In Situ Expression of Interleukin-10 in Noninflamed Human Gut and in Inflammatory Bowel Disease

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A dysregulated secretion of contra-inflammatory cytokines such as interleukin-10 (IL-10) could play a role in the pathogenesis of inflammatory bowel disease (IBD). We have investigated the expression of IL-10 in gut tissues from patients with Crohn's disease (CD), ulcerative colitis (UC) and controls by mRNA *in situ* hybridization and immunohistochemistry. Intestinal epithelial cells were found to express IL-10 mRNA and IL-10 protein in all of the tissues investigated without any major differences in the expression patterns. However, compared with noninflamed gut, significantly increased numbers of mononuclear cells (MNCs) producing IL-10 were present in inflamed gut, both in CD and UC. This cytokine was expressed most prominently by inflammatory infiltrates enriched in macrophages, although T cells seem to contribute to its production as well. Elevated IL-10 expression in IBD was mainly detected in the submucosa, whereas IL-10 production by lamina propria cells remained comparably low. In contrast, the expression of IL-1 β mRNA was preferentially increased in the lamina propria. Our data argue against a general deficiency in IL-10 production in IBD. The results suggest rather that the local production of IL-10 by mucosal MNCs in IBD is insufficient to down-regulate pro-inflammatory cytokines such as IL-1 β in the lamina propria compartment. (*Am J Pathol* 1998, 153:121–130)

Crohn's disease (CD) and ulcerative colitis (UC) represent the two major forms of chronic inflammatory bowel disease (IBD). Although their etiology is unknown and possibly heterogeneous, a disturbance in intestinal immune regulation is believed to be an important factor involved in their pathogenesis.¹ Immune homeostasis in the gut depends on a fine-tuned balance between immunological nonresponsiveness to the multitude of exogenous antigens present in the gut lumen (especially food

antigens and the bacterial microflora) and the ability of the mucosal immune system to mount protective responses against potential pathogens. Recent data suggest that this homeostasis is broken in IBD, resulting in an elevated immune reactivity against components of the resident intestinal microflora and/or environmental antigens with the consequence of inflammation.^{2–4} Investigations in knock-out mice indicate that changes in the local cytokine network represent an important factor contributing to such an enhanced immune reactivity.^{5,6}

Several studies have shown that the local production of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- α is considerably increased in IBD.^{7–12} This could be due to a dysregulated secretion of contra-inflammatory cytokines, such as IL-10. IL-10 is thought to be produced mainly by activated monocytes/macrophages,¹³ but production by activated T and B cells has also been reported.^{14–16} IL-10 effectively inhibits the antigen-presenting function of monocytes/macrophages and their production of pro-inflammatory cytokines (eg, IL-1, IL-6, and TNF- α). Conversely, IL-10 enhances the production of the anti-inflammatory IL-1 receptor antagonist.^{17–19} Co-stimulatory functions of macrophages for mitogen-induced T cell proliferation are also inhibited by IL-10.²⁰ In T cells, IL-10 particularly down-regulates the production of the T helper 1 cytokines interferon (IFN)- γ and IL-2, whereas T helper 2 cytokines, such as IL-4 and IL-5, seem to be less affected.^{21,22} Moreover, IL-10 can induce an antigen-specific anergic state in human CD4-positive T cells.²³ In B cells, IL-10 stimulates immunoglobulin production, in particular, IgA synthesis by CD40-activated cells.²⁴ In view of these functional properties, IL-10 might play a significant role in maintaining a noninflammatory immune status in the normal intestine. Using reverse transcription polymerase chain reaction (RT-PCR), basal production of IL-10 mRNA has been reported to exist in normal bowel tissues.²⁵ However, the cellular sources are unclear at present.

In IBD, initial reports of increased production of IL-10 mRNA exclusively in UC, but not CD tissues,²⁶ have not been confirmed by subsequent studies, which demon-

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strated increased mRNA levels in both active UC and CD²⁵ or even decreased production in UC.²⁷ Serum levels of IL-10 protein are also increased in active UC and CD.²⁸ However, concerning local IL-10 bioactivity, it was demonstrated that an elevated production of pro-inflammatory cytokines by IBD mononuclear phagocytes could be down-regulated by external IL-10 *in vitro* and, even more important, also *in vivo* by topical treatment with IL-10 enema preparations.²⁹ This indicates that the intrinsic intestinal bioactivity of IL-10 might be insufficient to control local inflammation, despite elevated mRNA levels.

