**EPITHELIAL CELL BIOLOGY**

Key role of the sympathetic microenvironment for the interplay of tumour necrosis factor and interleukin 6 in normal but not in inflamed mouse colon mucosa

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**Background:** In the intestinal tract, the role of sympathetic neurotransmitters has been largely ignored in mucosal neuroimmunology.

**Aim:** Our aim was to investigate the influence of the sympathetic microenvironment on the mucosal interplay of tumour necrosis factor (TNF) and interleukin 6 (IL-6).

**Methods:** Colon strips of normal and colitic BALB/c mice were superfused in vitro. Tissue was electrically stimulated to investigate the influence of endogenous norepinephrine (NE) on secretion of IL-6, with or without anti-TNF antibodies (anti-TNF) and adrenoceptor antagonists. IL-6 was secreted from macrophages.

**Results:** Superfusion with anti-TNF stimulated IL-6 secretion in normal but not in colitic colon (p<0.005). Parallel superfusion with a β-adrenergic antagonist abrogated this phenomenon. Anti-TNF increased release of NE from normal colonic strips (p<0.05), which demonstrates TNF-induced inhibition of preterminal NE release. In colitic mice, anti-TNF did not change NE release. In the presence of anti-TNF, exogenous and endogenous NE stimulated colonic IL-6 secretion via β-adrenoceptors in normal (p<0.001) but not in colitic mice. In the absence of anti-TNF, endogenous and exogenous NE inhibited IL-6 secretion via the β-adrenoceptor in normal but not in colitic mice (p<0.01). Colitic mice demonstrated loss of sympathetic nerve fibres.

**Conclusions:** Modulation of mucosal IL-6 is largely dependent on the sympathetic microenvironment and availability of local TNF in normal but not in colitic mice. Anti-TNF strategies may lead to an increase in the proinflammatory cytokine depending on adrenergic tone. This would be relevant with normal sympathetic innervation, which is lost in colitic mice. We present a model of sympathetic regulation of colonic macrophage TNF and IL-6 secretion.

Using a superfusion technique, we were able to characterise sympathetic nerve-immune cell interplay in spleen slices (reviewed by Straub). Electrical field stimulation induced release of endogenous neurotransmitters. Studies with specific antagonists revealed that the endogenous sympathetic neurotransmitters NE, endogenous opioids, and neuropeptide Y modulate IL-6 and TNF secretion of splenic macrophages. These studies demonstrated that IL-6 is the downstream readout parameter of adrenergic TNF regulation when TNF is switched on during bacterial infection. In this situation, IL-6 downstream of TNF is inhibited via βAR. In contrast, if TNF secretion is negligible, adrenergic regulation of IL-6 is reversed and its secretion is stimulated via βAR. This demonstrates the intricate interrelation of NE and these two cytokines under immune stimulated and unstimulated conditions.

This present study aimed to investigate the major source of IL-6 in the intestinal wall. In addition, we aimed to study the neuroimmune interplay of endogenous NE, TNF, and IL-6 using a superfusion technique with colon strips from normal and colitic BALB/c mice. We focused on βAR mediated effects of NE for this interplay between TNF and IL-6. Furthermore, we examined the influence of TNF on neuronal NE release in normal and inflamed mouse colon mucosa.

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**Revised version received 25 February 2005**
**Accepted for publication 4 April 2005**
**Published online first 21 April 2005**

Abbreviations: βAR, β-adrenoceptors; anti-TNF, neutralising anti-TNF antibodies; DSS, dextran sodium sulphate; ELISA, enzyme linked immunosorbent assay; HPLC, high performance liquid chromatography; IFN-γ, interferon-γ; IL, interleukin; NE, norepinephrine; PBS, phosphate buffered saline; SAP, saporin; TBS, Tris buffered saline; TNF, tumour necrosis factor.
and studied sympathetic innervation of the colon in normal and colitic mice. In our discussion we link the results of this study to anti-TNF therapy in patients.

**MATERIALS AND METHODS**

**Animals**

A total of 100 female BALB/c mice (8–10 weeks; 26–34 g; Charles River, Sulzfeld, Germany) were used. The exact numbers of animals used are given in the figure legends. Animals were fed a standard laboratory chow diet. All procedures were in accordance with the guidelines for the care and use of laboratory animals approved by the University of Regensburg Institutional Animal Care and Use Committee (Government of Oberpfalz AZ 621-2531.1-22/02).

