Therapeutic Administration of a Selective Inhibitor of Nitric Oxide Synthase Does Not Ameliorate the Chronic Inflammation and Tissue Damage Associated with Adjuvant-Induced Arthritis in Rats

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ABSTRACT

Up-regulation of the inducible isoform of nitric oxide synthase (iNOS) was determined during the development of adjuvant-induced arthritis in the rat. iNOS enzymatic activity, measured in spleen tissue, appeared and increased coincidentally with the appearance and degree of paw swelling and joint destruction in this arthritis model, when measured on days 0 through 21 subsequent to inoculation of the rats with adjuvant. The increase in enzymatic activity was paralleled by an increase in the plasma nitrite/nitrate (NOx) level and the appearance of immunoreactive iNOS, as measured by Western immunoblot, in the spleens of these rats. Prophylactic administration of N-iminoethyl-L-lysine (L-NIL) completely abolished iNOS activity (plasma NOx elevation) and effectively reduced both the swelling and radiographic changes in the joint tissues of the noninjected paw measured on day 21. However, therapeutic administration of L-NIL beginning on day 14 had no effect on the inflammatory or arthritic changes measured on day 21, even though plasma NOx levels were reduced to that of the naive controls. These results suggest that iNOS may be involved with the initial stages of the immune response to adjuvant injection, but its product, NO, does not mediate the chronic inflammation and joint destruction which occur during the later phase in this model.

Nitric oxide is a gaseous, free radical that mediates several diverse biological events. Being readily diffused through tissues and short-lived because of its high chemical reactivity, NO is eminently suitable for a role as a biological mediator (Stefanovic-Racic et al., 1993). NO is rapidly reduced to nitrate forms in tissues, which are then carried in the blood for elimination in the urine or the gastrointestinal tract (Stichotenoth et al., 1994). It is produced by the enzyme NOS, during the conversion of arginine to citrulline, through the oxidative removal of the terminal guanidino nitrogen of arginine (Kwon et al., 1990). NOS occurs in at least three isoforms. NOS(1) (neNOS), which plays a part in neurotransmission, especially in the central nervous system, or NOS(3) (ecNOS), which is involved in maintenance of vascular tone through the vasodilatory effects of NO, are constitutively active. NOS(2), or iNOS, is only found in tissues after its induction, such as in response to the inflammatory stimuli endotoxin or cytokines (Corbett et al., 1991). Increased production of NO, after iNOS up-regulation has been suggested as an antimicrobial or antitumor mechanism (Stefanovic-Racic et al., 1993; Adams, 1996). In addition, NO has been implicated extensively as a mediator of inflammatory and immunological diseases (Farrell et al., 1992; Ialenti et al., 1993; Jacob et al., 1992; Zheng et al., 1993; Evans et al., 1995). Evidence of this involvement has relied mainly on the determination of increased levels of the NO end product, nitrates (NOx), in various biological fluids. Increased NOx levels have been found in blood serum after injection of mice with endotoxin (Tracey et al., 1995; Florquin et al., 1994), in the plasma and urine of rats during adjuvant-induced arthritis (Stichotenoth et al., 1994; Cannon et al., 1996; Connor et al., 1995; Stefanovic-Racic et al., 1994), and in the serum of rats during allograft rejection (Langrehr et al., 1992). A marker of NO production, 3-nitrotyrosine, has been shown to be increased in the serum and synovial fluids of patients with rheumatoid

ABBREVIATIONS: NO, nitric oxide; NOS, nitric oxide synthase; NOx, total nitrate/nitrate; L- or D-NIL, N-iminoethyl-(L or D)-lysine; L-NAME, Nω-nitro-L-arginine methyl ester; L-NMMA, Nω-monomethyl-L-arginine; RAD, radiographic; DTT, dithiothreitol; FAD, flavin-adenine-dinucleotide; FMN, flavin-mononucleotide; NADPH, β-nicotinamide adenine dinucleotide phosphate; COX, cyclooxygenase; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; LPS, lipopolysaccharide.

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arthritides (Kaur and Halliwell, 1994). Also, NOS inhibitors, such as l-NAME, l-NMMA and aminoguanidine, have been reported to ameliorate a variety of immunologically induced experimental inflammatory diseases in animals, such as inflammatory bowel disease in rats (Miller et al., 1993; Rachmilewitz et al., 1995), adjuvant-induced arthritis in rats (Ialenti et al., 1993; Stefanovic-Racic et al., 1994), carrageenan-induced edema in mice (Ialenti, et al., 1992) and streptococcal cell-wall-induced arthritis in rats (McCartney-Francis et al., 1993). In addition, transgenic mice lacking iNOS exhibited less paw swelling in response to carrageenan injection than wild-type controls (Wei et al., 1995). The selective iNOS inhibitor, L-NIL (Moore et al., 1994), was effective in reducing the paw swelling associated with adjuvant-induced arthritis in rats (Connor et al., 1995).

