High Expression of Inducible Nitric Oxide Synthase Correlates with Intestinal Inflammation of Interleukin-2-Deficient Mice

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Interleukin-2-deficient mice [IL-2(–/–)] spontaneously develop colitis and other autoimmune defects when kept under conventional conditions[1] or following immunization with a given antigen (2,4,6-trinitrophenol, coupled to keyhole limpet hemocyanin).[2] With increasing age, animals develop colitis and chronic diarrhea in parallel between weeks 6 and 15 of age. The absence of IL-2 in mice leads to the development of activated autoreactive CD4-positive lymphocytes, which are considered pivotal for the development of colitis.[3] In support of this hypothesis, transfusion of isolated lymphocytes from IL-2(–/–) mice into wild-type mice leads to colitis in these animals.[4] CD4-positive lymphocytes of IL-2(–/–) mice show a strong Th1-secretion pattern, which is characterized by high levels of interferon-γ (IFNγ) in the presence of very low levels of IL-4.[4] IFN γ is one of the most potent inducers of the macrophage-type nitric oxide synthase (inducible NO synthase, iNOS, NOS type II), which in turn generates high amounts of NO. Besides being a potent vasodilator and a neurotransmitter, NO can also act as a cytotoxic agent.

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In addition, NO may influence T-cell differentiation and was shown to inhibit leukocyte infiltration into the tissue.[5] The immunomodulating properties of NO in inflammation may be both anti- and pro-inflammatory, depending on the type and phase of the inflammatory reaction.[5]

Based on the observed highly elevated IFN γ levels in IL-2(–/–) mice it is postulated that IFN γ may induce iNOS gene expression in the inflamed intestine of IL-2(–/–) mice. This, in turn, may lead to high tissue levels of NO. On the basis of this hypothesis, we established a semiquantitative RT-PCR method to analyze iNOS mRNA expression in the inflamed tissue and investigated in parallel the iNOS expression at the protein level.

MATERIALS AND METHODS

Colonic segments (ascending colon, a.c.; transverse colon, t.c.; descending colon, d.c.) were studied in IL-2(–/–) mice (animals 1–4) and wild-type mice (animals 5–8).

Total RNA and protein from indicated colonic segments were prepared in parallel using the Trizol reagent (Gibco BRL, Eggenstein, Germany) according to the instructions of the manufacturer. To prevent DNA contamination, RNA samples were digested with DNase I (Boehringer, Mannheim, Germany). First-strand cDNA for PCR was generated by random primed reverse transcription (RT) using 1 μg total RNA and 200 U M-MLV reverse transcriptase (Gibco BRL), in the presence of 20 U RNasin (Promega, Madison, WI).

A modified PCR protocol according to Schönfelder *et al.*[7] was used to amplify the cDNA. Water or RT mixtures without M-MLV were used as negative controls. Briefly, the reaction was performed in a final volume of 50 μl, containing 1.5 mM $MgCL_2$, 150 μM of each dNTP, 0.3 μM of each specific primer (sense, position 1490–1509, 5′-GCAGAATGTGACCATCATGG-3′ and antisense; position: 1915–18, 5′-ACAACCTTGGTGTTGAAGGC-3′). After initial denaturation at 94°C, 1.5 units Taq polymerase (Promega) were added (hot-start technique), and the amplification was carried out either as kinetic (consecutive cycles) or as dilution analysis at the following temperature profile: 94°C for 30 sec, 60°C for 60 sec, and 72°C for 90 sec.

PCR resulted in amplification products of the predicted size of 426 bp for iNOS cDNA, analyzed by means of ethidium bromide-stained 2% agarose gel electrophoresis. To assess initial levels of cDNA, control RT-PCR for β-actin as a housekeeping gene was performed using ITC Clontech (Heidelberg, Germany) control amplimer pairs. For quantification of PCR products, the ethidium bromide–stained gels were examined by still video gel documentation and subsequent densitometric analysis using the public domain Image Tool software (Version 1.25; University of Texas, San Antonio, TX).

Histological changes of the colonic tissue samples were assessed on hematoxylin/eosin-stained kryostat sections using the following degrees of

inflammation: 0, no signs of inflammation; 1, very low level of inflammation; 2, low level of infiltration by white blood cells (WBC); 3, high level of infiltration by WBC, thickening of the colonic wall; 4, transmural infiltration, loss of goblet cells, thickening of the colonic wall.

At the protein level, iNOS was detected by means of Western blotting as described in detail elsewhere.[7] Protein samples from the Trizol method were subjected to SDS polyacrylamide gel electrophoresis (8% gels) and transferred to nitrocellulose membranes. Blocked nitrocellulose membranes were incubated consecutively with monoclonal antibodies against iNOS (Transduction Laboratories, USA, dilution 1/1000) and horseradish peroxidase–coupled secondary anti-mouse antibodies (Dianova, Hamburg, Germany, 1/5000). After a final washing of the nitrocellulose membranes, immunodetection was performed using the ECL solutions according to the manufacturer's instructions (Amersham, Braunschweig, Germany).

