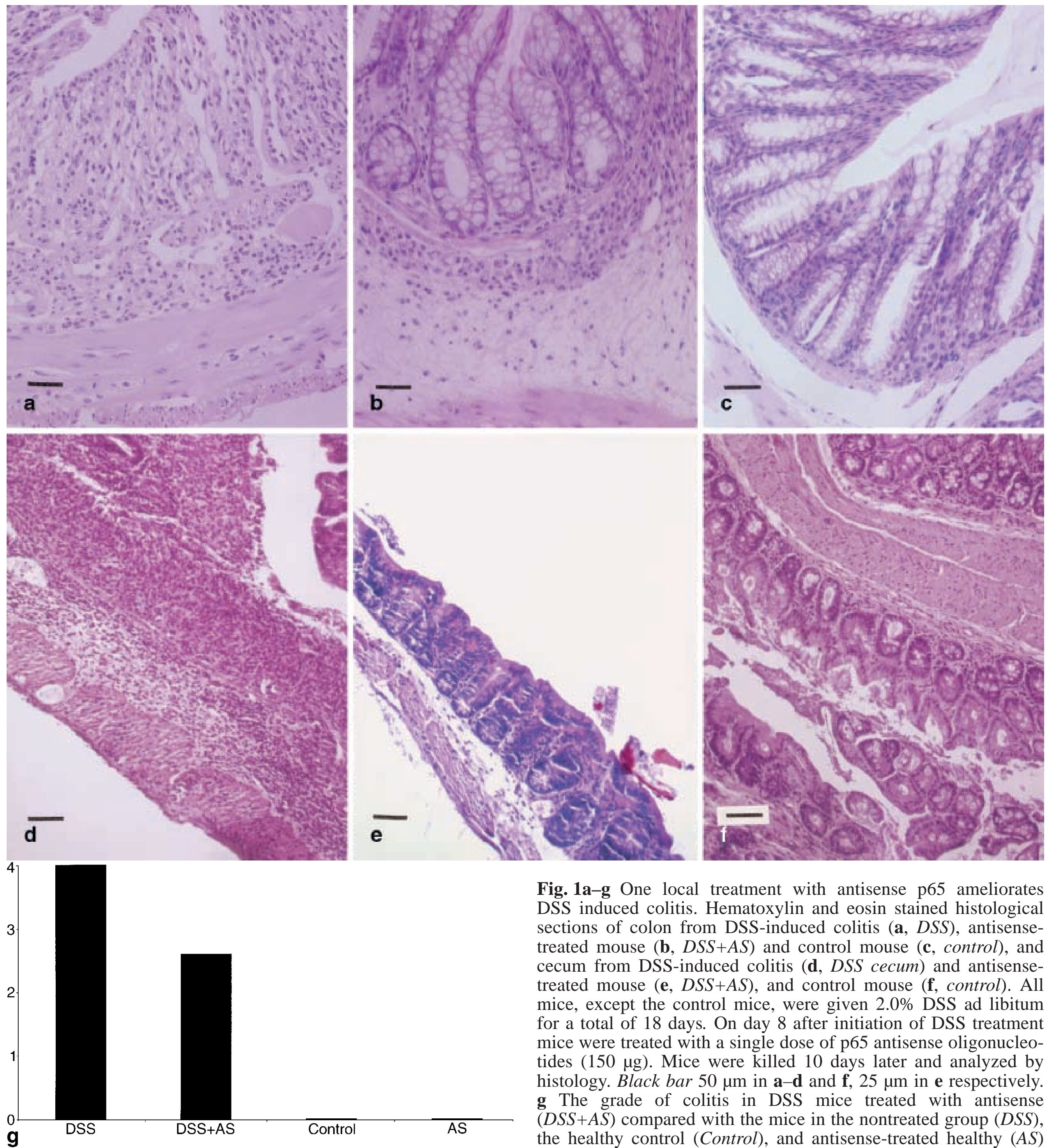
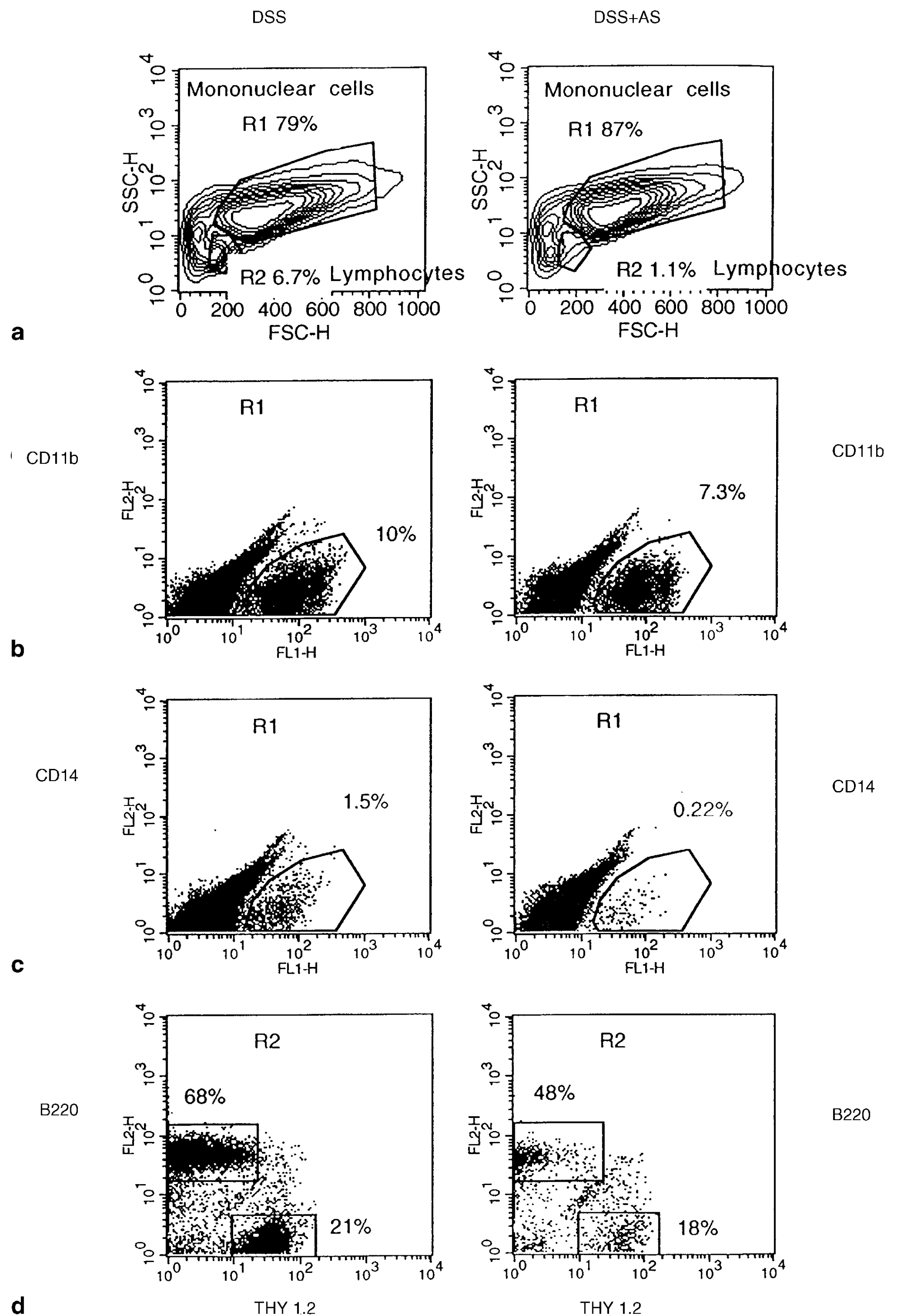