In this article, we characterize the induction of iNOS during the development of adjuvant-induced arthritis in rats and demonstrate its relationship with the multifaceted clinical aspects of this disease. We have also examined the effects of both prophylactic and therapeutic administration of the selective iNOS inhibitor, L-NIL, on the development of the inflammation and joint damage associated with this model.

Materials and Methods

All animal procedures were approved by the Merck Co. Laboratory Animal Care and Use Committee in accordance with National Institutes of Health and US Department of Agriculture guidelines. Adjuvant arthritis. Female Lewis rats (Charles River, Raleigh, NC), 7.5 weeks old (140–160 g), were weighed, ear marked and assigned to groups of 10 animals. Body weights and paw volumes of both hind feet were taken before injection of adjuvant (day 0). Rats were then anesthetized with an intramuscular injection of a combination of ketamine (87 mg/kg) and xylazine (13 mg/kg) in a total volume of 0.03 to 0.10 ml, and lateral to medial radiographs of the hind paws were taken. Each rat was then injected with adjuvant in the left hind foot. A group of noninjected rats served as normal age-matched controls. On various days after adjuvant injection, body weights and foot volumes were obtained on groups of rats before sacrifice via carbon dioxide. Blood was then collected from the caudal vena cava and placed in heparinized tubes. Plasma was prepared by centrifugation of whole blood for 10 min at 2,000 × g for 1 hr at 4°C, and the protein concentrations of the plasma samples were determined. A linear response was obtained with use of standards consisting of 0 to 200 µM sodium nitrate or sodium nitrite added to 2 µl rat plasma. Conversion of nitrate to nitrite was 90% based on comparison of these standards. The mean ± S.D. coefficient of variation between duplicates was 13.4 ± 9.1% in a typical assay of 25 experimental samples containing 10 to 70 µM nitrate.

Foot volume. Foot volumes were determined by mercury displacement plethysmography. The change in foot volume was calculated as the difference between day 0 and the day of sacrifice. Adjuvant protocol. Adjuvant was made by grinding Mycobacterium butyricum in a mortar and adding to it light mineral oil so that the final concentration was 5 mg/ml. Rats received a subplantar injection of 0.1 ml of adjuvant in the left hind paw by inserting a 25-gauge 0.5-inch needle between the second and third digit into the dorsum of the hind paw.

Radiographic analysis. Lateral radiographic projections of the tarsus of each rat were obtained at time 0 and at 14 and 21 or 22 days postinduction. A Faxitron X-ray system (Hewlett-Packard, Buffalo Grove, IL) with a 0.5-mm focal spot and beryllium window and nonscreen Kodak X-OMAT TL film were used. The focal film distance was 61 cm, and exposures were made during 30 sec at 45 peak keV and 3 mA. All radiographs were evaluated by a board-certified radiologist without knowledge of the assignment of treatment groups. RAD scores were assigned according to an adaptation of a previously described method (Clark et al., 1979). The following radiographic changes were graded numerically according to severity: increased soft tissue volume (0–4), narrowing or widening of joint spaces (0–5), subchondral erosion (0–3), periosteal reaction (0–4), osteolysis (0–4), subluxation (0–5) and degenerative joint changes (0–5). Specific criteria were used to establish the numerical grade of severity for each radiographic change. The maximum possible score per foot was 26.

Plasma NOx assay. Nitrites in plasma were reduced enzymatically to nitrite with nitrate reductase. The total amount of nitrite was then determined by reaction with 2,3-diaminonaphthelene, according to the method of Misko et al. (1993), which was modified by us for use in 96-well plates. Two microliters of plasma was incubated for 5 min at room temperature in a total of 30 µl of 20 mM Tris/HCl buffer, pH 7.6, containing 40 µM NADPH and 14 µM nitrate reductase (Aspergillus niger, Sigma, St. Louis, MO). Fifty micromolar of 0.12 N HCl containing 0.01 mg/ml 2,3-diaminonaphthelene was then added. After 10 min, 10 µl 1.4 N NaOH was added. Fluorescence (excitation: 360 nm/emission: 425 nm) was measured with a microplate fluorometer (Cambridge Technologies, Inc., Watertown, MA). Samples were run in duplicate, and background fluorescence (wells containing no sample) was subtracted from each average reading. The concentration of nitrite in each plasma sample was calculated by comparison of the absorbance of each with that obtained with a set of sodium nitrate standards (0–200 µM) in water, which was run on each plate. The fluorescence value of each nitrate standard dilution was the same when assayed with or without 2 µl rat plasma, after subtraction of the background, which indicates that this small amount of plasma did not affect the assay. A linear response was obtained with use of standards consisting of 0 to 200 µM sodium nitrate or sodium nitrite added to 2 µl rat plasma. Conversion of nitrate to nitrite was >90% based on comparison of these standards. The mean ± S.D. coefficient of variation between duplicates was 9.1% in a typical assay of 25 experimental samples containing 10 to 70 µM nitrate.

