Early Bacterial Dependent Induction of Inducible Nitric Oxide Synthase (iNOS) in Epithelial Cells upon Transfer of CD45RB^{high} CD4^{+} T Cells in a Model for Experimental Colitis

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Background: Both the role of inducible nitric oxide synthase (iNOS) in the development of inflammatory bowel disease (IBD) as well as the molecular details governing its mucosal induction remain unclear.

Methods: In the present study we evaluated the role of the residing intestinal microflora in the induction of epithelial iNOS upon transfer of CD45RB^{high} CD4^{+} T cells to SCID mice. CB-17 SCID mice were reared with conventional flora (CNV) or germfree CB-17 SCID mice were monoassociated with *Helicobacter muridarum, act A(−)* mutant *Listeria monocytogenes*, segmented filamentous bacteria (SFB), or *Ochrobactrum anthropi*.

Results: Within 2 weeks CNV SCID mice injected with CD45RB^{high} CD4^{+} T cells showed a focal, epithelial iNOS expression on the apical site of villi that preceded the infiltration of CD4^{+} T cells and cytokine production followed by extension of this expression to the entire surface along the whole crypt axis as the colitis progressed. SCID mice monoassociated with *H. muridarum* developed a severe colitis and showed high epithelial iNOS expression. CNV-SCID mice without T cells and SCID mice monoassociated with SFB did not show any iNOS expression, whereas SCID mice monoassociated with *act A(−)* mutant *L. monocytogenes* and *O. anthropi* showed some scattered epithelial iNOS staining on the apical site of a few villi, but none of these mice developed colitis.

Conclusions: These findings demonstrate that the expression of epithelial iNOS is highly bacterium-specific and correlates with the severity of disease, suggesting an important role for this enzyme in the development of IBD.

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Key Words: IBD, iNOS, SCID, CD45RB^{high} CD4^{+} T cells, microflora

The inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are chronic inflammatory conditions of the human gastrointestinal tract. Studies in animal models suggest that IBD is due to an aberrant mucosal T-cell response to gut bacteria. Several animal models have shown that both T helper cells and bacterial flora are needed to induce disease.[1] The bacterial species and lymphoid interactions involved, however, remain subject to intensive discussion. In a monoassociation study we showed that out of 5 nonpathogenic bacteria, only *Helicobacter muridarum* was capable of provoking an accelerated colitis when compared to conventionally reared (CNV) mice after transfer of CD45RB^{high} CD4^{+} T cells to severe combined immunodeficient (SCID) mice.[2] A striking feature of *H. muridarum* in comparison to many other bacterial species is that its ecological niche is in close contact with the epithelial cells of the crypts.[3] Therefore, we hypothesized that epithelial cell activation by *H. muridarum* might be an important trigger in the development of colitis in *H. muridarum*-associated mice.

In patients with active IBD, there is a strong expression of inducible nitric oxide synthase (iNOS) at the apical side of epithelial cells.[4] The induction of iNOS is mediated by the nuclear factor κB (NF-κB) and it is known that some bacteria can directly activate NF-κB, whereas other bacteria silence

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TABLE 1. Primer Pairs for RT-PCR

	Sense	Antisense	Bp
iNOS	5′-ggtatgctgtgtttggcctt-3′	5′-ggctggacttttcactctgc-3′	390
TNF-α	5′-ctcttcaagggacaaggctg-3′	5′-cggactccgcaaagtctaag-3′	253
IL-1β	5′-caggcaggcagtatcactca-3′	5′-aggccacaggtattttgtcg-3′	350
IFN-γ	5′-actggcaaaaggatggtgac-3′	5′-tgagctcattgaatgcttgg-3′	237
Gro	5′-gctgggattcacctcaagaa-3′	5′-tggggacacctttagcatc-3′	169
MMIP	5′-agtgaactgcgctgtcaatg-3′	5′-ttcagggtcaaggcaaactt-3′	153
β-actin	5′-cctaaggccaaccgtgaaaag-3′	5′-tcttcatggtgctaggagcca-3′	672

the epithelial NF-κB response to avoid epithelial cell activation.[5] It is at present not known whether this induction is an important causative factor for the disease nor is it clear whether bacterial colonization per se is associated with epithelial iNOS expression or whether the induction of iNOS is associated with specific bacterial types.

These considerations prompted us to compare the induction of iNOS after colonization of the mouse intestine by different microflora. To this end we investigated whether epithelial iNOS expression and the development of colitis in CNV-reared and monoassociated SCID mice after $CD45RB^{high}$ $CD4^+$ T-cell transfer and generated a kinetic study relating epithelial iNOS expression to T-cell infiltration and proinflammatory cytokine production in CNV and monoassociated SCID mice. We demonstrate an early focal expression of iNOS in epithelial cells preceding the T-cell infiltration and proinflammatory cytokine production in CNV SCID mice after transfer of naïve T cells. At the terminal stages of colitis both CNV SCID mice as well as *H. muridarum* monoassociated SCID mice showed strong iNOS expression along the whole crypt axis. In contrast, there was no or very low expression of iNOS in epithelial cells in SCID mice monoassociated with bacteria that did not induce colitis upon transfer of naïve T cells. These results demonstrate that bacteria-specific epithelial cell activation and subsequent iNOS induction and NO production are early events in the development of colitis in the $CD45RB^{high}$ $CD4^+$ T-cell transfer model of IBD.

