Differential Expression of Nitric Oxide Synthase in Experimental Uveoretinitis

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Purpose. To investigate the site and the cellular source of inducible nitric oxide synthase (iNOS) expression in human S-antigen peptide–induced experimental autoimmune uveoretinitis (EAU).

Methods. Twenty-one Lewis rats were sensitized with human S-antigen peptides. Three rats were killed each consecutive day from day 6 through day 12 after sensitization. Frozen sections of the enucleated eyes were analyzed for iNOS by the dual immunohistochemical method. Primary antibodies included rabbit anti-mouse iNOS combined with anti-human endothelium NOS, anti-rat lysosomal protein (ED1), or anti-rat major histocompatibility complex class II molecule (OX6) monoclonal antibodies. Secondary antibodies were fluorescein-conjugated anti-mouse IgG and streptavidin rhodamine-labeled anti-rabbit IgG. The adjacent sections were separately stained with ED1, iNOS, and glial fibrillary acidic protein (GFAP). The mouse macrophage cell line RAW 264.7 was exposed to either interferon (IFN)γ/lipopolysaccharide (LPS) or S-antigen and to interphotoreceptor retinoid-binding protein (IRBP), myelin basic protein, and bovine serum albumin for 12 hours. Cells were harvested for detection of iNOS expression by northern blot analysis hybridization and detection of protein by immunohistochemistry.

Results. In the retina of eyes with EAU, ED1+/iNOS+ and OX6+/iNOS+ cells were first detected on day 9 after sensitization. These iNOS+ cells increased in number on subsequent days in parallel with the increasing severity of retinal damage. Most of the cells localized around the outer retina. In contrast, a large number of ED1+ and OX6+ cells that were localized in the uvea and conjunctiva were negative for iNOS. Retinal pigment epithelial cells did not stain for iNOS. Macrophages exposed to IFNγ/LPS, S-antigen, and IRBP showed expression of iNOS mRNA and the protein.

Conclusions. Macrophages are an important source of NO production in eyes with EAU. These macrophages preferentially express iNOS in the retina. Such a differential expression of iNOS by the macrophages appears to be related to retinal soluble proteins. (*Invest Ophthalmol Vis Sci.* 1999;40:1899–1905)

Nitric oxide (NO) has been widely studied by those attempting to elucidate the mechanism of inflammation and tissue destruction.[1] The cytotoxic molecule NO is synthesized by an inducible NO synthase (iNOS); once this synthase is expressed, NO is produced for a period of several hours to several days. In contrast, constitutive NO synthase (cNOS) generates NO for short periods of only a few seconds to a few minutes. Cytotoxicity is usually associated with the products of iNOS rather than the products of cNOS.[1] Increased production of NO is found in many endotoxin-induced acute inflammations, including endotoxin-induced uveitis (EIU).[2–4] The release of NO has also been noted in various experimental autoimmune inflammatory disorders, such as experimental arthritis and certain neurologic inflammations.[5,6] Such experimentally induced inflammations have shown that NO production is involved in the enhancement of inflammation through its effects on vasodilatation, neutrophil adhesion, and alteration of vascular permeability leading to tissue damage.

Unlike EIU, experimental autoimmune uveoretinitis (EAU) can be readily induced by several retinal proteins, such as retinal soluble antigen (S-antigen), interphotoreceptor retinoid-binding protein (IRBP), rhodopsin, and others. Experimental autoimmune uveoretinitis is clinically and histologically different from EIU. In the latter, the inflammatory process is mild and involves primarily the anterior uvea. In contrast, the inflammation of EAU is characterized by retinitis, marked retinal damage, and uveitis. The retinal disease is mainly localized in the outer retina, and the ensuing retinal damage could be caused by the formation of oxygen metabolites, including peroxynitrite.[7,8] In the present study, we attempted to investigate the cellular source of and the role of retinal proteins in the expression of iNOS and NO generation that occurs with S-antigen peptide–induced uveoretinitis.

Methods

Twenty-one Lewis rats (aged 6–8 weeks) were immunized by a hind footpad injection of 0.1 ml synthetic human S-antigen

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Table 1. Histologic Determination of EAU Severity and Immunohistochemical Colocalization of ED1+, OX6+, and iNOS+ Infiltrating Cells in the Retina and Uvea