Fig. 1a–g One local treatment with antisense p65 ameliorates DSS induced colitis. Hematoxylin and eosin stained histological sections of colon from DSS-induced colitis (**a**, *DSS*), antisense-treated mouse (**b**, *DSS+AS*) and control mouse (**c**, *control*), and cecum from DSS-induced colitis (**d**, *DSS cecum*) and antisense-treated mouse (**e**, *DSS+AS*), and control mouse (**f**, *control*). All mice, except the control mice, were given 2.0% DSS ad libitum for a total of 18 days. On day 8 after initiation of DSS treatment mice were treated with a single dose of p65 antisense oligonucleotides (150 µg). Mice were killed 10 days later and analyzed by histology. *Black bar* 50 µm in **a**–**d** and **f**, 25 µm in **e** respectively. **g** The grade of colitis in DSS mice treated with antisense (*DSS+AS*) compared with the mice in the nontreated group (*DSS*), the healthy control (*Control*), and antisense-treated healthy (*AS*) mice. Grades presented are mean values of all scored animals. The tissues were scored in a blinded fashion on a scale from 0 to 4. Intensity of lesions: 0=normal, 1=mild, 2=moderate, 3=severe, and 4=very severe including edema, ulceration, leukocytic infiltration, transmural inflammation and mitoses/hyperplasia in the epithelium

