

Effects of Nitric Oxide on the Induction of Experimental Allergic Orchitis in Guinea Pig

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ABSTRACT

Background: Production of nitric oxide (NO) by inducible NO synthase (iNOS) has been implicated in the pathology of autoimmune disease. It is unknown whether iNOS expression is increased within testes and whether iNOS and NO have essential roles in the pathogenesis of EAO. **Methods:** EAO was induced in guinea pig testes at 17 days after secondary immunization by administration of crude extract (CE) and purified glycoprotein 1 (GP1) from normal guinea pig testes. iNOS gene expression was assessed by RT-PCR and Northern blot analysis in testes. Localization of iNOS and Mac-1 and the indicator of NO-mediated tissue injury, nitrotyrosine, were detected in the testicular lesion by immunohistochemistry. **Results:** In control testes, inflammation and iNOS gene expression were not detected, whereas, in CE- and GP1-injected testes, inflammation and marked iNOS gene expression were evident at day 17 after secondary immunization. Immunohistochemistry of Mac-1 showed the colocalization with iNOS protein and nitrotyrosyl proteins in intertubules, suggesting that NO produced by infiltrated macrophages may be involved in inflammatory lesions of intertubules. Intraperitoneal administration of aminoguanidine significantly prevented EAO with reduction of inflammation, iNOS expression and nitrotyrosine formation. **Conclusion:** These results suggest that NO production by macrophages may be important in the pathogenesis of CE- and GP1-induced EAO. Furthermore, this study demonstrated the therapeutic potential of iNOS inhibitor in the treatment of inflammatory and autoimmune mediated-diseases. (*Immune Network* 2004;4(2):108-115)

Key Words: Autoimmune disease, peroxynitrite, nitrotyrosine, aminoguanidine

Introduction

Experimental allergic orchitis (EAO) is a well-established model of an autoimmune disease characterized by inflammation of the seminiferous tubule, generally accompanied by aspermatogenesis (1), that closely resembles the human disease, clinically and pathologically. EAO is induced in the guinea pig following immunization with highly purified testes acrosomal glycoprotein (2). Delayed type hypersensitivity (DTH) response against testicular antigens has been described both in EAO and infertile patients with a history of mumps orchitis and azoospermia (3,4). CD4⁺Th1 cells and their cytokines such

as interleukin-2 (IL-2) and interferon- γ (INF- γ) might be implicated in the pathogenesis of testicular autoimmunity. In particular, INF- γ is a powerful activator of CD8 cytotoxic T cells and macrophages, and these cells also induce major histocompatibility complex (MHC) class II antigen (5,6). Moreover, locally produced IFN- γ might exert inhibitory effect on testicular function, as shown by its ability to impair sperm motility and to decrease testosterone production by Leydig cells in response to luteinizing hormone (LH) (7). Although INF- γ is essential for the development of the murine EAO (8), the immunopathogenic mechanism responsible for this autoimmune process is not fully understood.

Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase (NOS) family of enzyme, is a highly reactive free radical involved in physiologic homeostasis and immune response. Increasing evi-

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dences suggest that NO is also involved in the inflammatory and autoimmune-mediated disease processes (9). The inducible NOS (iNOS) were first isolated from murine macrophages but have since been shown to be expressed in many different cell types following exposure to immunologic stimuli and inflammatory cytokines. iNOS is induced as a first line of defense, mediating the nonspecific cytotoxicity of macrophages against invaded pathogens (10,11) or tumor cells (12). However, the high levels of NO produced by iNOS may also damage surrounding healthy tissues either directly or by the generation of cytotoxic products such as peroxynitrite (13). Peroxynitrite (ONOO⁻) is formed by the diffusion-limited reaction of superoxide and NO *in vivo* and modifies tyrosine residues in proteins to form nitrotyrosine. It therefore has been suggested that peroxynitrite is responsible for an inflammatory damage attributed to NO (14). Increased NO production has been associated with a range of inflammatory and autoimmune-mediated disease processes including rheumatoid arthritis (15), experimental allergic encephalomyelitis (EAE) and experimental allergic uveoretinitis (EAU) (16,17).

Recent studies using competitive inhibitors of NOS have greatly advanced in our understanding of the role of NO in the pathogenesis of several diseases. Animals treated with NOS inhibitors developed significantly less inflammation not only in the acute inflammatory response but also in the chronic inflammatory response. Inhibition of NO production by NOS inhibitor prevented arthritis (18), ileitis (19) and EAE (20), further suggesting a role for NO in the pathogenesis of autoimmunity.