		Retina†			Choroid†			Iris/Ciliary Body†		
Postimmunization	**EAU Severity***	**ED1+**	**OX6+**	**iNOS+**	**ED1+**	**OX6+**	**iNOS+**	**ED1+**	**OX6+**	**iNOS+**
Day 0	0	−	−	−	1+	1+	−	1+	1+	−
Day 6	0	−	−	−	1+	1+	−	1+	1+	−
Day 7	0	−	−	−	1+	1+	−	1+	1+	−
Day 8	0	1+	1+	−	1+	1+	−	1+	1+	−
Day 9	Mild/moderate	2+	2+	2+	2+	2+	−	2+	2+	−
Day 10	Mild/moderate	2+	3+	2+	2+	2+	−	2+	3+	−
Day 11	Moderate	3+	3+	3+	3+	3+	+/−	3+	3+	+/−
Day 12	Moderate/severe	3+	3+	3+	3+	3+	+/−	3+	3+	+/−

* EAU severity based on hematoxylin and eosin staining. 0, No infiltrating cells in the eye; mild, infiltrating cells limited to the retina, anterior chamber, iris, and ciliary body; moderate, infiltrating cells present in the retina and choroid; severe, infiltrating cells present in retina, uvea, and sclera.

† Immunohistochemistry. − no positive-staining cells. 1+, < 10 cells/HP; 2+, 10–20 cells/HP; 3+, > 20 cells/HP; +/−, weak staining. HP, high-power field (×40).

peptide (100 μg) in Freund's complete adjuvant containing 4 mg/ml of heat-killed Mycobacterium tuberculosis. Three rats were killed on each day, from day 6 to day 12 after immunization. Three naive rats served as control subjects. All animal procedures used in this study were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antigen Preparation

Human S-Antigen Peptide. The sequence of human S-antigen peptide, 307 to 326 DTNLASSTIIKEGI DRTVLG,[9] was synthesized by an automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, CA), desalted on a Sephadex G-10 column (Sigma, St. Louis, MO), and purified by reversed-phase high-performance liquid chromatography (RP 304 Column; Bio-Rad Laboratories, Hercules, CA). This peptide is highly identical with the amino acid sequence of bovine S-antigen and is uveitogenic for Lewis rats.[10]

Bovine S-Antigen and IRBP. Bovine S-antigen and IRBP were isolated according to a procedure previously described.[7,11] Briefly, each antigen was precipitated from bovine retinal extract by half-saturated ammonium sulfate, then purified by gel filtration chromatography (Ultrogel AcA 34 followed by HAultrogel; IBF Biotechnics, Savage, MD). Both antigens were identified in elutes by the Ouchterlony immunodiffusion test (Biowhittaker, Walkersville, MD). On 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, isolated bovine S-antigen or IRBP revealed one band of approximately 48 kDa in S-antigen and one of approximately 130 kDa in IRBP. The uveitogeneity was separately confirmed by immunization of Lewis rats.

Immunolocalization of iNOS in the Ocular Tissues

The enucleated eyes were embedded in frozen tissue specimen medium, and 7-μm frozen sections were obtained from the globes. These sections were fixed in acetone and exposed to various antibodies. The primary antibodies used included rabbit anti-mouse macrophage iNOS polyclonal antibody (1:50) and mouse anti-human endothelium NOS monoclonal antibody (cNOS, 1:50), both from Transduction Laboratories (Lexington, KY), and mouse anti-rat lysosomal membrane (ED1, 1:100) and mouse anti-rat major histocompatibility complex class II common determinant (OX6, 1:100) monoclonal antibodies (Serotec). Each section was incubated with one of the following combinations of primary antibodies: iNOS/cNOS, iNOS/ED1, and iNOS/OX6 at 4°C overnight. After a phosphate-buffered saline (PBS) wash, the sections were incubated with two different secondary antibodies, goat anti-mouse fluorescein-conjugated IgG (1:200; Vector Laboratories, Burlingame, CA) and goat anti-rabbit streptavidin rhodamine-labeled IgG (1:100; Southern Biotech, Birmingham, AL), consecutively at room temperature for 2 hours. After a PBS wash, the specimens were mounted and examined under a confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). For control, mouse IgG (20 μg/ml) and normal rabbit serum (1:750) were substituted for the primary antibodies. The adjacent sections of each eye were stained with hematoxylin and eosin.

The adjacent sections were separately incubated with antibodies against ED1, iNOS, and rabbit anti-cow glial fibrillary acidic protein serum (GFAP, 1:100, Dako, Carpinteria, CA). The following secondary antibodies were used: biotin-conjugated anti-rabbit immunoglobulins for iNOS (1:300, Dako), anti-mouse immunoglobulins for ED1 (1:100, Dako, preabsorbed with normal rat serum), and goat anti-rabbit streptavidin rhodamine-labeled IgG (1:100, Southern Biotech) for GFAP. The antigen–antibody binding was detected by avidin-biotinylated horseradish peroxidase (Vector Laboratories), then with 3-amino-9-ethyl-carbazole (Sigma). The sections were briefly immersed in hematoxylin for counterstaining and observed under light microscope.