**Animal model of chronic colitis**

The model of chronic colitis has been described previously. Dextran sodium sulphate (DSS, molecular weight 40 000; ICN, Inc., Eschwege, Germany) chronic colitis was induced by feeding 2% DSS over four cycles. In parallel, mice received a standard laboratory chow diet. One cycle consisted of feeding 2% DSS in drinking water for seven days followed by a period of 10 days drinking water without DSS. Thirty days after the last cycle, animals were killed and the colon was removed for further investigation (superfusion technique, see below). Of the 100 BALB/c used, a total of 25 were included in animal experiments with chronic colitis (Government of Oberpfalz AZ 621-2531.1-22/02).

**Immunohistochemical localisation of IL-6 secreting cells and macrophages in the mucosa**

Colon strips were transferred to superfusion chambers and superfused for two hours with 10 µg/ml Brefeldin A (Sigma-Aldrich, Deisenhofen, Germany) in order to prevent cytokine excretion (superfusion technique, see below). Strips were removed from the superfusion chambers and embedded in Tissue-Tek (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, the Netherlands) and snap frozen. Tissue samples were cut into 5 µm thick sections. Several pairs of consecutive cryosections were placed on different precoated slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany) and placed in a solution with 0.5% H2O2 in 0.05 M Tris buffered saline (TBS). Tissue sections were incubated in a humid chamber for 12–18 hours with purified polyclonal rabbit antibodies against IL-6 (dilution 1:250; Acris via DPC Burlingame, California, USA) and the AEC substrate kit for localization of peroxidases (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany). After washing, small strips of the entire colonic wall with a size of approximately 25 mm² were cut and randomly transferred to superfusion chambers. These chambers had a volume of 80 µl and were equipped with platinum electrodes forming the bottom and top of each chamber, respectively. We used colon strips from different parts of the colon which were randomly transferred to the superfusion chambers in a blinded manner.

**Superfusion medium, preparation of the tissue, superfusion protocol, and standardisation of the tissue**

The colon (including the ascending, transverse, and half of the descending colon) of the above mentioned BALB/c mice was carefully removed after cervical dislocation and opening of the peritoneal cavity. The colon was opened using scissors to cut the intestinal wall in a longitudinal direction. The opened colon was washed several times in ice cold culture medium (RPMI 1640, 25 mM HEPES, 5% fetal calf serum, 30 µM mercaptoethanol, 0.57 mM ascorbic acid, 1.3 mM calcium, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Sigma), and 8 µg/ml ciprofloxacin (Bayer, Leverkusen, Germany)). After washing, small strips of the entire colonic wall with a size of approximately 25 mm² were cut and randomly transferred to superfusion chambers. These chambers had a volume of 80 µl and were equipped with platinum electrodes forming the bottom and top of each chamber, respectively. We used colon strips from different parts of the colon which were randomly transferred to the superfusion chambers in a blinded manner.

The superfusion method has been described previously. Briefly, superfusion was performed for 5.5 hours at a temperature of 37°C and a flow rate of 66 µl/min (one strip per chamber, 4×8 = 32 chambers in parallel; one mouse with 32 strips per day). During the first 2.5 hours of the superfusion period, all strips were superfused with culture medium without any additional drugs or electrical stimulation. Between 120 and 150 minutes, superfusate was collected to determine IL-6 secretion (ELISA technique, see above). During the second part of the superfusion period (3rd–5th hour), drug, electrical stimulation, or both, were applied to modulate IL-6 secretion. Between the 300th and 330th minute, superfusate was collected a second time to determine IL-6, IL-6 at three hours was used to standardise the IL-6 secreting capacity of the different strips. The dimensionless ratio \( \psi = 100 \times (IL-6_{3\text{h}}/IL-6_{3\text{h}}) \) was used to standardise IL-6 secretion of each strip at 5.5 hours. In experiments with spleen slices, this standardisation technique was found to be superior to standardisation using the leucocyte count of the slice, wet weight, dry weight,
Tissue viability of colon strips was excellent after 5.5 hours, as estimated by NE release, histology, and lactate dehydrogenase (LDH) release.

Experiments with adrenergic agonists and adrenergic antagonists, and electrical stimulation

To study the effects of adrenergic agonists, NE (Sigma) and isoproterenol (β1/β2 adrenergic agonist; Sigma) were used. In all experiments the substances were added between the 170th and the 330th minute of superfusion without additional electrical stimulation.

