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Time Course and Cellular Localization of Interleukin-10 mRNA and Protein Expression in Autoimmune Inflammation of the Rat Central Nervous System

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Experimental autoimmune encephalomyelitis of the Lewis rat is a T-cell-mediated autoimmune disease of the central nervous system characterized by a self-limiting monophasic course. In this study, we analyzed the expression of the anti-inflammatory cytokine interleukin (IL)-10 at the mRNA and protein level in experimental autoimmune encephalomyelitis actively induced with the encephalitogenic 68–86 peptide of guinea pig myelin basic protein. Semiquantitative reverse transcriptase-polymerase chain reaction revealed that IL-10 mRNA expression peaked during the acute phase of the disease at days 11 and 13. IL-10 mRNA was synchronously induced with mRNA for the proinflammatory cytokine interferon-γ. Immunocytochemistry with a monoclonal antibody against rat IL-10 showed that the peak of IL-10 mRNA was accompanied by an abundant expression of IL-10 protein during the acute stage of the disease. Both *in situ* hybridization and double labeling immunocytochemistry in combination with confocal microscopy identified T cells, macrophages/microglia, and astrocytes as major cellular sources of IL-10 *in vivo*. The early peak of IL-10 production was unexpected in light of its well-documented anti-inflammatory properties. Additional studies are required to determine whether endogenous IL-10 contributes to rapid clinical remission typical for Lewis rat experimental autoimmune encephalomyelitis or if it plays other, yet undefined, roles in central nervous system autoimmunity. *(Am J Pathol 1998, 152:975–982)*

Experimental autoimmune encephalomyelitis (EAE) of the Lewis rat is a widely used model for autoimmune central nervous system (CNS) disease.[1,2] EAE can be induced by active immunization with various myelin constituents or the adoptive transfer of *in vitro*-generated autoantigen-specific T-cell lines. Pathoanatomically, the disease is characterized by extensive spinal cord infiltration by T cells and activated macrophages. After several days of progressive deterioration, the animals recover completely and are resistant to additional attempts to reinduce the disease. Obviously, effective mechanisms must exist that control inflammatory activity in this monophasic CNS disease.

Recently, evidence from the study of *in vitro*-cloned T-cell lines indicated that T-helper (Th) cells can be subdivided into Th1 and Th2 subsets, each producing distinctive sets of pro-and anti-inflammatory cytokines, respectively.[3] According to this concept, EAE is regarded as a classical Th1-mediated delayed type hypersensitivity reaction induced by autoaggressive T cells secreting interferon (IFN)-γ and interleukin (IL)-2. At least *in vitro*, Th1-dependent effector mechanisms are effectively down-regulated by Th2-derived cytokines like IL-4 and IL-10.[4–7] Based on the observation by reverse transcriptase-polymerase chain reaction (RT-PCR) that the mRNA for IL-10 is induced in the course of EAE,[8] this cytokine holds considerable promise as an endogeneous immunosuppressant contributing to recovery from CNS autoimmune disease. The cellular sources of IL-10 mRNA *in vivo* and the extent to which IL-10 mRNA is actually translated into protein are presently unknown. In this study, we therefore used a combined RT-PCR, *in situ* hybridization and immunocytochemical approach to analyze the spatiotemporal pattern of IL-10 expression in EAE.

Materials and Methods

Animals

EAE was induced in 10- to 14-week-old female Lewis rats (Charles River Laboratories, Kisslegg, Germany) by immunization with a synthetic peptide (25 μg per animal) corresponding to the amino acids 68–86 of guinea pig myelin basic protein (MBP68–86).[9] High performance

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Table 1. Primers Used for the Amplification of cDNAs

cDNA (accession-no.)	Sequence (5′ to 3′)	Location in cDNA (nt)
Rat IFN-γ	ATCTGGAGGAACTGGCAAAAGGACG (se)	160–184
(M29316; M29317)	CCTTAGGCTAGATTCTGGTGACAGC (as)	447–423
Rat IL-10	GAAAACAGAGCTTCAGCATGCTTGG (se)	28–52
(X60675)	TTTGAGTGTCACGTAGGCTTCTATGC (as)	569–544
Rat GAPDH	CCTTCATTGACCTCAACTACATGGT (se)	131–155
(M17701)	TCATTGTCATACCAGGAAATGAGCT (as)	974–950

The IFN-γ primers were purchased from Clontech, Palo Alto, CA.
Abbreviations: se, sense primer; as, antisense primer.

liquid chromatography-purified MBP68–86 peptide was purchased from the Department of Biochemistry, Georg-August-University, Göttingen, Germany (Dr. B. Schmidt).