Cell Culture and Induction

Mouse macrophage cell line RAW264.7 (American Type Culture Collection, Rockville, MD) was used in vitro to detect the expression of iNOS in the presence of retinal proteins (S-antigen and IRBP). The cells were plated on a six-well culture plate (Becton Dickinson, Lincoln Park, NJ) with a density of 10^6 cells/well. Culture media consisted of minimum essential medium (MEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Gibco), and 2 mM L-glutamine (JRH Biosciences, Lenexa, KS).

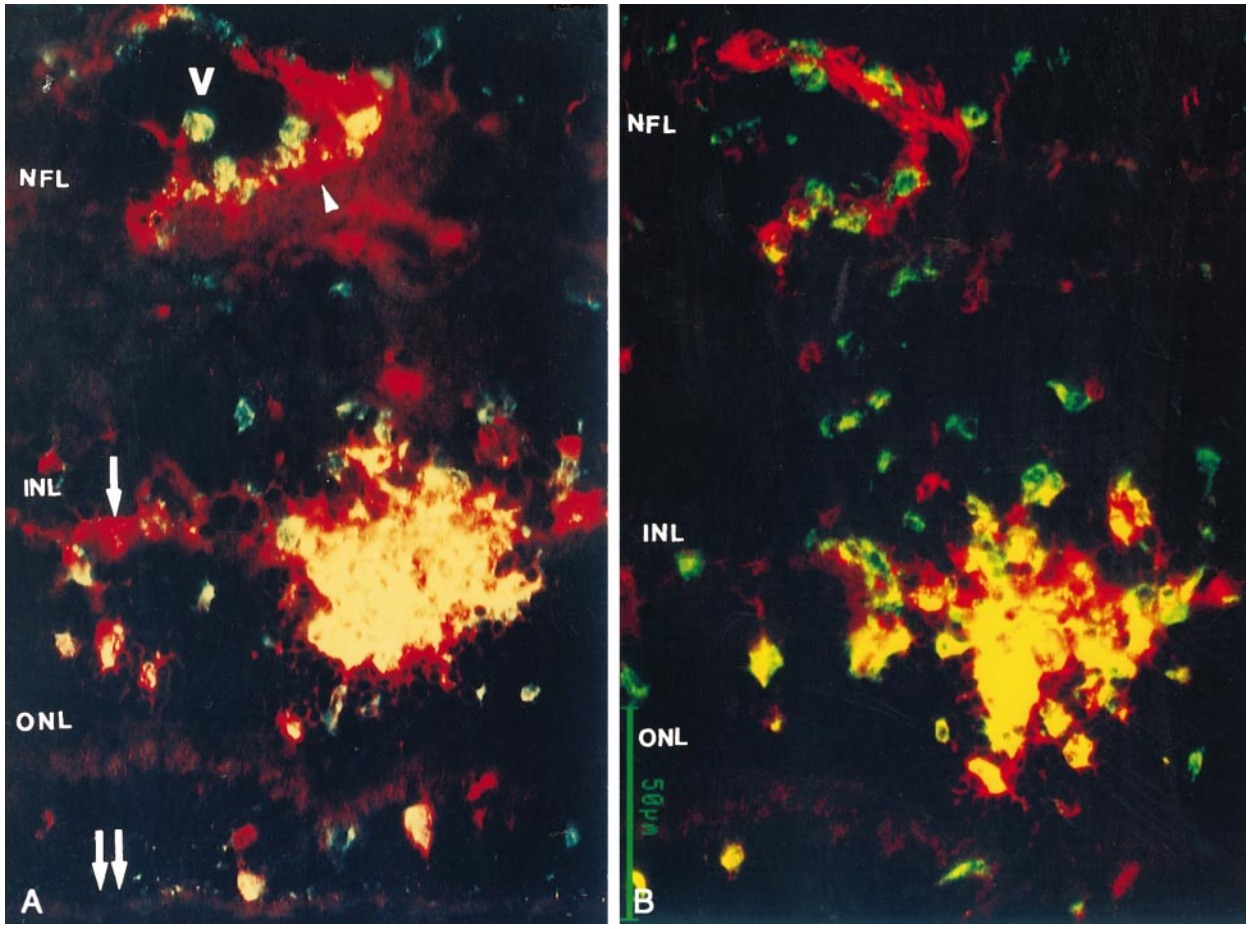

FIGURE 1. (**A**) Colocalization of OX6+/iNOS+ cells in the retina on postimmunization day 12. OX6+/iNOS+ cells (*yellow*) appeared in the outer plexiform layer, inner plexiform layer, outer nuclear layer (ONL), and subretinal space. A few OX6+/iNOS− cells (*green*) were seen intravascularly and perivascularly in the nerve fiber layer (NFL). The processes of perivascular macroglia (*arrowhead*) and the microvascular endothelia (*arrow*) showed iNOS-positive staining (*red*). Retinal pigment epithelial cells were negative for OX6 or iNOS staining (*double arrows*). (**B**) Colocalization of ED1+/iNOS+ cells in the retina on postimmunization day 12. The distribution of ED1+/iNOS+ cells (*yellow*) was similar to that of OX6+/iNOS+ cells in the retina in the adjacent section. More ED1+/iNOS− cells (*green*) were noted in the NFL and inner plexiform layer. INL, inner nuclear layer.