In this study, we showed the first *in vivo* evidence that infiltrated macrophages into the testes of crude extract (CE) or purified glycoprotein 1 (GP1)-treated guinea pig produced NO, which is implicated in the pathogenesis of EAO through peroxynitrite formation.

Materials and Methods

Animals. Male adults syngenic Denver strain 13 guinea pigs at 8–10 weeks of age weighting 350 to 500 g were used. The original stock of the guinea pig strains was purchased from the Jackson laboratory (Bar Harbor, ME, USA). All of the animal experiments were carried out in accordance with institutional guidelines for animal care.

Antigen preparation. A highly purified GP1 isolated from normal guinea pig testes was used in immunization to animals. The isolation procedures have been reported by Hagopian et al. (21). In brief, the testes were mixed with chilled methanol and homogenized. And two volumes of chilled chloro-

form were added and aqueous layer was filtered on double-thickness Whatman no.41 paper. For acid extraction, the residue from the defatting process was suspended in 20 to 50 volumes of H₂O adjusted to pH 3.0, and the residue was removed by centrifugation. The filtrate was adjusted to pH 6.0 with concentrated ammonium hydroxide and then, precipitated by addition of solid ammonium sulfate to 85% saturation. Trichloroacetic acid (50%) was added to solubilized ammonium sulfate precipitate to final concentration of 5%, and centrifuged and resuspended in water. The supernatant fluid was dialyzed and lyophilized. Lyophilized fraction was resuspended in water and performed to Bio-Gel P-2 column with a bed volume 5 to 10 times. Protein and glycoprotein were detected by monitoring at 280 nm and 220 nm. A Bio-Gel A-1.5-m column was equilibrated with 0.1 M ammonium acetate (pH 7.0). Further purification of the glycoprotein fraction was achieved by isoelectrofocusing. Preparative isoelectrofocusing was performed in a 440-ml LKB electrofocusing column by using a linear sucrose density gradient (0 to 50%) containing 6% ampholytes, pH 3 to 6. As a final purification step following electrofocusing, the fractions containing glycoproteins were pooled and applied to Model 491 Prep Cell (Bio Rad) at 12 watts constant power. Cooling the lower buffer and running the gel in the cold room helped to maintain the biological activity of proteins. The samples were applied to the gels in 10% glycerol and overlaid with buffer, and the migration of protein run in Ornstein-Davis gels at 12 watts. An elution flow rate of 0.75–1.0 ml/min was adopted. Fractions were collected following elution of the ion front.

Experimental allergic orchitis (EAO). To induce EAO, guinea pigs were immunized by subcutaneous (s.c.) inoculation at the four footpads with 20 µg CE or 20 µg GP1 in a 0.1 ml emulsion with an equal volume of phosphate buffered saline (PBS) and complete Freund's adjuvant (CFA) supplemented with killed *Mycobacterium tuberculosis*, strain H37Ra (Sigma chemical Co., MO, USA), at a concentration of 5 mg/ml. A total of 0.05 ml was injected into each four footpads (n=10, respectively). In control experiments guinea pigs received only CFA or PBS (n=5, respectively). After 8 days from primary immunization, the animals were boosted with 20 µg of CE and GP1 in a 0.1 ml emulsion with an equal volume of PBS and CFA. After 17 days from secondary immunization, the animals were sacrificed and then the experiment was performed.

***In vivo* blocking of NO production.** After guinea pigs were immunized with CE and GP1, to block NO production, guinea pigs were injected intraperitoneally with aminoguanidine (Sigma Chemical Co., MO, USA)

dissolved in PBS at doses 100, 200 and 400 mg/kg every two day from day 0 until the day they were killed, i.e, day 25 (n=10, respectively). A similar dose has previously been shown to prevent increased NO production in vivo and successfully ameliorate disease (22). Controls were injected intraperitoneally with PBS only (n=5).

Histology. Guinea pig testes were removed, fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin-eosin for histologic evaluation.

Preparation of primer. Primer sequences were designed on the basis of Gene Bank Data (accession number M87039). Primers were chemically synthesized by using DNA synthesizer (Pharmacia, Ltd., USA) and purified by using NAP-10 column (Pharmacia, Ltd., USA) Primer sequences of purified iNOS3 were as follows: sense oligonucleotide was 5'-CAGAAGCA GAATGTGACCATC-3' and antisense oligonucleotide was 5'-CTTCTGGTCGATGTCATGAGC-3'.

