Cutting Edge: Antisense Knockdown of Inducible Nitric Oxide Synthase Inhibits Induction of Experimental Autoimmune Encephalomyelitis in SJL/J Mice

Minzhen Ding, Ming Zhang, Joyce L. Wong, Norma E. Rogers, Louis J. Ignarro and Rhonda R. Voskuhl

*J Immunol* 1998; 160:2560-2564; ;
http://www.jimmunol.org/content/160/6/2560
Cutting Edge: Antisense Knockdown of Inducible Nitric Oxide Synthase Inhibits Induction of Experimental Autoimmune Encephalomyelitis in SJL/J Mice

Minzhen Ding, Ming Zhang, Joyce L. Wong, Norma E. Rogers, Louis J. Ignarro, and Rhonda R. Voskuhl

We used an antisense oligodeoxynucleotide (ODN) complementary to inducible nitric oxide synthase (iNOS) to inhibit experimental autoimmune encephalomyelitis (EAE) in female SJL/J mice, an animal model for multiple sclerosis. The antisense ODN was administered intraventricularly to mice daily for 10 days beginning at the time of adoptive transfer of myelin basic protein-specific T lymphocytes. The antisense ODN treatment significantly reduced the clinical score of EAE and blocked iNOS mRNA and protein synthesis, as well as iNOS enzyme activity within the central nervous system. The levels of nitric oxide and cyclic guanosine monophosphate were also significantly reduced by the antisense ODN treatment. Neither sense nor random ODN affected clinical EAE or iNOS expression. Moreover, the protein and enzyme activity level of constitutive neuronal nitric oxide synthase was not affected by the antisense ODN. Thus, we have shown that the iNOS antisense ODN specifically blocked iNOS expression and ameliorated the induction of EAE. The Journal of Immunology, 1998, 160: 2560–2564.

Nitric oxide mediates functions as diverse as vasodilation (1, 2), neurotransmission (3), and immune-mediated cytotoxicity (4). The synthesis of NO is catalyzed by NO synthases of which there are at least three isoforms (5–7). Inducible nitric oxide synthase (iNOS)-derived NO has been implicated in the pathogenesis of multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system (CNS) characterized by autoimmune-mediated destruction of myelin. A significant increase in NO production and a higher concentration of neopterin, a precursor of a cofactor for iNOS, have been found in the serum and cerebrospinal fluid of MS patients (8, 9). Also, mRNA levels of iNOS and NOS catalytic activities have been shown to be elevated in MS lesions (10), and an elevated titer of Abs against conjugated nitro-saminio acids in MS sera has been reported (11). In addition, cytokines such as IFN-γ and TNF-α that induce NO induction are thought to play an immunopathogenetic role in MS (12). Finally, in animals with experimental autoimmune encephalomyelitis (EAE), a model for MS, increased levels of NO and iNOS mRNA have been detected in the CNS (13–15), and the administration of iNOS inhibitors or NO scavengers has inhibited disease (16, 17).

Selective blockade of iNOS production has been considered as a novel therapeutic approach in MS. Unfortunately, current generation of substrate-based pharmacologic iNOS inhibitors lack high selectivity for different isoforms of NO synthases (18). In contrast, antisense oligodeoxynucleotide (ODN) knockdown strategy has the unique potential to be a highly selective tool for arresting iNOS mRNA translation into functional enzyme. Extensive studies have demonstrated that a short synthetic ODN, complementary to a specific mRNA, can enter cells by receptor-mediated endocytosis and stop protein translation either by blocking the translocation of ribosomes (19, 20) or by destroying the target mRNA through a RNase H-mediated degradation process (21). Furthermore, recent in vivo studies have suggested that it is a viable and powerful technique for treating systemic disease (22).

Our previous studies have shown that an antisense ODN, complementary to mouse iNOS mRNA, significantly inhibited LPS- and IFN-γ-induced iNOS and NO production in adult SJL mouse glial cultures (23), thereby demonstrating the efficacy of the antisense ODN. Additionally, we have shown that highly susceptible female SJL mice with severe EAE expressed higher levels of iNOS and NO in the CNS than less susceptible male mice with mild EAE (15, 24). In this report, we aimed to specifically inhibit iNOS in the CNS of female SJL mice through the intraventricular administration of iNOS antisense ODN, hypothesizing that this might ameliorate EAE.
Materials and Methods

Animals

Female SJL/J mice, age 8 wk, were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained according to National Institutes of Health guidelines.

Immunization and adoptive EAE induction

Adoptive EAE was induced through the adoptive transfer of 18.5-kDa myelin basic protein-specific T lymphocytes as described (15, 24).

Clinical assessment of disease

Animals induced to develop EAE were examined daily for clinical signs of disease and graded in a blinded fashion on a scale of 0 to 5 as follows: 0, normal; 1, limp tail; 2, difficulty righting; 3, partial paralysis of one hind limb; 4, complete paralysis of at least one hind limb; 5, moribund.