On reaching confluence, the macrophage cultures were exposed to media containing one of the following agents (50 μg/ml of each): bovine retinal S-antigen, IRBP, bovine serum albumin (BSA; Sigma); bovine brain myelin basic protein (MBP; Sigma) and a mixture of lipopolysaccharide (LPS; 100 ng/ml, Sigma) with recombinant murine IFNγ (100 U/ml; Gibco). The culture media, retinal proteins, BSA, and MBP were passed through endotoxin-removing gel (Detoxi-Gel; Pierce, Rockford, IL). All were incubated at 37°C with 10% CO_2 in an air atmosphere for 12 hours. For negative control samples, cells were incubated without any of the reagents mentioned.

Detection of iNOS mRNA Expression in RAW 264.7 Cell Line

The remaining macrophages were collected for isolation of total RNA. A total RNA isolation kit (CLONsep; Clontech, Palo Alto, CA) protocol was followed. Total RNA (10 μg per lane) was subjected to electrophoresis on a 1% agarose gel containing 18% formaldehyde. RNAs were blot transferred to nylon membrane (Amersham Life Science, Arlington Heights, IL) and UV autocrosslinked. Membrane was hybridized with iNOS cDNA probe (Cayman Chemical, Ann Arbor, MI) for 20 hours at 45°C. The hybridization buffer contained 50% deionized formamide, 4× SSC, 1× Denhardt's, 0.05% SDS, and denatured salmon sperm DNA (100 μg/ml, Sigma). The cDNA probe (10^6 cpm/ml) was labeled with [α-^{32}P] dCTP (Amersham Life Science) by random priming. The hybridized membrane was washed at room temperature in 2× SSC/0.1% SDS, followed by 1× SSC/0.1% SDS, and then by 0.1× SSC/0.1% SDS at 65°C for 30 minutes each. Autoradiography was performed by exposure to x-ray film (X-Omat; Eastman Kodak, Rochester, NY) at −70°C for 12 hours in the presence of intensifying screens. Relative mRNA levels were revealed by subsequent stripping and rehybridized with [α-^{32}P] dCTP-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

Detection of iNOS Expression in RAW 264.7 Cell Line

The RAW 264.7 macrophages were plated on two-well chamber slides at a density of 10^2 cells/well. These cells were

