Brief Definitive Report

Suppression of Arthritis by an Inhibitor of Nitric Oxide Synthase

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Summary

Nitric oxide (NO), a toxic radical gas produced during the metabolism of L-arginine by NO synthase (NOS), has been implicated as a mediator of immune and inflammatory responses. A single injection of streptococcal cell wall fragments (SCW) induces the accumulation of inflammatory cells within the synovial tissue and a cell-mediated immune response that leads to destructive lesions. We show here that NO production is elevated in the inflamed joints of SCW-treated rats. Administration of N^G-monomethyl-L-arginine, an inhibitor of NOS, profoundly reduced the synovial inflammation and tissue damage as measured by an articular index and reflected in the histopathology. These studies implicate the NO pathway in the pathogenesis of an inflammatory arthritis and demonstrate the ability of a NOS inhibitor to modulate the disease.

Nitric oxide (NO) participates in intercellular signaling when produced in small amounts by constitutive NO synthase (cNOS) in neurons and endothelial cells (1, 2). Immunologic and inflammatory stimuli, however, induce an isoform of NOS (inducible NOS [iNOS]) that can produce much larger amounts of NO over longer periods (1). In the latter circumstances, NO can exert cytostatic or cytotoxic effects against microbes, tumor cells, macrophages, and lymphocytes, as well as β cells in the pancreas (1). At the same time, NO can suppress the proliferation of T cells (3–5) and the emigration of neutrophils (6). Thus, while diseases such as experimental acute encephalomyelitis (7), rabies (8), and graft-vs.-host disease (9) are accompanied by induction of iNOS and/or production of NO, it is difficult to predict whether sustained generation of NO is more likely to exacerbate chronic inflammation or alleviate it.

Here we present the first evidence that inhibition of iNOS can markedly suppress tissue destruction resulting from chronic inflammation. Susceptible rats, exposed to streptococcal cell wall fragments (SCW), develop an acute inflammation of the joints, followed by a chronic, erosive arthritis (10). This bimodal pattern of arthritis occurs coincidently with the deposition and persistence of SCW antigens in the synovium. Whereas the early phase of the response is neutrophil driven, the chronic phase has been identified as a T cell– and monocyte-mediated immune response and is characterized by infiltration of mononuclear cells with release of inflammatory mediators, hyperplasia of the synovium, and erosive destruction of the subchondral and periarticular bone and cartilage (11, 12). The development of arthritis was accompanied by induction of iNOS and production of NO both in synovial tissue and blood mononuclear cells. Administration of an inhibitor of NOS, N^G-monomethyl-L-arginine (NMMA) (13), greatly reduced the accumulation of inflammatory leukocytes and erosion of the joint. NMMA was effective even when its administration was delayed until after the onset of arthritis.

Materials and Methods

Arthritis Induction. Arthritis was induced in pathogen-free LEW female rats (~100 g) (Harlan Sprague Dawley, Indianapolis, IN) by intraperitoneal injection of peptidoglycan-polysaccharide fragments (20 μg rhamnose/g body mass) derived from group A streptococcal cell walls (11, 12). Each of the four distal joints was scored on a scale of 0–4 for severity of arthritis on the basis of swelling, erythema, and distortion (11). The individual joint scores were summed to determine the articular index (AI), with a possible maximum score of 16. The indices for a group of animals were averaged and reported as the mean AI ± SEM. Statistical significance was determined using the unpaired, two-tailed *t* test. NMMA (Calbiochem Corp., San Diego, CA) was injected intravenously daily for specified intervals up to 26 d at a dosage of 30 mg/kg body weight. The NMMA stock solution was prepared in PBS (100 mg/ml) and contained ≤12 pg endotoxin/ml as determined by the limulus assay.