Fig. 2a–d Local administration of p65 antisense oligonucleotides inhibits lymphocyte infiltration and reduces the number of CD14+ lamina propria mononuclear cells in DSS mice. Lamina propria mononuclear cells were prepared and stained for flow cytometry analysis as described in the text. Cells from eight mice in each group were pooled and aliquots were stained as indicated in the figure. Dead cells were excluded from the analysis by uptake of propidium iodide. *Left* Cells from a pool of mice treated with DSS only; *right* cells from a pool of mice treated with both DSS and p65 antisense oligonucleotides. **a** Probability contour plot. **b–d** Dot plots in which 60,000 events of live cells were collected and from the different subpopulations were analyzed. **b**, **c** Cells from gate R1 were analyzed for expression of CD11b (**b**) or CD14 (**c**). **d** Cells from gate R2 were analyzed for expression of Thy 1.2 or B220



Results

Colitis

Following administration of DSS in the drinking water 16 mice were monitored daily for signs of intestinal inflammation such as weight loss and diarrhea. On day 8 after initiation, when inflammation was apparent macroscopically, one randomly divided group of eight DSS drinking mice was treated locally with 150 μg p65 antisense oligonucleotides; 10 days later both groups of mice were killed, and intestines were removed. Specimens of colon and cecum were saved for histological examinations (Fig. 1a–f). Colon from DSS-treated mice