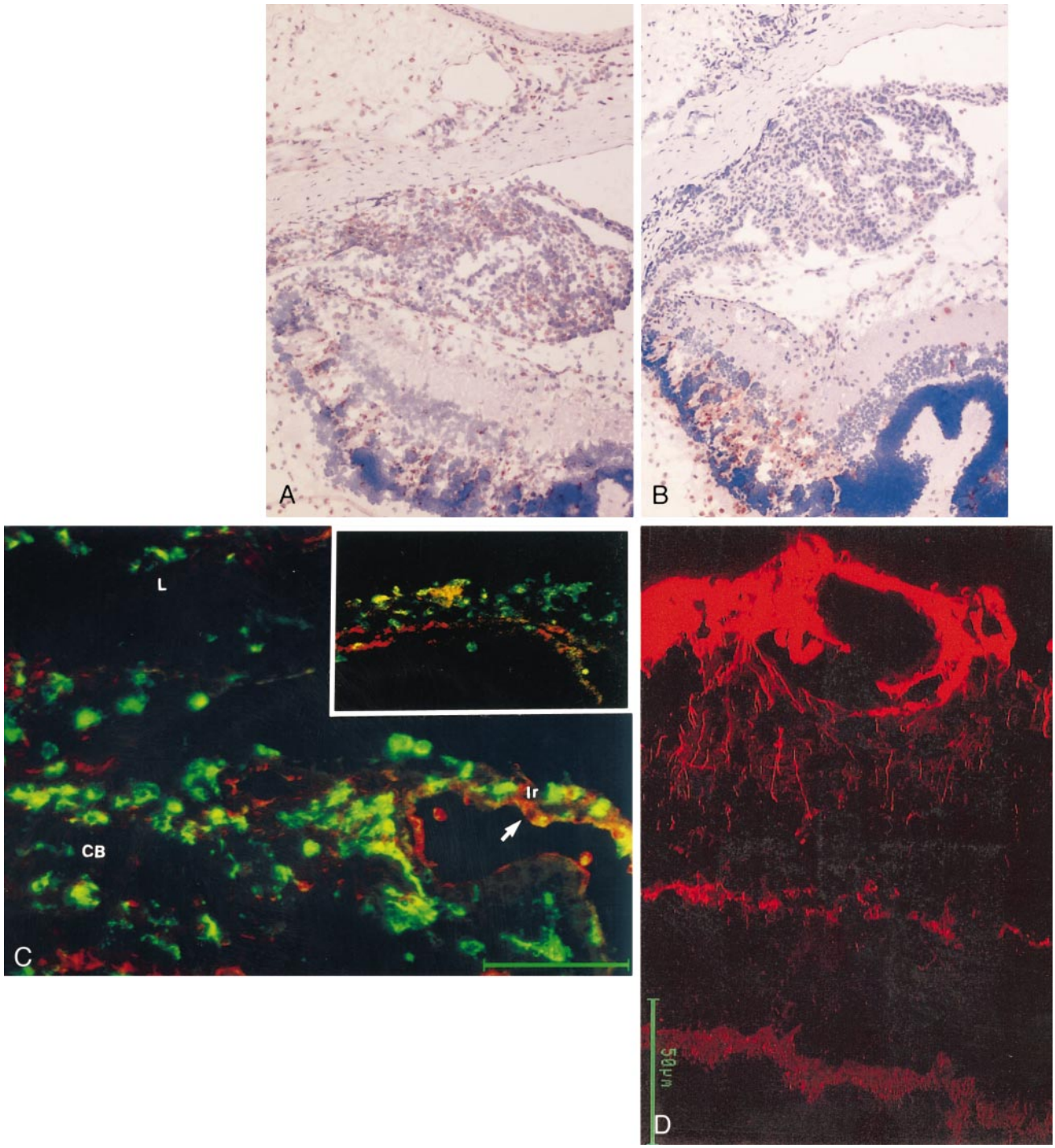

FIGURE 2. (**A**) Many ED1+ cells were seen in the stroma of iris, ciliary body, limbal area, and peripheral retina. (**B**) On the adjacent sections, iNOS+ cells were sparse in the iris (ir), ciliary body (CB) and limbal (L) area, but abundant in the retina. (**C**) iNOS+ staining was noted in the iris epithelial cells (*arrow*). *Insert* shows that there were many OX6+/iNOS− cells (*green*) and occasional OX6+/iNOS+ cells (*yellow*) in the iris stroma. iNOS+ staining (*red*) was seen in the epithelial cells (immunofluorescence). (**D**) GFAP+ staining was noted in the processes of astrocytes and Müller cells. (**A**, **B**) 3-Amino-9-ethyl-carbazole; magnification ×125.

incubated with S-antigen, IRBP, or BSA, at a dose of 100 μg/ml each, or with IFNγ/LPS (100 U/100 ng/ml) at 37°C with 10% CO_2 for 12 hours. The slides were fixed by 100% acetone at 4°C for 5 minutes and submitted for immunohistochemistry using iNOS polyclonal antibody, as described earlier.

RESULTS

Histopathologic analysis of the hematoxylin and eosin preparations revealed the presence of retinal perivasculitis, infiltrating cells in the anterior chamber and iris and ciliary body on