Assay for iNOS enzymatic activity. iNOS activity in spleen homogenates was measured by an assay in which the radiolabeled substrate, [3H]arginine, is converted to the radiolabeled product, [3H]citrulline (Robertson et al., 1993), and which was modified by K. R. Silverman and Dr. R. B. Lingham (Merck & Co., Rahway, NJ). Spleens were homogenized for 1 min by a Polytron homogenizer in an ice-cold inhibitor solution (0.2 ml/mg tissue/ml) consisting of 20 mM Tris/HCl buffer, pH 7.5, containing 25 µg/ml each of antipain, aprotinin, chymotrypsin, pepstatin A, leupeptin, phenylmethylsulfonyl fluoride, 2 mM DTT, 5 µg/ml FAD, 100 µM tetrahydrobiopterin and 10% glycerol. The homogenates were then centrifuged at 100,000 × g for 1 hr at 4°C, and the protein concentrations of the supernatants were determined. Biopterin and its metabolites were separated by reverse-phase high-performance liquid chromatography. The homogenates were then centrifuged at 100,000 × g for 1 hr at 4°C, and the protein concentrations of the supernatants were determined. Biopterin and its metabolites were separated by reverse-phase high-performance liquid chromatography. The supernatants were then assayed. Ten microliters of spleen supernatants were incubated for 2 hr at 20°C in 100 µl total volume of 0.1 M Tris/HCl buffer, pH 7.5, containing 5 µM FAD, 5 µM FMN, 2.5 mM DTT, 1 µM tetrahydrobiopterin, 0.5 mg/ml bovine serum albumin, 1 mM EDTA, 60 mM valine, 0.1 mM NADPH and 1 µM arginine containing 0.04 µCi [14C]arginine. Samples were run in triplicate, with or without the inclusion of 0.1 mM l-NAME in the reaction mixture. To separate substrate from product, 200 µl Dowex AG50X8 resin (H+) in 20 mM NaOAc, pH 5.5, containing 1 mM L-citrulline and 2 mM EDTA was added to the reaction mixture for 10 min at room temperature. After centrifugation at 100,000 × g for 10 min at room temperature, an aliquot of each supernatant was incubated with 25 ml/mg with inhibitor solution, they were stored at −70°C until assayed. Ten microliters of spleen supernatants were incubated for 2 hr at 20°C in 100 µl total volume of 0.1 M Tris/HCl buffer, pH 7.5, containing 5 µM FAD, 5 µM FMN, 2.5 mM DTT, 1 µM tetrahydrobiopterin, 0.5 mg/ml bovine serum albumin, 1 mM EDTA, 60 mM valine, 0.1 mM NADPH and 1 µM arginine containing 0.04 µCi [14C]arginine. Samples were run in triplicate, with or without the inclusion of 0.1 mM l-NAME in the reaction mixture. To separate substrate from product, 200 µl Dowex AG50X8 resin (H+) in 20 mM NaOAc, pH 5.5, containing 1 mM L-citrulline and 2 mM EDTA was added to the reaction mixture for 10 min at room temperature. After centrifugation at 100,000 × g for 10 min at room temperature, an aliquot of each supernatant, which contained the reaction product, [3H]citrulline, was mixed with scintillation fluid and dpm were determined in a liquid scintillation counter. The dpm of an aliquot of the original substrate solution were also determined. The dpm of a blank (reagents but no enzyme, which routinely averaged about 7% of the total counts) were subtracted from each sample and the amount of conversion of substrate to product was calculated. The iNOS activity is expressed as picomoles of citrulline produced per milligram of protein per hour, and is described as that activity...
inhibited by L-NAME, but not by EDTA and valine. This amounted to >90% of the activity in these spleen preparations. Turnover of substrate with samples containing the highest amount of iNOS activity was never more than 10% of total. Under the conditions of the assay, 100% of the radioactivity was accounted for in the arginine and citrulline peaks obtained on high-performance liquid chromatography analysis of the reaction mixture when rat liver homogenate was used as the enzyme source. In addition, 100% of the radiolabeled citrulline added to rat liver homogenate under the conditions of the assay was recovered in the citrulline peak on analysis by high-performance liquid chromatography (personal communication, B. Green and S. Grant, Merck & Co., Rahway, NJ).

