The effects of NO synthase inhibitors on murine collagen-induced arthritis do not support a role of NO in the protective effect of IFN-γ

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Abstract: DBA/1 mice deficient in expressing the interferon-γ (IFN-γ) membrane receptor (IFN-γR KO mice) are more susceptible to collagen-induced arthritis (CIA) than wild-type mice, indicating that endogenous IFN-γ plays a protective role in the pathogenesis of CIA. In IFN-γR KO mice, nitric oxide (NO) production during CIA is impaired. Because NO is known to exert immunosuppressive and anti-inflammatory effects in certain model systems, the protective effect of IFN-γ might be mediated by NO. Here, we tested in wild-type mice whether inhibition of NO production by metabolic inhibitors, aminoguanidine (AG) and L-N-(1-iminoethyl)lysine (L-NIL), could mimic the ablation of the IFN-γ receptor. A high-dose regimen of AG supplied in the drinking water inhibited NO production, disease development, and anticollagen antibody production but was also associated with transient body weight loss. At a dose and time regimen that still inhibited NO production but did not cause body weight loss, AG failed to affect disease scores. Treatment with L-NIL, which more specifically than AG affects inducible NO production, caused a slight increase in anticollagen antibody production although not significantly affecting disease occurrence. These data indicate that the diminished capacity of the IFN-γR KO mice to produce NO following immunization with collagen is unlikely to account for their higher susceptibility to CIA. J. Leukoc. Biol. 68: 119–124; 2000.

Key Words: interferon-γ · nitric oxide · collagen-induced arthritis

INTRODUCTION

Collagen-induced arthritis (CIA) is a chronic inflammatory arthropathy that is induced in susceptible rodents by immunization with collagen type II in complete Freund’s adjuvant. CIA in DBA/1 mice has clinical, histological, and immunological features similar to human rheumatoid arthritis and has therefore been regarded as a relevant model for this disease.

The role of interferon (IFN)-γ in CIA was recently addressed by studies on DBA/1 mice deficient in expressing the ligand-binding peptide of the IFN-γ membrane receptor (IFN-γR KO mice). It was found that these mutant mice are highly susceptible to CIA; the disease was significantly accelerated and the incidence of arthritis was found to be higher than in wild-type mice [1, 2]. A similarly increased susceptibility to CIA was found in wild-type mice that had been pretreated with IFN-γ neutralizing antibodies. These results point to a disease-protective role of endogenous IFN-γ in the pathogenesis of CIA.

Recently, we reported evidence that this protective effect derives from inhibition of myelopoiesis, in particular of reduced generation of Mac-1+ cells [3].

IFN-γ is well-known to be a potent inducer of nitric oxide (NO), in vitro and in vivo [reviewed in ref. 4]. IFN-γ-induced NO production has been found to suppress mitogen-induced T-cell proliferation [5] and has been proposed to account for the biological activity of “suppressor” macrophages [6]. Furthermore, we recently presented evidence for a protective role of IFN-γ-induced NO in the anti-CD3-induced cytokine release syndrome [7]. In view of these effects of NO, we investigated whether the IFN-γ-mediated protection in CIA might be mediated by the production of NO. We reasoned that if this was the case, treatment of mice with inhibitors of NO synthase should increase the incidence and/or the severity of CIA.

In the present study, aminoguanidine (AG) was used, because it has been shown to be an inhibitor of NO synthase (NOS) [8–10]. The effect of L-N-(1-iminoethyl)lysine (L-NIL), which has recently been described as a more selective iNOS inhibitor [11], was also tested in our CIA model. We found that, whereas treatment with these inhibitors did reduce NO production in vivo, it failed to augment disease parameters.

MATERIALS AND METHODS

Mice and experimental conditions

The generation and the basic characteristics of the mutant mouse strain (129Sv/Ev) with a disruption in the gene coding for the α-chain of the IFN-γR have been described [12]. These IFN-γR KO mice were back-crossed with DBA/1 wild-type mice for 10 generations to obtain the DBA/1 IFN-γR KO mice used in the present study. All mice were bred in the Experimental Animal Centre of the University of Leuven. The experiments were performed in 8- to 12-week-old mice, but in each experiment, the mutant and wild-type mice were

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age-matched with a 5-day limit. The male and female ratio was kept between 0.8 and 1.3 in each experimental group.

