

## **Increased Expression of Blood Mononuclear Cell Nitric Oxide Synthase Type 2 in Rheumatoid Arthritis Patients**

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### **Summary**

Nitric oxide (NO) is an important inflammatory mediator in nonhuman animal models of rheumatoid arthritis (RA). The purpose of the present study was to determine whether blood mononuclear cells from patients with active RA (as compared to control subjects) have higher levels of NO synthase type 2 (NOS2) and produce more NO in vitro. Leukocytes from 25 RA patients and 20 normal subjects were examined. Arthritis activity was assessed by tender and swollen joint counts, duration of morning stiffness, patient assessment of pain, physician and patient global assessment of disease activity, the modified Stanford Health Assessment Questionnaire, and by blood levels of acute phase reactants. Blood mononuclear cell NOS enzyme activity/antigen content and nitrite/nitrate formation in vitro were measured. Blood mononuclear cells from RA patients had increased NOS activity and increased NOS2 antigen content as compared to those from normal subjects, and responded to interferon- $\gamma$  with increased NOS expression and nitrite/nitrate production in vitro. NOS activity of freshly isolated blood mononuclear cells correlated significantly with disease activity, as assessed by tender and swollen joint counts. Our results demonstrate that patients with RA have systemic activation for NOS2 expression, and that the degree of activation correlates with disease activity. Increased NOS2 expression and NO generation may be important in the pathogenesis of RA.

Rheumatoid arthritis (RA) is a chronic systemic disease of unknown etiology that is characterized by joint inflammation and progressive loss of articular cartilage and subchondral bone (1). Prominent pathologic features of joint inflammation are synovial proliferation and mononuclear cell infiltration, which are believed to result from dysregulated production of cytokines, growth factors, cell adhesion molecules, and nonprotein mediators, such as arachidonic acid metabolites and reactive oxygen species (2–4).

Nitric oxide (NO) may be a critical mediator in this inflammatory cascade (5, 6). NO is generated endogenously from L-arginine by oxidation to L-citrulline and NO. This reaction is catalyzed by a group of three related NO synthase (NOS) enzymes that are encoded by separate genes (neural NOS [NOS1], endothelial NOS [NOS3], and inducible NOS or NOS type 2 [NOS2]). Increased NOS2 expression and NO production have been noted in induced and spontaneous nonhuman animal models of arthritis, and inhibitors of NOS can reduce arthritis in these conditions (6–12). Moreover, serum and synovial fluid from humans with inflammatory arthritides have increased levels of NO catabolites (13–15). Also, synovial tissue from hu-

mans with inflammatory arthritis express NOS2 mRNA and protein, and generate NO in vitro (16).

The purpose of the present study was to determine whether blood mononuclear cells from patients with active RA have enhanced expression of NOS2 and enhanced capability to produce NO in vitro. We report results of work that couples detailed clinical and laboratory rheumatological studies with research laboratory measurements of NOS expression and NO production.

### **Materials and Methods**

**Subjects.** 25 patients who met the American Rheumatism Association 1987 revised criteria for the classification of RA (17) were recruited from the rheumatology outpatient clinics at the Duke University Medical Center (DUMC). To be eligible for this study, doses of prednisone ( $\leq 10$  mg/d) and nonsteroidal anti-inflammatory drugs (NSAIDs) in RA patients must have been stable for at least 2 wk before entry. Concomitant use of second-line drugs such as methotrexate, gold, sulfasalazine, hydroxychloroquine, and azathioprine was permitted if the doses had been stable for at least 4 wk before the study. 20 age ( $\pm 5$  yr)- and gen-

**Table 1.** Characteristics of Study Subjects

|   | Patients<br>(n = 25) | Controls<br>(n = 20) |
|---|----------------------|----------------------|
| Age (yr)*                                   | 58 (14)              | 56 (23)              |
| Female gender                               | 17 (68%)             | 14 (70%)             |
| Disease duration (yr)                       | 9 (10)               |                      |
| Subcutaneous nodules                        | 12 (48%)             |                      |
| Functional class                            |                      |                      |
| I   | 3 (12%)              |                      |
| II  | 15 (60%)             |                      |
| III   | 7 (28%)              |                      |
| Positive RF                                 | 23 (92%)             |                      |
| Previous second-line drugs                  | 18 (72%)             |                      |
| Current NSAID users                         | 13 (52%)             | 2 (10%)              |
| Current prednisone users                    | 16 (64%)             | 0 (0%)               |
| Mean dose (mg/d)                            | 8.5 (5.0)            |                      |
| Current second-line drug <sup>‡</sup> users | 18 (72%)             |                      |
| Current methotrexate users                  | 9 (36%)              |                      |