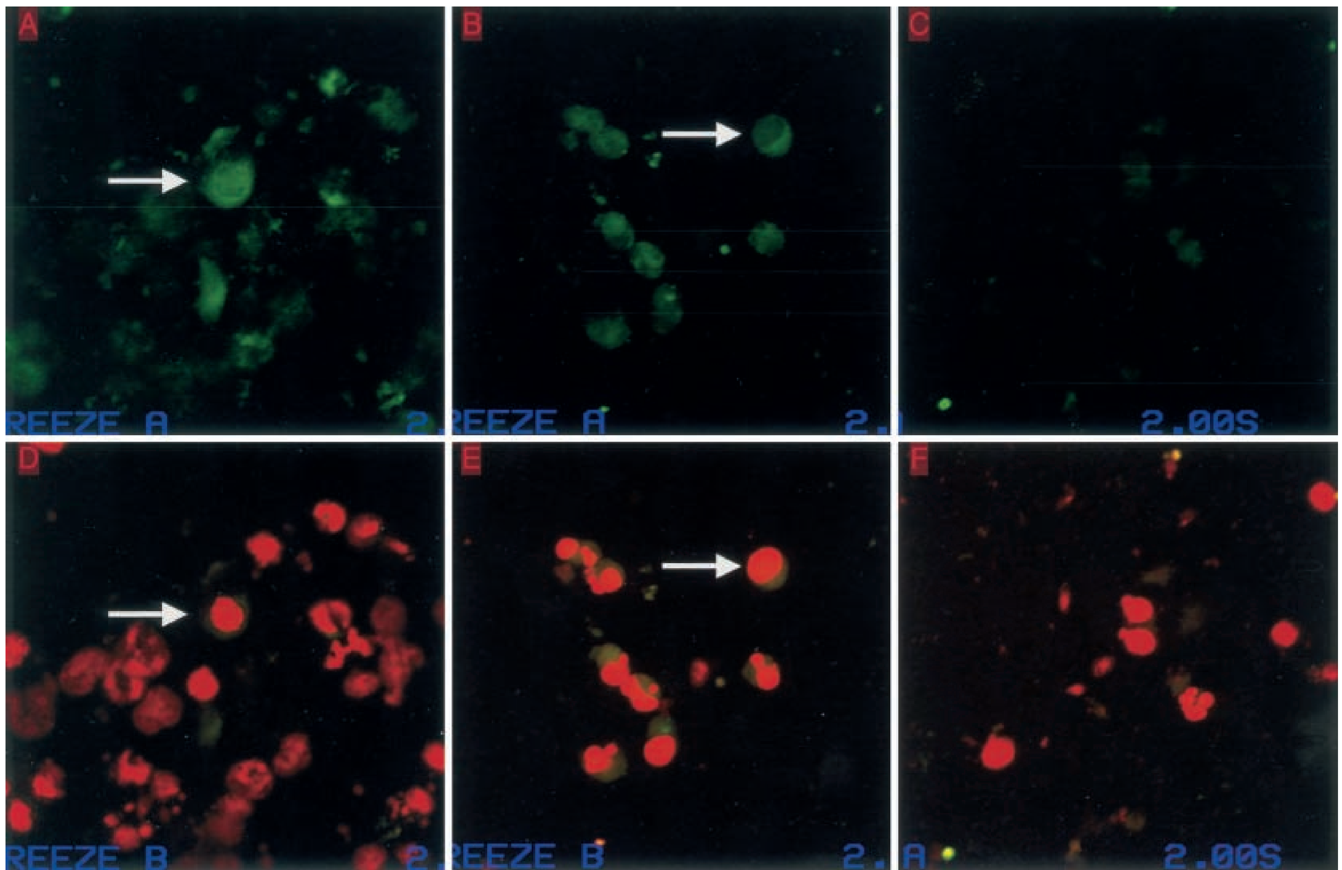


Fig. 3a–f Antisense p65 oligonucleotides reverses the elevated levels of p65 protein expressed in lamina propria macrophages of DSS colitis mice. Intracellular immunofluorescent staining with FITC-labeled antibodies against p65. **a** Dextran sulfate sodium colitis mouse lamina propria macrophages with elevated levels of nuclear NF-κB p65 (*arrow*). **b** Antisense p65-treated DSS colitis mouse lamina propria macrophages with blocked nuclear translocation of NF-κB p65 (*arrow*). **c** Normal mouse lamina propria macrophages. **d–f** Nuclear counterstaining of the same cells as shown in **a–c**