Western immunoblot analyses. Spleen homogenate supernatants were diluted to the same protein concentration as described for the iNOS enzymatic assay. A Novex PAGE apparatus was used to produce immunoblots of the samples, observing the manufacturer’s methods. Supernatants were diluted 1:1 in SDS sample buffer containing 100 mM DTT (Novex) and placed in a boiling water bath for 10 min. These samples (20 μL) were run on SDS-PAGE with 4 to 12% gels for 90 min at 125 V and transferred to nitrocellulose for 60 min at 100 V. Immunoblots were prepared by sequential incubations with rabbit polyclonal antibody to mouse iNOS (Transduction Laboratories, Lexington, KT) and goat anti-rabbit IgG alkaline phosphatase (Promega, Madison, WI) and developed in BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) substrate (Sigma, St. Louis, MO). Band authenticity was confirmed running an iNOS standard prepared from LPS-activated RAW 264.7 (ATCC TIB71) cells (Transduction Laboratories, Lexington, KT) on the same gel as the spleen samples.

Compound administration. L-NIL, D-NIL or indomethacin were orally administered twice daily, at 8:30 A.M. and 4:30 P.M., in 1 ml 0.5% methocel/water vehicle. Compounds were administered beginning either on the day of immunization with adjuvant (prophylactic regimen), or 14 days later (therapeutic regimen), and continuing until sacrifice of the animals on day 21. On day 21, before compound administration, animals were anesthetized with Metofoe and heparinized blood was obtained by cardiac puncture (16 hr postdose). When they were recovered from the anesthesia, animals were given the final dose of compound and blood was obtained after sacrifice 4 hr later (4 hr postdose). Thus, NOx levels were determined on plasma samples obtained 4 and 16 hr after oral administration of vehicle or compounds. Dose levels of L-NIL were chosen for these experiments with consideration for the ability of this compound to inhibit LPS-induced plasma NOx elevations in rats. This animal model for testing inhibitors of iNOS has been described in detail by Tracey et al. (1995). In our hands, L-NIL administered orally to rats 16 hr before injection of LPS reduced the plasma NOx response with an ED50 of 3 mg/kg and an ED90 of 30 mg/kg. Administration of a single oral dose of 10 mg/kg L-NIL to rats 4 or 16 hr before injection of LPS resulted in, respectively, 93 ± 2% and 63 ± 3% suppression of plasma NOx elevation.

Statistical analysis. Changes in body weight, foot volumes, thymus and spleen weights, as well as plasma NOx levels were analyzed by a two-factor (treatment and time) analysis of variance applied to the change of individuals from base line. RAD scores were analyzed by a two-factor (treatment and time) analysis of variance applied to the rank-transformed scores. The Dunnett’s test was used to compare the effect of treatments with the vehicle-treated or naive control groups. All values are expressed as the mean ± S.D. (n = 10); *P < .05 and **P < .01 compared with the vehicle control group.

Results

Clinical characteristics of the adjuvant model. Swelling with erythema was evident within 1 day in the injected hind paw, which peaked on day 3 and began to decline through day 8 (fig. 1). Little change in volume was noticed through day 8 in the contralateral, noninjected paw, which also exhibited no pathology in radiographs (fig. 2). Rats exhibited a gradual increase in spleen weight compared with naive rat controls during the first 8 days, whereas thymus weights initially dropped, then partially recovered by day 8 (fig. 3). Animals lost weight during the first 3 days after immunization, after which weight gain returned to normal up to day 8 (fig. 4).

A secondary, chronic phase of the inflammatory response began to occur after day 8 after adjuvant injection. During this time, animals exhibited renewed swelling of the injected paw, a progressive swelling in the phalangeal and tarsal joints of the noninjected paws (fig. 1) and swelling in other joint areas such as along the vertebrae of the tail. Also during this time, spleen weight increased more dramatically, thymus weight loss was renewed and total body weight again began to decline (figs. 3 and 4). All these changes occurring during the secondary phase of the adjuvant response appeared to reach maximum and level off between days 15 and 22. Histological sections through the involved, noninjected paws on day 22 showed the presence of a chronic inflammation, with neutrophilic and monocytic infiltration of the subcutaneous tissue (fig. 6).
synovial tissues. Synovial edema, proliferation of synovocytes and fibroblasts, erosion of cartilage, subchondral and metaphyseal bone and pannus invasion of the joint space was present at this time. Increasing osteoblastic proliferation, as well as osteoid formation, was noted on the periosteal surfaces (results not shown). By the 22nd day deformities, especially in the hind paws, were evident on visual inspection of the animals. Arthritic changes were very evident in radiographs of both the injected and the contralateral, noninjected hind paws, as illustrated by the increasing RAD scores of these animals during days 12 through 22 (fig. 2). The incidence of disease in our experiments was 100%.