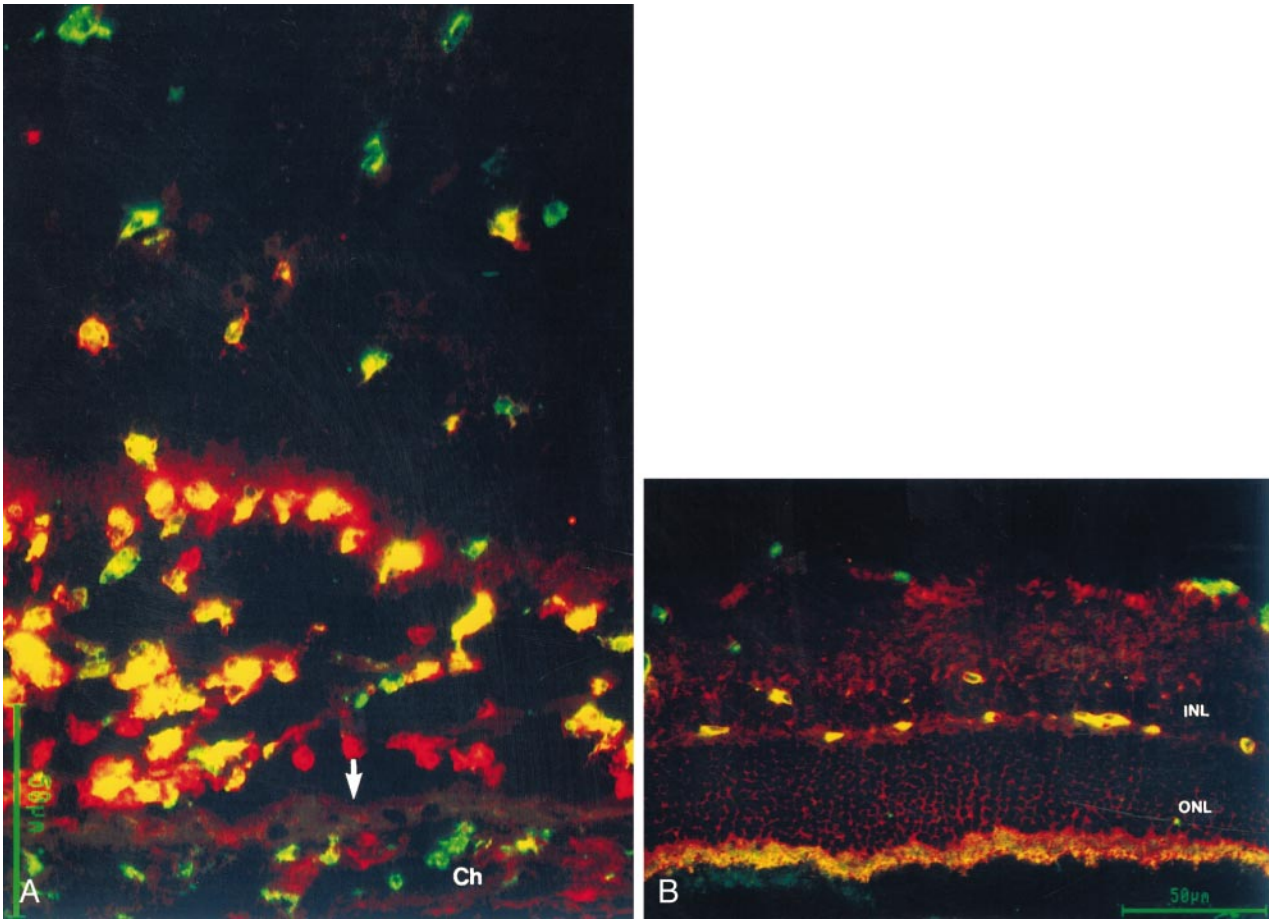

FIGURE 3. (**A**) ED1+/iNOS− cells (*green*) existed in the inner part of the retina and choroid (Ch). Many ED1+/iNOS+ cells (*yellow*) and a few ED1−/iNOS+ cells (*red*) appeared in the outer segment and subretinal space. Retinal pigment epithelium was negative for ED1 or iNOS staining (*arrow*). (**B**) Coexpression of cNOS and iNOS in endothelial cells of retinal vessels by dual immunohistochemistry. Microvascular endothelial cells in the inner nuclear layer (INL) and the inner plexiform layer were stained positively by both iNOS and cNOS (*yellow*). The endothelia in the nerve fiber layer were stained positively by cNOS (*green*). ONL, outer nuclear layer.

postsensitization day 9. The inflammation peaked on day 12, and the inflammatory infiltration was present in the inner and outer retina, which consisted of monocytes, neutrophils, and lymphocytes. In addition, the infiltration was present in the subretinal space, iris, ciliary body, choroid, conjunctiva, and limbus. Retinal damage included focal edema and exudation in the inner and outer nuclear layers and destruction of photoreceptor cells. Exudative retinal detachment also was noted. The severity of EAU at various intervals is shown in Table 1.

Immunolocalization of iNOS in the Ocular Tissue

On postimmunization day 8, occasional ED1+ and OX6+ cells were seen inside and around the vessels of the nerve fiber layer. These cells were negative for iNOS staining. On day 9, iNOS+ cells were detected in the retina, particularly surrounding the vessels in the inner nuclear layer, and at the site of photoreceptor cells, which were disrupted. The iNOS+ macrophages (OX6+) in the outer retina increased, paralleling the severity of the inflammation. Dual immunohistochemical staining showed that these cells were iNOS+/ED1+ and iNOS+/OX6+ (Figs. 1A, 1B). Although there were many ED1+ and OX6+ infiltrating cells in the iris, ciliary body, choroid, and conjunctiva, only occasional cells