\*Values are expressed as the median and interquartile range for continuous variables (age, disease duration, and mean dose), and as the number and percent of subjects for categorical variables.

<sup>‡</sup>Second-line drugs include methotrexate, hydroxychloroquine, sulfasalazine, parenteral gold salts, azathioprine, and cyclosporine. RF, rheumatoid factor; NSAID, nonsteroidal antiinflammatory drugs.

der-matched control subjects without RA were recruited by newspaper advertising. The study protocol was approved by the DUMC Institutional Review Board. Informed consent was obtained from each subject before participation.

Patients and controls were excluded if they had a coexisting chronic inflammatory condition, active infection, malignancy, cirrhosis, or a serum creatinine >2.5 mg/dl. Pregnant women were excluded because of reports of elevated NO production in pregnancy (18). Use of nitroglycerin, other nitrate-containing medications, and tobacco products was prohibited during the study.

**Study Design.** Eligible subjects were evaluated in the General Clinical Research Center (GCRC) at DUMC. A medical history and physical examination were done on admission to confirm subject eligibility. RA patients underwent a clinical arthritis assessment using the following disease measures: tender and swollen joint count (with a maximum of 68 tender and 66 swollen joints), duration of morning stiffness (minutes), patient assessment of pain (10 cm visual analogue scale), physician and patient global assessment of disease activity (10 cm visual analogue scale), and the modified Stanford Health Assessment Questionnaire (HAQ; 19).

A complete blood count and routine laboratory values, including serum creatinine and albumin, were obtained upon entry. The erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), indices of RA disease activity, were measured in both patients and controls.

**Blood Mononuclear Cell Preparation.** 40 ml of blood was drawn into lithium heparin. Anticoagulated blood was spun for 25 min at 150 g, and platelet-rich plasma was discarded. Mononuclear cells were then separated by centrifugation over Ficoll/Hypaque