iNOS up-regulation and expression during adjuvant arthritis. During the first 8 days after immunization with adjuvant, plasma NOx levels were elevated to about twice the normal level of the naive controls (fig. 5). Plasma NOx levels were elevated 4 to 5 times normal during the secondary phase of adjuvant-induced arthritis (days 12–22). iNOS activity, measured by enzymatic assay, was found in the spleens of rats immunized with adjuvant but not in those of naive controls (fig. 5). The amount of NOx found in the plasma paralleled the elevations in iNOS enzymatic activity in the spleens of the rats during the 22-day course of the experiment. Also, immunoreactive iNOS was detected, by analysis of the soluble fraction of splenic homogenates by SDS-PAGE/Western blot, only after immunization of the rats with adjuvant (fig. 6A). Immunoreactive iNOS protein appeared in spleen samples from rats immunized with adjuvant, concurrently with detection of iNOS enzymatic activity and plasma NOx elevations. The iNOS bands, although not subjected to densitometer scanning, were heaviest in the samples containing the highest amount of enzymatic activity (days 12–18). This period coincided with the secondary phase of the disease, when swelling was greatest in the noninjected feet.

Effects of prophylactic administration of l-NIL. Oral administration of l-NIL twice each day during the 21 days of adjuvant-induced arthritis development caused a dose-related reduction in plasma NOx elevations compared with the vehicle control group (table 1). l-NIL administered at 20 mg/kg/day suppressed plasma NOx to the level of the naive controls. Neither the prophylactic nor the therapeutic regi-

Fig. 3. Splenomegaly and thymic involution after immunization of rats with adjuvant. Organs were removed and weighed after sacrifice of animals on the days indicated. Values are the mean ± S.D. (n = 10) for the spleen (△—△) and thymus (■—■) wet weights from naive control rats and spleen (△—△) and thymus (■—■) wet weights from adjuvant-immunized rats.

Fig. 4. Weight loss after immunization of rats with adjuvant. Values are the mean ± S.D. (n = 10) of the total body weights for the naive control group (■—■) and adjuvant-immunized group (●—●).

Fig. 5. Up-regulation of iNOS after immunization of rats with adjuvant. Rats were sacrificed on the days indicated after the injection of adjuvant. Values are the mean ± S.D. (n = 10) for the plasma NOx levels in immunized (■—■) or naive control (■—■) rats and specific iNOS enzymatic activity (●—●) or total iNOS enzymatic activity (△—△) in spleen tissue.

Fig. 6. Western immunoblot analysis of soluble spleen homogenates prepared from rats (A) 0 to 22 days after immunization with adjuvant and (B) on day 21 after immunization with adjuvant, after prophylactic or therapeutic treatment with vehicle or l-NIL, as described in the text.
men of L-NIL administration affected the induction of iNOS in spleen tissues, as measured by Western immunoblot (fig. 6B). The decrease in plasma NOx caused by L-NIL was mirrored by a dose-related decrease in swelling of the noninjected, secondary paw, measured on days 14 and 21. The ID₅₀ values for inhibition of swelling of the secondary paw were calculated to be 7.6 and 12.3 mg/kg/day L-NIL, determined on days 14 and 21, respectively. Maximum inhibition of secondary paw swelling was only slightly reduced in rats that received the highest dose of L-NIL, and the reduction was not statistically significant. Indomethacin treatment also suppressed the changes in spleen tissues, as measured by Western immunoblot (fig. 6B). The decrease in plasma NOx caused by L-NIL was mirrored by a dose-related decrease in swelling of the noninjected, secondary paw, measured on days 14 and 21. 