To further investigate the potential role of IL-10 in non-inflamed intestine and in IBD, we have studied the tissue-specific expression of IL-10 at the mRNA and protein levels by *in situ* hybridization (ISH) and immunohistochemistry (IHC), respectively. In addition, IL-10 mRNA was compared with the local expression of IL-1 β mRNA.

Materials and Methods

Tissues

Thirty intestinal tissue samples from 24 different IBD patients (12 with CD, 12 with UC) undergoing surgical resection of diseased gut were investigated. Diagnosis was established by conventional clinical and histopathological criteria. Disease duration in patients ranged from 4 weeks to 20 years in CD (median, 8 years; 10 female, 2 male) and from 2 to 27 years (median, 12 years; 4 female, 8 male) in UC. The mean age at surgery was 38 years (range, 21 to 67 years). Seven CD patients and eleven UC patients received immunosuppressive therapy before the operation (corticosteroids; azathioprin in one case). Immediately after resection, small samples of tissue were dissected out, oriented on cork-plates, snap-frozen in liquid nitrogen, and stored at -70°C . Transmural sections 4 μm thick were cut at -20°C and used for IHC and ISH. All UC samples investigated ($n = 14$) originated from diseased colon. In CD, 12 samples derived from ileum and 4 from colon ($n = 16$). This included samples from inactive or virtually noninflamed areas in both CD ($n = 5$) and UC ($n = 5$). All other samples histologically had inflammatory disease, mostly including erosions, fissures, or larger ulcerations ($n = 20$). As normal controls, nonaffected ileum ($n = 2$) and colon ($n = 8$) from 10 patients undergoing bowel resection for colon cancer or functional disorder (1 case) were obtained. As inflammatory controls, samples from patients with recurrent sigma diverticulitis ($n = 2$), nonspecific ileitis ($n = 1$), ischemic enteritis ($n = 1$, ileum), recurrent appendicitis ($n = 1$), and thrombangiitis obliterans with chronic ulcerative enteritis ($n = 1$, ileum) were obtained.

Immunohistochemistry

The following monoclonal primary antibodies (MAbs) were used in the study: mouse anti-human IL-10, 1:50 (MAB 217, clone 23738.11, R&D Systems, Biermann, Bad Nauheim, Germany); mouse anti-human CD68 (macrophage marker), 1:100 (clone KP-1, Dako, Hamburg,

Germany); mouse anti-human CD20 (B cell marker), 1:50 (clone L-26, Dako); mouse anti-human CD3 (T cell marker), 10 $\mu\text{g}/\text{ml}$ (OKT-3, Ortho, Neckargemünd, Germany); mouse anti-human CD14 (clone MEM 14, kindly provided by Dr. V. Horejsi, Prague, Czech Republic); mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgM negative control MAb (Dako, Dianova, Hamburg, Germany).

Immunostaining of IL-10 was performed using the paraformaldehyde-saponin procedure (PSP).³⁰ After cutting, native cryostat sections were fixed in 2% paraformaldehyde (PFA; Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS) for 20 minutes at room temperature, followed by a wash in Earle's balanced salt solution (EBSS; Gibco, Life Technologies, Eggenstein, Germany) supplemented with 0.01 mol/L HEPES buffer (Gibco). Permeabilization was performed by incubation of the slides in 0.1% saponin (Riedel de Haen AG, Seelze, Germany)/EBSS/0.01% HEPES. This buffer was used for all washings and dilutions of MAbs and secondary reagents. The primary MAbs were incubated overnight at room temperature with the addition of 2.5 mg/ml normal human immunoglobulins (γ -venin, Behring, Marburg, Germany). After washing, the sections were incubated for 20 minutes with 10% inactivated normal sheep serum diluted in permeabilization buffer. After flicking off the blocking serum, biotinylated sheep anti-mouse Ig (1:200; Amersham, Braunschweig, Germany) was added for 30 minutes, followed by streptavidin complexed with biotinylated alkaline phosphatase for 30 minutes (complex prepared after the manufacturer's instructions (Dako)). Blocking of endogenous biotin was performed in some experiments but later was omitted as it did not influence the results. After subsequent washes with permeabilization buffer, EBSS/0.01 HEPES without saponin, and finally water, the color reaction was developed using naphthol AS-bisphosphate (Sigma, Deisenhofen, Germany) and new fuchsin (Merck, Darmstadt, Germany) as chromogen.³¹ After counterstaining with hematoxylin, the sections were mounted in glycerol-gelatin.