RNA extraction and Northern blotting. Total RNA (25µg) was prepared by using the LiCl-urea method (23), subjected to electrophoresis in 1.2% agarose-formaldehyde gels, and transferred to nylon membrane by capillary action in 20X SSC (1X SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.2). After prehybridization, these membranes were hybridized with random [α -³²P]dCTP-labeled probes having specific activity of 1 to 5×10⁸ cpm/µg in 10% dextran sulfate, 50% formamide, 4X SSC, 1X Denhardt's solution, and 10µg/ml salmon sperm DNA for 24 hr at 42°C. Then, these membranes were washed, dried, and examined by autoradiography.

Reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription of RNA was carried out in a 20µl of final volume containing 2.5µl of 10 mM dNTP mix, 4µl of 5×RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 5 mM MgCl₂), 2µl of random primer (25 pmoles/25µl), 1µl of RNase inhibitor (20 U/ml), 3µl (1µg/ml) of denatured total RNA (70°C, 5 min), 6.5µl of DEPC-treated water. After adding 1µl of reverse transcriptase (200 U/ml) the mixture was spun down, and incubated at 37°C for 1 hr. The reaction was heated at 95°C for 5 min, and then quickly chilled on ice. PCR was performed by using capillary thermal cycler (DeaHan Medical Co., Ltd, Seoul, Korea). The synthesized cDNA (2µl) was mixed with 1µl of dNTPs (2.5 mM), 1µl of 10× PCR buffer (100 mM Tris-Cl, 30 mM MgCl₂, 2.5 mg/ml BSA), 1µl of primer pair (10 pmole/10µl), 5µl DEPC-treated distilled water, and 1µl of Taq polymerase (0.2 U/µl). After an initial incubation at 94°C for 20 sec., denaturation at 94°C for 5 sec., annealing at 55°C for 5 sec., elongation at 72°C for 10 sec., and post denaturation at 94°C for 20 sec., were

carried out. The number of cycle was 45. The PCR products from tissue were separated by electrophoresis.

Immunohistochemistry. Paraffin-embedded testis sections (4µm thick) were prepared and stained with DAKO kit (DAKO Co., Denmark) using the labelled step-tavidin-biotin (LSAB) method. Endogenous peroxidase activity can be quenched by first incubating the specimen for 5 min in 3% hydrogen peroxide. Nonspecific staining was blocked by 5 min incubation with blocking reagents. The specimen was then incubated with an characterized and diluted polyclonal anti-iNOS antibody (1 : 100) (Santacruz Biotechnology, CA, USA) or polyclonal anti-nitrotyrosine antibody (1 : 100) (Upstate Biotechnology Incorporated, VA, USA), followed by sequential incubation with biotinylated link antibody and peroxidase-labeled streptavidin for 10 min. The specimen was stained by incubation with substrate chromogen solution, and then counterstained with hematoxylin. The expression level of iNOS-stained cells at light microscopy was compared in different specimens by three separate observers.

Statistical analysis. Group differences in a number of inflammatory foci and a number of immunohistochemically positive cells were analyzed by the unpaired nonparametric Mann-Whitney two sample test and all values were expressed as mean±S.D. The differences between groups were considered to be significant at $P < 0.05$.

Results

Induction of EAO by the injection of CE or GP1 and histologic evaluation of EAO. The CE and GP1 were used for EAO induction. It has been reported that GP1 elicited EAO in guinea pig at 9µg and thus proved to be a highly effective antigen (21). At a dose 9µg, however, some mild infiltration of inflammatory cell was observed. Thus we injected with PBS, CFA, 20µg CE or 20µg GP1 as primary and secondary immunogens. All 10 guinea pigs treated with GP1 developed EAO. Similarly, CE-treated guinea pigs were shown to induce EAO in all 10 animals. To examine the histologic evaluation of EAO, tissue sections were taken from the testes at day 17 after secondary injection and were stained with hematoxylin and eosin. Control testes injected with PBS or CFA did not develop EAO and show any signs of inflammation in the intra- and intertubules. Moreover, it was maintained normal seminiferous tubules containing mature sperms in the intratubular lumen (Fig. 1A, B). But 12 (80%) and 13 (86.6%) of respective 15 CE- and GP1-treated testes developed EAO. Infiltrated cells, primarily small lymphocytes and macrophages, were seen in perivascular and inter-