**TABLE 1**

Effect of prophylactic L-NIL treatment on secondary paw swelling and RAD scores, and plasma NOx levels during adjuvant-induced arthritis in the rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma NOx (day 21)</th>
<th>Change in Volume</th>
<th>RAD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hr postdose</td>
<td>16 hr postdose</td>
<td>Day 14</td>
</tr>
<tr>
<td>Naive</td>
<td>16 ± 5</td>
<td>17 ± 5</td>
<td>62 ± 52</td>
</tr>
<tr>
<td>Vehicle</td>
<td>39 ± 9</td>
<td>56 ± 18</td>
<td>1069 ± 154</td>
</tr>
<tr>
<td>L-NIL (mg/kg/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>38 ± 6</td>
<td>51 ± 9</td>
<td>1148 ± 542</td>
</tr>
<tr>
<td>0.6</td>
<td>31 ± 9</td>
<td>45 ± 9</td>
<td>1032 ± 347</td>
</tr>
<tr>
<td>2</td>
<td>28 ± 8*</td>
<td>36 ± 9**</td>
<td>799 ± 428</td>
</tr>
<tr>
<td>6</td>
<td>16 ± 14**</td>
<td>26 ± 11**</td>
<td>597 ± 206*</td>
</tr>
<tr>
<td>20</td>
<td>10 ± 13**</td>
<td>20 ± 6**</td>
<td>190 ± 170*</td>
</tr>
<tr>
<td>D-NIL (20 mg/kg/day)</td>
<td>44 ± 3</td>
<td>55 ± 9</td>
<td>1367 ± 475</td>
</tr>
<tr>
<td>Indomethacin (1 mg/kg/day)</td>
<td>41 ± 9</td>
<td>49 ± 11</td>
<td>309 ± 88*</td>
</tr>
</tbody>
</table>

* P < .05, ** P < .01 compared with the vehicle control group.

**TABLE 2**

Effect of prophylactic L-NIL treatment on primary paw swelling during adjuvant-induced arthritis in the rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 4</td>
</tr>
<tr>
<td>Naive</td>
<td>9 ± 42</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1503 ± 396</td>
</tr>
<tr>
<td>L-NIL (20 mg/kg/day)</td>
<td>1433 ± 349</td>
</tr>
<tr>
<td>Indomethacin (1 mg/kg/day)</td>
<td>555 ± 183**</td>
</tr>
</tbody>
</table>

* P < .05, ** P < .01 compared with the vehicle control group.

**Effects of therapeutic administration of L-NIL.** Twice daily administration of L-NIL at doses up to 60 mg/kg/day beginning on day 14 did not affect the paw swelling, RAD scores or any other clinical parameters measured, even though plasma NOx levels determined on day 21 were reduced to the background levels of the naive controls (table 4). 

**Discussion**

The model of adjuvant-induced arthritis in the rat has been used for many years for evaluation of anti-arthritic/anti-inflammatory agents (Watnich, 1975; Winder et al., 1969), and is well characterized. In this model, rats develop a chronic swelling in multiple joints, with influx of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling. These inflammatory changes ultimately result in the complete destruction of joint integrity and function in the affected animal. Anti-inflammatory treatments which have been useful for the treatment of human disease (e.g., COX inhibitors, such as indomethacin) ameliorate the joint inflammation in this rat model (Winder et al., 1969; Vaillant and Armand, 1976; Benslay and Nickander, 1982). The rat adjuvant model has also been useful for development of new anti-inflammatory agents, most recently the COX2 inhibitors (Visco et al., 1996). Therefore, we chose this model to examine the role of iNOS in the various aspects of the inflammatory process.

Previous reports have implied that iNOS has a role in the development of inflammation based on the prophylactic effects of relatively nonspecific inhibitors of NOS, such as the arginine analogs, l-NAME (Ialenti et al., 1993) and l-NMMA (Stefanovic-Racic et al., 1994), which serve as competitive substrates for all NOS isoforms, and of the more specific iNOS inhibitors such as L-NIL and aminoguanidine (Connor et al., 1995). Decreasing plasma or urinary NOx levels after administration of these inhibitors correlated with a decrease in inflammatory parameters, such as swelling at the site of inoculation of carrageenan (Ialenti et al., 1992), or immunization with adjuvant (Connor et al., 1995). That inflammation-induced elevations in plasma NOx are a direct result of iNOS up-regulation, and not caused by increases in constitutive activity of eNOS or nNOS, has been demonstrated in genetically altered, iNOS-deficient mice. These mice develop...
and succumb to hypotensive shock in response to endotoxin (LPS) injection without the typical increase in plasma NOx levels seen when LPS is injected into normal wild-type controls (MacMicking et al., 1995). In this paper, we have demonstrated a direct parallel between the development of arthritic symptomology in the rat and the appearance and activity of iNOS. We have also shown that plasma NOx levels provide an accurate measurement of iNOS activity in this model. The clinical manifestations of disease development in the model of rat adjuvant-induced arthritis have already been described in great detail (Pearson, 1963; Chang et al., 1998). We have confirmed and extended these findings by correlating the timing of the inflammatory changes (e.g., paw swelling, RAD score, splenomegaly and thymic involution) with the appearance and enzymatic activity of iNOS. Our results demonstrate that iNOS is indeed up-regulated in this model in a manner consistent with its product, NO, being implicated as an inflammatory mediator, which suggests that this model is useful for the testing of the effects of the selective iNOS inhibitors on inflammation.