To indirectly study the effect of electrically released endogenous NE, nadolol (β1/β2 adrenergic receptor antagonist; Sigma) was used at the concentrations indicated. Dilutions of the drugs were prepared immediately before the experiments. In experiments to study transmitter effects, drugs were added at 150 minutes until the end of superfusion. After a drug equilibration period of 20 minutes between the 150th and the 170th minute, slices were electrically stimulated using one train of monophasic rectangular pulses (2 ms, 5 Hz, 43 mA, 45 000 pulses) which lasted until the end of superfusion.

Experiments with the monoclonal neutralising anti-TNF antibody V1qH8

As it is known that secretion of TNF precedes that of IL-6, the significance of TNF for IL-6 secretion was characterised using the monoclonal anti-TNF antibody V1qH8. In these experiments, purified V1qH8 was used at the concentrations indicated, between the 150th and the 330th minute of superfusion. Control experiments were performed with a respective IgG.

Determination of superfusate norepinephrine

NE was determined by high performance liquid chromatography (HPLC) with electrochemical detection. NE was isolated from superfusate using a commercially available kit for the determination of catecholamines in plasma (Recipe, Munich, Germany). The HPLC system consisted of a pump model K-1001 (Knauer, Berlin, Germany), an autosampler model SIL-9A (Shimadzu, Duisburg, Germany), an analytical column (length x inner diameter 150 x 4.6 mm) filled with Luna C18(2) 5 μm silica gel (Phenomenex, Aschaffenburg, Germany), an electrochemical detector model L-3500A (Recipe), and the class 10 integration software (Shimadzu, Duisburg, Germany). Analytical column and detector cell...
(model sputnik with glassy carbon working electrode set at 0.50 V v Ag/AgCl; Recipe) were mounted in a high pressure liquid chromatography thermostat HT3000 (Recipe) maintained at 25˚C. The mobile phase was mixed with 6.90 g NaH2PO4·H2O, 1.80 g NaOH, 100 mg sodium octane sulphonate, 50 mg Na2EDTA, 3.0 ml acetic acid, 1000 ml water, 40 ml acetonitrile, and 20 ml methanol (resulting pH 5.4). At a flow rate of 1.0 ml/min, NE eluted after 4.5 minutes and the internal standard dihydroxy benzylamine after 7.9 minutes. Using 2.5 ml of perfusate, the limit of quantitation was 5 pg/ml for NE.

Immunohistochemistry of sympathetic nerve fibres in the colon
Colon pieces from normal and colitic animals were washed and immediately fixed after sacrifice in 4% formalin for 12 hours. Tissue was then washed in phosphate buffered saline (PBS) and incubated in 20% sucrose in PBS for another 12 hours. Thereafter, tissue was embedded in protective freezing medium (Tissue-Tek; Sakura Finetek Europe, Zoeterwoude, the Netherlands) and quick frozen floating on liquid nitrogen for nerve fibre staining or embedded in paraffin for standard haematoxylin-eosin staining. Cryosections were immunostained with primary antibodies against tyrosine hydroxylase (Chemicon, Temecula, California, USA), the key enzyme for NE production in sympathetic nerve endings, according to a protocol described previously. An Alexa 546 conjugated secondary goat antirat antibody (Molecular Probes, Leiden, the Netherlands) was used to achieve immunofluorescent staining. Under control conditions, the respective isotype was used in the above mentioned protocol.
Statistical analysis
All data are given as mean (SEM). In order to compare entire curves over time, the general linear model procedure was used (SPSS for Windows V11.5.1; SPSS Inc., Chicago, Illinois, USA). The unpaired t test (SPSS) was used to compare group means. A p value of <0.05 was considered significant.

RESULTS
Allocation of cellular IL-6 to cells in the intestinal wall
In order to visualise IL-6 secreting cells, a pair of consecutive cryosections were immunohistochemically stained for either IL-6 or Mac-3. IL-6 immunohistochemistry stained large cells in and close to the muscularis mucosa and also between crypts (fig 1A). In the consecutive cryosection, Mac-3 positive cells matched to the area of IL-6 staining (compare fig 1B with fig 1A).