**Table 2.** Disease Activity Measures in Patients with RA

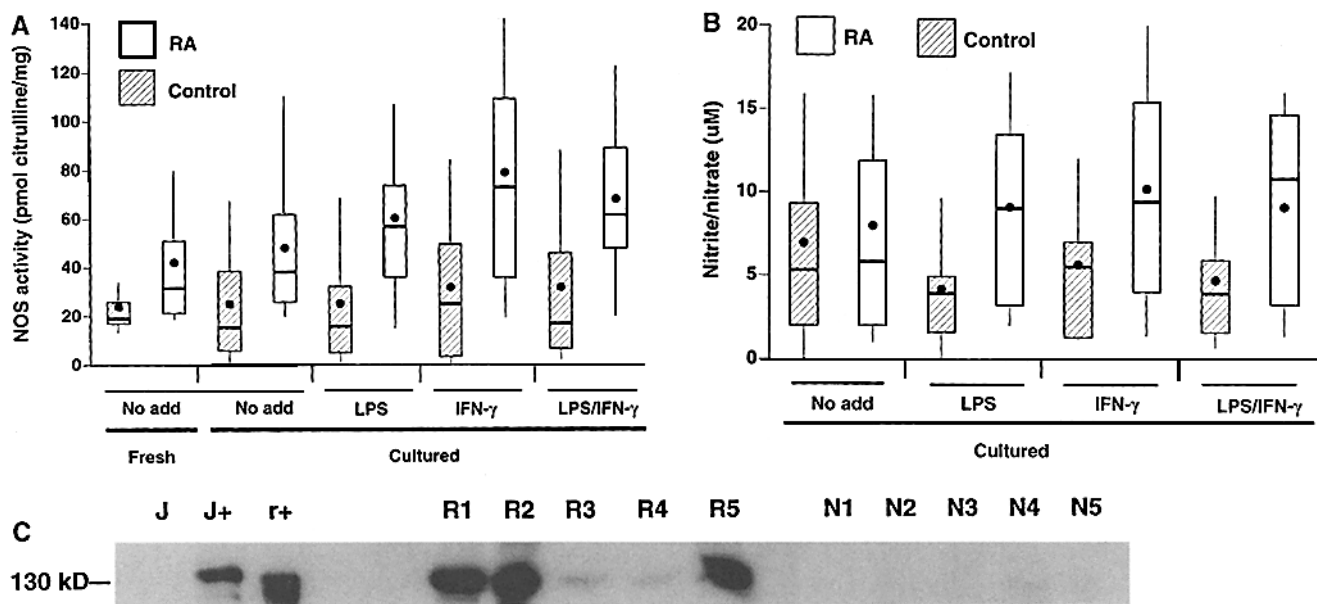
| Disease measure                 | Median (IQR) | Range     |
|---------------------------------|--------------|-----------|
| Number of tender joints         | 31 (22)      | 0–63      |
| Number of swollen joints        | 28 (17)      | 5–44      |
| Morning stiffness (min)         | 60 (105)     | 0–1,440   |
| Physician assessment of disease | 41 (39)      | 3–88      |
| Patient pain score              | 46 (42)      | 11–98     |
| HAQ score                       | 1.62 (0.88)  | 0.25–2.88 |
| ESR (mm/h)*                     | 26 (51)      | 5–108     |
| CRP (mg/liter)*                 | 13 (33)      | 0–96      |
| Hemoglobin (g/dl)*              | 12.9 (1.8)   | 10.4–16.3 |
| Serum albumin (mg/d)*           | 4.0 (0.4)    | 3.1–4.6   |

\*Normal values: ESR, 0–30; CRP, 0–4; hemoglobin, 13.5–17.0 for males and 11.4–15.2 for females; and serum albumin, 3.5–5.5. IQR, interquartile range.

(specific gravity = 1.077). Leukocyte-free platelets were prepared as noted before (20). Cytocentrifuge preparations of the cells were analyzed by Wright and nonspecific esterase stains (21). Cells were then cultured at  $5 \times 10^5$  cells per 6-mm diameter microtiter plate well in 0.2 ml of DMEM with 10% heated (56°C 30 min) normal, pooled human serum for 5 d without additives, with recombinant IFN- $\gamma$  (500 U/ml; Genzyme, Cambridge, MA) alone, with LPS (1  $\mu$ g/ml; *Escherichia coli* 0128:B12; Sigma Immunochemicals, St. Louis, MO) alone, or with IFN- $\gamma$  and LPS together. Some aliquots of cells were rapidly frozen for subsequent analyses. After 5 d of culture, supernatant media were saved for nitrite/nitrate measurement, and cell lysates were processed for measurement of protein, NOS enzyme activity, and NOS2 antigen.

**Nitrite/nitrate, NO Synthase Enzyme Activity, and Antigen Analyses.** Since NO is rapidly oxidized to nitrite/nitrate, stable inorganic nitrogen oxides, levels of these catabolites have been used as an indicator of NO production. Nitrite/nitrate levels in supernatant media were measured as described (22, 23). Cellular extracts were prepared and analyzed for NOS activity (14-C-L-arginine conversion to 14-C-L-citrulline) and antigen content by immunoblot, as described earlier (23). Immunoblots were done using a monoclonal anti-NOS2 antibody (Transduction Laboratories, Lexington, KY; 23) and the enhanced chemiluminescence reagents from Amersham (Arlington Heights, IL). For known negative and positive extracts, we used untreated cells from the murine macrophage cell line J774 and cells of the murine macrophage cell lines J774 and RAW 264 treated with murine rIFN- $\gamma$  (200 U/ml) and LPS (200 ng/ml) for 3 d. 50  $\mu$ g protein from the human and murine cells was used in the individual lanes. A “positive immunoblot” for NOS2 was one in which a clear band was visible at 130–131 kD.