RT-PCR

MBP68–86-immunized rats on days 8 ($n = 5$), 11 ($n = 5$), 13 ($n = 5$), 15 ($n = 5$), 18 ($n = 3$), 21 ($n = 3$), and 28 ($n = 1$) after immunization were killed by an overdose of ether. The lumbar and lower thoracic spinal cord (approximately 300 mg of wet weight per animal) was rapidly prepared by air insufflation and immediately homogenized in 5 ml of TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD). Total RNA was isolated according to the manufacturer's protocol. One μg of RNA isolated from each spinal cord was reverse transcribed using oligo$(dT)_{20}$-primers and SuperscriptII-reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's protocol, essentially. cDNA equivalent to 20 ng of total RNA was subjected to subsequent PCR analysis in a total volume of 30 μl containing 25 pmol of each primer (Table 1), 10 mmol/L Tris-HCl, pH 8.3, at 25°C, 50 mmol/L KCl, 10% dimethyl sulfoxide, 1.25 mmol/L $MgCl_2$, 250 μmol/L each of dATP, dCTP, dGTP, and dTTP, and 1.5 units AmpliTaq DNA-polymerase (Perkin-Elmer, Oak Brook, IL). The reaction was performed in a TRIO-Block thermocycler (Biometra, Göttingen, Germany) at the following conditions: 1) 2 minutes at 93°C; 2) 30 seconds at 93°C, 30 seconds at 62°C (IL-10), 60°C (IFN-γ), or 58°C (GAPDH), 45 seconds at 72°C for 30 cycles (IL-10 and IFN-γ) or 20 cycles (glyceraldehyde-phosphate dehydrogenase); and 3) 10 minutes at 72°C. Preliminary experiments had shown that for each gene product PCR amplification of cDNA was in the linear range under these cycling conditions (data not shown). PCR products were analyzed on 1.2% agarose gels containing 10 μg/ml ethidium bromide. The gels were photographed using a CSC-camera (Cybertech, Berlin, Germany), and densitometric analysis was performed with the Tina 2.1 software (Raytest, Straubenhardt, Germany). Controls included RNA subjected to the RT-PCR procedure without addition of reverse transcriptase and PCR performed in the absence of cDNA, which always yielded negative results. To ascertain the identity of the specific amplification products representative samples were sequenced on an ABI PRISM 310 genetic analyzer (Perkin–Elmer).

Detection of IL-10 mRNA by Nonradioactive in Situ *Hybridization (ISH)*

Diseased animals were killed on day 11, 12, 13, 14, 15, 18, 21, and 28 after immunization. The lumbar spinal cord was rapidly removed and frozen in isopentane precooled to −70°C. Five-μm cryostat sections were fixed in 4% paraformaldehyde (5 minutes, 4°C), dehydrated, air-dried, and acetylated using 0.1 mol/L triethanolamine, pH 8, for 10 minutes at room temperature. After washing, sections were prehybridized for 4 hours at 50°C in hybridization solution containing 50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris/Cl, pH 8.0, 1 mmol/L EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 500 μg/ml tRNA. After a brief wash in 4× SSC, sections were dehydrated and air-dried again and hybridized overnight at 50°C in hybridization solution containing 2 ng/ml digoxigenin-labeled RNA probe specific for rat IL-10. Probe synthesis and labeling was done as described previously.[10] Rat IL-10 cDNA[11] was generously provided by Dr. R. Bell (Ithaca, NY). After hybridization, washing, and RNase A digestion, digoxigenin detection was performed using the alkaline phosphatase-conjugated Fab fragment of sheep anti-digoxigenin, (Boehringer Mannheim, Mannheim, Germany), and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate as described previously.[10] Hybridizations with sense probe, which were always run in parallel to the antisense probe hybridization, gave no signal. In addition, selected slides were digested with RNase before ISH, which led to the complete disappearance of the signal.