MATERIALS AND METHODS

Animal Model

We used the $CDRB45^{high}$ $CD4^+$ T-cell transfer model in SCID mice as described earlier.[2] Briefly, C.B-17 SCID mice were reared under conventional (CNV) or monoassociated conditions. Three weeks after stable colonization of germfree C.B17 SCID mice ($>10^{10}$ bacteria/g feces) with Segmented Filamentous Bacterium, an avirulent actA (−) mutant DP-L1942 of *Listeria monocytogenes*, *Ochrobactrum anthropi*, or *H. muridarum* 5–10 $\times$ 10^5 $CDRB45^{high}$ $CD4^+$ T cells were injected intraperitoneally into the monoassociated SCID mice. Of these bacterial strains only *H. muridarum* is able to induce a severe colitis upon transfer of T cells, as discussed by Jiang et al.[2] For the kinetic study of epithelial iNOS expression in CNV-reared SCID mice 2–3 mice were sacrificed 1, 2, 3, 4, 5, 6, 7, 8, 10, and 13 weeks after T-cell transfer. Monoassociated animals were sacrificed at 11 weeks (SFB, *L. monocytogenes* and *O. anthropi*) after T-cell transfer, except for mice that were monoassociated by *H. muridarum* that were sacrificed at 6 weeks because of the development of severe colitis.[2] Normal BALB/c, conventionally and germfree-reared SCID mice that did not receive T cells were used as controls. All experiments were approved by the animal welfare board.

Histology

Specimens of the large intestine were embedded in OCT compound (Miles, Elkhart, IN) and frozen in 2-methylbutane with dry ice. Longitudinal sections (4 μM) were fixed with 4% paraformaldehyde for immunohistochemistry and 10% formalin for silver staining followed by rinsing in phosphate-buffered saline (PBS), and staining with hematoxylin-eosin.

Immunohistochemistry

For immunohistochemistry, 7-μm cryostat sections were cut, dried, and fixed in acetone for 10 minutes at room temperature. For iNOS detection a rabbit polyclonal antibody developed in our laboratory was used.[6] For CD4 detection a monoclonal biotin-conjugated antibody clone H129.19 (Pharmingen, San Diego, CA) was used. Slides were incubated with the polyclonal iNOS antibody (1:300) or monoclonal CD4 antibody (1:50) in PBS containing 1% bovine serum albumin (BSA) for 60 minutes at room temperature. Subsequently, endogenous peroxidase activity was blocked by incubating for 30 minutes in PBS containing 0.075% H_2O_2. For iNOS detection, peroxidase-conjugated goat antirabbit Ig (1:50) and peroxidase-conjugated rabbit antigoat Ig (1:50), all from Dako (Glostrup, Denmark), were used as secondary and

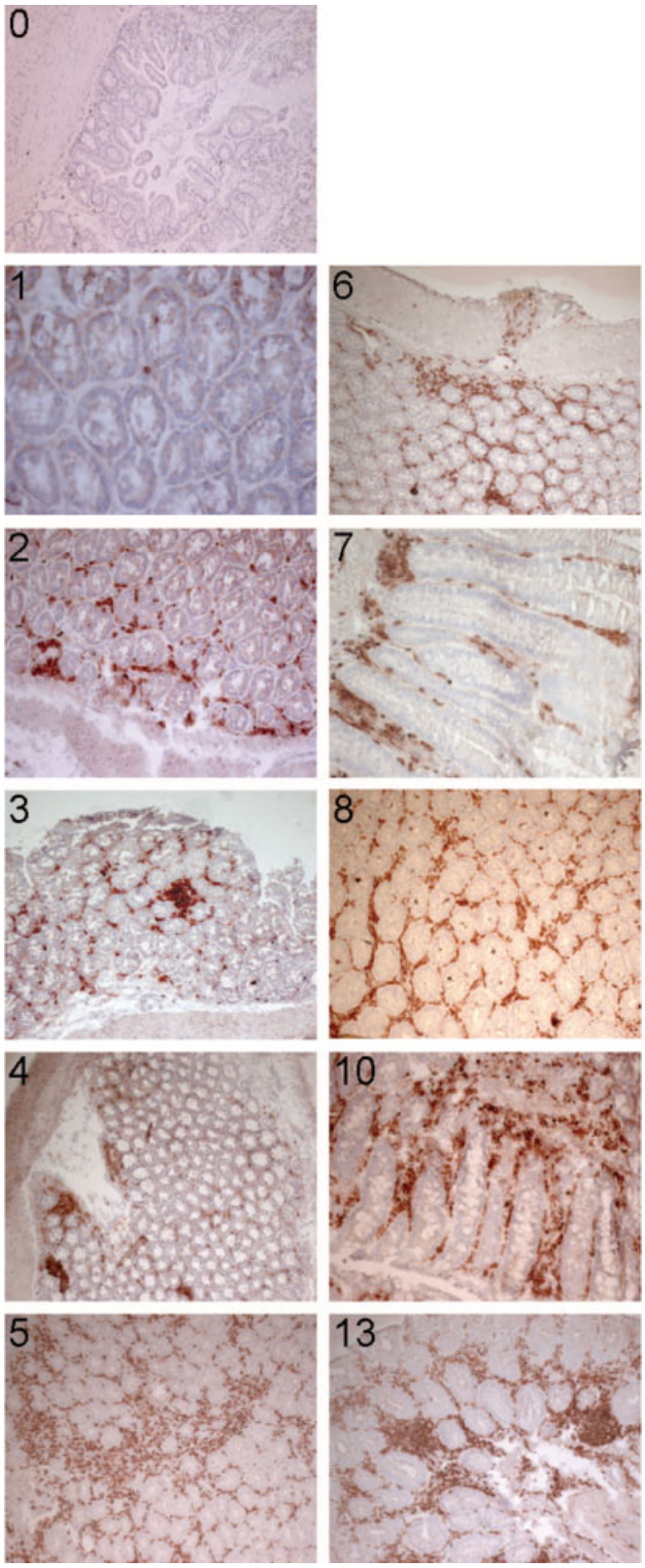


FIGURE 1. Immunohistochemistry of the colon for CD4 at 1, 2, 3, 4, 5, 6, 7, 8, 10, and 13 weeks after transfer of CD45RB^{high} CD4^{+} T cells into SCID mice reared with conventional flora. For comparison a control CNV-SCID mouse without T cells is also shown (SCID) (number-0). CD4^{+} T cells are first present near the muscularis propria (right panel) and expand into the lamina propria after 4 weeks. After 8 weeks the lamina propria is filled with conglomerates of CD4^{+} T cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