To further investigate the role of macrophages for IL-6 secretion, experiments with antibodies conjugated to the ribosome inactivating protein SAP were carried out. In experiments without rat IgG preloading, administration of anti-Mac-1 IgG-SAP, or rat IgG-SAP demonstrated marked inhibition of IL-6 secretion in relation to administration of anti-Mac-1 antibodies alone (similar to the control curve without any substance; not shown, fig 1B left panel). This indicates that administration of saporin conjugated antibodies eliminates approximately 66% of IL-6 secreting cells. However, the effect seemed to be unspecific because rat IgG-SAP, which does not specifically bind to macrophages, also showed a similar effect (fig 1B left panel). In order to avoid the unspecific effect, mice were pretreated with a high dose of rat IgG intraperitoneally for five days prior to sacrifice. Preloading with rat IgG avoided the unspecific effect with rat IgG-SAP (fig 1B right panel). Both experiments indicate that administration of saporin conjugated anti-Mac-1 IgG-SAP, or rat IgG-SAP demonstrated marked inhibition of IL-6 secretion in relation to administration of anti-Mac-1 antibodies alone (similar to the control curve without any substance; not shown, fig 1B left panel). This indicates that administration of saporin conjugated antibodies eliminates approximately 66% of IL-6 secreting cells.

Role of TNF for colonic IL-6 secretion and influence of endogenous βAR pathways
Experiments with anti-TNF antibodies demonstrated an increase in IL-6 secretion from colonic strips (fig 2A). This
was surprising because TNF normally stimulates downstream IL-6 secretion and thus we expected a decrease in IL-6 secretion, as demonstrated previously in murine spleen slices. In animal with chronic colitis, anti-TNF antibodies did not modulate spontaneous IL-6 secretion (fig 2C). In order to study the influence of the βAR pathway, the βAR antagonist nadolol was used. Nadolol completely inhibited the increase in colonic IL-6 secretion in the presence of anti-TNF (fig 2B). This indicates that the increase in IL-6 secretion in the absence of TNF is mediated by endogenous NE via βAR. However, in the absence of anti-TNF, nadolol increased IL-6 secretion (fig 2B), which indicates that under these conditions IL-6 is inhibited via βAR pathways. These effects were not observed in animals with chronic colitis (fig 2D).

To corroborate these findings, the influence of NE on colonic IL-6 secretion was investigated. NE dose dependently inhibited colonic IL-6 secretion in the absence of anti-TNF (fig 3A), which was not observed in chronic colitic mice (fig 3A). As NE at high concentrations (it then binds with high affinity to βAR) demonstrated the strongest inhibition of IL-6 secretion under these conditions, we assumed a role for βAR. Indeed, in the presence of anti-TNF, the βAR agonist isoproterenol dose dependently increased IL-6 secretion (fig 3B). In chronic colitic animals, this effect was markedly reduced (fig 3B). This indicates a switch in the influence of βAR in the absence and presence of TNF in normal but not in colitic mice. Furthermore, these experiments also suggested an inhibitory influence of TNF on NE secretion.
NE secretion under the influence of TNF and electrical stimulation

Superfusion experiments in the presence of anti-TNF demonstrated a dose dependent increase in NE secretion from colonic strips in normal mice (fig 3C) but not in colitic mice (fig 3C), which indicates that TNF inhibits preterminal NE release in normal mice. The constant increase in NE under control conditions most likely represents spontaneous NE release from nerve terminals or NE producing cells (fig 3C).

In the above experiments, we demonstrated the influence of exogenous β-adrenergic agonists and antagonists. In order to study the influence of endogenously released NE, colon strips were electrically stimulated in order to release NE. NE was secreted on electrical stimulation in normal mice only and not in colitic mice (fig 3D). Interestingly, the level of NE secretion was somewhat increased in colitic mice but electrical stimulation did not change the outflow (fig 3D).

Sympathetic innervation of the colon in normal and colitic mice

Differences in the presence and absence of sympathetic innervation are quite apparent under control conditions in normal and colitic mice. Sympathetic innervation was not detectable in colon tissue from colitic mice because the latter animals have lost sympathetic innervation as a result of chronic inflammation (similar to the infected spleen, see Straub and colleagues24). In the colon, sympathetic innervation is not colitis specific because it has been demonstrated in diabetic rats.37 In the spleen of NMRI and BALB/c mice, conditions in the spleen.26 As demonstrated here in normal mice, the presence of anti-TNF strategy can have large effects in the colon but to inhibition of IL-6 in the spleen (spleen data reviewed by Straub24). In the colon, sympathetic regulation of TNF is contrary to regulation of other stimuli constantly stimulate TNF secretion. This is different from the spleen because the role of TNF on downstream IL-6 secretion is much smaller under sterile conditions in the spleen.26 As demonstrated here in normal colon, and in earlier studies,19 elevated TNF in the colon inhibits sympathetic NE release which probably leads to upregulation of BAR pathways under normal conditions (sensitisation). After TNF neutralisation, increased release of endogenous NE now stimulates IL-6 secretion via the sensitised BAR. This only happens in normal but not in colitic mice because the latter animals have lost the sympathetic innervation in the inflamed tissue. Loss of sympathetic innervation is not colitis specific because it has also been demonstrated in rheumatoid arthritis25 and insulinitis in diabetic rats.27 In the spleen of NMRI and BALB/c mice, TNF neutralisation inhibits IL-6 secretion which is completely independent of BAR.26 Thus in the spleen, sensitisation of the BAR pathway does not exist.