Double staining for IL-10 protein and CD3 or CD68 was also performed using the PSP method. After simultaneous incubation of the slides with anti-IL-10 (IgG2b isotype) plus anti-CD3 (IgG2a isotype) or anti-CD68 (IgG1 isotype), a combination of biotinylated goat anti-mouse IgG2b (Dunn, Asbach, Germany) and peroxidase-labeled goat anti-mouse IgG2a or IgG1 (Dunn) was used, followed by streptavidin-alkaline phosphatase. The alkaline phosphatase reaction (blue color) was developed first using the Fast Blue detection kit (Vector/Camon, Wiesbaden, Germany). 3,3'-Diaminobenzidine (Dako) was used as the substrate for peroxidase (brown color). The slides were mounted directly without further counterstaining.

Single staining of mononuclear cells (MNCs) for CD3, CD16, CD20, and CD68 was performed on native cryostat sections fixed in cold (-20°C) acetone for 10 minutes after a protocol similar to the PSP method, except that Tris-buffered saline (TBS; no saponin) with the addition of 0.2% bovine serum albumin (BSA; Sigma) was used as the diluent and washing medium.

In Situ Hybridization

A 477-bp fragment of human IL-1 β cDNA (bases 11 to 487 of the sequence published by Nishida et al).³² was subcloned into the *Bam*HI/*Hind* III restriction site of pBluescript II SK(+) (Stratagene, Heidelberg, Germany). A *Bam*HI/*Ssp*I fragment of human IL-10/pCDSR α cDNA (number 68192, American Type Culture Collection, Rockville, MD) containing a fragment (base pairs 1 to 691) of IL-10 cDNA³³ was subcloned into the *Bam*HI/*Eco*RV restriction site of pBluescript II KS(-) (Stratagene). The identity of these constructs was confirmed by sequencing.

Full-length antisense and sense (negative controls) cRNA probes of high specific activity ($>10^8/\mu\text{g}$ RNA) were transcribed *in vitro* from linearized templates in the presence of ^{32}P -labeled uridine triphosphate (UTP; >1000 Ci/mmol) (Amersham or DuPont NEN, Bad Homburg, Germany) with T7 and T3 RNA polymerases using an RNA transcription kit (Stratagene). Limited alkaline hydrolysis of the resulting cRNA probes was performed to obtain fragments ranging in length from approximately 50 to 300 bp.³⁴

In situ hybridization was performed as described recently,⁷ with slight modifications. Under standard precautions to avoid RNase contamination, cryostat sections were placed onto 3-aminopropyltriethoxysilane-treated slides (Histo-bond slides, Marienfeld, Heidelberg, Germany) and fixed in 4% PFA buffered with 2X SSPE, pH 7.4 (1X SSPE contains 0.15 mol/L NaCl, 0.01 mol/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 mmol/L EDTA) plus 5 mmol/L MgCl_2 for 15 minutes. Sections were dehydrated (70%/100% ethanol), air dried, and stored at -70°C until use or directly processed for ISH. Rehydrated slides were digested with proteinase K (Boehringer, Mannheim, Germany), 0.5 mg/ml, for 15 minutes at 37°C and rinsed with 0.1 mol/L glycine (Merck) in 2X SSC (1X SSC contains 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate). After post-fixation (5 minutes at room temperature) with 4% PFA and treatment with iodoacetamide (0.1 mol/L in 0.01 mol/L triethanolamine, pH 8.2; Sigma) for 30 minutes, the slides were acetylated in 0.1 mol/L triethanolamine with 0.25% acetic anhydride (Merck) for 10 minutes and equilibrated with 50% deionized formamide (FA; Fluka, Neu-Ulm, Germany) in 2X SSPE for at least 15 minutes. After shaking off excess solution, semi-dry sections were prehybridized with hybridization mix containing 50% FA/2X SSPE, 10 mmol/L Tris/HCl, pH 7.4, 0.1% sodium dodecyl sulfate (SDS; Serva), 10% dextran sulfate (molecular weight, 500,000; Pharmacia, Uppsala, Sweden), 1X Denhardt's solution (Merck), 500 $\mu\text{g}/\text{ml}$ tRNA from brewer's yeast (Boehringer), 500 $\mu\text{g}/\text{ml}$ poly A (Boehringer), irrelevant RNA probe labeled with cold UTP, and 100 $\mu\text{g}/\text{ml}$ denatured, sonicated herring sperm DNA (Promega, Madison, WI) at 50°C in a sealed chamber filled at the bottom with 50% FA/2X SSPE. After 3 hours, 2×10^6 cpm of [^{32}P]UTP-labeled RNA probe was added and hybridized overnight at 50°C . Post-washings were performed in a shaking waterbath with 50% FA/2X SSPE for 30 minutes at 50°C ; 2X SSC/0.1% SDS for 10 minutes at 37°C ; 2X SSC/0.1% SDS plus 50 $\mu\text{g}/\text{ml}$ RNase A and 10 U/ml RNase T1 (Boehringer) for 30 minutes at 37°C ; 50%