Double-Labeling ISH/Immunocytochemistry

Astrocytes were identified on serial sections using a rabbit polyclonal antibody against S100 (1:10,000 dilution, Sigma, Deisenhofen, Germany) followed by affinity-purified biotinylated goat anti-rabbit IgG and the ABC Elite kit with diaminobenzidine as the substrate (Vector Laboratories, Burlingame, CA). Macrophages and T cells were identified by performing indirect immunofluorescence on the same section after *in situ* hybridization. For this purpose, mAb ED1 against macrophages (1:1000 dilution, Serotec, Oxford, UK) or mAb 15-6A1 against rat T cells (1:500 dilution, Holland Biotechnology, Leiden, The Netherlands) were applied simultaneously with the alkaline

phosphatase-labeled anti-digoxigenin antibody, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:50 dilution, Jackson ImmunoResearch, West Grove, PA). Sections were mounted using Vectashield mounting medium (Vector Laboratories).

Detection of IL-10 Protein by Immunocytochemistry

Fresh frozen cryostat sections were fixed in acetone (for 10 minutes, at −20°C), air-dried, and incubated with the mAb A5–4 against recombinant rat IL-10 (5 μg/ml, Pharmingen, San Diego, CA) followed by biotinylated, rat-absorbed horse anti-mouse IgG and the ABC Elite kit with diaminobenzidine as substrate. No immunostaining appeared on sections incubated with nonspecific IgG2b myeloma protein at 5 μg/ml (MOPC 141, Sigma) as an isotype-matched control.

Double-Labeling Immunofluorescence

In the double-labeling immunofluorescence experiments, mAb A5–4 was used at 10 μg/ml and detected using isotype-specific, biotinylated goat anti-mouse IgG2b (1:50, Southern Biotechnology Associates, Birmingham, AL) in combination with Texas Red-avidin (1:100, Vector Laboratories). Cell type-specific markers of different isotypes were applied simultaneously with the IL-10 antibody and detected by the appropriate fluorescein isothiocyanate-labeled, isotype-specific goat anti-mouse antibody (1:50, Southern Biotechnology Associates). Primary antibodies used were (isotype and dilutions in parenthesis): mAb ED1 against rat macrophages (mouse IgG1, 1:500), mAb Ox-42 (Serotec) against rat CD11b/c (mouse IgG2a, 1:50), and mAb R1–3B3 (Seikagaku Kogyo, Tokyo, Japan) against rat CD5 (mouse IgG2a, 1:200). Polyclonal rabbit anti S-100 was used at 1:1000 and detected with a fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody (1:50, Jackson ImmunoResearch). After immunostaining, sections were postfixed with 4% formalin in phosphate-buffered saline and mounted using Vectashield mounting medium. Double-stained slides were examined by confocal microscopy using a Bio-Rad MRC 1024 confocal laser scanning system connected to a Nikon DIAPHOT 300 inverted microscope. The excitation light source was provided by an Argon-Krypton laser. Images were acquired through the *x*, *y* focal plane of the tissue sections from two channels at 488- and 568-nm wavelengths. To analyze the localization of different antigens in double-stained samples, images obtained from the appropriate excitation wavelength were collected and merged.

Results

Clinical Course of EAE

MBP68–86-immunized Lewis rats exhibited a paresis of the tail as the first clinical sign of EAE at day 11. Thereafter, the animals developed a progressive paraparesis that was usually complete at day 15. Then they started to recover gradually. On day 22, all animals were asymptomatic. The animals showed a highly homogenous disease course and the manifestation rate was 100%.