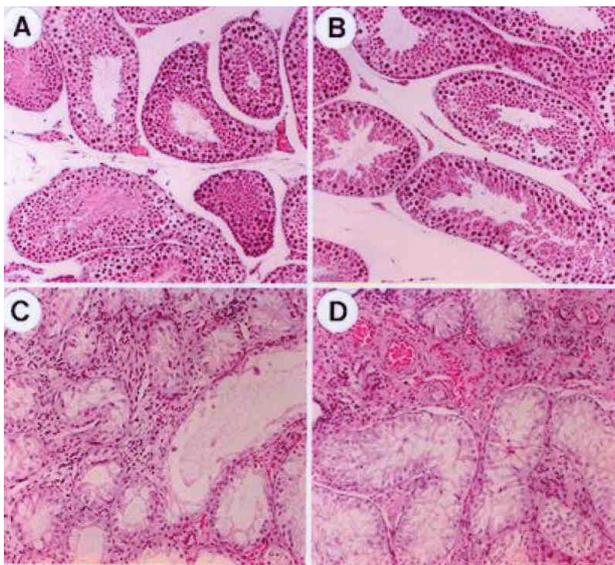


Figure 1. Histopathology of EAO in guinea pig testes. Guinea pigs were primarily immunized with PBS (A), CFA (B), CE (C), and GP1 (D) and after 8 days animals were injected with the same secondary immunogens as primary ones. Testes were collected for histopathology 17 days after secondary immunization. These results are representative of the changes seen in all animal testes and the sections of testes were embedded in paraffin and stained with H & E. (magnification $\times 100$)

tubular location. It has been shown that spermatogenesis was eventually blocked by the invasion of these inflammatory cells into the tubules, showing an essentially empty seminiferous tubules with only bordering Sertoli cells remaining intact (Fig. 1C, D). These histological appearances of the testes represent typical of the well characterized features of EAO. *Expression of iNOS mRNA in EAO following injection with CE or GP1.* To determine whether the induction of EAO by GP1 is correlated with iNOS mRNA expression during the development of EAO, we examined iNOS mRNA expression in the testes. Total RNAs extracted from testes of PBS-, CFA-, CE-, GP1-injected animals were subjected to reverse-transcriptase PCR and Northern blot hybridization. In PBS- and CFA-injected control testes, iNOS mRNA was not detectable by RT-PCR (Fig. 2A, lane 2 and 3). In contrast, iNOS mRNA expression was readily detected in CE- or GP1-injected testes (Fig. 2A, lane 4 and 5). However, there was no difference in iNOS mRNA expression between CE- and GP1-treated testes. It was previously reported that induction of EAO by GP1 was virtually identical to those produced by whole testes crude extract and purified acrosomal protein 1 (24).

Also, we examined iNOS mRNA contents by Northern blot with radiolabeled cDNA probe of iNOS. On the basis of Northern blot hybridization

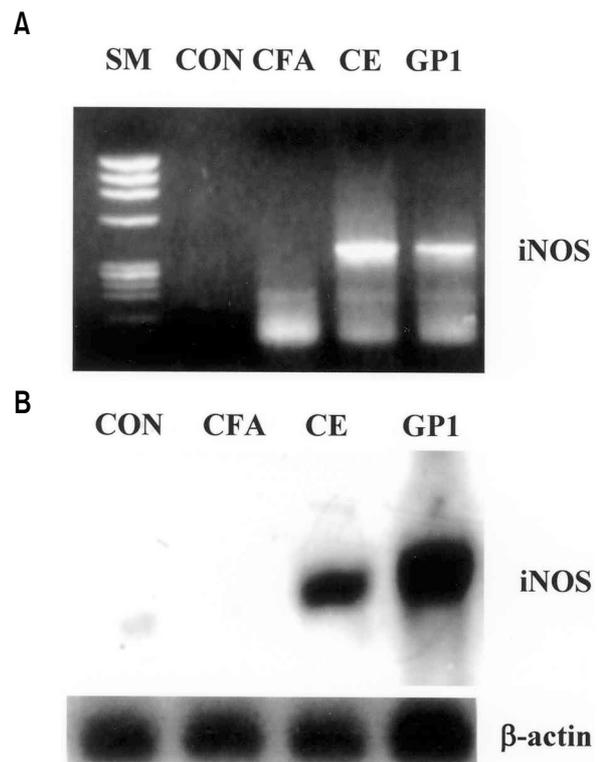


Figure 2. Effects of CE and GP1 treatment on iNOS gene expression in the testes during EAO. Guinea pigs were immunized as described in Fig.1. Testes were collected 17 days after secondary immunization and then, total RNAs were prepared. iNOS gene expression was analyzed by RT-PCR (A) using a 560 base primer to a conserved region of iNOS and Northern blot hybridization (B) using a [32 P]dCTP-radiolabeled cDNA probe of macrophage iNOS. Bands corresponding to iNOS were detected in testes from every EAO-induced guinea pig. These results are representative of the changes seen in all animal testes (SM indicate size marker of ϕ X174/HaeIII).

data, CE and GP1 autoantigens induced iNOS mRNA expression (Fig. 2B, lane 3 and 4), but injection with either PBS or CFA had no effect on the expression of iNOS mRNA (Fig. 2B, lane 1 and 2).