displayed colitis, varying in intensity from moderate to very severe, profound colitis with ulcers extending into the submucosa (Fig. 1a). Hyperplastic epithelium was evident as well as recruitment of numerous inflammatory cells such as macrophages and mononuclear cells, compared to colon from a normal mouse (Fig. 1c). Macrophages frequently displayed large, foamy cytoplasms. After antisense treatment, however, no ulcers were apparent, significantly fewer inflammatory cells were present, and the inflammation was reversed (Fig. 1b). Similarly, considerably less inflammation was seen in cecum from DSS-treated mice after antisense treatment (Fig. 1e) than in DSS mice that had not received antisense treatment, in which a massive infiltration of inflammatory cells and disruption of the architecture was evident (Fig. 1d, compare with antisense-treated, panel e, and normal cecum, panel f). In a blind study histology from all mice in the healthy group were considered noninflamed (0) by an experienced mouse pathologist (Fig. 1g) and in the group of DSS-treated mice, all mice exhibited very severe inflammation (4). On the other hand, in the antisense-treated group one-half of the mice were scored between 1 and 2 (total mean value 2.6; Fig. 1g) and only one of the eight antisense-treated DSS mice was scored 4 (very severe inflammation). Histology from healthy control animals treated only with antisense were all noninflamed (0; Fig. 1g). A similar improved histology pattern in colon was also evident after one local antisense treatment on day 14 after onset with DSS when mice were killed on day 23 (data not shown). The fact that DSS-induced inflammation in cecum could be recovered after a single local antisense treatment indicates that the p65 antisense oligonucleotide has anti-inflammatory effects that are not confined to colon in the gastrointestinal system.

Cellular findings

Mononuclear cells were prepared from lamina propria and analyzed by FACS for the presence of various cell populations (Fig. 2). In DSS-treated mice a major popu-

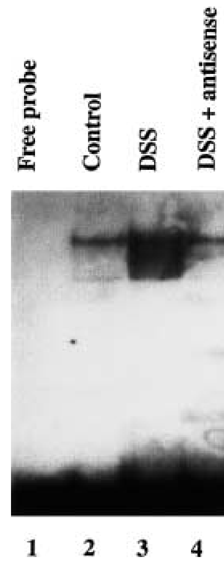


Fig. 4 Antisense p65 treatment downregulates the DNA binding activity of NF-κB in lamina propria macrophages of DSS-induced colitis mice. Nuclear extracts of lamina propria macrophages were prepared from a pool of eight mice within each test group, and levels of NF-κB p65 binding activity were determined in an electromobility shift assay. Elevated levels of DNA binding activity was apparent in DSS drinking animals (*lane 3, DSS*) as compared to healthy littermates (*lane 2, control*). One local antisense treatment resulted in DNA binding activity at control levels (*lane 4, DSS+antisense*; compare with *lane 2*, control animals, *lane 3*, DSS-induced colitis mice)

lation of large and granulated cells (as revealed by forward- and side-light scattering properties) was evident as well as a distinct population of smaller more dense cells. The major population contains cells such as macrophages, confirmed by 10% of them being Mac-1 positive (Fig. 2b), and the remaining cells are probably granulocytes and eosinophils. The more dense population of cells are lymphocytes, 90% of them being positive for a B cell marker (B220) or a T cell marker (Thy-1; Fig. 2c). p65 antisense treatment resulted in a small decrease in number of macrophages but a more substantial decrease in number of CD14-expressing cells. CD14 binds LPS with high affinity and is involved in lipopolysaccharide (LPS) mediating LPS responses. Binding of LPS to CD14 requires the serum factor LPS-binding protein, which delivers LPS to CD14-expressing monocytes/macrophages [29, 30]. Interestingly, following p65 antisense treatment the lymphocyte population and CD14 expression were drastically reduced to about 15% of expression levels. This may reflect an indirect effect of the treatment since targeting the macrophages may result in reduced cytokine production, which affects recruitment of the adaptive immune system (i.e., lymphocytes).

NF-κB activation

To measure the level of NF-κB activation in lamina propria inflammatory cells from the different treatment groups we performed intracellular immunofluorescent analyses. High cytoplasmic and nuclear levels of p65 were displayed in cells from DSS-treated mice (Fig. 3a). Opposed to this, in cells from the antisense-treated animals the nuclear and the overall p65 staining was less intense (Fig. 3; compare panels b and a). In lamina propria cells from normal mice, where no inflammation was present, the total (Fig. 3c) p65 staining was weak. As a control the lower panels of Fig. 3 (d, e, f) represent nuclear counterstaining of the p65 stained cells in the upper panels (a, b, c). In parallel, NF-κB site-specific binding activity was determined by EMSA in nuclear extracts prepared from lamina propria mononuclear cells from the indicated mice. Cells from DSS-treated mice displayed higher levels of binding activity than healthy littermates (Fig. 4, lanes 2 and 3). One local dose of antisense p65, however, decreased the NF-κB binding activity to the levels of healthy control mice (Fig. 4, lanes 2–4).