FA/0.5X SSC/0.1% SDS for 30 minutes at 37°C ; and 0.1X SSC for 30 minutes at 37°C . Slides were then dehydrated in 50/70/95/100% ethanol containing 0.3 mol/L ammonium acetate (Merck), air dried, dipped in Kodak NTB-2 emulsion (Technomara, Fernwald, Germany) for autoradiography, and exposed at 4°C in the dark for 6 weeks, followed by development in Kodak D-19, fixing in Kodak AL-4 (both from Technomara), and finally staining with hematoxylin and eosin (H&E). Autoradiographs were viewed with a Zeiss photomicroscope (Zeiss, Oberkochen, Germany). Cells with grain numbers of more than three times the background (~ 20 grains/cell) were considered positive. Incubations with sense cRNA probes served as negative controls.

Slide Evaluations and Statistical Analysis

mRNA

The number of positive cells expressing mRNA for IL-10 and IL-1 β was independently assessed by two of the authors, one of which (B. Helmke) was unaware of the diagnosis. The slides were analyzed using a Zeiss photomicroscope equipped with a video camera (XC-003P, Sony Corp., Köln, Germany) and a color monitor (SyncMaster 17 GLi, Samsung Electronics, Steinbach, Germany). In each case, fractions of 500 nucleated cells were counted in two different representative areas in the lamina propria as well as in the submucosa over adjacent high-power fields (objective $\times 25$) using a calibrated grid (total grid area, 0.01 mm^2), and the relative percentage of mRNA-expressing cells to the total number was determined. The lamina propria was assessed from the area immediately below the surface epithelium down to the deeper lamina propria. The submucosa was analyzed in a similar fashion from below the lamina muscularis mucosa down to the muscularis propria border. As all nucleated cells were counted, the enumeration included both leukocytes and endothelial cells, fibrocytes, and other cell types that sometimes cannot be distinguished with certainty from leukocytes under the given staining conditions. Typical inflammatory dense lymphoid aggregates, which occurred in the submucosa in highly inflamed cases, were analyzed separate from the remaining submucosa, which contained focally accentuated infiltrates of heterogeneous inflammatory cells (probably pre-existing germinal lymphofollicular complexes at characteristic locations in the mucosa or at the mucosal/submucosal border were not counted). Thus, a total number of up to 3000 cells per case was evaluated. For technical reasons, exact cell counting was considered unreliable at ulcerative sites, and they were excluded from the quantitative analysis. Epithelia were also excluded from this evaluation.

Median values of the respective results were obtained for statistical evaluation, which was performed using the SAS program (Statistical Analysis System for Windows, version 6.11, SAS Institute, Cary, NC). The inter-observer variability was analyzed using the paired *t*-test (no significant differences were noted). The Wilcoxon's two-tailed test for unpaired samples was used for comparative sta-

tistical analysis of the mRNA expression in different disease groups. Differences in expression between lamina propria and submucosa in these groups were analysed using the Wilcoxon matched pairs signed rank test. *P* values of <0.05 were considered to be significant.