RESULTS

To analyze iNOS mRNA expression in IL-2(–/–) mice with colitis, we developed a semiquantitative RT-PCR method based on a procedure described by Murphy *et al*.[6] To adjust the PCR cycle number for subsequent dilution analysis, we first performed PCR at consecutive cycles (see FIG. 1**A**). As a second step, the PCR was applied to serially diluted cDNA from 1/1 to 1/1048 in 1/2 dilution steps (nominally 50 ng initial RNA to nominally 48.8 pg). Densitometric peak area values were plotted on semi-log scale against serial dilutions (FIG. 1**B**). To assess the differences in mRNA expression levels, the linear ranges of the resulting curves (exponential range of amplification) were compared and used to calculate density values for the estimation of the relative expression (FIG. 1**C**). Relative expression was calculated as the relation of the density of the sample to the mean density of wild-type mouse samples. The results of the semiquantitative iNOS RT-PCR were normalized to the relative β-actin expression to take into account different initial amounts of RNA.

The increased expression of iNOS in the inflamed colonic tissue of IL-2 (–/–) at mRNA and protein level is shown in FIGURE 2. The iNOS mRNA expression level is elevated in the range of 5.5-fold in sample a.c.-3 and 19-fold in sample t.c.-1, compared to iNOS expression in the wild-type mice (WT) (FIG. 2**A**). The observed increases of iNOS mRNA levels in tissue samples (FIG. 2**A**) also corresponded to the iNOS-protein levels as detected by Western blotting (FIG. 2**B**).

To correlate the increased iNOS expression with the degree of inflammation, we assessed the histological changes of the tissue samples on hematoxylin/eosin-stained kryostat sections. Increased expression of iNOS mRNA correlated with the severity of inflammatory changes (FIG. 2**A**, *filled triangles*), whereas relative β-actin mRNA expression, which was used as a control, was found at constant levels in wild-type as well as IL-2 (–/–) mice independent of the degree of inflammation.

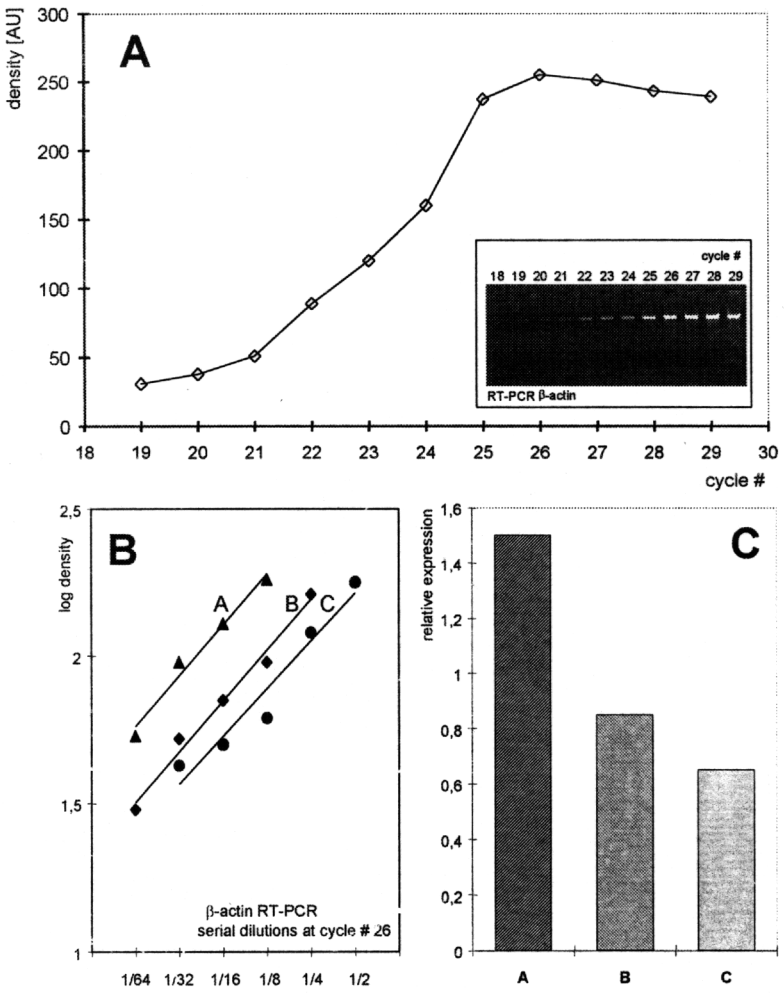

FIGURE 1. Representative semiquantitative RT-PCR using β-actin-specific primers and wild-type mouse cDNA. (A). Kinetic PCR analysis. Kinetic analysis at consecutive cycles was used to estimate the PCR cycle number for subsequent dilution analysis (top of the exponential amplification phase). *Inset*: Ethidium bromide–stained agarose gel (2%), amplification products for β-actin cDNA were identified at the predicted size of 838 bp. **(B). Evaluation of dilution PCR analysis.** For quantification of PCR products from dilution PCR analysis, the ethidium bromide–stained gels were examined by still video gel documentation and subsequent software-based densitometry. Densitometric peak area values were plotted on semi-log scale against serial dilutions. To assess the differences in mRNA expression levels, the linear ranges of the resulting curves (exponential range of amplification) were compared and used to calculate values for sample densities. **(C). Calculated relative expression values**. Relative expression was calculated as the relation of the density of the respective sample to the mean density of samples A, B, and C.