Induction and clinical assessment of arthritis

Native chicken collagen type II (Elastin Products, Owensville, MO) was dissolved in 0.05 M acetic acid at 2 mg/ml by stirring overnight at 6°C and emulsified in an equal volume of complete Freund’s adjuvant (CFA) containing 1.5 mg/ml heat-killed Mycobacterium butyricum (Difco, Detroit, MI). Mice were sensitized with a single 100 μl intradermal injection of the emulsion at the base of the tail. From day 10 postimmunization, mice were examined daily for signs of arthritis. The disease severity was recorded following a scoring system for each limb: 0, normal; 1, redness and/or swelling in one joint; 2, redness and/or swelling in more than one joint; 3, redness and/or swelling in the entire paw and 4, deformity and/or ankylosis.

Measurement of body weight

At regular time points, the weight of the individual animals was measured. The change in body weight was calculated by comparison with the initial weight at the beginning of each experiment; i.e., [weight on indicated time point (−day) x] minus initial weight/initial weight.

NOS inhibitors and treatment schedule

AG (Sigma Chemical Co., St. Louis, MO) and L-NIL (Bachem, Bubendorf, Switzerland) were the NOS inhibitors used in this study. AG and L-NIL were administered in the drinking water, given ad libitum at doses indicated in Results.

Determination of plasma nitrite levels

NO production was determined by measuring its stable degradation products, nitrate and nitrite. Nitrate was detected after reduction to nitrite using nitrate reductase. Total nitrite was then determined spectrophotometrically, as previously described [13]. Briefly, blood samples were taken from the orbital sinus and were collected in tubes containing heparin. Plasma was centrifuged within the beginning of each experiment; i.e., [weight on indicated time point (−day) x] minus initial weight/initial weight.

Measurement of serum anti-CII antibodies

Blood samples were taken from the orbital sinus and were allowed to clot for 1 h at room temperature and at 4°C overnight. Individual sera were tested for antibodies directed to chicken CII by enzyme-linked immunosorbent assay (ELISA), as described [1]. A serial two-fold dilution series of a purified standard preparation was included in each experiment to allow for calculation of the antibody content. The standard material was purified by affinity chromatography from pooled sera obtained from several IFN-γR KO and wild-type arthritic mice, and contained 0.5 mg/ml immunoglobulin G (IgG).

RESULTS

Plasma nitrite levels in wild-type and IFN-γR KO mice before and during CIA development

Groups of IFN-γR KO and wild-type mice were immunized with CII in CFA on day 0 and were evaluated for clinical signs of arthritis. As was the case in our previously reported experiments [1], the mutant mice developed signs of arthritis more rapidly and with a higher frequency than wild-type counterparts. Plasma samples from both types of mice were collected before and at two time points after immunization and were analyzed for the presence of total nitrite. The levels on day 0 were 35.4 ± 6.3 μM (n=3) and 99.6 ± 20.3 μM (n=3) for the IFN-γR KO and wild-type mice, respectively. On days 8 and 57 postimmunization, we found plasma nitrite levels of 43.6 ± 16.4 μM (n=3) and 30.2 μM (n=2) for the mutant mice, and 123.8 ± 8.1 μM (n=3) and 110.8 ± 20.8 μM (n=3) for wild-type animals. Thus, at all time points, the total plasma nitrite levels were lower in IFN-γR KO mice than in corresponding wild-type ones.

Effect of peroral AG on the course of CIA

To assess whether the increased sensitivity to CIA in IFN-γR KO mice might be related to their decreased production of NO, wild-type mice were treated with AG, an inhibitor of NO synthase.

In a first experiment, AG was administered in the drinking water at a dose of 2% (wt/vol); this route of delivery and dose of AG had been shown to be effective in abrogating NO production in mice infected with M. tuberculosis [14]. Wild-type mice not treated with AG and IFN-γR KO mice were included for comparison. All mice were immunized with CII in CFA and were evaluated daily for signs of arthritis. The cumulative incidences, nitrite levels, anti-CII antibody serum levels and body weight are shown in Figure 1. Arthritis signs started to appear in IFN-γR KO mice as early as day 17, against day 45 in the corresponding wild-types (Fig. 1A). Final cumulative incidences of arthritis in IFN-γR KO and wild-type mice were 70% and 46%, respectively. The increased sensitivity of the mutant mice was associated with decreased NO production, as evident from significantly lower nitrite levels at all investigated time points (Fig. 1B). A similar decrease in NO production was observed in wild-type mice that were treated with the 2% AG regimen. However, clinical symptoms of arthritis were not detectable at all in these mice. Because the severity of CIA has been found to depend, in part, on the extent of the humoral immune response against collagen II [15], CII-specific antibodies were analyzed in the sera of the mice. As evident from the data shown in Figure 1C, the anti-CII antibody response was significantly reduced in the wild-type mice that had received the 2% AG treatment.

