Nitric Oxide-Derived Species in Synovial Fluid from Patients with Juvenile Idiopathic Arthritis

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ABSTRACT. ***Objective.*** To evaluate superoxide anion (O_2–), nitrite/nitrate (NO_2–/NO_3–), and nitrotyrosine (NT) production and the contribution of myeloperoxidase (MPO) to the production of NT-containing proteins in the synovial fluid (SF) of patients with juvenile idiopathic arthritis (JIA). The affected tissues in inflammatory arthritis produce large amounts of nitric oxide (NO) or peroxynitrite (ONOO–) but there are no reports of NO or ONOO– participation in JIA. We also attempted to correlate our findings with variables of disease activity and articular damage.

Methods. We analyzed 40 patients with JIA, mean age 12.7 years, mean disease duration 7.8 years. O_2– production was measured by cytochrome C reduction after incubation of 10^6 synovial fluid (SF) cells with or without phorbol myristate acetate (PMA), formyl-methionyl-leucyl-phenylalanine (FMLP) or opsonized zymosan. SF and serum NO_2–/NO_3– levels were measured by Griess reaction; NT was detected by Western blot. Myeloperoxidase (MPO) activity was estimated spectrophotometrically. Clinical and laboratory variables [erythrocyte sedimentation rate, C reactive protein (CRP), and radiological score] and interleukin 6 (IL-6) levels were evaluated.

Results. NO_2–/NO_3– production was greatly enhanced in the joints of JIA patients (54.6 ± 3.2 µM) when compared with serum (13.9 ± 0.6 µM; $p < 0.001$). NO_2–/NO_3– levels in SF were positively correlated with the number of infiltrating lymphomononuclear cells. NT-modified proteins detected in the SF showed a high correlation with radiological score, disease duration, CRP, and IL-6.

Conclusion. Our results confirm the increased oxidative stress in children with JIA, suggesting a high *in situ* production of NO. The positive correlation between the expression of NT-modified proteins and variables of disease activity and damage is additional evidence that nitrogen and oxygen species may be involved in the joint destruction seen in patients with JIA. (J Rheumatol 2004; 31:992–7)

Key Indexing Terms:
JUVENILE IDIOPATHIC ARTHRITIS NITRIC OXIDE SUPEROXIDE
NT-MODIFIED PROTEINS SYNOVIAL FLUID MYELOPEROXIDASE

Juvenile idiopathic arthritis (JIA) is an autoimmune disease characterized by chronic inflammation in the joints with consequent destruction of articular tissue. The participation of reactive oxygen species in intraarticular inflammatory processes has been fully described in experimental models and adult patients with rheumatoid arthritis (RA). Nitric oxide (NO) is produced by chondrocytes, synoviocytes, and osteoblasts[1,2]. Increased concentrations of the NO end products nitrite/nitrate (NO_2–/NO_3–) have been found in the joint fluids of RA patients[3,4]. Moreover, Sakurai, *et al*[5] verified inducible NO synthase (iNOS) expression in both RA and OA synoviocytes and chondrocytes by *in situ* hybridization and immunohistochemistry studies. The administration of NOS inhibitor in experimental arthritis models was followed by reduction of intraarticular accumulation of leukocytes, swelling, and histopathological abnormalities[6-8].

Endogenous release of NO enhances macrophage cyclooxygenase (COX) activity that may result in the production of pro-inflammatory prostaglandins[9,10]. NO has the important property of reacting with superoxide anion O_2–. Although this was originally thought to provide a protective function, especially in response to ischemia and reperfusion, newer evidence suggests that this reaction can generate further destructive species, particularly the peroxynitrite anion (ONOO–)[11]. Once protonated, peroxynitrite can decompose to form the highly reactive hydroxyl radical