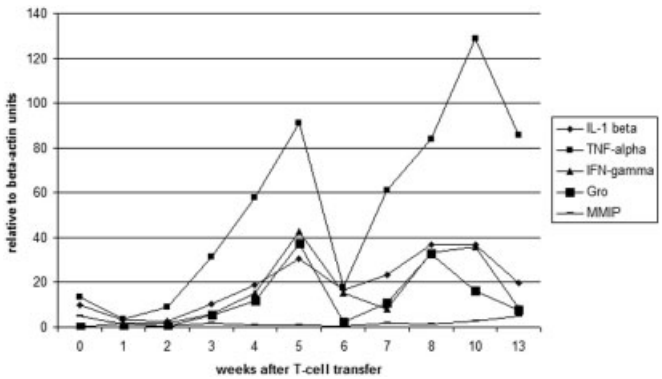


FIGURE 2. RT-PCR for mRNA of IL-1β, TNF-α, IFN-γ, and the chemokines Gro and mMIP. The mRNA levels of the proinflammatory cytokines IL-1β, TNF-α, and INF-γ start to increase 3 weeks after T-cell transfer and showed a biphasic response with high peaks around 5 and 8 weeks. The chemokine Gro showed the same pattern, whereas another chemokine, mMIP, increased only in the terminal phase of colitis. Numbers on the x-axis represent the weeks after CD4^{+} T-cells transfer to SCID mice.

tertiary antibodies. For CD4 detection streptavidin-peroxidase (Southern Biotechnology, Birmingham, AL) was used as a secondary step. Color was developed using 3-amino-9-ethylcarbazole (10 mg / 2.5 mL dimethylformamide in 50 mL 0.05 mol/L acetate buffer, pH 4.9) containing 0.03% H_2O_2 for 10 minutes at room temperature. Counterstaining was performed with hematoxylin and the slides were covered with Kaiser's glycerin-gelatin. Silver staining was used to show the presence of *H. muridarum* inside the colon according to Scanziani et al.[7]

RNA Isolation and Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated from tissue specimens using Trizol reagent (Sigma-Aldrich, Zwijndrecht, Netherlands) according to the manufacturer's instructions. RT was performed on 5 μg of total RNA using Oligo-dT primers (Invitrogen, Breda, Netherlands) in a final volume of 30 μL. PCR on cDNA was performed with Taq polymerase (Invitrogen) on the Biometra PCR system. The PCR primers for mice iNOS, TNF-α, IL-1β, IFN-γ, Gro, mMIP, and β-actin were selected from multiple exons and are depicted in Table 1. The cycling

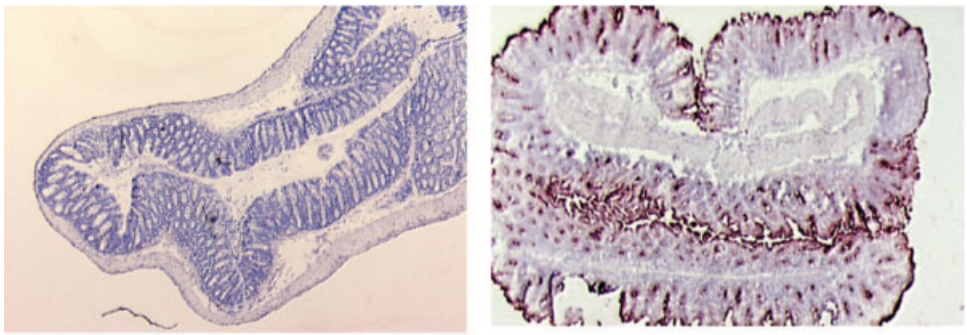


FIGURE 3. Immunohistochemistry of the colon for iNOS without (left panel) and 8 weeks after (right panel) transfer of CD45RB^{high} CD4^{+}T cells into SCID mice reared with conventional flora (CNV). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

program was 94°C for 2 minutes, 58°C for 60 seconds, 72°C for 60 seconds for the first cycle, and 94°C for 30 seconds, 58°C for 60 seconds, 72°C for 60 seconds for 30 cycles. The level of mRNA expression is expressed relative to the β-actin level.