However, we also noted a rather severe body weight loss in the AG-treated mice (Fig. 1D). Although equal or higher doses have been used in other studies, without any report of toxicity [14, 16], the treatment appeared to be toxic in DBA/1 mice. Therefore, in a next experiment, we reduced the dose AG in the drinking water to 0.5%. Control groups of IFN-γR KO and wild-type mice were included. The incidences, plasma nitrite levels, and anti-CII antibody serum levels are shown in Figure 2. It can be seen that the NOS inhibitor significantly down-regulated the plasma nitrite levels (Fig. 2B). IFN-γR KO mice developed signs of arthritis from day 17 onward, whereas symptoms of disease appeared in the wild-type control mice on day 41 after immunization (Fig. 2A). The mutant mice reached an incidence of 60%, against 33% in the wild-type controls. Disease development in 0.5% AG-treated wild-type animals was indistinguishable from that in untreated controls [mean day of onset±SEM: 50±2 for the AG-treated mice (n=10) vs. 47.7 ± 4.4 for the control group (n=9)]. The humoral response against collagen also remained unaffected by the
treatment (Fig. 2C). Thus, in contrast to a dose of 2% AG, a dose of 0.5% could not change the development of arthritis nor the anti-CII antibody profile, although nitric oxide plasma levels were clearly inhibited.

We also examined whether arthritis development would be affected by AG, given at 4 mg intraperitoneally, a regimen that has been found suitable in other studies on the role of NO in experimental autoimmune diseases [17, 18]. However, in our model,
this treatment did not significantly affect plasma nitrite levels nor did it influence the course of the disease (unpublished results).

A final experiment was done to test the effect of high-dose AG treatment using a time schedule that might avoid toxicity. In the previous experiments, body weight loss was most obvious during the first 3 weeks of AG treatment and, thus, coincided with the induction phase of CIA. Reduced food and water intake might have been responsible for the observed effects on CIA. Therefore, in the next experiment, a 2% AG pretreatment was initiated 20 days before immunization to allow the mice to adapt to the treatment. Figure 3 shows body weight, cumulative incidence, nitrite levels, and development of humoral immunity. Determination of plasma nitrite levels indicated that with this altered treatment regimen, NO production during the disease was effectively reduced (Fig. 3C). Body weight loss was evident only in mice that received AG in the drinking water (Fig. 3A). However, they had recovered and reached their original body weight after 20 days, i.e., the time point of immunization. Pretreatment with 2% AG did not result in a complete inhibition of CIA, as was the case when treatment was started on day 0 (Fig. 1A). However, the onset of the disease was still significantly delayed [mean day of onset ± SEM: 53±3 (n=9) vs. 34±2 (n=9) for wild-type controls; P<0.002 by Student’s t-test, comparing AG-treated mice with control]. A possible explanation for this retardation of CIA development might be the effect of AG on anti-CII antibody production. Indeed, the mice that received AG displayed a clearly diminished humoral response against CII on day 33 postimmunization (Fig. 3D). However, at the end of the experiment (day 70), the level of anti-CII antibody of these mice reached a level comparable with that in controls.

**Effect of peroral L-NIL on the course of CIA**

Because AG is known to inhibit not only the IFN-γ-inducible NO synthase but also constitutive NO synthases and certain other enzymes, we tested the effect of a more selective iNOS-inhibitor, L-NIL. A dose of 0.1% in the drinking water was used, because in a preliminary experiment (unpublished results), this appeared to be required to inhibit the production of NO; also, this regimen was not associated with body weight loss.