IL-10 Protein

Although reliable protein staining for IL-10 could be achieved in all cases using the PSP method, cell-to-cell enumeration of positive MNCs proved to be difficult because significant immunohistochemical staining was also observed in spaces between MNCs, especially in densely inflamed areas, most probably due to the existence of secreted cytokine in the interstitium. Thus, a score was used for a rough estimation of positive cells. Fractions of 200 nucleated cells were assessed at three different representative sites in the lamina propria and the submucosa and scored as follows: negative, 0; single positive or less than 10% positive cells, 1; $>10\%$ to 50% positive cells, 2; $>50\%$ positive cells, 3. Differences between median values of the respective results in the different disease groups were statistically analyzed using the Fisher's exact test (two-tailed). *P* values of <0.05 were considered as significant.

Results

IL-10

Noninflamed Gut

In noninflamed normal gut, intestinal epithelial cells were found to express IL-10 mRNA in a diffuse fashion. Some intestinal MNCs in the noninflamed lamina propria also gave positive hybridization signals, but epithelial expression predominated (Figure 1, A and B). Local production of IL-10 in noninflamed gut was also detected at the protein level by IHC, where again epithelia diffusely stained in all cases. In contrast to epithelia, IL-10 protein was expressed at rather low levels by lamina propria MNCs (Figure 2, A and B). An exception occurred at sites of organized mucosal lymphoid follicles, where most MNCs regularly stained for IL-10 protein.

Inflamed Gut

Epithelia were found to diffusely express both IL-10 mRNA and protein in inflamed gut in all investigated samples, showing no major differences in expression patterns between the different disease groups. However, in addition to this epithelial expression, significantly increased numbers of MNCs expressing IL-10 were observed in inflamed tissues, both in CD and UC as well as in cases of other types of gut inflammation (inflammatory controls). Quantitative assessment of IL-10 mRNA compared with IL-1 β mRNA and the median score values for IL-10 protein are summarized in Figure 3, A and B, and Table 1.

Figure 1. IL-10 mRNA expression in noninflamed gut. **A:** *In situ* hybridization of noninflamed colon demonstrates IL-10 mRNA expression by intestinal epithelial cells with accumulation of white autoradiographic grains. Only a few lamina propria MNCs display positive signals. **B:** Negative control with use of the sense cRNA probe. Dark-field micrographs; magnification, $\times 40$.

At the mRNA level, increased IL-10 production was mainly apparent in the submucosal compartment of the gut wall, where significantly elevated numbers of IL-10 mRNA-expressing cells were observed in both active UC and CD (respective medians of 6.55% and 11.75% positive cells compared with 0.4% in normal controls; $P < 10^{-4}$; Figure 4 A–C, eg, shows ISH for IL-10 mRNA in a case of UC). Expression of IL-10 mRNA was especially prominent on heterogeneous accumulations of perivas-

Figure 2. IL-10 protein expression in noninflamed gut. **A:** Immunohistochemical detection of IL-10 protein with strong staining (red) of intestinal epithelia in noninflamed normal colon. Only a few lamina propria MNCs are faintly stained. **B:** Corresponding negative control with omission of the primary antibody. PSP method; alkaline phosphatase; magnification, $\times 40$.

cular MNCs as well as scattered inflammatory cells. Parallel immunostaining of tissue sections for CD68 and CD14 indicated that such infiltrates were rich in macrophages, suggesting that IL-10 mRNA is mainly expressed by mononuclear phagocytes (Figure 4D). In addition, CD3-positive T cells (Figure 4E), but virtually no CD20-positive B cells (Figure 4F), were present within such infiltrates. Thus, the former likely contribute to local IL-10 production as well.

Few IL-10 mRNA-producing cells were observed inside submucosal dense lymphoid aggregates (medians of 0.6%, 0.5%, and 0% positive cells in active UC, active CD, and inflammatory controls, respectively). Positive cells were rather clustered in the periphery of such foci (Figure 5, A and B) and were co-localized with CD14- and CD68-positive MNCs, suggesting that macrophages are the main source of IL-10 mRNA at these peripheral sites (Figure 5C). However, despite the lack of IL-10 mRNA expression, most MNCs inside of dense lymphoid aggregates stained for IL-10 protein by IHC (Figure 5D). Parallel immunostaining for CD20 revealed that dense lymphoid aggregates often contained large numbers of B cells in addition to T cells (Figure 5E).