Immunohistochemical examination of iNOS in EAO. To examine the localization of iNOS enzyme throughout the development of EAO, testes from guinea pigs injected with PBS, CFA, CE or GP1 were immunostained with antibodies to iNOS and Mac-1 macrophage marker. iNOS was not detected in the PBS- and CFA-injected testes (Fig. 3A, B). Immunostaining for iNOS was also negative in the preclinical stages of EAO (day 8 postimmunization) and there were no signs of inflammation in intertubular spaces (data not shown). Since iNOS expression is abundant in macrophages after stimulation with immunologic stimuli, we examined whether macrophages were

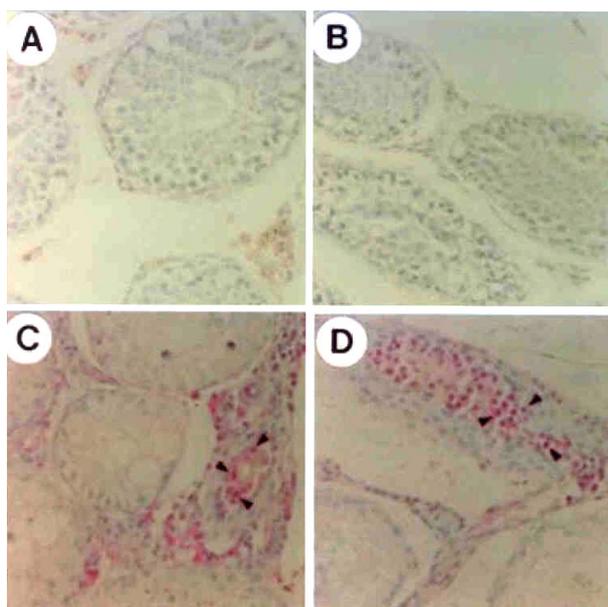


Figure 3. Immunohistochemical localization of iNOS in the testes during EAO. Guinea pigs were immunized as described in Fig. 1. Sections of paraffin-embedded testes from a normal (A) and CFA-injected (B) guinea pigs. Control testes showed only background staining for iNOS (A and B, magnification $\times 100$). Guinea pigs were immunized with $20\mu\text{g}$ of CE (C) and $20\mu\text{g}$ GP1 (D), respectively. CE- and GP1-injected testes showed iNOS protein expression throughout the intertubular spaces (C and D, arrowhead, (magnification $\times 200$). iNOS was detected in testes from every EAO-induced guinea pig. These results are representative of the changes seen in all animal testes and the data represent the results of three similar immunohistochemical experiments.

infiltrated into the testes of EAO, using antibody for Mac-1 macrophage marker. Infiltrated macrophages were not detected in the control testes (Fig. 4A, B). At day 17 postimmunization, infiltration of inflammatory cells in CE- and GP1-injected testes was evident and iNOS enzyme was strongly detected in the perivascular, intertubular, and rarely intratubular spaces (Fig 3C, D). The pattern of staining for iNOS was similar to that for the Mac-1 positive macrophages (Fig. 4C, D).

Immunohistochemical localization of nitrotyrosine in EAO. Since it was found previously that peroxynitrite formed by interaction of NO with superoxide plays a key role in pathogenesis of inflammatory autoimmune disease such as experimental allergic encephalomyelitis (EAE) (16), we suspected that peroxynitrite might be produced via NO generation and induce EAO in animal model. To confirm this hypothesis, we assessed nitrotyrosine by immunohistochemistry. Formation of nitrotyrosine was detected in CE- and GP1-injected testes and the positive staining for nitrotyrosine was particularly detected in perivascular and intertubules (Fig. 5C, D). In contrast, nitro-