Three independent experiments were done, the results of which are shown in Figure 4. NO production in the sera of treated animals was significantly decreased at all investigated time points (Fig. 4A). The incidence of arthritis in L-NIL-treated mice followed a time course that was not significantly different from that in controls (Fig. 4B); if anything, the treated mice developed disease more slowly than the controls but reached the same final incidence [13 out of 28 (46.4%) for the L-NIL-treated mice, against 12 out of 30 (40%) for the controls]. Also, the disease scores of affected animals did not differ (unpublished results). Finally, analysis of the anti-CII antibodies revealed a slight but statistically significant up-regulation (on average 1.7-fold) of the humoral response against CII in the L-NIL-treated mice (Fig. 4C).

Thus, whereas blocking of IFN-γ resulted in increased disease manifestations and reduced anti-CII antibody formation, blocking of NO production by L-NIL had the opposite effects, indicating that endogenous NO production is unlikely to be involved as a mediator of the protective effect of endogenous IFN-γ.

**DISCUSSION**

The purpose of the present study was to test the hypothesis that induction of NO production by IFN-γ might explain the protective effect of endogenous IFN-γ in certain models of autoimmune disease in mice. In murine EAE and EAU, blockage of endogenous NO production was indeed found to be associated with augmented disease manifestation [19–25], suggesting that NO,
Contrary to what our hypothesis predicted, AG at the higher dose (2%) in the knock-out animals. Therefore, it seemed a plausible hypothesis that NO would mediate the protective effect of IFN-γ in CIA.

First, it was verified that enhanced CIA occurrence in IFN-γR KO mice was associated with reduced plasma levels of nitrite, the terminal break-down product of NO. When this was found to be the case, we tested in wild-type mice whether two inhibitors of NO synthase, AG and L-NIL, could mimic the effect of IFN-γ ablation. AG is known to inhibit not only the IFN-γ-inducible NO synthase [8–10] but also constitutive NO synthases [28–30] and certain other enzymes [reviewed in ref. 31]. Three parameters were studied: (1) incidence, kinetics, and severity of CIA; (2) plasma level of NO derivatives; and (3) serum levels of antibodies against CII. In wild-type mice in which CIA was induced, 0.5% or 2% AG in the drinking water caused a reduction in nitrite levels. Contrary to what our hypothesis predicted, AG at the higher dose (2%) completely blocked occurrence of CIA in these mice, and the lower (0.5%) dose had no effect. Significantly, however, the 2% regime caused severe but transient body weight loss and reduction in anti-CII antibody levels, raising concern that the blocking of CIA was a result of pharmacological effects unrelated to inhibition of NO synthases. When the 2% AG regime was started earlier so that the mice had adapted to the treatment and had regained weight by the time of auto-immunization, the treatment still reduced nitrite levels, as well as antibody levels and CIA scores. However, the effect was much less pronounced than when AG treatment was started at the time of immunization, again strengthening the presumption of an aspecific or even toxic effect.

The second inhibitor tested, L-NIL, is known to more specifically address inducible NOS [11, 32]. Its administration in the drinking water at 0.1% inhibited circulating NO release, although not causing signs of toxicity. This treatment had little effect on the course of CIA. If anything, it was associated with slightly increased anti-CII antibody levels and resulted in a trend toward delayed disease development.

In summary, our study shows that neither AG nor L-NIL, when administered in regimens that inhibit circulating NO release without causing gross physiological changes, mimics the effect of IFN-γ ablation on the pathogenesis of CIA. Whereas IFN-γ ablation was associated with increased disease signs, treatment with the inhibitors tended to retard disease appearance. Therefore, we conclude that lower levels of systemic NO release during CIA in IFN-γR KO, as opposed to wild-type controls, cannot account for the higher disease scores in the knock-out animals.

It remains possible but unlikely that the NOS inhibitors were less efficient than the IFN-γR knock-out mutation in lowering NO production levels at certain local sites, and thereby failed to achieve a comparable effect on critical steps in the pathogenesis, such as the generation of effector or regulator cells.
Therefore, it may be of interest to see how a knock-out mutation of the iNOS gene affects the susceptibility of mice to CIA (J. S. Mudgett, personal communication, reporting no difference between iNOS knock-out and wild-type mice) [26]. The role of the decreased anti-CII antibody response, as a possible factor in the increased CIA susceptibility of the IFN-γ KO mice, could not be clarified by the results obtained with NOS inhibitors, because one of the inhibitors (AG) inhibited, whereas the other one (L-NIL) up-regulated the antibody response. A lack of correlation between humoral responses and disease severity has also been noted by others [33, 34].

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