Discussion

IBD comprises various chronic inflammatory conditions in which acute flare-up phases alternate with periods of remission. Due to the diversity of the disease and the unknown cause it is difficult to approach early stages of inflammation. Previous studies have demonstrated that DSS-induced IBD can be provoked and maintained predominately by macrophagelike cells which are abundant in inflamed areas both in rodents and in humans. Glucocorticoids and glucocorticoid analogues are widely used for the suppression of inflammation in chronic inflammatory diseases. Studies of the molecular mechanism behind the effects of glucocorticoids on inflammation display strong evidence that they inhibit the action of transcription factors such as activator protein 1 and NF-κB [2, 31, 32, 33, 34, 35]. The DSS-induced inflammation in mice has been shown to respond positively to treatment with the anticolitis drugs mesalazine, sulfasalazine, and olsalazine [36] that are commonly used to treat human chronic IBD. In addition, it is known that sulfasalazine is a potent and specific inhibitor of NF-κB activation [5]. Recently ligands for the peroxisome proliferator-activated receptor γ (PPAR-γ) were shown to inhibit the expression of various cytokines in monocytes and macrophages by preventing NF-κB activity through an unknown mechanism [37, 38]. Moreover, thiazolidinedione ligands for PPAR-γ markedly reduced colonic inflammation in a DSS mouse model [39, 40]. It would be interesting to evaluate the expression levels and to screen for any mutations in the PPAR-γ gene as this gene has been linked to the pathogenesis of colon cancer, diabetes and hypertension [41]. Mutations in PPAR-γ would make these persons more prone to sustained NF-κB activity, as is the case in chronic inflammation.

The use of antisense oligonucleotides represents an alternative strategy for inhibiting gene function, and they have been reported to successfully inhibit genes in both in vivo and in vitro models [42, 43]. Antisense oligonucleotides are usually around 20 bases in length, designed

to hybridize to pre-mRNA or mature mRNA and hence block expression of the targeted protein [42, 44]. Phosphorothioate antisense oligonucleotides, in which one of the nonbridging oxygens in the backbone of DNA is replaced by sulfur to stabilize the structure, are among the most useful and the best characterized classes of oligonucleotides used as antisense agents [45, 46].

In this study we aimed to target NF-κB in mice with established DSS induced colitis, using phosphorothioate antisense oligonucleotides directed against the p65 subunit. We found that this treatment ameliorates the macroscopic signs of colitis as well as the nuclear accumulation and DNA binding activity of p65 protein, measured by LSC and EMSA, respectively. The LSC experimental set-up is novel for this kind of application, and it should prove instrumental for determining NF-κB activation at the cellular level, especially at early flare-up stages in various inflammatory conditions. To be used on patient material this system needs to be further established. EMSA displayed higher nuclear levels of p65 in nuclear cell extracts from lamina propria mononuclear cells of DSS-treated mice than in healthy littermates. Treating DSS colitis mice with antisense oligonucleotides against p65 resulted in NF-κB DNA binding activity similar to healthy controls. This agrees with the results of a previous study in which inhibition of NF-κB abrogated clinical and histological signs of inflammation in two independent animal models of intestinal inflammation [1]. Histological examinations revealed that the inflammation in colon DSS-treated mice can be reversed by administration of one local dose of antisense p65 oligonucleotides (Fig. 1a–c). Interestingly, histology showed that local, lumenal antisense treatment can also reverse the DSS-induced inflammation in cecum (Fig. 1d, f). This indicates that local p65 antisense treatment acts not only locally in colon but also in other inflamed gastrointestinal areas.