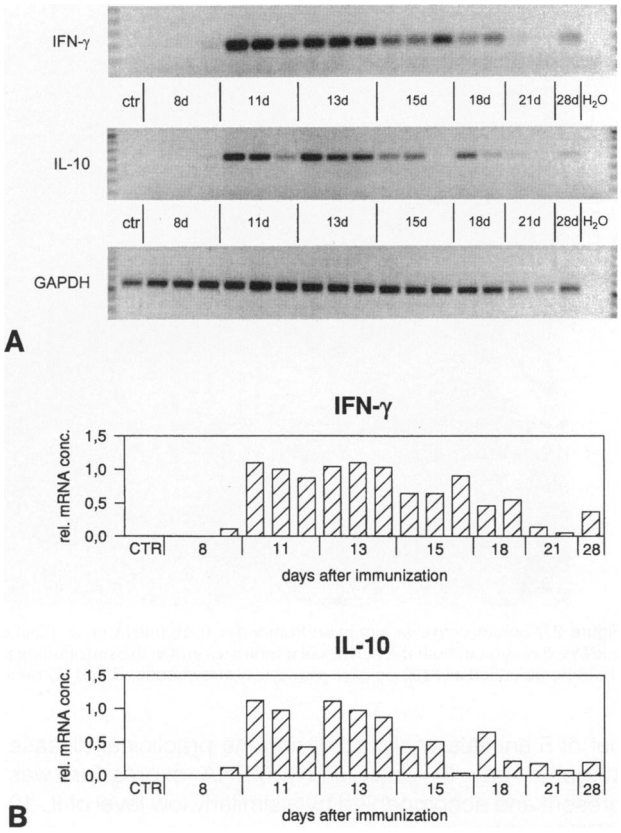

Figure 1. Time course of IL-10 mRNA expression in EAE analyzed by RT-PCR. **A:** Representative results from the analysis of total spinal cord RNA from 18 of 27 rats analyzed at the indicated intervals after immunization. Each RT-PCR experiment was repeated as least once with essentially identical results. **B:** Densitometric analysis of IFN-γ and IL-10 mRNA levels relative to the expression of the housekeeping gene GAPDH. As principal finding, the mRNAs for both IFN-γ and IL-10 show a highly synchronous time course of expression that peaks at days 11 and 13 after immunization and decreases thereafter.

Time Course of IL-10 mRNA Expression

To assess the overall time course of IL-10 induction during EAE we performed RT-PCR analysis of total spinal cord RNA isolated at days 8, 11, 13, 15, 18, 21, and 28 after immunization. For comparison, we additionally amplified the mRNA for the proinflammatory cytokine IFN-γ.

Both messages were readily detectable in samples obtained from animals in the acute stage of EAE (Figure 1A). DNA sequencing of representative PCR products confirmed their identity as IFN-γ and IL-10 cDNA, respectively. For semiquantitative evaluation, IL-10 and IFN-γ expression levels were normalized against those of the housekeeping gene GAPDH (Figure 1B). Both IFN-γ and IL-10 mRNA were maximally expressed at days 11 and 13 after immunization and decreased thereafter. In only 1

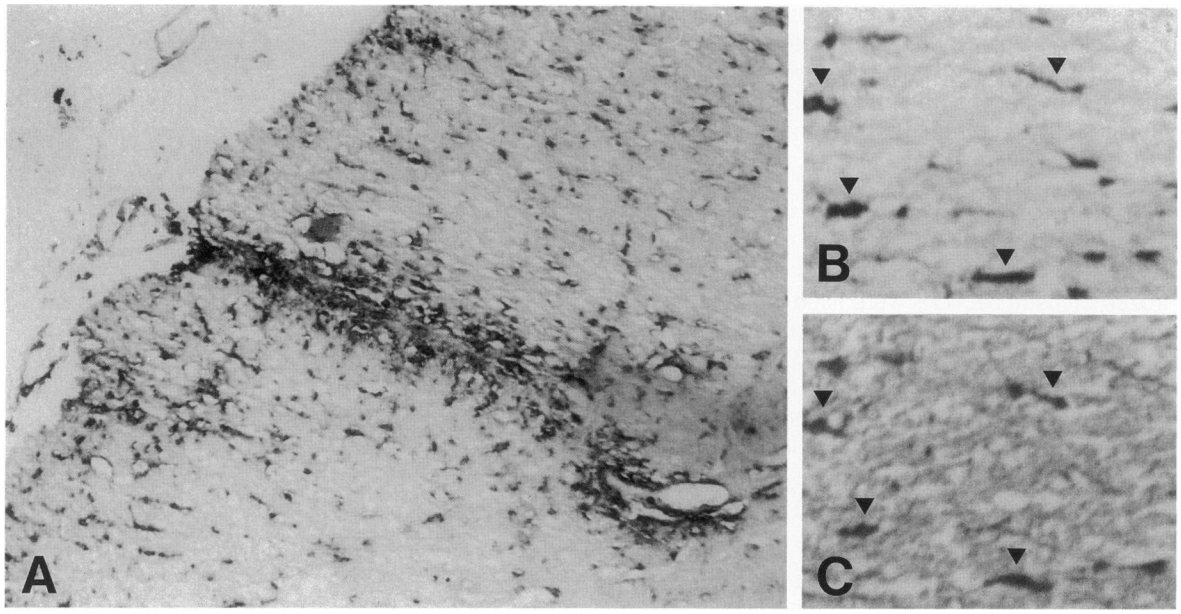

Figure 2. Nonradioactive *in situ* hybridization for IL-10 mRNA on a spinal cord section of an EAE animal 14 days after immunization showing abundant IL-10 mRNA expression in both the perivascular infiltrates and in the surrounding parenchymal cells (**A**). A proportion of the parenchymal IL-10 mRNA-expressing cells could be identified as S100-positive astrocytes (**arrowheads**, **B** and **C**) on a serial section. Magnification, ×82 (**A**), ×260 (**B** and **C**).

out of 5 animals analyzed during the preclinical disease stage at day 8, weak IFN-γ mRNA expression was present and accompanied by a similarly low level of IL-10 mRNA expression.