Prophylactic treatment of rats with l-NIL was efficacious in preventing much of the inflammation and significantly lessened the joint destruction that occurred during the development of adjuvant arthritis, which confirms an earlier report (Connor et al., 1995). In addition, the degree of efficacy of l-NIL to prevent the inflammatory and arthritic changes was dose related and correlated with the ability of l-NIL to inhibit iNOS activity in this model. However, therapeutic administration of l-NIL, beginning on day 14 and using doses higher than necessary to inhibit arthritic development in the prophylactic protocol, was without effect on paw swelling or the subsequent development of joint tissue destruction. Inflammation continued unabated even though iNOS activity was completely abolished by treatment with l-NIL. These results clearly indicate that the critical time for iNOS involvement in the development of arthritis in this model is during the initial immunization stage after adjuvant injection. This appears to be so even though we found that iNOS activity increased more dramatically and prospectively during the secondary stage of the disease (days 8–22). The iNOS of neutrophils, chondrocytes and synovocytes is known to be up-regulated during inflammation (Stefanovic-Racic et al., 1993). Thus, the relatively large increase in plasma NOx levels during the later stage of adjuvant-induced arthritis may be indicative of the activity of these cells, as well as the joint damage and remodeling which appears during this stage of the disease. Even so, this damage was not ameliorated by therapeutic administration of an iNOS inhibitor in our studies, whereas this same inhibitor was effective when administered prophylactically. That iNOS inhibition affects mainly the initial cellular response to adjuvant injection has been suggested by others (Connor et al., 1995). In addition, results of investigations of the cellular responses during rat adjuvant arthritis, and of the cells capable of being used for passive transfer of the disease to naive host animals, suggest that a unique subpopulation of CD4-bearing T lymphocytes found in lymph and spleen tissues are responsible for initiation of the arthritis and are capable of conferring arthritogenicity to a host animal upon donation (Van De Langerijt et al., 1994; Taurog et al., 1983). This conclusion has been reinforced further by the report that injection of rats with antibody to αβ T-cell receptor depletes T cells and suppresses adjuvant-induced arthritis only when given before or at the time of peak swelling of the secondary paw, and it is not effective when injected later (Yoshino et al., 1990). Thus,

### TABLE 3
Effect of prophylactic l-NIL treatment on whole-body and organ weights during adjuvant-induced arthritis in the rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Change in Body Weight</th>
<th>Spleen</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
<td>Day 21</td>
<td>Day 21</td>
</tr>
<tr>
<td>Naive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>−16.2 ± 5.2</td>
<td>−13.6 ± 7.1</td>
<td>734 ± 108</td>
</tr>
<tr>
<td>l-NIL (mg/kg/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>−10.6 ± 3.0</td>
<td>−8.0 ± 7.6</td>
<td>855 ± 114*</td>
</tr>
<tr>
<td>0.6</td>
<td>−9.8 ± 7.0</td>
<td>−8.4 ± 8.0</td>
<td>828 ± 110</td>
</tr>
<tr>
<td>2</td>
<td>−12.1 ± 6.9</td>
<td>−13.3 ± 4.3</td>
<td>762 ± 116</td>
</tr>
<tr>
<td>6</td>
<td>−7.4 ± 7.3</td>
<td>−2.5 ± 7.3*</td>
<td>717 ± 73</td>
</tr>
<tr>
<td>20</td>
<td>5.7 ± 11.3**</td>
<td>14.5 ± 13.3**</td>
<td>636 ± 52</td>
</tr>
<tr>
<td>Indomethacin (1 mg/kg/day)</td>
<td>−2.6 ± 10.7*</td>
<td>0.7 ± 6.2*</td>
<td>670 ± 141</td>
</tr>
</tbody>
</table>

* P < .05, ** P < .01 compared with the vehicle control group.

### TABLE 4
Effect of therapeutic l-NIL treatment on adjuvant-induced arthritis in the rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma NOx</th>
<th>Change in Volume</th>
<th>RAD Score</th>
<th>Change in Body Weight</th>
<th>Thymus Weight</th>
<th>Spleen Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µl</td>
<td>Primary paw</td>
<td>Secondary paw</td>
<td>Primary paw</td>
<td>Secondary paw</td>
</tr>
<tr>
<td>Naive</td>
<td>26 ± 7</td>
<td>57 ± 59</td>
<td>58 ± 58</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>49.4 ± 11.2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>76 ± 10</td>
<td>1844 ± 412</td>
<td>1280 ± 495</td>
<td>13.2 ± 3.3</td>
<td>9.6 ± 4.5</td>
<td>13.9 ± 4.5</td>
</tr>
<tr>
<td>l-NIL (60 mg/kg/day)</td>
<td>26 ± 6**</td>
<td>2395 ± 547</td>
<td>1142 ± 525</td>
<td>14.1 ± 4.2</td>
<td>10.5 ± 5.3</td>
<td>8.2 ± 7.3</td>
</tr>
</tbody>
</table>

* P < .05, ** P < .01 compared with the vehicle control group.
iNOS inhibition may affect the function of T cells during the elicitation of the immune response necessary for development of chronic inflammation and arthritis.