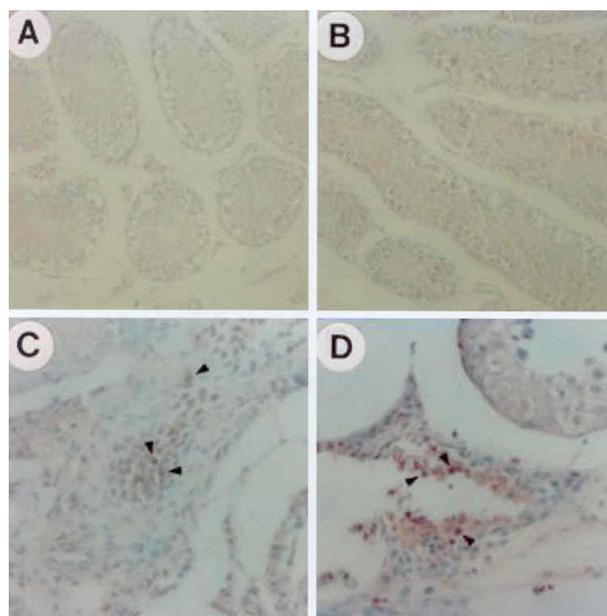


Figure 4. Immunohistochemical localization of Mac-1 in the testes during EAO. Guinea pigs were immunized as described in Fig.1. Section of paraffin-embedded testes from normal (A) and CFA-injected (B) guinea pigs testes showed only background staining for Mac-1. Guinea pigs were immunized with $20\mu\text{g}$ CE (C) and $20\mu\text{g}$ GP1 (D), respectively. A large proportion of Mac-1 positive macrophages were infiltrated in intertubular spaces of CE- and GP1-treated testes (C and D, arrowhead). Mac-1 was detected in testes from every EAO-induced guinea pig. These results are representative of the changes seen in all animal testes and the data represent the results of three similar immunohistochemical experiments. (magnification $\times 200$).

tyrosine was not detected in PBS- and CFA- injected testes. Fig. 3, 4 and 5 show the colocalization patterns of iNOS, Mac-1 and nitrotyrosine stainings in perivascular and intertubules in EAO.

Effects of aminoguanidine on EAO. iNOS was only detected in areas of inflammation in CE- and GP1-injected testes, suggesting that NO production may be directly involved in EAO development. To determine whether the administration of aminoguanidine (AG: $100\sim 400\text{ mg/kg}$), a selective inhibitor of iNOS, affects the pathogenesis of EAO, we immunized guinea pigs with AG for 25 days and then examined inflammation, iNOS expression and nitrotyrosine formation. Testes from 10 high dose AG-treated animals (200 mg/kg and 400 mg/kg , respectively), 10 low dose AG-treated animals (100 mg/kg) and 5 PBS-treated animals obtained on day 17 after secondary immunization were studied. Treating guinea pigs with AG suppressed EAO development in 5, 8 and 8 of 10 animals (100 , 200 and 400 mg/kg respectively), whereas in the PBS-treated group, all animals developed EAO ($p < 0.005$). Fig. 6 shows the effect of AG treatment (400 mg/kg) on the development

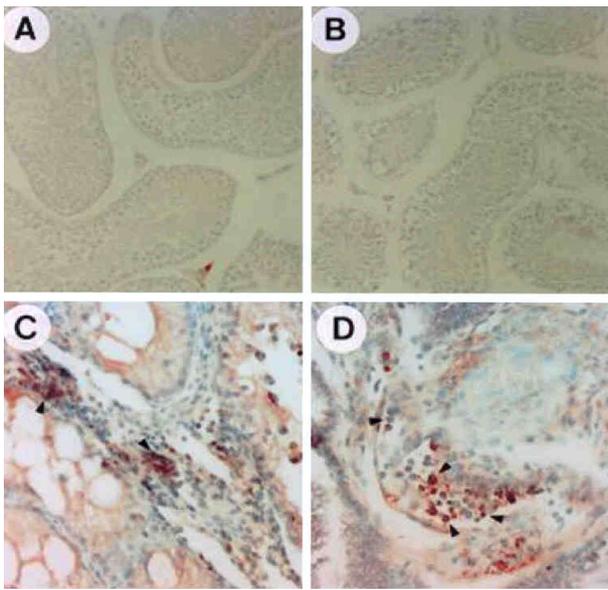


Figure 5. Immunohistochemical localization of nitrotyrosine in the testes during EAO. Guinea pigs were immunized as described in Fig. 1. Section of paraffin-embedded testes from a normal (A) and CFA-injected (B) guinea pigs testes showed only background staining for nitrotyrosine (A and B, magnification $\times 100$). Guinea pigs were immunized with 20 μ g CE (C) and 20 μ g GP1 (D), respectively. Nitrotyrosine was detected in the intertubular space of CE- and GP1-treated testes (C and D, arrowhead, magnification $\times 200$). Nitrotyrosine was detected in testes from every EAO-induced guinea pig. These results are representative of the changes seen in all animal testes and the data represent the results of three similar immunohistochemical experiments.