Cellular Localization of IL-10 mRNA by in Situ *Hybridization*

In line with the RT-PCR findings, nonradioactive ISH demonstrated numerous IL-10 mRNA-positive cells around day 13 after immunization. IL-10 mRNA was expressed by both perivascular and parenchymal cells (Figure 2A). On serial sections, some of the parenchymal IL-10-mRNA-positive cells could be identified as S100-positive astrocytes (Figure 2, B and C). For cellular localization of IL-10 mRNA in the inflammatory infiltrates, we combined ISH with indirect immunofluorescence labeling of T cells and macrophages on the same sections. IL-10 mRNA was present in proportions of both T cells (Figure 3, A to D) and macrophages (Figure 3, E to H).

Time Course and Cellular Localization of IL-10 Protein Expression in EAE

To analyze the expression of IL-10 protein in EAE we performed immunocytochemistry with a monoclonal antibody specific for rat IL-10. IL-10 immunoreactivity was absent in normal control spinal cord but was abundantly expressed in both perivascular and parenchymal cells at day 13 after immunization (Figure 4). After day 15, IL-10-immunoreactivity was overall decreasing. At days 21 and 28, only few IL-10-positive cells remained (not shown).

For cellular localization, the IL-10-specific mAb (IgG2b-isotype) was combined with either macrophage-specific markers (Ox-42 (IgG2a) or ED1 (IgG1)) or the mAb R1–3B3 against rat T cells (IgG2a), followed by detection with fluorescent isotype-specific secondary antibodies. In addition, a rabbit polyclonal antibody against S-100 was used to identify astrocytes.

IL-10 immunoreactivity was partly expressed by CD11b-positive cells recognized by mAb Ox-42 (Figure 4, A and D to F). Double-positive cells were concentrated around vessels. In addition, numerous parenchymal cells with short processes, most likely activated microglia, expressed IL-10-immunoreactivity. Similar results were obtained using the mAb ED1 as macrophage marker (Figure 4G). Strikingly, IL-10 immunoreactivity and the lysosomal ED1 antigen were localized to distinct intracellular compartments within the same cells. Prominent IL-10 expression was, in addition, found in perivascular T cells (yellow in Figure 4B) and astrocytes (Figure 4C). Most T cells located distant to vessels in the parenchyma and the meninges were IL-10-negative (green in Figure 4B), whereas a considerable proportion of parenchymal astrocytes displayed IL-10 immunoreactivity (Figure 4, C and H).

Discussion

As principal finding, this study shows a strong induction of IL-10 mRNA and protein during the acute stage of Lewis rat EAE and identifies infiltrating T cells and macrophages as well as resident astrocytes and microglia as cellular sources of IL-10 *in vivo*.

In our RT-PCR experiments, we found the strongest expression of IL-10 mRNA between days 11 and 13 after immunization. Since at that time the animals were still progressing to maximum disease severity, the peak expression of IL-10 mRNA occurred during the active phase of EAE. In contrast, after day 15 when the rats