Statistical Analysis

Significant differences between mean values were determined by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Kinetics of T-cell Infiltration, Cytokine Production, and Epithelial iNOS Induction in CNV-reared SCID Mice After CD45RB^{high} CD4^{+} T-cell Transfer

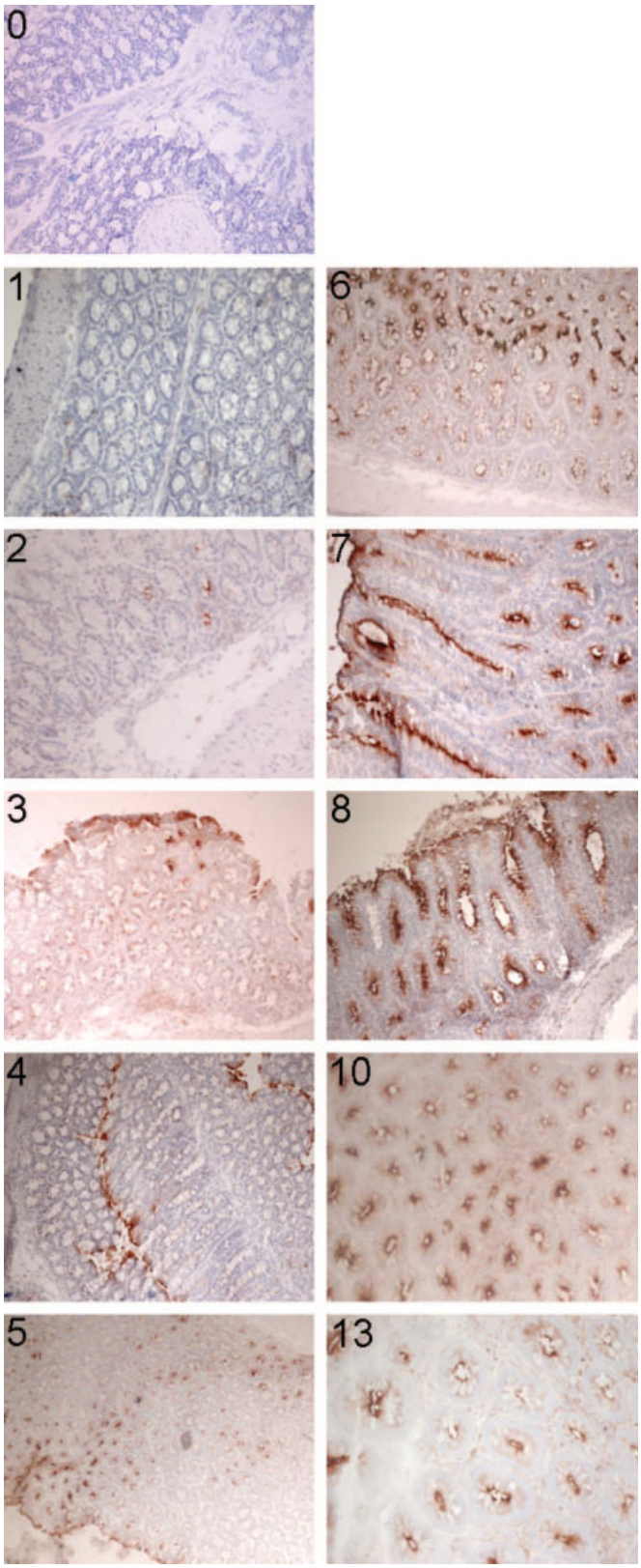
T-cell transfer in SCID mice is generally considered a rodent model for IBD, mimicking important aspects of the disease. No CD4^{+} T cells were observed in the lamina propria of CNV SCID mice. Upon T-cell transfer, CNV SCID mice show small aggregates of CD4^{+} T cells near the muscularis propria after 1–2 weeks (Fig. 1). After 4 weeks the cells are no longer present near the muscularis but now small aggregates are present in the lamina propria, sometimes close to the epithelial cells. At 8 weeks, substantially more and larger lamina propria conglomerates of CD4^{+} T cells are observed. The end-stage of colitis is characterized by a lamina propria completely filled with conglomerates of CD4^{+} T cells. Thus, the histology observed in this model is consistent with an IBD-like inflammatory reaction in the colon.

This notion is supported by RT-PCR experiments for the proinflammatory cytokines IL-1β, TNF-α, IFN-γ, and the chemotactic cytokines Gro and mMIP. In these experiments we observe equal levels of TNF-α and IFN-γ in BALB/c and SCID mice that did not receive CD4^{+} T cells. IL-1β levels are reduced in untreated SCID mice compared to BALB/c mice (data not shown). The mRNA of the proinflammatory cytokines IL-1β, TNF-α, and INF-γ are significantly raised from 4 weeks on after T-cell transfer ($P < 0.05$, weeks 4–13 compared to control and weeks 1–2) and shows a biphasic response with high peaks around 5 and 8–10 weeks (Fig. 2). The chemotactic cytokine Gro shows the same pattern, whereas another chemokine, mMIP, shows only an increase in the terminal phase of colitis (Fig. 2). Subsequently, experiments were initiated to investigate the expression of iNOS during such inflammation.

Despite a critical function of iNOS in mucosal immune responses, the relationship between its expression and the composition of the gut flora is poorly understood. CNV SCID mice do not show any iNOS expression in their colon tissue (Figs. 3, 4). CNV SCID mice show focal epithelial iNOS 2 weeks after T-cell transfer (Fig. 4). The iNOS expression is on the apical site of the enterocytes on the top of the villi and not in the crypt epithelial cells. In weeks 3 and 4 after T-cell transfer larger areas of epithelial cells show iNOS expression but it is still confined to the top of the villi. From week 5 on crypt epithelial cells also show iNOS expression (Fig. 4). At week 8 the intensity of the iNOS expression is further increased and involves almost the whole epithelial surface (Fig. 3, right panel, Fig. 4). At the end-stage of colitis the whole epithelial surface shows a diffuse but intense iNOS expression along the whole crypt axis. Inflammatory cells show no iNOS expression. In control CNV SCID mice we did observe some iNOS signal that we did not observe in our samples at weeks 1 and 2 after T-cell transfer; RT-PCR analysis (Fig. 5) is in general in line with the immunohistochemistry results from week 3 on, although the RT-PCR for iNOS became positive shortly after the focal epithelial staining that was seen at 2 weeks. At the end-stage of the colitis we did not observe a positive signal by RT-PCR, while we still see clear staining of the iNOS protein. This lack of iNOS mRNA could be caused by the severe damage of the epithelial cells, while the iNOS protein was still present. In general, the inflammatory reaction in the colon following T-cell transfer correlates with the induction of epithelial iNOS expression. Thus, expression of iNOS seems to correlate with the severity of the mucosal inflammatory reaction.