Synovial Tissue Culture. Synovial tissues were excised from control and arthritic rats, finely minced, and equivalent amounts based on wet weights were cultured in eight-well slide chambers (Nunc,

Inc., Naperville, IL) in 0.2 ml RPMI 1640 (GIBCO BRL, Gaithersburg, MD) containing gentamicin sulfate (50 μg/ml), 2 mM glutamine, 100 μM L-arginine, and supplemented with [guanido-^{14}C]L-arginine (50 nCi/well; DuPont Co., Wilmington, DE). Labeled urea (arginase pathway) and citrulline (NO pathway) in the supernatants were separated by HPLC using an automated amino acid analyzer (Dionex, Sunnyvale, CA). Results are reported as the fraction (%) of total radioactivity found in these compounds at the end of culture. The nitrite content of the supernatants was assayed with Griess reagent (14). Samples were tested in triplicate and reported as the mean concentration ± SEM.

Cell Isolation and Culture. PBMC were isolated from heparinized blood of control and SCW-injected animals by density gradient centrifugation through Ficoll (Histopaque 1083; Sigma Chemical Co., St. Louis, MO). Cells were suspended in arginine-supplemented DME (phenol red–free; Mediatech, Inc., Washington, DC) in 24-well plates (Costar Corp., Cambridge, MA) and cultured for 24 h in the presence or absence of SCW and NMMA.

RNA Isolation and Northern Blot Analyses. Total cellular RNA was extracted from cells or homogenized tissue using the guanidine thiocyanate/phenol/chloroform procedure (15). RNA was separated on 1% agarose gels containing 1.2 M formaldehyde and transferred to nitrocellulose (BA-S; Schleicher and Schuell, Keene, NH). The blots were probed with a radiolabeled 645-bp HindIII/BamHI fragment of the iNOSL3, a derivative of the macrophage NOS clone (16). The blots were exposed to phosphor screens and analyzed by a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software. The blots were then stripped and screened with a probe encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed housekeeping gene (17).

Histopathology. Joint tissues from control and arthritic rats were excised and fixed in 10% buffered formalin, decalcified in 10% EDTA, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results and Discussion

Production of NO in SCW-induced Arthritis. To test whether NO was associated with the evolution of chronic inflammation, susceptible rats were injected with SCW to induce an acute inflammation of the joints, followed by a chronic, erosive arthritis (11). Synovial tissue excised from inflamed joints and placed in culture secreted NO, as measured by formation of nitrite (NO_2^-), a stable decomposition product of NO, during the acute and the chronic phases of disease (Fig. 1 *A*), in contrast to control synovium. Moreover, the inflamed