To understand the cellular characteristics of DSS-induced inflammation and the active mechanism of p65 antisense treatment we determined the cellular content of inflammatory cells derived from the lamina propria of DSS or DSS plus p65 antisense-treated mice by flow cytometry. A large population of infiltrating macrophages was apparent after DSS treatment as well as a defined population of B and T lymphocytes. Interestingly, a substantial fraction of the macrophagelike cells expressed CD14 on the cell surface. CD14 is the recognition receptor for the LPS-binding protein [29, 47] and is expressed on myeloid-lineage cells following activation. In humans lamina propria macrophages of uninflamed mucosa express very low if any levels of CD14 [48] whereas a large fraction of macrophages recovered from sites of active inflammation are $CD14^+$. Whether CD14 is induced on lamina propria macrophages by the abundant proinflammatory cytokines during active inflammation, or whether $CD14^+$ macrophages are recruited from the circulation to the site of inflammation is presently unknown. Furthermore, the signals regulating CD14 expression are largely unknown. However, CD14 lacks intracellular signaling properties (no cytoplasmic tail), and it was recently demonstrated that LPS-induced signaling is also dependent on members of the Toll-like receptor (TLR) family. Different TLRs are likely to recognize different pathogen-associated molecular patterns, mediating specificity of the innate immune system [49]. Indeed, it has recently has been shown that the naturally occurring TLR4 mutation of C3H/HeJ mice causes their unresponsiveness to LPS [50].

Considering that CD14 expression requires induction, and that macrophages from uninflamed human mucosa are $CD14^-$ [48], it is tempting to speculate that the DSS-induced inflammation results in recruitment of $CD14^+$ cells or alternatively induces CD14 expression on resident macrophages. Either way, we now know that cells are present, at the site of inflammation, with increased responsiveness to bacterial endotoxins by expression of surface CD14. Whether these cells also express the TLR2 and/or TLR4 or other TLRs is presently unknown, but we find it likely that the bacterial load of the intestine would coregulate CD14 and TLR expression. If we were able to block expression of either of these receptors, production of the hazardous inflammatory mediators (e.g., tumor necrosis factor α) that are induced by the CD14/TLR signaling pathway would be avoided. Indeed, in this report we demonstrate that local NF-κB p65 antisense treatment resulted in a drastically reduced population of CD14 expressing inflammatory cells. This finding may imply that NF-κB is, directly or indirectly, involved in regulation of CD14 expression.

Furthermore, p65 antisense treatment resulted in reduced accumulation of B and T lymphocytes. This is probably a secondary effect of the treatment, due to diminished production of cytokines, chemokines, and other inflammatory mediators from infiltrating macrophages. Altogether this indicates that p65 antisense treatment has a broad effect on the immune system, both at a molecular and a cellular level, which is of great value for the design of new drugs against inflammatory diseases.

Development of new agents to block various immunoregulatory molecules offers a therapeutic potential to inhibit the different signaling events that lead to chronic inflammation. Since it has been shown that a bacterial protein, YopJ, can downregulate the activation of important signaling pathways such as those of NF-κB [28] and p38 mitogen-activated protein kinase [51] it is most likely that certain pathogens can overcome the immune system of the host in a similar way. Knowledge about the way in which bacteria can outwit the host defense may provide new insights into the development and production of anti-inflammatory drugs of the future. Interestingly, a nonpathogenic strain of *Escherichia coli* was recently shown to be as effective as mesalazine in

maintaining remission of ulcerative colitis [52]. Since increasing data show NF-κB to be a central player in the proinflammatory events that can result in an inflammatory state, the further development of drugs that suppress NF-κB will be of great value.

Acknowledgements These studies were supported by the Swedish Medical Research Council, the Swedish Society of Medicine, and the Julius foundation. The authors very much appreciate the histopathological evaluation made by R. Feinstein, and thank Annika Samuelsson for much help, Ewa Österlund for excellent care of the animals, and Dr. Neil French for critically reading the manuscript.

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