Epithelial iNOS Induction in Monoassociated SCID Mice After CD45RB^{high} CD4^{+} T-cell Transfer

Subsequently, we investigated different flora components for their effect on iNOS expression in IBD. As shown



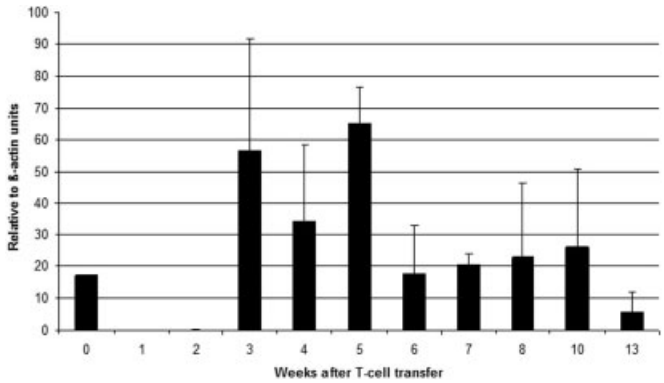


FIGURE 5. RT-PCR for iNOS (mRNA expression relative to expression of β-actin) of the colon from 2–3 CNV reared SCID mice colon samples at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 13 weeks after transfer of $CD45RB^{high}$ $CD4^{+}$ T cells. The iNOS mRNA is detectable as early as 3 weeks after T-cell transfer. Also, some iNOS expression is observed in the control CNV-SCID mice, although at weeks 1 and 2 after T-cell transfer we did not observe iNOS expression.

before, mice monoassociated with *Act A(−) L. monocytogenes, O. anthropi,* and SFB do not develop colitis, whereas mice monoassociated with *H. muridarum* develop a severe colitis already at 6 weeks after T cell transfer.[6] *Helicobacter muridarum* is closely associated with the colon epithelium within the crypts, as shown by silver staining (Fig. 6).

Mice monoassociated with SFB do not show any iNOS expression 11 weeks after T-cell transfer (Fig. 7). Mice monoassociated with *Act A(−) L. monocytogenes* and *O. anthropi* show very focal iNOS staining 11 weeks after T-cell transfer. The iNOS staining is confined to the apical site of the enterocyte and is only present in enterocytes on the top of a few villi (Fig. 7). However, 6 weeks after T-cell transfer, concomitant with clear clinical symptoms of IBD in mice monoassociated with *H. muridarum*[6] an intense and diffuse iNOS staining of enterocytes along the whole crypt axis is observed (Fig. 7). Hence, specific flora components are markedly different with respect to inducing iNOS expression and the extent of the iNOS expression correlates with the severity of the mucosal inflammation.

DISCUSSION

In this study we show that epithelial cells of CNV-reared SCID mice express iNOS early in the development of

FIGURE 4. Immunohistochemistry of the colon for iNOS at 1, 2, 3, 4, 5, 6, 7, 8, 10, and 13 weeks after transfer of $CD45RB^{high}$ $CD4^{+}$ T cells into SCID mice reared with conventional flora (CNV). For comparison a control CNV-SCID mouse without T cells is also shown (SCID) (number-0). The epithelial cells express iNOS 2 weeks after T-cell transfer. In the first weeks this expression is focal and confined to top of the crypts, in the weeks thereafter the iNOS expression is diffuse and also present in epithelial cells in the crypts. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

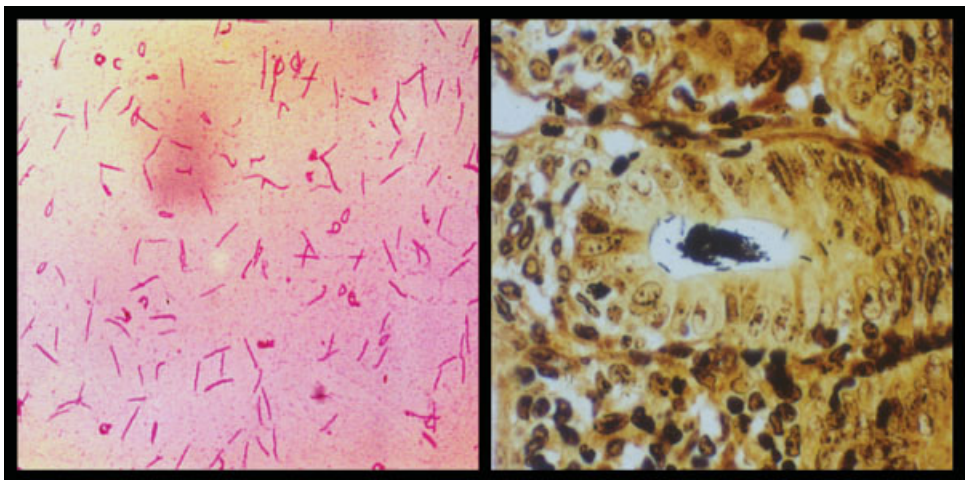


FIGURE 6. Silver staining of *H. muridarum* in close association with epithelial cells within the colon crypts in monoassociated SCID mice. The morphology of *H. muridarum* used for monoassociation is shown by Gram staining of cultured *H. muridarum*. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

colitis after CD45RB^{high} CD4^{+} T-cell transfer. The absence of epithelial iNOS expression in CNV-reared SCID mice that did not receive naïve T cells demonstrates that both the bacteria and T cells are needed for epithelial iNOS expression and the development of colitis. The monoassociation studies show that certain bacteria (e.g., SFB) do not induce iNOS and colitis, whereas other bacteria (e.g., *L. monocytogenes act A(−), O. anthropi*) induce a very low level of iNOS expression without causing full-blown colitis, whereas yet other bacteria (e.g., *H. muridarum*) give rise to strong epithelial iNOS induction and accelerated colitis as compared to CNV-reared SCID mice. Thus, iNOS expression in general correlates with the severity of mucosal inflammation.