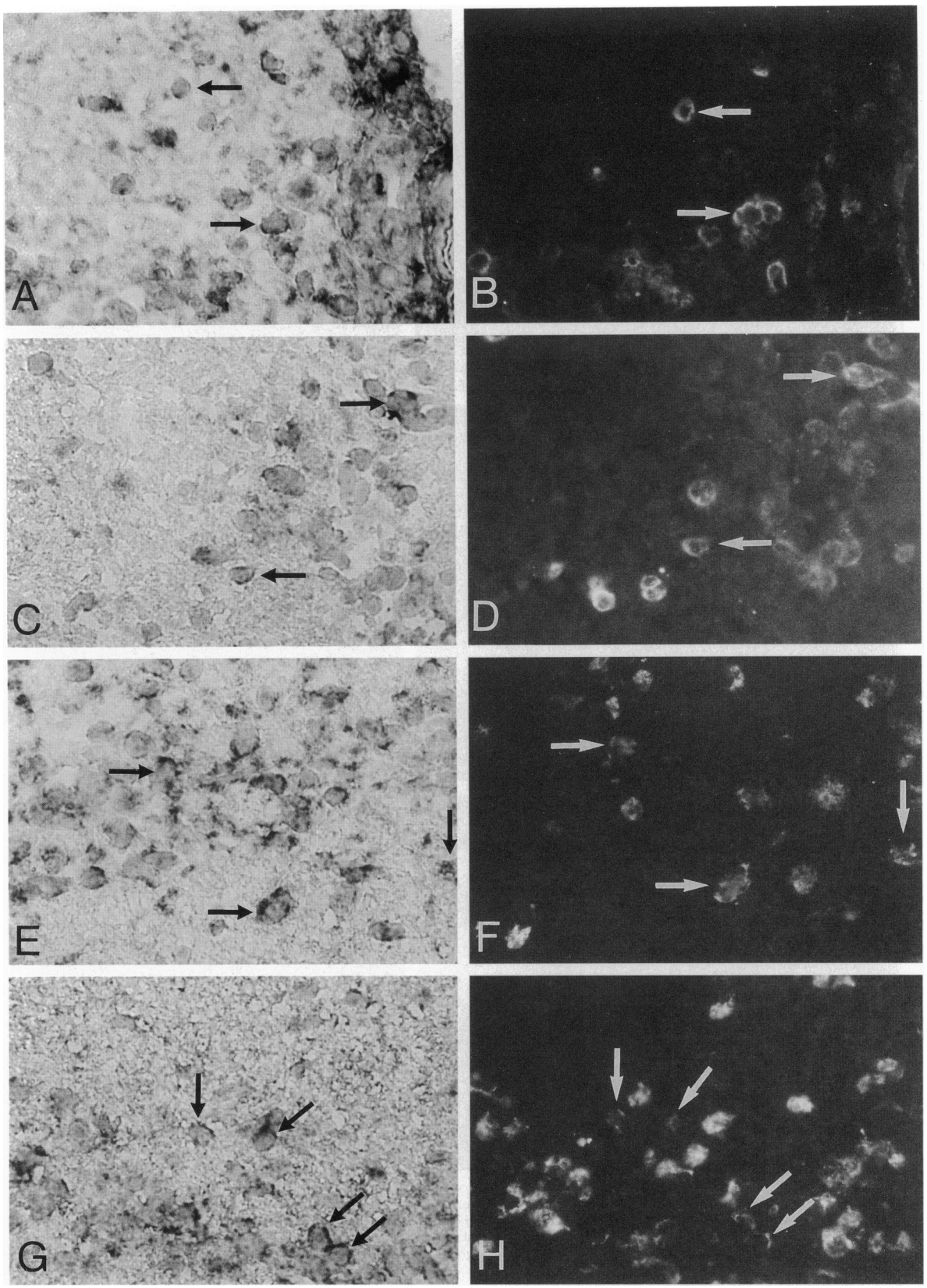

Figure 3. Nonradioactive *in situ* hybridization for IL-10 mRNA (**A, C, E,** and **G**) combined with immunofluorescent labeling for T cells (**B** and **D**) and ED1-positive macrophages (**F** and **H**) on the same section on day 14 after immunization. **Arrows** denote IL-10 mRNA-expressing T cells (**A** to **D**) and macrophages (**E** to **H**). Note that only proportions of both cell types express IL-10 mRNA. Magnification, ×330.