**Statistical Analyses.** Descriptive statistics are expressed in terms of the median and interquartile range for continuous variables and as the number and percent of subjects for categorical variables. Comparisons between cases and controls were made with the Wilcoxon rank-sum test for continuous variables and with a chi-square test of homogeneity for categorical variables. Within-subject comparisons used the Quade test (24), a generalization of the Wilcoxon signed-rank test.



**Figure 1.** (A) NOS activity in freshly isolated and cultured mononuclear cells from control subjects and RA patients. Blood mononuclear cells were prepared. Extracts from freshly isolated cells (*Fresh*) or from cells cultured 5 d with no additions, 1  $\mu\text{g}/\text{ml}$  LPS alone, 500 U/ml IFN- $\gamma$  alone, or 500 U/ml IFN- $\gamma$  and 1  $\mu\text{g}/\text{ml}$  LPS were assayed for NOS activity (ability to convert L-arginine to L-citrulline). Assays were done as six replicates for each individual subject. Results are shown as medians (*horizontal bar*), means (*circle*), the interquartile range (*box*), and the 10th to 90th percentile range (*vertical lines*). There were 20 control subjects and 25 RA patients. Using the Wilcoxon rank-sum test, RA patients' NOS activities differed significantly from control subjects' NOS activities in the categories *Fresh* ( $P < 0.003$ ), *No add* ( $P < 0.005$ ), *LPS* ( $P < 0.002$ ), *IFN- $\gamma$*  ( $P < 0.002$ ), and *LPS/IFN- $\gamma$*  ( $P < 0.002$ ). In analyses of cultured cells, the within-group comparison was significant for RA patients ( $P < 0.001$ ), but not for control subjects. Pairwise comparisons for cells from RA patients revealed significant differences for treatments that included IFN- $\gamma$  (*No add* vs. *IFN- $\gamma$*  [ $P < 0.003$ ], and *No add* vs. *LPS/IFN- $\gamma$*  [ $P < 0.003$ ]). (B) Nitrite/nitrate production by cultured mononuclear cells from normal subjects and RA patients. Blood mononuclear cells were prepared and cultured 5 d with no additions, 1  $\mu\text{g}/\text{ml}$  LPS alone, 500 U/ml IFN- $\gamma$  alone, or 500 U/ml IFN- $\gamma$  and 1  $\mu\text{g}/\text{ml}$  LPS. Supernatant media were then measured for nitrite/nitrate. Assays were done as six replicates for each individual subject. Results are shown as medians (*horizontal bar*), means (*circle*), the interquartile range (*box*), and the 10th to 90th percentile range (*vertical lines*). There were 20 control subjects and 25 RA patients. Using the Wilcoxon rank-sum test, RA patient's nitrite/nitrate levels differed significantly from control subjects' nitrite/nitrate levels in the categories *LPS* ( $P < 0.01$ ) and *LPS+IFN- $\gamma$*  ( $P < 0.02$ ). Within-group comparison was significant for control subjects ( $P < 0.02$ ), but not for RA patients. Pairwise comparisons for cells from control subjects revealed significant differences for *No add* vs. *LPS* ( $P < 0.02$ ) and *LPS* vs. *IFN- $\gamma$*  [ $P < 0.003$ ]). (C) Immunoblot analysis of mononuclear cells from normal controls and RA patients for NOS2 expression. Blood mononuclear cells were isolated, and extracts were analyzed for NOS2 antigen content using an NOS2-specific mouse monoclonal anti-NOS2 antibody. The NOS2 antigen has a molecular mass of  $\sim 130$  kD. Extracts from the murine macrophage cell line cells J774 and RAW 264 were used as negative and positive controls. 40  $\mu\text{g}$  of protein from the extracts were used in each lane. Parallel gels and blots done using isotype-specific control Ig showed no reactivity. J, J774 control; J+, J774 cells cultured with LPS + IFN- $\gamma$ ; r+, RAW 264 cells cultured with LPS + IFN- $\gamma$ ; R1-5, samples from five separate RA patients; N1-5, samples from five separate normal (control) subjects. While only 3 of 20 samples from controls were positive, 19 of 25 samples from RA patients were positive.