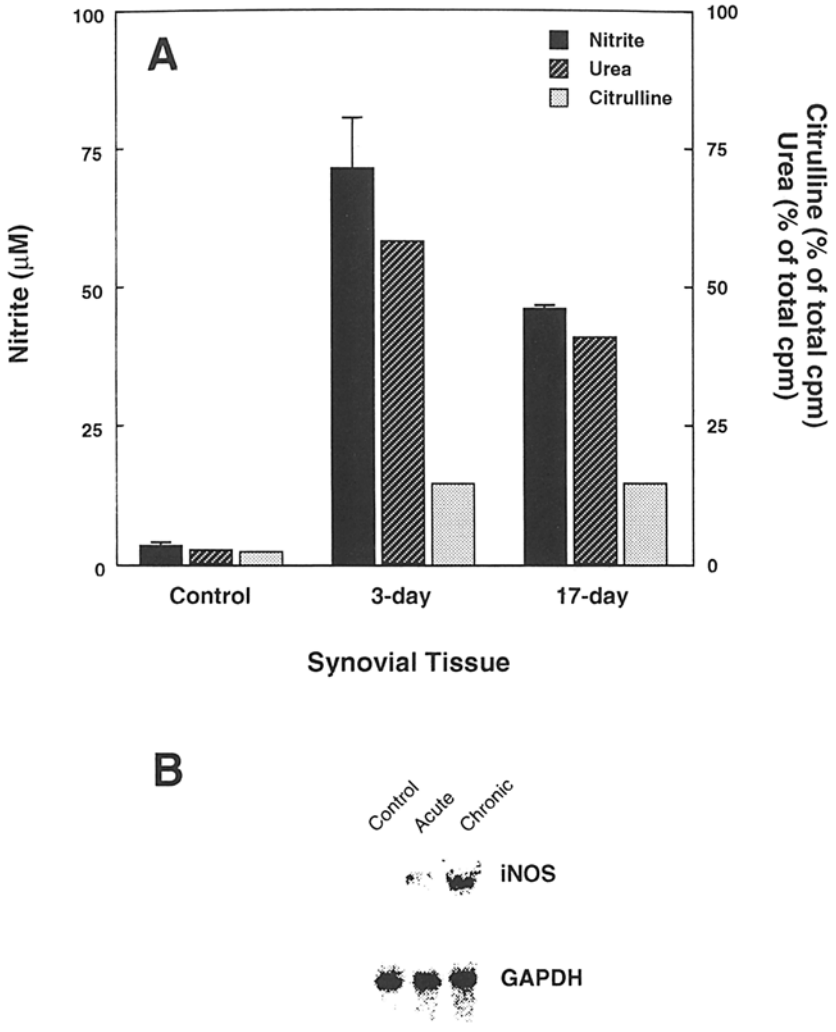

Figure 1. NO production in synovial tissues is associated with arthritis development. Animals received a single intraperitoneal injection of an arthropathic dose of SCW on day 0. Synovial tissues were excised from joints of nine control rats (AI = 0), two rats with acute inflammation (day 3, AI ⩾3/joint), and two rats with chronic inflammation (day 17, AI ⩾3/joint), and samples were pooled for each condition. Equivalent amounts of tissue based on wet weights (27 mg/0.2 ml) were cultured in duplicate for 24 h in the presence of [guanido-^{14}C]L-arginine. (*A*) Quantitation of nitrite by the Griess reaction and [^{14}C]urea and [^{14}C]citrulline by HPLC analysis of synovial tissue culture supernatants. (*B*) Northern blot hybridization of synovial tissue RNA from freshly isolated control and inflamed joints of representative animals with cDNA probes encoding macrophage iNOS and GAPDH.

tissues expressed augmented levels of RNA for iNOS (Fig. 1 *B*).

Culture of the synovial tissue in [guanido-^{14}C]L-arginine revealed metabolism by both arginase and NOS (Fig. 1 *A*). Greater than 14% of the arginine metabolized in the inflamed synovial tissues, but not in the control tissue (≤1%), passed through the NO pathway, as revealed by accumulation of [^{14}C]citrulline, providing evidence for inducible NO during both acute and chronic inflammation. Arginase accounted for a significant proportion of the arginine use as monitored by [^{14}C]urea levels. Arginase, produced by macrophages and reportedly high at sites of inflammation (18), could diminish arginine in the synovial fluids. Under conditions of limiting L-arginine concentrations, brain NOS has been shown to generate hydrogen peroxide and superoxide (19, 20), molecules that contribute to joint pathology in SCW-injected rats (12). It is possible that synovial iNOS uses both oxidative pathways in SCW-induced arthritis.

Systemic Effects of SCW Exposure on NO Production. PBMC isolated from SCW-injected arthritic animals released more nitrite than control PBMC (48 vs. 7 nmol nitrite/10^6 cells; $p \leq 0.004$) and, after exogenous addition of SCW in vitro, these levels were augmented in both populations (Fig. 2). Furthermore, PMN from arthritic animals secreted increased nitrite (data not shown). The specific NOS inhibitor NMMA blocked NO generation in the PBMC cultures (Fig. 2). Significant suppression was evident at 10 μM NMMA, with return to basal NO levels at 100–500 μM.

To establish whether the SCW could directly induce the NO pathway in phagocytic cells, resident peritoneal cells were isolated from control animals and exposed to SCW in vitro. SCW induced substantial NO synthesis (>120 nmol nitrite/10^6 cells) and high levels of [^{14}C]citrulline (>25%) with little arginase activity. Thus, macrophages and neutrophils, as well as chondrocytes and fibroblasts, which have been shown to express iNOS and/or release NO in response to cytokines (1, 21), may contribute to the elevated synovial NO levels in SCW-arthritic rats.