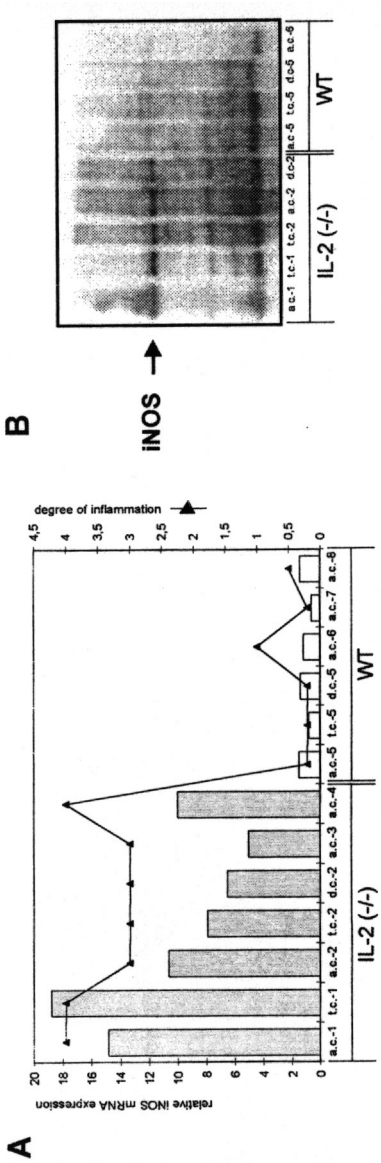

FIGURE 2. Analysis of iNOS expression in colonic sections from four IL-2 (–/–) mice (1, 2, 3, 4) and four WT mice (5, 6, 7, 8). Samples were described using the abbreviation of the respective colonic section and number of the respective mouse, for example, ascending colon of IL-2 (–/–) mouse 1, a.c.-1 or descending colon from WT mouse 5, d.c.-5. (**A**) **Relative expression of iNOS mRNA (bars) and degree of inflammation (filled triangles).** Relative expression was calculated as the relation of the density of the respective sample to the mean density of wild-type mouse samples. All samples were normalized to the relative β-actin expression to take into account different initial amounts of RNA. Degree of inflammation was assessed by means of histological changes on hematoxylin/eosin-stained kryostat sections of the colonic tissue samples. (**B**) **iNOS-specific antibodies detected a 130-kDa band on Western blots of protein from IL-2 (–/–) and wild-type mice corresponding to the iNOS protein**. Note that in tissue samples showing markedly increased iNOS mRNA levels (e.g., t.c.-1, a.c.-2) the corresponding Western blot analysis from the same tissue sample revealed strong iNOS protein bands (t.c.-1, a.c.-2).

SUMMARY AND CONCLUSIONS

Severely inflamed colonic sections of IL-2 (–/–) mice showed up to 19-fold increased iNOS mRNA levels. The level of iNOS protein expression corresponded to the increased iNOS mRNA levels as detected by means of Western blot analysis. There was a clear, positive relationship between the level of iNOS expression and the degree of inflammation in the colonic tissue of IL-2 (–/–) and wild-type mice.

Our data suggest that iNOS may play a key role in the pathogenesis of ulcerative colitis-like disease in IL-2 (–/–) mice. Further investigation should elucidate the impact of NO on the regulation of the inflammatory process in this model and might contribute to a better understanding of the role iNOS expression in human immune-mediated diseases.

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REFERENCES

1. SADLACK, B. *et al*. 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell **75**: 253–261.
2. EHRHARDT, R. O. *et al*. 1997. Induction and prevention of colonic inflammation in IL-2-deficient mice. J. Immunol. **158**: 566–573.
3. HORAK, I. *et al*. 1995. Immunol. Rev. **148**: 35–44.
4. LUDVIKSSON, B. R. *et al*. 1997. Dysregulated intrathymic development in the IL-2-deficient mouse leads to colitis-inducing thymocytes. J. Immunol. **158**: 104–111.
5. NATHAN, C. & Qiao Xie. 1994. Nitric oxide synthases: Roles, tolls, and controls. Cell **78**: 915–918.
6. MURPHY, L. *et al*. 1990. Use of the polymerase chain reaction in the quantitation of *mdr*-1 Gene Expression. Biochemistry **29**: 10351–10356.
7. SCHÖNFELDER, G. *et al*. 1996. Expression of inducible nitric oxide synthase in placenta of women with gestational diabetes. FASEB J. **10**: 777–784.