## Results

**Patient Characteristics.** 25 RA patients and 20 healthy controls were eligible for the study. Characteristics of the patients and controls are described in Table 1. RA patients generally had long-standing, severe disease, as evidenced by their use of multiple second-line drugs, and the relatively high prevalence of serum rheumatoid factor positivity and subcutaneous nodules. Most of the RA patients were receiving multiple arthritis medications. Approximately half of the RA patients were taking an NSAID, while only 2 of the 20 controls were taking a medication from this class of antiinflammatory agents. Of the 25 RA patients, 18 (72%) and 16 (64%) were receiving concomitant second-line drug and low dose prednisone therapy, respectively. Despite treatment with these agents, the RA patients exhibited moderate disease activity, as shown by high tender and swollen joints counts, long durations of morning stiffness, moderately high levels of functional disability, as measured

by the HAQ, and elevated levels of the acute phase reactants ESR and CRP (Table 2).

**NOS Enzyme Activity in Blood Mononuclear Cells.** NOS activity was measured in freshly isolated and cultured cells from RA patients and controls. Freshly isolated cells from RA patients had significantly higher levels of NOS activity than did the cells from control subjects ( $P < 0.004$ ) (Fig. 1 A). The numbers of monocytes in the freshly isolated mononuclear cells were essentially identical in the two groups: 59 (21)% (median [interquartile range]) in RA patients, and 55 [19]% in control subjects. It is very likely that the bulk of the NOS activity was present in monocytes and not lymphocytes or platelets. Analysis of leukocyte-free platelets demonstrated that  $< 9\%$  of the total NOS activity could be attributed to the small numbers of platelets in the preparations. Although human EBV infected lymphocytes may have very small amounts of NOS activity (25), normal

lymphocytes contain little or no NOS activity (5, 26). Inclusion of 2 mM  $N^G$ -monomethyl-L-arginine in the NOS assay reduced the conversion of L-arginine to L-citrulline by >90%.

To assess the responsiveness of blood mononuclear cells to immune stimulation, isolated cells from controls and RA patients were cultured without additives, with LPS alone, IFN- $\gamma$  alone, or the combination for 5 d. NOS activity in the cultured, untreated cells from RA patients was twofold greater than that in controls (Fig. 1 A), indicating that the cells from RA patients retained increased NOS activity, even after 5 d of in vitro culture. In agreement with our previous observations (23), treatment of cells from controls with LPS alone, IFN- $\gamma$  alone, or the combination in vitro did not augment NOS activity. However, cells from RA patients cultured in vitro with IFN- $\gamma$  alone or IFN- $\gamma$  + LPS exhibited significantly increased NOS activity (Fig. 1 A). Comparable to the increases in NOS activity in RA cells, supernatant media from the RA cells cultured with LPS or LPS + IFN- $\gamma$  contained more nitrite/nitrate than did those from control subjects (Fig. 1 B).

**NOS Antigen Expression.** Freshly isolated cells from each control subject and RA patient were analyzed by immunoblot for NOS2 antigen expression. Cell lysates from 19 of the 25 RA patients contained a protein (130–131 kD) that reacted with the anti-NOS2-specific antibody, while only 3 of 20 from controls displayed the antigen on blot ( $P < 0.0001$ ). There was no reaction with an isotype-matched (IgG2a) control Ig. Fig. 1 C shows examples from controls and RA patients.

**Relationship between NOS Activity, Medications, and Disease Measures.** RA patients had increased NOS activity relative to control subjects, despite concomitant use of medications that might inhibit this enzyme. The 13 patients taking NSAIDs had lower NOS activity (pmol citrulline/mg) in their freshly isolated cells (27 [15], median [interquartile range]) than did the 12 patients not taking NSAIDs (39 [32]). The 16 patients receiving prednisone had comparable NOS activity (31 [26]) to the 9 patients not receiving prednisone (32 [55]). NOS activity in the 9 RA patients taking methotrexate (39 [33]) was higher than that for the 16 patients not taking methotrexate (31 [22]). Because of the complexity of the drug regimens (several patients were taking multiple medications) and small numbers of subjects in the different groups, these results were not analyzed statistically.