The induction of iNOS is mediated by the nuclear transcription factor κB (NF-κB) in epithelial cells[8] and in other cells involved in colitis.[9] Our current data support this notion: iNOS expression correlates well with the severity of the colitis and the production of NF-κB-dependent cytokines. The observation that a combination of cytokines (IL-1β, IFN-γ, TNF-α) and endotoxin (LPS) is needed for the in vitro induction of iNOS in native colon cells and intestinal tumor cell lines may be interpreted as that epithelial cells of the

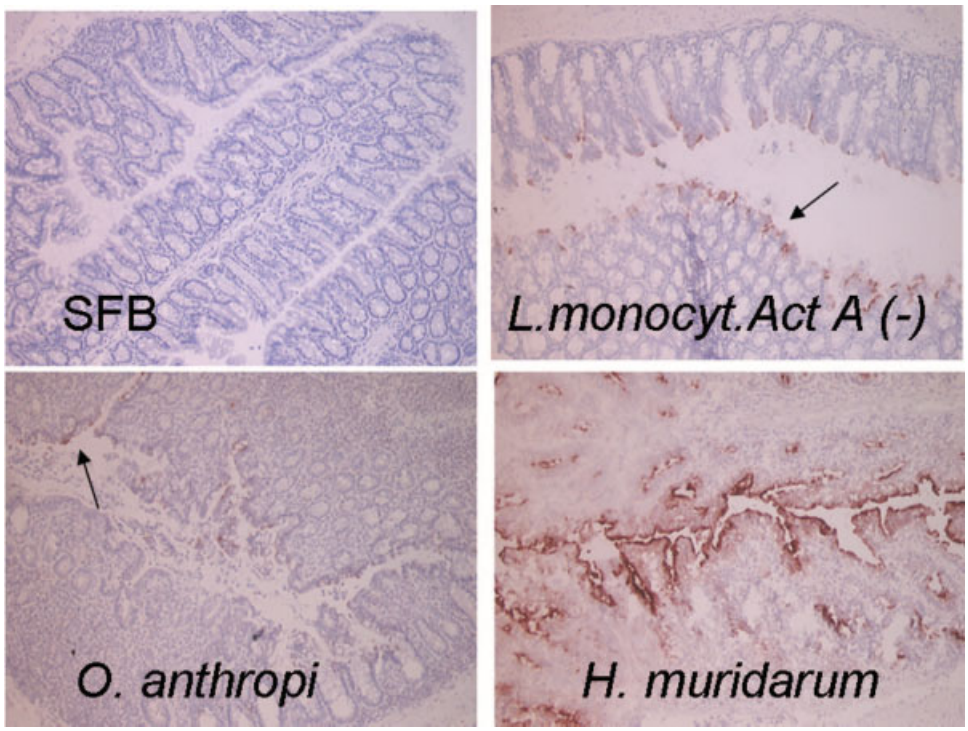


FIGURE 7. Immunohistochemistry of the colon for iNOS 11 weeks and 6 weeks (for *H.muridarum*) after transfer of CD45RB^{high} CD4^{+} T cells into SCID mice (monoassociated with SFB, *L. monocytogenes Act A (−), O. anthropi*, or *H. muridarum*). *SFB*-monoassociated mice did not induce iNOS expression 11 weeks after T-cell transfer. Mice monoassociated with *Act A(−) L. monocytogenes* and *O. anthropi* demonstrated a low level of very focal iNOS staining that was confined to the apical site of the enterocyte and was only present in enterocytes on the top of a few villi (arrows). However, mice monoassociated with *H. muridarum* at 6 weeks showed an intense and diffuse iNOS staining of enterocytes along the whole crypt axis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

colon are relatively resistant to cytokine-induced NF-κB activation.[10] More specifically, a decreased IκB kinase (IKK) activity and a consequent resistance to IκBα degradation has been postulated as a protective response of intestinal epithelial cells, enabling the cells to remain quiescent in the hostile strongly proinflammatory colon environment.[10] However, this relatively unresponsive state still allows several particularly pathogenic enteroinvasive organisms such as *Salmonella, Shigella, Listeria*, and *Helicobacter* species to directly activate NF-κB[5] and induce iNOS and proinflammatory cytokines in intestinal epithelial cells.[11] This may be an important defense response as the bacterial pathogenic genus *Yersinia* has developed delivery of virulence Yop factors capable of blocking the NF-κB-mediated production of proinflammatory cytokines, thus preventing an antibacterial epithelial cell response.[12] It will be of interest to investigate whether reduced iNOS expression as a consequence of impaired NF-κB activation in *Yersinia*-infected cells is important in this reduced antibacterial epithelial cell response.