Another explanation for the lack of therapeutic effect of L-NIL on adjuvant-induced arthritis is that, as suggested by Connor et al. (1995), tissue damage may result as an accumulative effect of NO production. Peroxynitrite, generated by the reaction of NO with superoxide radicals, is cytotoxic and can decompose to numerous products which are themselves inflammatory (e.g., hydroxide radicals; Kaur and Halliwell, 1994). It is possible that these inflammatory by-products of NO formation had accumulated sufficiently before L-NIL therapeutic administration to initiate the irreversible tissue damage that occurred during the later stages of arthritis in our experiments. Also, our studies did not address the possibility that iNOS inhibition therapy might be effective in reducing the chronic, cyclical episodes of inflammation which occur during the long-term course of arthritic disease. Perhaps related to this point, however, is the lack of therapeutic benefit obtained by administration of an iNOS inhibitor to rhesus monkeys that develop spontaneous, chronic, ulcerative colitis (Ribbons et al., 1997).

NO has been implicated in mediating both the acute and chronic inflammation associated with streptococcal cell wall-induced arthritis in rats, because L-NMMA suppressed the tissue damage which occurs in this model when administered either prophylactically and therapeutically (McCarty-Francis et al., 1993). This may result from the relative lack of selectivity of L-NMMA for iNOS, however. Inhibition of ecNOS, which would have generalized and localized effects on blood pressure (Moncada et al., 1991), affecting fluid exchange in tissues, could account for the decreased inflammatory readouts reported with nongeneric NO inhibitors such as L-NMMA. Decreases in inflammatory swelling responses are readily explained as secondary effects to the lack of blood flow produced during NO inhibitor-induced vasoconstriction. We have not been able to obtain a therapeutic effect in the streptococcal cell wall model in rats with L-NIL in our laboratories (personal communication, J. McDonnell, Merck & Co., Rahway, NJ). Oral administration of L-NIL to rats at the levels effective in our adjuvant-induced arthritis studies did not produce a change in blood pressure, and effects on blood pressure were seen only after intravenous injection of rats with high levels of L-NIL (32 mg/kg resulted in a 40% increase in blood pressure; personal communication, R. Meurer, Merck & Co., Rahway, NJ). In comparison, we found that L-NIL suppressed the elevations in plasma NOX levels in rats with an ED50 of 0.2 mg/kg when administered intravenously 2 hr before injection of LPS. In addition, Moore et al. (1994) reported that L-NIL is 28-fold more selective for mouse iNOS than rat brain nNOS. All these results indicate a high selectivity of L-NIL for iNOS over cNOS and suggest that the effects of L-NIL in our adjuvant studies were probably not caused by inhibition of constitutive NOS.

It is possible that other, nonspecific activities of L-NIL treatment were responsible for causing the amelioration of the inflammatory responses we observed in the rat adjuvant-induced arthritis model. The anti-inflammatory effects of NO inhibitors have been reported to include inhibition of prostaglandin synthesis and cell infiltration (Salvemini et al., 1993, 1995). Indomethacin, or the selective COX2 inhibitor, SC-58125, have been effective in reducing secondary paw swelling when administered therapeutically to rats with adjuvant-induced arthritis (Anderson et al., 1996). The lack of effect of therapeutic L-NIL treatment on secondary paw swelling that we observed in our experiments suggests that COX products were not inhibited at the levels of L-NIL used, and therefore, COX inhibition could not account for the efficacy of prophylactic L-NIL treatment in this model. In addition, our immunoblot results showed identical levels of iNOS in the spleens of rats treated with either vehicle or L-NIL. This suggests that the inhibitor did not affect the induction of iNOS which occurred in response to immunization with adjuvant.

In conclusion, whether iNOS up-regulation in rat adjuvant-induced arthritis is merely coincidental with joint swelling and destruction, or rather, an integral cause of the chronic, inflammatory response, remains to be proven conclusively. Our results suggest that iNOS may be essential only during the early phase of adjuvant-induced arthritis, during sensitization and activation of the immune system by the antigenic stimuli, and is not a critical agent in the development of the secondary phase of inflammation and joint destruction. Therefore, our results do not support the supposition that iNOS inhibitors would be of therapeutic value in the general treatment of chronic inflammatory disease.

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