NOS activity of freshly isolated cells was investigated as correlates of traditional clinical and laboratory indices of disease activity. No significant relationship was found between NOS activity and the HAQ score, physician and patient global assessment of disease activity, patient pain scale, or levels of the acute phase reactants ESR and CRP. NOS activity of freshly isolated blood cells, however, was positively correlated with the tender joint counts ( $r = 0.48$ ) and swollen joint counts ( $r = 0.47$ ) ( $P < 0.02$  for each), indicating a possible relationship between blood cell NOS activity and the extent of joint inflammation.

## Discussion

Our study documents that mononuclear blood cells from RA patients are activated for systemic NO overproduction. Patients have increased NOS enzyme activity and NOS2 antigen in freshly isolated blood mononuclear cells. Furthermore, when cultured in vitro, cells from RA patients respond to LPS, IFN- $\gamma$ , or the combination of both by increasing their levels of NOS activity and NO production, while cells from controls do not. Our data provide new information indicating the potential importance of NOS2 and NO in the pathogenesis of RA, and they raise the possibility that excess NOS2 expression and NO production could be targeted for therapy in humans with RA.

Although previous studies of blood monocytes in RA patients have noted systemic activation for the generation of reactive oxygen species generation, prostanoids, IL-1, TNF, and neopterin (27–29), our work is the first to demonstrate systemic activation of blood mononuclear cells in this disease for NOS expression and NO production. NO is an important inflammatory mediator in nonhuman animal models of inflammatory arthritis; these include adjuvant arthritis, collagen-induced arthritis, and spontaneous arthritis in autoimmune MRL-*lpr/lpr* mice (6, 11, 30). Likewise, NO participates in the pathogenesis of spontaneous myositis in the SJL mouse (31). NO has been reported to be increased in the synovial fluid and sera of patients with RA (13, 15, 32); however, some of these studies are difficult to evaluate because the subjects' oral intake of nitrite and nitrate (NO catabolites measured by the investigators) was not limited and quantitated. Kaur and Halliwell showed increased levels of nitrotyrosine (a product resulting from peroxynitrite action) in serum and synovial fluid from arthritis patients (14). Investigators have recently reported that synovial tissues from patients with RA and osteoarthritis produce NO in vitro, and express NOS2 mRNA (reverse transcription-PCR) and protein (immunoblot and immunohistology). NOS2 was associated primarily with CD14<sup>+</sup> cells (mononuclear phagocytes; 16). In RA, blood monocytes are recruited to synovial tissues. Recently emigrated monocytes may be activated for NOS expression and NO production in the synovium (16). Also, monocytes activated for NOS expression and NO production in the circulation may emigrate to the synovium where they may initiate and propagate inflammation.

NO has many actions that are appropriate for a proinflammatory agent. It is made by numerous cell types in sites of inflammation, and it increases blood flow and vascular permeability. NO has cell and tissue-destructive abilities; it can also induce cyclooxygenase, cause pain, destroy certain protease inhibitors, and enhance IL-1, TNF, and NADPH oxidase activities in myeloid cells (6, 33). NO production may be augmented by several substances, including cytokines, growth factors, immune complexes, and bacterial products. Since superoxide ( $O_2^-$ ) may convert NO to peroxynitrite (a potent proinflammatory molecule [34, 35]), coincident production of NO and  $O_2^-$  may further amplify the inflammatory state. In the joint, several cell types can

produce NO—mononuclear phagocytes, chondrocytes, endothelial cells, and possibly others. Mononuclear phagocytes and chondrocytes are the most likely sources in the joint. Human articular chondrocytes produce relatively high levels of NO in response to cytokines (11, 36, 37). If NO is

a vital mediator of joint inflammation in RA, then blocking NO production by NOS2-specific inhibitors and blocking NO effects by NO quenchers may have therapeutic roles in RA.

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We thank Ms. Edna Scarlett for assistance with study coordination, and the nursing and technical staff of the GCRC for assistance with subject monitoring and specimen collection.

This work was supported by the Veterans Affairs Research Service, National Institutes of Health grants AR-39162 and MO-1-RR-30 (GCRC), and the James R. Swiger Hematology Research Fund.

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Received for publication 22 May 1996 and in revised form 28 May 1996.

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