of GP1-induced EAO. The area of inflammatory lesions was significantly decreased (Fig. 6A, compared to Fig. 1C, D). Also, smaller number of macrophages (Fig. 6C, compared to Fig. 4C, D) were iNOS and nitrotyrosine positive in guinea pigs testes that had been administered AG than in those that had not been administered AG (Fig. 6B, D, compared to Fig. 3, 5). Table I shows that inflammation, iNOS expression, and nitrotyrosine formation were markedly reduced in all the AG-treated groups compared to PBS-injected group. The results of the study demonstrated that NO production by iNOS expression directly attributed to the pathogenesis of EAO.

Discussion

It was previously reported that the glycoproteins, GP1 and GP4, localized in the outer acrosomal surface were used as an aspermatogenic antigen and induced immunopathologic events (2). In this study, we used CE and GP1 for induction of EAO. Induction of inflammation elicited by CE and GP1 was observed, showing intertubular infiltration of mononuclear cells and destruction of germinal cells (Fig. 1). In the model of EAE and EAU, CD4⁺ T

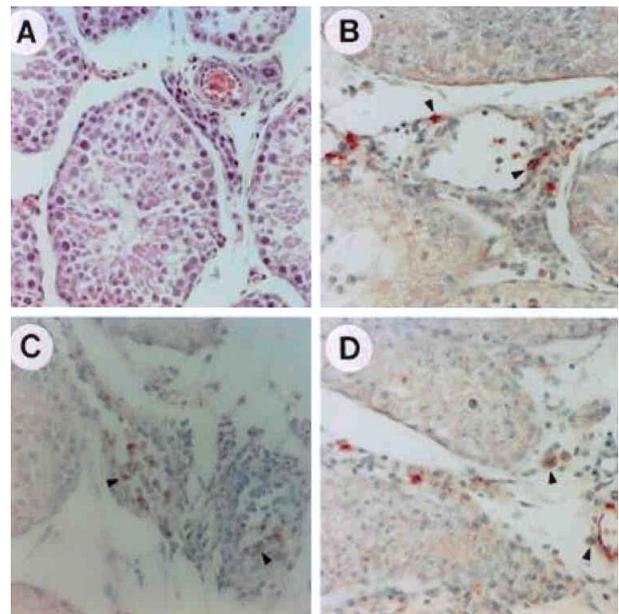


Figure 6. Effects of aminoguanidine treatment in GP1-induced EAO. Guinea pigs were immunized with GP1 (20 μ g) antigens and then, administered with AG (400 mg/kg) every two days for 25 days. Testes were collected for histopathology 17 days after secondary immunization. The sections of testes were embedded in paraffin. (A) Only focal infiltration of inflammatory cells is seen in intratubules, and intact seminiferous tubules are seen (H & E). Immunohistochemical staining for iNOS (B) and nitrotyrosine (D) shows in some positive focally infiltrating macrophages staining for Mac-1 (C). These results are representative of the changes seen in all animal testes and the data represent the results of three similar immunohistochemical experiments. (magnification $\times 200$).

Table I. Effect of AG on EAO

	PBS	GP1 (20 μ g)		
		AG (100 mg/kg)	AG (200 mg/kg)	AG (400 mg/kg)
Inflammation*	9.0 \pm 1.7	7.3 \pm 2.6	3.6 \pm 1.9	2.6 \pm 1.4
iNOS**	95.0 \pm 21.0	68.0 \pm 18.0	60.0 \pm 12.0	55.0 \pm 11.0
Nitrotyrosine**	64.0 \pm 16.0	32.0 \pm 15.0	26.0 \pm 13.0	22.0 \pm 10.0

*Data are expressed as mean \pm SD of the number of inflammatory foci in each section, consisting of at least 20 intertubular mononuclear cells. **Data are expressed as mean \pm SD of the number of positive immunostaining cells in each inflammatory foci, consisting of at least 20 intertubular mononuclear cells ($P < 0.05$). The testes tissue was obtained 17 day after 2^o immunization.

cell-mediated disease, macrophages are known to play a key role in the induction of autoimmune disease

(25-27). Using the model of CE- or GP1-induced EAO as used in this study, we observed the infiltration of macrophages in the interstitium of testes by detecting Mac-1 immunohistochemically (Fig. 4). However, the actual effector function of these macrophages, apparently central to development of autoimmune diseases such as EAO, is not yet defined.