Apart from pathogenic bacteria, there is also evidence that nonpathogenic bacteria in the normal gut can prevent epithelial NF-κB induction and hence contribute to the reduction of the mucosal immune response against normal gut bacteria. Therefore, the capability of bacteria to interfere with the epithelial NF-κB response might reflect its potential to induce or suppress IBD. Studies aimed at inhibition of NF-κB activation in IBD are promising[13,14] and the correlation observed in the present study between iNOS expression and the severity of the colitis calls for studies in which the importance of iNOS expression for mucosal inflammatory reaction in IBD is assessed directly. However, as shown by a conditional epithelial NF-κB knockout animal model, an adequate epithelial NF-κB response is also an important antiapoptotic response necessary for epithelial healing and repair; thus, strategies directly aimed at inhibiting iNOS downstream of its induction by NF-κB may be more promising as future therapeutic avenues for dealing with IBD.[15]

In this context it is important to keep in mind that although we observe a clear correlation between the epithelial iNOS response and the development of colitis, the exact role of iNOS and epithelial derived NO in the development of IBD is not known.[16] In relation to this, an increase in iNOS expression in colonic samples has been observed in some animal models.[17] However, studies using inhibitors of NOS in experimental colitis are conflicting and show little improvement,[18,19] no effects,[20,21] or even worse effects[22] on colitis probably due to the lack of iNOS specificity (i.e., also inhibition of endothelial NOS) of the inhibitors used. In addition, studies of experimental colitis in iNOS knockout mice also showed conflicting results even when the same experimental model was used.[23–29] IL-10 knockout mice develop colitis spontaneously. Colitis developed at the same rate and intensity in IL-10 knockout mice and IL-10/iNOS double knockout mice.[30] Considering the absence of macroscopic ulcerations in the presence of large amounts of NO in patients suffering from microscopic colitis, a role of NO in diarrhea and ulcer healing has been suggested.[31] Indeed, topical administration of the NOS inhibitor N^g-monomethyl-L-arginine (L-NMMA) reduced fluid secretion in patients with collagenous colitis[32] and an NO-donating mesalazine derivative had an additional beneficial effect on TNBS-induced colitis.[33] The reduced gastrointestinal toxicity of NO-donating nonsteroidal antiinflammatory drugs (NSAIDs)[34] and aspirin[35] are in agreement with a protective effect of NO on intestinal epithelial cells. Apart from the above-mentioned beneficial effects of NO in mucosal injury, NO can also inhibit NF-κB activation.[36] Therefore, high amounts of NO may participate in a negative feedback loop to block prolonged activation of NF-κB, thereby limiting chronic inflammation, in which case strategies aimed at inhibiting NO production may be self-defeating. In this context it must be kept in mind that NO itself is not toxic to many bacteria, as certain enteric bacteria contain nitrate reductase and produce NO on their own.[37] Importantly, however, as observed in septic patients, epithelial iNOS induction and NO production may cause increased intestinal permeability.[38] Indeed, selective inhibition of iNOS in endotoxemic rats ameliorated mucosal permeability for dextran (MW 4000)[39] and reduced bacterial translocation.[40] The absence of bacterial translocation in endotoxemic iNOS knockout mice further supports a pathogenic role of epithelial derived NO in sepsis. As long as there are no truly selective iNOS inhibitors available it will be hard to examine the exact role of epithelial iNOS induction and NO production in IBD. In this study, however, we provide evidence that epithelial iNOS expression is an early and bacteria-dependent event in the development of colitis in the $CD45RB^{high}$ $CD4^+$ T-cell transfer model of IBD and that bacteria that cause colitis also induce high epithelial iNOS expression.

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REFERENCES

1. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol.* 2002;20:495–549.
2. Jiang HQ, Kushnir N, Thurnheer MC, et al. Monoassociation of SCID mice with Helicobacter muridarum, but not four other enterics, provokes IBD upon receipt of T cells. *Gastroenterology.* 2002;122:1346–1354.
3. Phillips MW, Lee A. Isolation and characterization of a spiral bacterium from the crypts of rodent gastrointestinal tracts. *Appl Environ Microbiol.* 1983;45:675–683.
4. Dijkstra G, Moshage H, van Dullemen HM, et al. Expression of nitric oxide synthases and formation of nitrotyrosine and reactive oxygen species in inflammatory bowel disease. *J Pathol.* 1998;186:416–421.
5. Naumann M. Nuclear factor-kappa B activation and innate immune response in microbial pathogen infection. *Biochem Pharmacol.* 2000; 60:1109–1114.