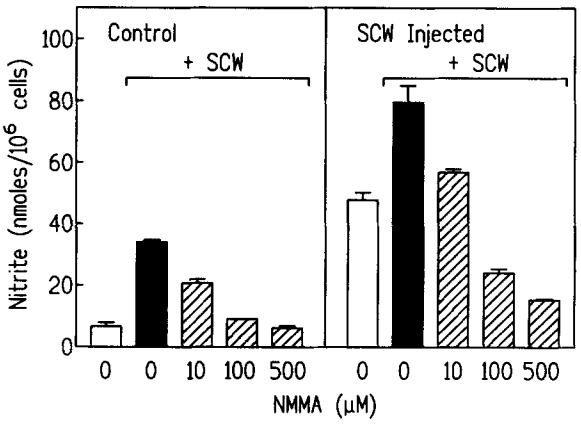

Figure 2. Inhibition of NO synthesis by NMMA. PBMC were isolated from control and SCW-injected rats (4 wk postinjection) and cultured for 24 h in the presence of SCW (3 μg/ml) and NMMA (0–500 μM). Nitrite content of the supernatants was determined and normalized to cell concentration. Data are from a representative experiment in which the individual means ± SEM were determined from duplicate cultures assayed in triplicate.

Suppression of Acute and Chronic Arthritis by NMMA, an Inhibitor of NOS. To determine whether the elevated NO levels were contributing to tissue pathology, female LEW rats were treated with NMMA after a single arthropathic dose of SCW. Control rats receiving only SCW developed typical acute and chronic arthritis (11, 12) (Fig. 3 *A* and *B*). Daily intravenous injections of NMMA at 30 mg/kg body weight beginning at day 0 resulted in a marked suppression of the cellular infiltrate (Fig. 3 *C*) as reflected by the AI and compared with the untreated arthritic animals (Fig. 3 *A*). While acute inflammation was suppressed clinically (30% on day 3; $p \leq 0.0001$), NMMA did not significantly inhibit acute leukocyte recruitment (not shown). However, NMMA virtually eliminated the joint swelling and distortion and greatly reduced the cellular infiltration during the T cell macrophage–dependent chronic phase of disease (on day 22, AI = 1.0 ± 0.8 vs. 9.1 ± 0.7 for SCW only; $p \leq 0.0002$). Moreover, the AI was also reduced significantly (80% on day 22, AI = 4.5 ± 1.5; $p \leq 0.0001$) in SCW-injected rats receiving NMMA beginning on day 12 as the acute response subsided and the chronic arthritic phase commenced. Rats receiving NMMA during the acute response only (days 0–4) also displayed reduced AI during the chronic phase, although the suppression was modest and variable (data not shown). NMMA did not appear toxic to the animals even after 26 d of treatment.

The systemic effects of SCW exposure were demonstrated by the induction of NO in circulating mononuclear cells (Figs. 2 and 4). However, constitutive nitrite production by PBMC obtained from NMMA-treated arthritic rats (days 0–26) was reduced 70% ($p \leq 0.001$) compared with arthritic rats. Treatment with NMMA during the chronic disease only (days 12–26) achieved similar suppression of nitrite production (70%; $p \leq 0.0005$), whereas limiting treatment to the acute phase (days 0–4) resulted in a smaller reduction in PBMC nitrite levels when evaluated on day 26.

By Northern analysis, PBMC of SCW-injected rats (days 3 and 21 postinjection) revealed increased levels of iNOS mRNA (~4.3 kb) compared with control rats (Fig. 4). As predicted, NMMA treatment of SCW-injected rats, while reversing the arthritis and inhibiting nitrite production, did not decrease levels of iNOS mRNA (<15% change at day 21). NMMA treatment alone did not influence iNOS mRNA expression.