The free radical NO is considered a major mediator in autoimmune reactions and mediates damage in inflammatory tissues (28), when activated macrophages to produce high levels of NO following induction of iNOS. In addition, expression of iNOS has been found in demyelinating lesions of multiple sclerosis and spinal cords of EAE (16) and uveitic eye of EAU (17).

The aims of this study were to demonstrate the importance of NO in the pathogenesis of EAO elicited by CE and GP1. Thus, by using reverse transcription polymerase chain reaction and Northern blot hybridization techniques, we for the first time revealed iNOS gene expression in the CE- or GP1-treated guinea pig testes but iNOS was not expressed in non-treated guinea pig testes (Fig. 2A, B). To further address the issue of the potential role of iNOS as a source of NO in this model, we performed iNOS immunohistochemistry. As a result, iNOS was detected predominantly in macrophages infiltrated into the intertubular spaces, as confirmed by Mac-1 immunohistochemistry, during the inflammatory stages of EAO (Fig. 3). Our observation supports the notion that NO is involved in inflammation and dysfunction in guinea pig testes with CE- and GP1-induced EAO. The results obtained in this study supply evidence that induction of iNOS in infiltrated macrophages is a critical event in the guinea pig model of EAO.

There is considerable evidence from *in vitro* experiments that iNOS-derived NO can modulate the cytokine response of macrophages, T cells, endothelial cells and fibroblasts. This might be due to its capacity to activate and inactivate ion channels, G protein, protein kinase, redox sensitive kinase, and transcription factors (29,30). NO also regulates lymphocytes proliferation and activation, which play a crucial role in the actiology of EAE (31). NO has the capacity to suppress INF- γ production by activated lymphocytes (32), whereas IL-10 and IL-12 are enhanced.

Peroxynitrite may exert its toxic effect as a factor in the pathogenesis of autoimmune diseases, such as EAE and EAU, consistent with the association of the iNOS induction. And the detection of peroxynitrite formation is through nitration of molecules such as tyrosine or possibly cysteine. Nitrotyrosine staining was virtually absent in control-noninflammatory testes. On the other hand, nitrotyrosine in CE- and

GP1-treated testes was detected in intertubular spaces (Fig. 5). The colocalization of iNOS and peroxynitrite suggests that peroxynitrite or other nitrating species generated from NO are formed in this model of EAO and may contribute to block spermatogenesis.

The efficacy of AG was initially reported that AG inhibits the development of clinical signs of adoptive EAE transferred in SJL mice and confirmed the beneficial effect of AG in adoptively transferred EAE in Lewis rats (33) and decrease the severity of the pathophysiological sequelae of excess NO production in uveitis (34) and EAE (31). NO was found to play an important part in the elimination of infiltrating inflammatory cells from lesions in the central nervous system in EAE (35). Evidence from our study supports the notion that the inhibition of iNOS by AG correlates with the inhibition of tissue inflammation, iNOS induction and nitrotyrosine formation induced by CE and GP1 administration. However, AG treatment did not completely abolish inflammation in this EAO model, but significantly reduced it and the number of infiltrating inflammatory cells was quite small in the AG-treated group (400 mg/kg, Fig. 6). Also, treatment with AG resulted in decreased induction of iNOS dose-dependently. This might be explained that AG contributes to induction of interleukin-10 (IL-10) (20). IL-10 is a product of Th2 cells and it suppresses secretion of proinflammatory cytokines by Th1 lymphocytes such as INF- γ (36), a known inducer of iNOS. Therefore, decreased INF- γ production may lead to down-regulated iNOS induction. In addition, we found decreased production of the NO metabolite, nitrotyrosine by AG treatment. Lower dose of AG (100 mg/kg) was less effective (Table I).

In conclusion, iNOS was highly expressed in testicular cells within the inflammatory lesion in CE- and GP1-induced EAO. The selective iNOS inhibitor AG prevented the progression of testicular lesion. Therefore, it is suggested that NO participated in the pathogenesis of testicular injury in this model of autoimmune orchitis. Agents that inhibit iNOS activity or expression offer great potential as a new therapeutic approach to these autoimmune diseases.

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