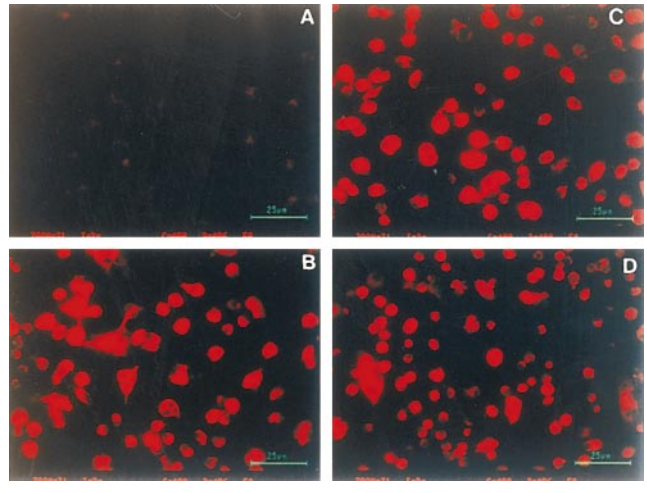

FIGURE 4. Expression of iNOS protein was detected by anti-mouse iNOS polyclonal antibody. Positive staining was seen in the cytoplasma of RAW 264.7 macrophages preincubated with IFNγ/LPS (**B**), S-antigen (**C**), and IRBP (**D**). A backgroundlike staining was seen in the macrophages incubated with BSA (**A**).

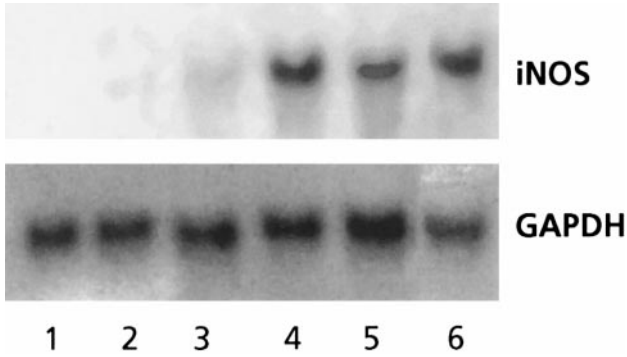

FIGURE 5. Expression of iNOS mRNA was detected by northern blot analysis hybridization with a mouse iNOS cDNA probe labeled with [α-^{32}P] dCTP (10^6 cpm/ml). iNOS mRNA was found in RAW 264.7 macrophages preincubated with IFNγ/LPS (*lane 4*), IRBP (*lane 5*), and S-antigen (50 μg/ml, *lane 6*). iNOS mRNA was not detected in the macrophages incubated with media only (*lane 1*), BSA (*lane 2*) or MBP (*lane 3*). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

stained positive for iNOS (Figs. 2A, 2B). The epithelial cells of iris and ciliary body, however, stained weakly for iNOS (Fig. 2C). A staining of iNOS and cNOS was noted in the capillary endothelia of the retina. The processes of astrocytes and Müller cells in the perivascular regions showed positive staining for iNOS. The staining pattern was similar to that of GFAP in the inner limiting membrane (Fig. 2D). Retinal pigment epithelia stained with neither anti-iNOS nor anti-cNOS antibody (Figs. 1A, 3). In the nonimmunized animals, the capillary endothelial cells in the inner plexiform layer and inner nuclear layer stained weakly with iNOS antibody. In contrast, all the vascular endothelia of the eye stained positively with cNOS antibody in uveitic and uninflamed eyes (Fig. 4).

Detection of iNOS and Its mRNA Expression in the RAW 264.7 Cell Line

Immunohistochemical analysis revealed iNOS in the cytoplasm of the macrophages exposed to IFNγ/LPS, S-antigen, and IRBP, but not in the cells exposed to BSA or in the unexposed control cells (Fig. 5). Similarly, expression of iNOS mRNA was found in the macrophages exposed to IFNγ/LPS, S-antigen, and IRBP, but not in the presence of BSA or in the unexposed control cells (Fig. 6).

DISCUSSION

In the present study, the expression of iNOS was seen in the extravasated mononuclear cells that expressed phenotypic markers for macrophages (ED1 and OX6). These cells were primarily present in the retina, particularly at the site of photoreceptor cell damage. Similarly, a colocalization of iNOS and ED1+ macrophages has been shown in the retina of animals with EAU, particularly in the rod outer segments.[12] In addition, we found that iNOS staining was predominant in infiltrates of the retina rather than those in the uvea, limbus, and conjunctiva, although the infiltrating cells, positive for class II molecules (OX6) and ED1, were seen in those sites. Such findings suggest that mononuclear phagocyte extravasation and display of class II molecules may not be sufficient for iNOS expression. Exposure to the local components, such as S-antigen, IRBP, or other retinal proteins may be required for the macrophages to express iNOS in EAU.