The oligodeoxynucleotide sequences

The iNOS antisense ODN sequence was a 21-base phosphorothioate-oligodeoxynucleotide (S-ODN) corresponding to bases 1 through 21 of the translation initiation site of mouse iNOS mRNA. The corresponding sense S-ODN and a random S-ODN sequence, each with the same base composition as the antisense S-ODN, were used as control ODNs. No sequence homology exists between these sequences and the rest of the known mouse sequences within GenBank. The ODN were synthesized by Oligos Etc. (Oregon, OR). Antisense: 5'-CAAGCCATGTCTGAGCTTTG-3'; sense: 5'-CAAAGTCTCAGACATGGCTTG-3'; random: 5'-GTCAAAGTAACCTTTGAGTTCC-3'.

Intraventricular injection of antisense ODN and preparation of tissue samples

SJL mice (6–8 wk old) underwent Metofane (Mallincrodt Veterinary, Mundelein, IL) inhalation anesthesia. Stereotaxic operations were performed in a Kopf small animal stereotaxic instrument (David Kopf, Tujunga, CA). The calvarium was exposed; a hole <0.5 mm in diameter was drilled with a dental drill at the coordinate of 1 mm lateral to the sagittal suture, 1 mm caudal to the bregma, and 2.5 mm below the surface of the skull. A 31-gauge cannula with dummy cannula (Plastic One, Roanoke, VA) was implanted at this site. The ODNs were dissolved in sterile 0.9% saline and injected into the ventricle through the cannula using a Hamilton syringe. Injections were administered from the day of T cell transfer to the appearance of severe clinical EAE in the control groups, 10 days posttransfer. Mice were killed, and the coordinates were checked with dye injection. The brains were removed, frozen in liquid nitrogen, and stored at −70°C.

Northern blot analysis for iNOS mRNA

Poly(A)+ RNA (5 μg/lane) from mouse cerebellum was extracted and subjected to Northern blot analysis as described (15). A [32P]dATP-labeled mouse macrophage iNOS cDNA fragment (obtained from Dr. D. Geller, University of Pittsburgh, Pittsburgh, PA) was used as the probe. A β-actin probe (Sal/BamHI 451-bp restriction fragment) was used to assure equal loading.

Determination of nitric oxide (NO2−) by Griess reaction and nitrate reductase

The NO2− level in the homogenates of mouse cerebellum was determined by measuring the levels of nitrite (NO2−) and nitrate (NO3−), stable oxidation products of NO. The concentrations of NO2− and NO3− were measured by Griess reaction as described (25).

Determination of cyclic guanosine monophosphate (cGMP) by radioimmunoassay

Mouse cerebellar tissues were homogenized in 1 ml of cold assay buffer (50 mM sodium acetate with 0.1% sodium azide, pH 6.2). The homogenates were centrifuged at 14,000 × g for 10 min at 4°C, and 100 μl of supernatant were then assayed in duplicate using cGMP radioimmunoassay as described (25, 26). Rabbit antiserum against cGMP was provided by Dr. S. Murphy (University of Iowa, Iowa City, IA).

Western blot analysis for iNOS protein

Total proteins from mouse cerebellar tissues were harvested and homogenized with 50 mM Tris buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 1 mM pepstatin A, and 2 mM leupeptin at 0–4°C. Homogenates were centrifuged at 20,000 × g for 60 min at 4°C. The protein concentration in the supernatant of the cellular homogenates was determined by the Bradford Coomassie brilliant blue method (Bio-Rad, Richmond, CA). Bovine serum albumin was used as the standard. The tissue lysates were diluted and subjected to Western blot analysis as described (25). The blot was sequentially probed with a polyclonal rabbit Ab specific for mouse iNOS peptide (1:500) and a mAb specific for mouse neuronal NOS (nNOS, 1:1000; Alexis, San Diego, CA).

Determination of iNOS enzyme activity by citrulline assay

The iNOS activity was measured by determining the conversion of L-[14C]arginine to L-[14C]citrulline as described (25, 27).

Statistical methods

Statistical analysis was performed using StatView SE+Graphics (Abacus Concepts, Berkeley, CA). This program performs ANOVA with factorial or repeated measures and expresses significance by Fisher’s pairwise least significant difference test.
Results
Reduction in the clinical severity of EAE by iNOS antisense ODN
To determine whether iNOS antisense ODN could affect the induction of clinical EAE, ODNs were delivered into mouse cerebrospinal fluid via daily intraventricular injection (10 μg/μl/injection/day) beginning on the day of adoptive transfer and continuing until saline-treated control mice demonstrated paralysis, day 10 posttransfer. Additional control groups included mice injected with sense or random ODNs. In the first experiment, there were three animals in each group. All mice in all control groups demonstrated signs of EAE by day 10, whereas none of the antisense ODN-treated mice developed paralysis. The clinical score of the antisense ODN-treated group was significantly lower than that of the saline-treated group (p < 0.001, Fig. 1A). In the second experiment, the sample size was increased to six mice per group. With this greater number of mice in each group, a minority of mice in the antisense-treated group demonstrated mild clinical sign of EAE (two of six mice, grade = 2), whereas the majority remained without sign of EAE (four of six mice, grade = 0). The mean clinical score of the antisense ODN-treated group was again significantly lower than that of the saline-treated control group (p < 0.01, Fig. 1B). In both experiments, the mean scores of mice treated with the sense and random ODNs were no different from those of mice treated with saline. No abnormal side effects of the therapy were observed.