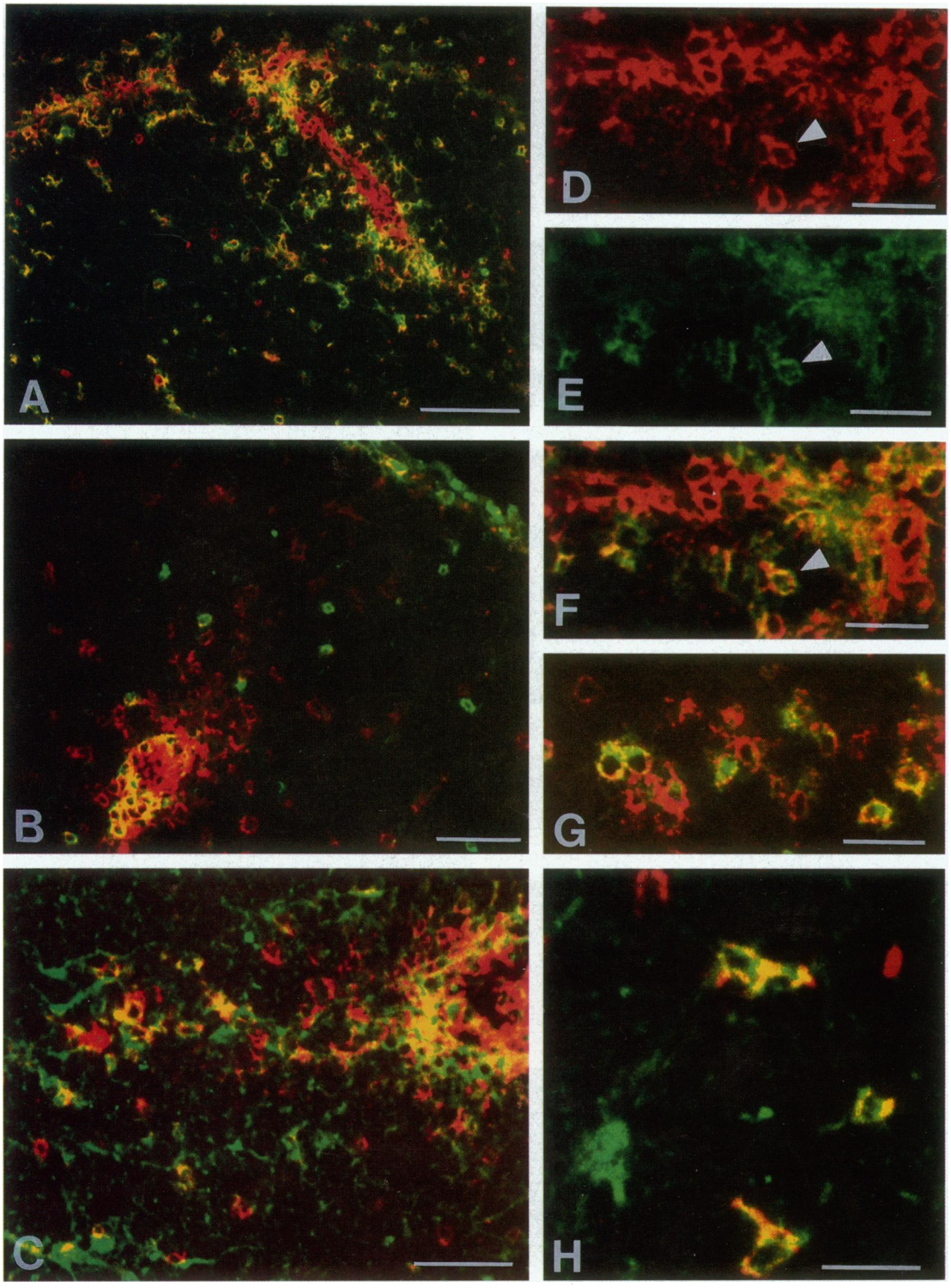

Figure 4. Cellular localization of IL-10 protein by double labeling immunofluorescence in combination with confocal microscopy. IL-10 immunoreactivity appears throughout in **red**. The IL-10 mAb was combined with the cell-type-specific markers (**green**) Ox-42 for microglia/macrophages (**A** and **D** to **F**), ED1 for phagocytic macrophages (**G**), CD5 for T cells (**B**), and S100 for astrocytes (**C** and **H**). **A** to **C** and **F** to **H** are superimposed images in which double-positive cells appear **yellow**. Within the microglia/macrophage lineage, IL-10 is localized to both perivascular cells, probably infiltrating macrophages, and parenchymal cells with short ramified processes, probably activated microglia (**A** and **D** to **F**). **D** and **E** are high magnification images from an IL-10/Ox-42 double staining that were obtained from single channel recordings at 568- and 488-nm wavelengths, respectively, showing IL-10 positive cells as **red** (**D**) and Ox-42 positive macrophages/microglia as **green** (**E**). **F** was obtained by superimposing **D** and **E**. A double-positive cell (**yellow** in **F**) is exemplified by **arrowheads** (**D** to **F**). Note that a dense accumulation of cells in the **upper left** is single **red**, ie, IL-10-positive but Ox-42-negative. IL-10-positive macrophages also express the ED1 marker (**G**). IL-10-positive T cells are found mainly in the perivascular infiltrates (**yellow** in **B**), whereas T cells in the parenchyma and the meninges are IL-10-negative (**green** in **B**). IL-10-expressing astrocytes are concentrated around inflamed vessels (**C**) but are also present in the parenchyma (**H**). Scale bars, 100 μm (**A**), 50 μm (**B** and **C**), 25 μm (**D** to **H**).