We conclude that NO production is necessary for the immunologically mediated joint destruction induced by SCW. The mechanisms by which NO promotes tissue damage in this setting have not been established. Direct effects might include toxicity, altered metabolism of synoviocytes and chondrocytes (21), increased permeability of the synovial microvasculature (22), or augmented reactive oxygen intermediate levels (19, 20). However, indirect effects, such as inhibition of suppressor T cells or antiproteases, might also play a role.

Blockade of the NO pathway has been suggested as a treatment for TNF-induced hypotension (23), toxic shock syndrome (24), and diabetes (25). The present data appear to

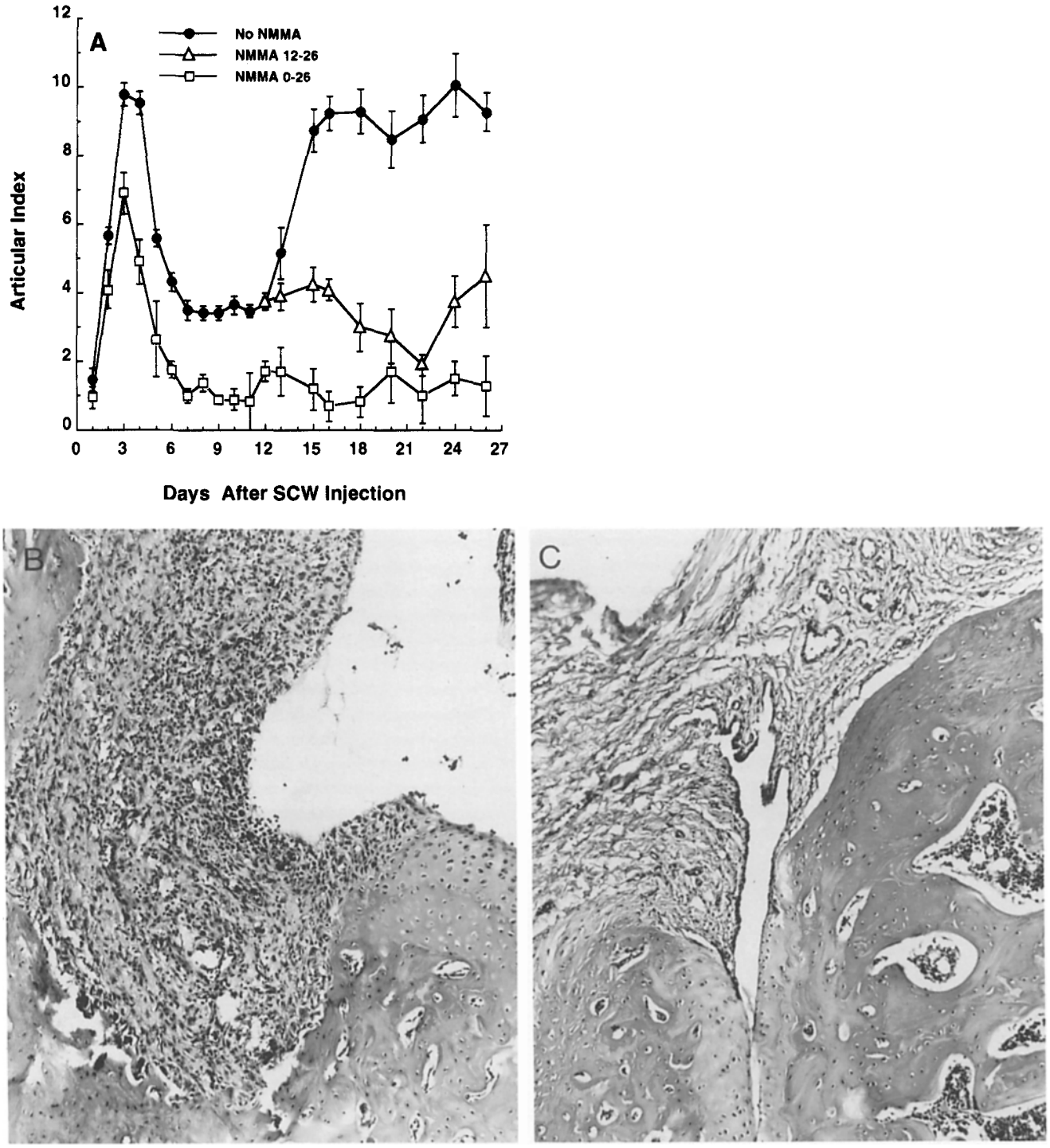

Figure 3. Suppression of SCW-induced arthritis by systemic administration of NMMA. Animals received an arthritogenic dose of SCW at day 0, and NMMA was injected intravenously daily for 26 d beginning on day 0 at a dosage of 30 mg/kg body weight. Some rats received NMMA on days 0–4 (data not shown); others received NMMA on days 12–26. (*A*) AI scores were determined at indicated intervals and data represents the mean ± SEM. All control animals (PBS, NMMA alone) had mean joint scores of zero throughout the experiment. Joints were excised and processed for histopathology on day 26. (*B*) Cellular infiltrate in the synovium of a SCW-injected animal that had evidence of bone erosion and cartilage degradation. (*C*) After NMMA therapy (days 0–26), a reduction in the cellularity of the synovium during the chronic phase was evident (original magnification, ×40).