Endotoxin and several cytokines are known to enhance in vitro expression of iNOS. In vivo, generation of NO has been demonstrated in EIU.[2–4] Based on such in vivo and in vitro studies, it appears that endotoxin mediates expression of iNOS through induction of various cytokines. However, which cytokine or cytokines could enhance iNOS production in EIU is not known. It is known that activated macrophages release tumor necrosis factor-α and interleukin-1 in the presence of endotoxin and that these cytokines may be inducers of iNOS expression.[13] However, in EAU, expression of iNOS in the class II molecule–positive macrophages was primarily seen in the retina, even though the uveal tract was similarly infiltrated by the macrophages. Such a differential expression may be caused by higher levels of cytokine released in the retina compared with that in the uveal tract. A previous report indicated that mRNA expression of IFNγ and iNOS was increased in the whole eyeball in EAU.[12]

The preferential expression of iNOS-positive macrophages in the retina also suggests either that stimulatory factors of iNOS exist in the retina or that there are inhibitory factors of iNOS in the uvea and in the limbal conjunctiva. TGF-β has been found to suppress NO release by macrophages and other cells.[14–18] In vivo, TGF-β is widely distributed in the retina, particularly in the photoreceptor layer, rather than in the iris and ciliary body.[19,20] Such a distribution pattern is expected to suppress the expression of iNOS in the retina, whereas the present results disclosed a preferential expression of iNOS in the retina and a scarcity of expression in the uvea and the conjunctiva. Such data suggest that TGF-β may not play a critical role in the modulation of iNOS expression in the macrophages infiltrating the retina in EAU.

The differential expression of iNOS in the inflammatory phagocytes in the retina compared with the uvea suggests that local factors are required for NO generation by the macrophages. The present in vitro studies reveal that soluble proteins such as S-antigen and IRBP could be local factors in the retina upregulating iNOS expression and NO release. It is plausible that other soluble proteins also may induce iNOS expression in the macrophages. Absence of such proteins in the uvea, limbus, and conjunctiva may explain the scarcity of iNOS-expressing macrophages in these inflamed tissues, despite the expression of class II molecules by such cells. Similar to the present results, thyroid autoantigens were recently found to induce expression of iNOS in monocytes isolated from patients with Graves' disease.[21] However, whether the retinal proteins can directly, or through cytokines, increase expression of iNOS is not clear and needs further investigation.

Coexpression of cNOS and iNOS was noted in the retinal microvascular endothelia, whereas endothelial cells in large vessels expressed cNOS only. In the early stages of EAU development, the infiltrating cells around the vessels were iNOS−/ED1+. As the inflammation progressed, the migrated ED1+ macrophages expressed iNOS, particularly at the outer retina, where S-antigen and IRBP are primarily localized. These findings also suggest the role of these autoantigens in the induction of iNOS within the activated macrophages.

The present study shows that iNOS and its mRNA can be induced in the RAW 264.7 cell line when exposed to S-antigen and IRBP. We also found that iNOS mRNA was not induced in these cells when exposed to MBP, one of the main proteins

consisting of myelin and a fairly small molecule (14 kDa) compared with S-antigen (48 kDa) and IRBP (130 kDa). A recent study has shown that phagocytosis of myelin, a receptor-mediated process, by rat peritoneal macrophages is poorer than that by microglia, but it can be increased in the presence of IFNγ or opsonized myelin. Such a phagocytic process could generate NO and could be inhibited by TGFβ.[22] It is conceivable that cytokines could enhance phagocytosis of MBP by microglia and macrophages in EAU and lead to release of NO.[22] Compared with this study our negative data may be because of our use of the purified MBP rather than myelin extract and because there was no exposure to opsonized MBP in the cells we used. These factors could have resulted in the absence of phagocytosis of MBP by macrophages and subsequent nitrite production.

In conclusion, the present study shows that in EAU, macrophages are an important source of NO production. iNOS was preferentially expressed in the macrophages infiltrating the retina but scarcely in the uvea. The retina-specific proteins were found to stimulate iNOS synthesis in the macrophages. Such findings suggest that in vivo expression of iNOS in the macrophages may require local signals (i.e., activation followed by exposure to cytokines and/or autoantigens).

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E R R A T U M

In the cover caption for the July 1999 issue of *IOVS*, reference to the related article was inadvertently omitted. The caption should have been: Retroillumination image of the posterior capsule after cataract extraction with IOL implant. A computer algorithm has outlined the areas of capsular opacity in red. See article by Friedman et al. on page 1715 of this issue.

The Journal regrets the omission and thanks the authors of this paper for providing the excellent cover for the July 1999 issue of *IOVS*.