started to recover, the IL-10 signal was significantly decreased. Based on the concept of a functional dichotomy between pro- and anti-inflammatory cytokines[3] it was surprising that the mRNAs for IFN-γ and IL-10 showed a highly parallel time course of induction and down-regulation during the active and remission phase, respectively. In only one of five animals analyzed at day 8, ie, during the presymptomatic disease stage, we found weak but significant expression of IFN-γ mRNA. However, even this very early induction of IFN-γ gene expression was accompanied by a similar rise in IL-10 mRNA. Thus, we did not find a sequential pattern with Th1-like proinflammatory cytokines preceding Th2-like counter-regulation. Furthermore, our immunocytochemical data suggest that the synthesis of IL-10 protein equally peaks before the animals enter into the remission phase of the disease and essentially parallels the expression of IFN-γ immunoreactivity in this disease model.[12] Taken together, we present combined evidence from the analysis of mRNA and *in vivo* protein expression showing that IL-10 up-regulation is a relatively early event in this model of self-limiting autoimmune CNS disease.

Our findings are at variance with two previous studies that relied exclusively on the analysis of mRNA to describe the time course of IL-10 expression in the effector phase of EAE.[8,13] Issazadeh et al[13] found the peak of IL-10 mRNA expression in Lewis rat EAE during the late chronic phase of the disease. As the authors used a basically nonquantitative *in situ* hybridization technique to analyze IL-10 mRNA in tissue sections, methodological reasons may account for the discrepancies to our present results. In contrast, Kennedy et al[8] used semi-quantitative RT-PCR comparable with our present study to analyze the time course of IL-10 mRNA in the SJL/J mouse model of EAE. These authors found that IL-10 gene expression peaked during the early recovery phase and was delayed by approximately 4 days relative to the peak of IFN-γ expression. Interestingly, in contrast to the Lewis rat the SJL/J mouse exhibits a chronic relapsing course of EAE.[14] If differences in the sequential appearance of pro- and anti-inflammatory cytokines account for the different course of EAE in the Lewis rat and SJL/J mouse model remains to be studied.

IL-10 was originally identified *in vitro* as a product of mouse Th2-cell lines inhibiting the proliferation and various effector functions of Th1 cells.[4] Subsequent studies showed that macrophages are equally potent producers of IL-10 *in vitro*.[6] The anti-inflammatory properties of IL-10 were related to its down-regulatory influences on proinflammatory cytokine production as well as the expression of MHC class-II antigens and co-stimulatory molecules on antigen-presenting cells.[4–7] Our data suggest that in addition to hematogenous macrophages and T-cells, resident glial cells such as microglia and astrocytes constitute major sources of IL-10 *in vivo*. In line with our findings, IL-10 mRNA and protein have been detected in mouse astrocytes in culture,[15] and astrocytic IL-10 immunoreactivity has been described in multiple sclerosis lesions.[16] Interestingly, both activated microglia and astrocytes have recently been suggested to down-regulate rather than promote T-cell responses in the CNS.[17–19] One might therefore speculate that IL-10 produced by CNS cells early in the course of EAE may contribute to the rapid resolution of clinical signs in this prototypic model of monophasic CNS autoimmune disease. However, in contrast to the considerable *in vitro* evidence suggesting an inhibitory effect of IL-10 on Th1-mediated immune responses, conflicting data concerning the actual *in vivo* role of IL-10 in autoimmune diseases have been reported. IL-10 exerted paradoxical disease-accelerating effects in experimental autoimmune diabetes in mice.[20] Attempts to neutralize endogenous IL-10 by monoclonal antibody treatment in adoptively transferred EAE in mice failed to show a significant effect on the course of the disease.[21] In a primate model of EAE, immune deviation with a shift from a Th1- to a Th2- dominated cytokine response led to a fatal demyelinating form of the disease that may be related to the capacity of IL-10 to enhance the production of pathogenic autoantibodies.[22] In contrast, recombinant IL-10 administered exogenously during the induction phase of Lewis rat EAE greatly diminished the severity of subsequent disease.[23] In the peripheral nervous system counterpart of EAE, experimental autoimmune neuritis, IL-10 treatment was beneficial even after the establishment of clinical disease.[24] Our study represents the first comprehensive analysis of the spatiotemporal expression pattern of IL-10 in EAE and may serve as a basis for future investigations addressing the complex functional role of this cytokine in CNS autoimmunity.

Acknowledgments

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