In CD, inflammatory infiltrates with high IL-10 mRNA expression were distributed throughout all layers of the submucosa (and deeper layers), whereas in UC they were accentuated in the upper submucosa near the mucosal border (predominant mucosal inflammation in UC). However, moderately increased numbers of submucosal IL-10 mRNA-expressing cells were also observed in cases with other types of gut inflammation (inflammatory controls; median of 4.0% positive cells *versus* 0.4% in normal controls; $P < 10^{-3}$). Both in UC and CD, the elevated submucosal mRNA production significantly correlated with active disease ($P < 0.011$) and was some-

what higher in active CD compared with active UC ($P < 0.015$).

Compared with the submucosa, the number of MNCs expressing IL-10 mRNA was considerably lower in the inflamed lamina propria, even in cases with high inflammatory activity (medians of 2.85% and 3.45% positive cells in the lamina propria in active UC and CD compared with 6.55% and 11.75% in the submucosa; $P < 0.01$ and < 0.001 , respectively; Figures 3 and 4A). In all disease groups investigated, only marginally elevated numbers of positive MNCs were observed in the lamina propria, which did not differ significantly from normal controls (except for a slight elevation in active CD; $P < 0.03$).

Staining for IL-10 protein in inflamed gut correlated with the pattern of mRNA expression but was generally observed on a larger number of MNCs (Figure 6A shows an example in a case of CD). Again, the highest IL-10 expression was observed in the inflamed submucosa where the majority of infiltrating MNCs stained for IL-10, both in IBD and in inflammatory control cases (median score of 3.0 in inflamed CD, UC, and inflammatory controls; Table 1). In highly inflamed areas, the staining reaction included infiltrating MNCs and intercellular spaces, most probably due to the presence of cytokines in the interstitium. Positive staining included dense lymphoid aggregates as well as macrophage-enriched, mixed inflammatory infiltrates. In such infiltrates, immunohistochemical double staining revealed IL-10 immunoreactivity on both CD68-positive macrophages and CD3-positive T cells (Figure 6, B and C). The numbers of MNCs that stained for IL-10 protein were considerably lower in the inflamed lamina propria compared with the submucosa (median score of 3.0 in the submucosa *versus* 1.0, 2.0, and 1.5 in the lamina propria in active CD, UC, and inflammatory controls, respectively; Table 1).

Figure 3. Expression of IL-10 mRNA (A) and IL-1 β mRNA (B) by nonepithelial cells of noninflamed and inflamed gut. The number of positive cells expressing mRNA was independently assessed by two observers. Fractions of 500 nucleated cells were counted in two different representative areas in the lamina propria and the submucosa and within dense lymphoid aggregates, and the relative percentage of mRNA-expressing cells to the total number was determined. The box plot diagrams represent median values of the respective results (median, 25th and 75th percentile). Minimum and maximum are indicated by dotted lines.

However, at the protein level the number of IL-10-positive cells was higher statistically in all disease groups compared with normal controls, especially in UC and inflammatory controls ($P < 0.05$). Concerning the lamina propria, IL-10 protein staining of inflammatory MNCs was higher in UC than in CD whereas no statistical difference was observed in the submucosa.

IL-1 β mRNA

In noninflamed normal gut, IL-1 β mRNA was expressed by rather few lamina propria MNCs in subepithelial regions (median of 0.2% positive cells; Figure 3B) whereas significantly higher numbers of IL-1 β mRNA-expressing cells were detected in inflamed gut. There, elevated numbers of cells expressing IL-1 β mRNA were observed mainly in the lamina propria compartment, whereas the expression in the submucosa was significantly lower (Figure 3B; $P < 0.01$ and <0.001 in active UC and CD, respectively). In the inflamed lamina propria, IL-1 β was expressed by focal clusters of intensively labeled MNCs, presumably macrophages. The highest expression was

observed in active IBD, without significant differences between UC and CD (medians of 2.45% and 3.45% positive lamina propria cells in active UC and CD compared with 0.2% in noninflamed controls). The number of IL-1 β -positive mucosal cells in IBD was significantly higher than in inflammatory controls not related to IBD (median of 0.7% in the lamina propria). As recently demonstrated,⁷ strong expression of IL-1 β mRNA was also found along the surface of mucosal ulcerations, where positive cells included both endoluminal granulocytes and macrophages. Submucosal dense lymphoid aggregates contained only a few IL-1 β -positive cells (similar to IL-10 mRNA).