DISCUSSION

This study in the colon of mice demonstrates the opposing BAR effects of endogenous NE on IL-6 secretion in the absence and presence of TNF in normal but not in colitic mice. Sympathetic regulation of TNF is contrary to regulation of other stimuli constantly stimulate TNF secretion. This is different from the spleen because the role of TNF on downstream IL-6 secretion is much smaller under sterile conditions in the spleen.26 As demonstrated here in normal colon, and in earlier studies,19 elevated TNF in the colon inhibits sympathetic NE release which probably leads to upregulation of BAR pathways under normal conditions (sensitisation). After TNF neutralisation, increased release of endogenous NE now stimulates IL-6 secretion via the sensitised BAR. This only happens in normal but not in colitic mice because the latter animals have lost the sympathetic innervation in the inflamed tissue. Loss of sympathetic innervation is not colitis specific because it has also been demonstrated in rheumatoid arthritis25 and insulinitis in diabetic rats.27 In the spleen of NMRI and BALB/c mice, TNF neutralisation inhibits IL-6 secretion which is completely independent of BAR.26 Thus in the spleen, sensitisation of the BAR pathway does not exist.

From our point of view, this has an important implication for TNF neutralising strategies because anti-TNF would lead to an increase in IL-6 in the colon but to inhibition of IL-6 in the spleen. Thus anti-TNF strategies can have largely different effects depending on the micromilieu in a certain...
tissue. Immune stimuli together with the sympathetic microenvironment can thus alter the influence of cytokine neutralising strategies. We do not know if this alteration leads to an unfavourable outcome in patients with colonic inflammation, such as ulcerative colitis. Several uncontrolled studies have indicated that anti-TNF strategies are not impressively effective in ulcerative colitis. Whether or not studies have indicated that anti-TNF strategies are not inflammatory, such as ulcerative colitis. Several uncontrolled neutralising strategies. We do not know if this alteration tissue. Immune stimuli together with the sympathetic Norepinephrine modulates mucosal TNF and IL-6

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Similar contextual phenomena probably occur with other even elicit a proinflammatory milieu. The reactions described effects, anti-TNF strategies can be favourable or unfavourable Depending on the balance of pro- and anti-inflammatory situation which depends on local noradrenergic tone. anti-TNF strategies may even cause a proinflammatory inflammatory pathways. This study demonstrates that anti-TNF strategies may lead to an increase in concentration of the proinflammatory cytokine IL-6 in the normal colon. Thus anti-TNF strategies may even cause a proinflammatory situation which depends on local noradrenergic tone. Depending on the balance of pro- and anti-inflammatory effects, anti-TNF strategies can be favourable or unfavourable in a given disease. This study provides clues as to whether or not, under certain circumstances, anti-TNF strategies can even elicit a proinflammatory milieu. The reactions described herein largely depend on the micromilieu in a specific tissue. Similar contextual phenomena often occur with other neutralising drugs. This study may help in understanding tissue specific modulation of cytokine secretion which depends on neuronal innervation.

ACKNOWLEDGEMENTS

This work was supported by the DFG (Deutsche Forschungsgemeinschaft: Sonderforschungsbereich 585, B8) and by the institution. We thank Nicole Steigerwald and Melanie Grünebeck for excellent technical assistance.

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Conflict of interest: None declared.

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EDITOR’S QUIZ: GI SNAPSHOT

An unusual case of biliary pain

Clinical presentation
A 62 year old woman presented with a one month history of increasing postprandial epigastric pain radiating through to her back. Her medical history included endoscopic sphincterotomy of the papilla with laparoscopic cholecystectomy two years earlier for biliary lithiasis. Her clinical evaluation was normal. White cell count was 14600/mm³, and liver aminotransferases, serum lipase, and amylase were all normal. A stress test and echocardiography were normal. No abnormalities were found at gastroscopy, on abdominal ultrasound, or after tomography. An endoscopic ultrasound was performed (fig 1). What does it show?

Question
What is the differential diagnosis?
See page 1145 for answer
This case is submitted by:

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doi: 10.1136/gut.2004.059659

Figure 1 Endoscopic ultrasound.