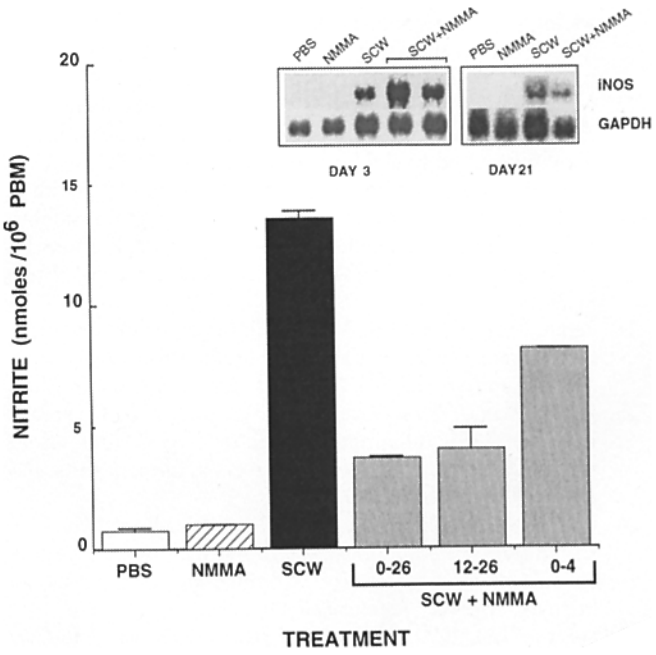

Figure 4. Differential effects of NMMA treatment on NO production and iNOS gene expression. Total RNA isolated from PBMC on days 3 and 21 post-SCW injection was analyzed by Northern hybridization with the iNOS and GAPDH probes. After normalization, little or no decrease in the NOS/GAPDH ratios was observed in the SCW + NMMA samples as compared with SCW alone. Nitrite levels were determined in 24-h culture supernatants of PBMC (2×10^6/ml) isolated on day 26 post-SCW injection.

be the first demonstrating a beneficial effect of blockade of the NO pathway in destructive arthritic lesions. NMMA reduced the inflammation and joint erosion even when the treatment was delayed until after the initiation of the chronic arthritic response. NMMA is not selective for the different isoforms of NOS. Interference with neuronal and endothelial cNOS could affect cell–cell communication, vascular tone, and neurotransmission, although the rats treated with NMMA did not display any obvious detrimental effects, consistent with other studies (26). As the incidence of rheumatoid arthritis is approaching 1% worldwide (27), new therapies are needed. Recent identification of nitrite in synovial fluid of arthritic patients (28, and McCartney-Francis et al., unpublished observations) further suggests that inhibitors selective for iNOS may prove useful in the long-term treatment of arthritides and perhaps other inflammatory diseases.

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