Discussion

The present results demonstrate that IL-10 is constitutively expressed in normal human gut tissue and that intestinal epithelial cells (IECs) represent a major source of this cytokine in noninflamed gut. However, production of IL-10 by IECs could also be demonstrated in inflamed tissues, both at the mRNA and protein levels, without major differences in expression pattern between the different disease groups. The fact that IL-10 is produced by IECs supports recent evidence that these cells exert important regulatory influences on the mucosal immune system.^{35–37} In view of its potent inhibitory properties on macrophage and T cell functions,^{13,17–23} IL-10 derived from the epithelia and, to a lesser extent, from mucosal MNCs might contribute to the specialized immune status in the intestine. Down-regulated responsiveness of local T cells to antigen receptor triggering^{38–42} as well as diminished co-stimulatory activities of intestinal macrophages⁴³ by IL-10 could prevent unwanted local immune responses against luminal antigens. Epithelial IL-10 is likely to influence the function of intraepithelial lymphocytes but may in addition act on mucosal MNCs after being released into the lamina propria. Moieties released into the gut lumen could possibly exert effects after backcrossing the epithelial barrier, for example, by receptor-bound transport, which has been described for certain

Table 1. Expression of IL-10 Protein by Nonepithelial Cells of Noninflamed and Inflamed Gut

Tissue	Number of investigated samples	Lamina propria, median score	Submucosa, median score
Normal controls	10	1	0
Crohn's disease	16		
Noninflamed/inactive	5	1	0
Inflamed	11	1	3
Ulcerative colitis	14		
Noninflamed/inactive	4	2	0
Inflamed	10	2	3
Inflammatory controls	6	1.5	3

The number of positive nucleated cells stained for IL-10 protein was semiquantitatively assessed on fractions of 200 nucleated cells at three representative sites in the lamina propria and submucosa and scored as follows: negative, 0; single positive or less than 10% positive cells, 1; >10% to 50% positive cells, 2; >50% positive cells, 3. Epithelia were excluded from this evaluation.

Figure 4. IL-10 mRNA expression in IBD. **A:** Only a few MNCs in the inflamed mucosa express IL-10 mRNA (white grains) in a case of UC (arrowheads), whereas epithelia diffusely express the signal. **B and C:** In comparison, many scattered inflammatory MNCs in the adjacent submucosa, including perivascular and perineural MNCs strongly express IL-10 mRNA in the same case. **D to F:** Immunohistology of the same area as in **B** and **C** reveals that many inflammatory cells express the macrophage marker CD68, suggesting that IL-10 mRNA is expressed by such cells (**D**). However, some CD3-positive T cells (**E**), but virtually no CD20-positive B-cells (**F**), are present at this site. **A and B:** *In situ* hybridizations; dark-field micrographs; magnification, $\times 40$. **C:** Corresponding bright-field of **B** (grains hardly visible at this magnification, $\times 40$). **D:** CD68; **E:** CD3; **F:** CD20; alkaline phosphatase; magnification, $\times 40$.

growth factors,⁴⁴ or after uptake by M cells. Some preliminary work has reported the production of IL-10 by isolated human IECs⁴⁵ as well as the human intestinal epithelial cell line C-205,⁴⁶ which is in line with our results. One recent *in situ* study with gut tissues from celiac disease patients failed to demonstrate epithelial IL-10.⁴⁷ However, autoradiographs were incubated for only 5 days in this study, which might be too short to detect the mRNA signal. Furthermore, IHC was performed with a different MAb on acetone-fixed slides. In our hands, such treatment abolishes the staining for IL-10 protein almost

completely in contrast to the PSP method employed here (data not shown).

We detected an elevated intestinal expression of IL-10 by MNCs in IBD. Significantly increased numbers of IL-10-producing inflammatory cells exist in the gut wall in actively inflamed cases, both in UC and CD. Our results are in line with data demonstrating increased IL-10 serum levels in UC as well as CD, correlating with active disease,²⁸ and argue against a general deficiency in the ability to produce IL-10 by these patients. Local MNCs secrete IL-10 presumably with the aim to control the