6. Vos TA, Gouw AS, Klok PA, et al. Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. *Gastroenterology.* 1997;113:1323–1333.
7. Scanziani E, Simpson KW, Monestiroli S, et al. Histological and immunohistochemical detection of different Helicobacter species in the gastric mucosa of cats. *J Vet Diagn Invest.* 2001;13:3–12.
8. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem.* 1994; 269:4705–4708.
9. Comalada M, Camuesco D, Sierra S, et al. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. *Eur J Immunol.* 2005;35:584–592.
10. Jobin C, Haskill S, Mayer L, et al. Evidence for altered regulation of I kappa B alpha degradation in human colonic epithelial cells. *J Immunol.* 1997;158:226–234.
11. Witthoft T, Eckmann L, Kim JM, et al. Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. *Am J Physiol.* 1998;275:G564–G571.
12. Schesser K, Spiik AK, Dukuzumuremyi JM, et al. The yopJ locus is required for Yersinia-mediated inhibition of NF-kappaB activation and cytokine expression: YopJ contains a eukaryotic SH2-like domain that is essential for its repressive activity. *Mol Microbiol.* 1998;28:1067–1079.
13. Neurath MF, Pettersson S, Meyer Zum Buschenfelde KH, et al. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. *Nat Med.* 1996;2:998–1004.
14. Dijkstra G, Moshage H, Jansen PL. Blockade of NF-kappaB activation and donation of nitric oxide: new treatment options in inflammatory bowel disease? *Scand J Gastroenterol Suppl.* 2002;37–41.
15. Chen LW, Egan L, Li ZW, et al. The two faces of IKK and NF-kappaB inhibition: prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nat Med.* 2003;9:575–581.
16. Grisham MB, Pavlick KP, Laroux FS, et al. Nitric oxide and chronic gut inflammation: controversies in inflammatory bowel disease. *J Investig Med.* 2002;50:272–283.
17. Camuesco D, Comalada M, Rodriguez-Cabezas ME, et al. The intestinal anti-inflammatory effect of quercitrin is associated with an inhibition in iNOS expression. *Br J Pharmacol.* 2004;143:908–918.
18. Hogaboam CM, Jacobson K, Collins SM, et al. The selective beneficial effects of nitric oxide inhibition in experimental colitis. *Am J Physiol.* 1995;268:G673–684.
19. Rachmilewitz D, Karmeli F, Okon E, et al. Experimental colitis is ameliorated by inhibition of nitric oxide synthase activity. *Gut.* 1995; 37:247–255.
20. Ribbons KA, Currie MG, Connor JR, et al. The effect of inhibitors of inducible nitric oxide synthase on chronic colitis in the rhesus monkey. *J Pharmacol Exp Ther.* 1997;280:1008–1015.
21. Conner EM, Chen Y, Gronberg A. Effect of nitric oxide synthase (NOS) inhibition on dextran sulfate sodium (DSS)-induced colitis in rats and mice. Gastroenterology 1995;100:A801.
22. Pfeiffer CJ, Qiu BS. Effects of chronic nitric oxide synthase inhibition on TNB-induced colitis in rats. *J Pharm Pharmacol.* 1995;47:827–832.
23. McCafferty DM, Mudgett JS, Swain MG, et al. Inducible nitric oxide synthase plays a critical role in resolving intestinal inflammation. *Gastroenterology.* 1997;112:1022–1027.
24. Zingarelli B, Szabo C, Salzman AL. Reduced oxidative and nitrosative damage in murine experimental colitis in the absence of inducible nitric oxide synthase. *Gut.* 1999;45:199–209.
25. McCafferty DM, Miampamba M, Sihota E, et al. Role of inducible nitric oxide synthase in trinitrobenzene sulphonic acid induced colitis in mice. *Gut.* 1999;45:864–873.
26. Vallance BA, Dijkstra G, Qiu B, et al. Relative contributions of NOS isoforms during experimental colitis: endothelial-derived NOS maintains mucosal integrity. *Am J Physiol Gastrointest Liver Physiol.* 2004; 287:G865–G874.
27. Hokari R, Kato S, Matsuzaki K, et al. Reduced sensitivity of inducible nitric oxide synthase deficient mice to chronic colitis. *Free Radic Biol Med.* 2001;31:153–163.
28. Krieglstein CF, Cerwinka WH, Laroux FS, et al. Regulation of murine intestinal inflammation by reactive metabolites of oxygen and nitrogen: divergent roles of superoxide and nitric oxide. *J Exp Med.* 2001;194: 1207–1218.
29. Beck PL, Xavier R, Wong J, et al. Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. *Am J Physiol Gastrointest Liver Physiol.* 2004;286:G137–G147.
30. McCafferty DM, Sihota E, Muscara M, et al. Spontaneously developing chronic colitis in IL-10/iNOS double-deficient mice. *Am J Physiol Gastrointest Liver Physiol.* 2000;279:G90–G99.
31. Lundberg JO, Herulf M, Olesen M, et al. Increased nitric oxide production in collagenous and lymphocytic colitis. *Eur J Clin Invest.* 1997;27: 869–871.
32. Perner A, Andresen L, Normark M, et al. Expression of nitric oxide synthases and effects of L-arginine and L-NMMA on nitric oxide production and fluid transport in collagenous colitis. *Gut.* 2001;49:387–394.
33. Wallace JL, Vergnolle N, Muscara MN, et al. Enhanced anti-inflammatory effects of a nitric oxide-releasing derivative of mesalamine in rats. *Gastroenterology.* 1999;117:557–566.
34. Elliott SN, McKnight W, Cirino G, et al. A nitric oxide-releasing nonsteroidal anti-inflammatory drug accelerates gastric ulcer healing in rats. *Gastroenterology.* 1995;109:524–530.
35. Fiorucci S, Antonelli E, Santucci L, et al. Gastrointestinal safety of nitric oxide-derived aspirin is related to inhibition of ICE-like cysteine proteases in rats. *Gastroenterology.* 1999;116:1089–1106.
36. Matthews JR, Botting CH, Panico M, et al. Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic Acids Res.* 1996;24:2236–2242.
37. Brittain T, Blackmore R, Greenwood C, et al. Bacterial nitrite-reducing enzymes. *Eur J Biochem.* 1992;209:793–802.
38. Johnston JD, Harvey CJ, Menzies IS, et al. Gastrointestinal permeability and absorptive capacity in sepsis. *Crit Care Med.* 1996;24:1144–1149.
39. Unno N, Wang H, Menconi MJ, et al. Inhibition of inducible nitric oxide synthase ameliorates endotoxin-induced gut mucosal barrier dysfunction in rats. *Gastroenterology.* 1997;113:1246–1257.
40. Sorrells DL, Friend C, Koltuksuz U, et al. Inhibition of nitric oxide with aminoguanidine reduces bacterial translocation after endotoxin challenge in vivo. *Arch Surg.* 1996;131:1155–1163.