Inhibition of iNOS mRNA and protein expression by iNOS antisense ODN
To determine the effect of iNOS antisense ODN treatment on iNOS mRNA and protein expression, Northern and Western analyses were performed on cerebellar tissues derived from mice with representative scores from each group (control groups, grade 3; antisense group, grade 0). Northern blots of poly(A)^+ RNA were probed with a murine macrophage NOS probe, and the expected 4.1-kb band was detected in control treated mice but not in iNOS antisense ODN-treated mice (Fig. 2A). Western blots of total cerebellar proteins using mouse iNOS-specific Ab demonstrated a reduction in iNOS protein level in the iNOS antisense ODN-treated mice as compared with mice in the three control groups. Importantly, the nNOS-specific Ab detected bands of equal intensity between all groups (Fig. 2B).

Inhibition of iNOS enzyme activities by antisense ODN treatment
To determine the effect of iNOS antisense ODN on iNOS enzyme activity, we assessed the ability of cytosolic iNOS, extracted from mouse cerebellum, to convert l-arginine to l-citrulline (27). Three

FIGURE 2. Inhibition of iNOS mRNA and protein in mouse cerebellum by iNOS antisense ODN. A, Northern blot. Electrophoreses with 5 μg of poly(A)^+ RNA per lane was performed. β-Actin mRNA was used as a loading control. B, Western blot analysis. One hundred micrograms of total protein were loaded per lane. The blot was probed with Abs specific for murine iNOS and nNOS. The NOS proteins were visualized by chemiluminescence. Northern and Western data were derived from a single mouse, with three different mice examined for a total of three repeated experiments with representative data shown. –, saline; R, random ODN; S, iNOS sense ODN; A, iNOS antisense ODN.

FIGURE 3. Inhibition of iNOS enzyme activity in mouse cerebellum by iNOS antisense ODN. Cytosolic iNOS was extracted from cerebellum homogenates and analyzed for its efficiency in converting l-arginine to l-citrulline. Means ± SD are shown for one of three repeated experiments, each on a single mouse. The antisense treatment significantly reduced iNOS enzyme activity compared with other control groups (*p < 0.05).
mRNA, protein, enzyme activity, and NO and cGMP production. In contrast, neither sense nor random ODN affected EAE induction or iNOS expression. Importantly, the level of nNOS protein was not affected by antisense ODN treatment, indicating high selectivity of the iNOS antisense ODN. Together these data have demonstrated that iNOS antisense ODN treatment ameliorated EAE by specifically inhibiting iNOS expression.

Despite the success of using antisense ODN to inhibit gene expression, the precise molecular mechanisms involved are still not fully understood. The inhibition of target mRNA translation by bound antisense ODN may involve the ubiquitous enzyme RNase H, which hydrolyzes the RNA of the RNA-DNA duplex, leading to a decrease in target RNA levels (21). Alternatively, the formation of the RNA-DNA duplex may block ribosome binding and translocation along the mRNA, thereby preventing the continued synthesis of the target protein (20). In the present study, Northern blot analysis indicated a significant decrease of iNOS mRNA in the CNS of mice treated with iNOS antisense ODN. These results support the mechanism of RNase H-mediated degradation of mRNA in DNA/RNA hybrids. Nevertheless, Western blot analysis revealed a significantly reduced level of iNOS protein, thereby demonstrating that the translation of iNOS protein was specifically blocked by the antisense treatment.

Although we have observed that continuous administration of iNOS antisense ODN significantly inhibited the induction of EAE, it remains to be determined whether clinical signs of EAE will appear following termination of iNOS antisense ODN treatment. This will be important in advancing toward the use of antisense ODN as a novel therapeutic approach in MS.

Finally, antisense ODN knockdown strategy may be implemented to examine the pathogenic role of the expression of other genes in EAE. In contrast to gene knockouts, the outcome of knockdown strategy is not complicated by the generation of functionally redundant pathways that may arise developmentally.

Acknowledgments
We thank Dr. Sean Murphy at Iowa University (Iowa City, IA) for the kind gift of cGMP Ab and help on cGMP radioimmunoassay.

References
circulating antibodies directed against conjugated S-nitrosocysteine. J. Neuroimmunol. 60:117.