Figure 5. Expression of IL-10 mRNA and protein in dense lymphoid aggregates in IBD. **A and B:** Corresponding bright-field (**A**) and dark-field (**B**) micrographs of a submucosal dense lymphoid aggregate in a case of CD (ileum) hybridized for IL-10 mRNA demonstrate strong expression of white autoradiographic signals (**B**) on inflammatory MNCs at the periphery of the focus, whereas most cells inside the lymphoid aggregate do not express the signal. *In situ* hybridization; magnification, $\times 40$. **C:** Immunohistochemistry with demonstration of CD14-positive MNCs predominantly at the periphery of a lymphoid aggregate in the same case (red) suggests that macrophages produce the IL-10 mRNA at such sites. **D and E:** However, despite the lack in mRNA expression, most MNCs inside a dense lymphoid aggregate are stained for IL-10 protein in the same case (**D**). Such aggregates are shown to contain many CD20-positive B cells (**E**). **C:** CD14; **D:** IL-10 protein; **E:** CD20; alkaline phosphatase; magnification, $\times 40$.

inflammatory process. In this regard, IL-10 is able to efficiently down-regulate enhanced secretion of IL-1 β by intestinal MNCs in IBD (while inducing IL receptor antagonist).²⁹ We have found that elevated expression of IL-10 by inflammatory MNCs in IBD is mainly restricted to the submucosal compartment of the gut wall, both at the mRNA and protein levels, whereas production by lamina propria MNCs remains comparably low. In the same tis-

sue samples, production of IL-1 β mRNA was significantly up-regulated in the inflamed lamina propria, whereas expression of this pro-inflammatory cytokine was comparably low in the submucosa. Thus, our data suggest an inverse relationship in the local expression of IL-10 *versus* IL-1 β mRNA in these two compartments in IBD.

Concerning the lamina propria, our data indicate that in IBD the extent of IL-10 production by mucosal MNCs

Figure 6. IL-10 protein expression in IBD. **A:** Many submucosal inflammatory cells in a case of CD strongly stain for IL-10 protein (red), whereas only few MNCs in the adjacent lamina propria are weakly positive. Note that the gut epithelium also stains for IL-10 protein in inflamed gut. PSP method; alkaline phosphatase; magnification, $\times 25$. **B and C:** Immunohistochemical double staining of inflammatory cells in a case of UC at a perivascular site in the submucosa. Weak to moderate stainings for IL-10 can be observed on most inflammatory cells (blue color, no nuclear counterstain), which contain both CD3-positive T cells (**B**) as well as CD68-positive macrophages (**C**) (brown color). PSP method; alkaline phosphatase and peroxidase; magnification, $\times 100$.

may not be sufficient to control the production of pro-inflammatory cytokines such as IL-1 β at this site. The beneficial effect of topical treatment of IBD patients with IL-10 enema preparations²⁹ could thus at least in part be explained by compensating this insufficiency. Reasons for the lack of sufficient IL-10 production by lamina propria MNCs are unclear at present. A constant exposure of mucosal cells to epithelial-derived IL-10 might play a role, as this probably down-regulates IL-10-synthesis by MNCs via autoregulative feedback mechanisms.¹³ Low production of IL-10 by MNCs in the mucosa, but moderately elevated expression in the submucosa, was also found in inflammatory control cases and thus does not appear to be a specific phenomenon of idiopathic IBD. However, the mucosal expression of IL-1 β was not up-regulated as strongly as in IBD, which in such cases might contribute to a more limited disease course.

Our data do not confirm a predominant activation of IL-10 in UC *versus* CD. Although IL-10 protein staining on MNCs was higher in UC, this difference applied only for the mucosa but not for the submucosa, and no difference was observed in the mucosa at the mRNA level (submucosal mRNA was even somewhat higher in active CD). Concerning the types of intestinal MNCs, our results indicate that IL-10 mRNA and protein are expressed most prominently by macrophages as well as by T cells. Protein staining basically correlated with the patterns of mRNA expression. A different situation exists at sites of dense lymphoid aggregates, where virtually all cells stain for IL-10 protein but do not express the mRNA. Such sites contain many CD20-positive B cells, and the immunohistochemical reaction most likely detects cell-bound soluble IL-10, which is produced by macrophages in the

periphery of such aggregates. In view of the strong epithelial IL-10 expression, one might wonder why lamina propria MNCs do not display a secondary protein staining of this type. One explanation could be higher turnover rates of the cytokine due to the presence of proteolytic enzymes in the mucosa. Alternatively, cells within dense lymphoid aggregates might express higher amounts of IL-10 receptor compared with lamina propria cells.

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