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(OH). The attack of ONOO– or its decomposition products acts upon aromatic amino acids leading to their nitration. The myeloperoxidase (MPO) released by infiltrating cells may contribute to the nitrotyrosine (NT) formation through the reaction of MPO derived hypochlorous acid with nitrite[12]. The resulting nitrated proteins are relatively stable and their occurrence in tissues is strong evidence of the formation of ONOO– *in vivo*. Sources of these reactive oxygen species (ROS) in inflammatory joints are numerous. Infiltrating neutrophils are a particular source of superoxide[11]. In this way, the production of such species during inflammation may be linked to tissue destruction, mainly cartilage, which has also been shown to be sensitive to degradation by ROS[13]. Compounds that mimic superoxide dismutase (SOD) reduce NT formation and attenuate the degree of chronic inflammation with avoidance of damage in experimental arthritis[14]. Thus, from the clinical point of view, NT-containing proteins could be considered a marker of tissue destruction derived from oxidative stress present in inflammatory processes. In biological fluids such as synovial fluid (SF) from adult rheumatic patients, the analysis of NT is highly indicative of the presence of both ROS and NO, which can react to form peroxynitrite, a potent oxidizing agent that contributes to joint damage.

The levels of extracellular superoxide dismutase in SF from patients with RA are significantly lower than those found in SF from healthy subjects[15], leading to increased superoxide formation. Drugs that reduce disease activity interfere with the production of superoxide anion[16]. Additionally, recent studies have shown that in the inflamed synovium from adults with RA, immunoreactivity to 3-NT was found in vascular smooth muscle cells and macrophages expressing CD68 and iNOS[17]. A decrease in SOD activity (and a consequent enhancement of superoxide anion production) in serum samples from patients with JIA has also been shown[18-21]. However, there are no available studies reporting NO or peroxynitrite production in SF from children with JIA.

We studied the production of both oxygen- and nitrogen-derived reactive species in SF obtained from children with JIA, and determined if any correlation exists between these measurements and variables of disease activity and articular damage.

MATERIALS AND METHODS

Patient selection. Patients (n = 40; 30 female) aged 12.7 ± 0.8 years (mean ± SEM; range: 2 - 22) with diagnosis of JIA (according to ILAR criteria[22]) with indication of intraarticular corticosteroid therapy were selected for the study. Eleven patients had systemic, 10 had polyarticular (rheumatoid factor- negative) and 19 had oligoarticular onset. The mean duration of disease was 7.5 ± 1.3 years (range: 0.3 - 20).

The appropriate institutional ethics review committee approved the study and a written informed consent was obtained from each patient or their parents before study entry. The patients were treated with combined therapy: disease modifying antirheumatic drugs (DMARD: methotrexate, sulfasalazine, chloroquine, and cyclosporine), nonsteroidal antiinflammatory drugs (NSAID), and corticosteroids (prednisone < 10 mg/day). Before knee articular aspiration, the children were evaluated clinically. Number of tender and swollen joints, duration of morning stiffness, duration of the disease, and functional class (according to the Steinbrocker classification[23]) were recorded. The following laboratory tests were performed: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), and antinuclear antibody (ANA). Radiological knee assessment was also performed and the radiological score was determined by Steinbrocker method[23].

Superoxide anion production by SF cells. Immediately after the articular aspiration, total cells were counted in a Newbauer chamber and adjusted to 1×10^6 cells/assay tube. For differential count, smears were prepared from a cell pellet and stained with Giemsa solution. Superoxide anion production was measured spectrophotometrically as the SOD inhabitable reduction of cytochrome c. The assay mixture contained 10^6 stimulated or unstimulated cells contained in 100 µl of SF and 100 µmol of cytochrome c (type VI from horse heart, Sigma, St. Louis, MO, USA). Control tubes were incubated in the presence of 90 UI of SOD (Sigma), and the reagent blanks were incubated in the absence of cells. The cells were incubated during 20 min at 37ºC in the presence of phorbol-myristate acetate (PMA, 50 ng/ml), formyl-methionyl-leucyl-phenylalanine (FMLP, 10^{-7} M) or opsonized zymosan (ZY, 1 mg/ml); basal superoxide anion production was estimated in the absence of any stimuli. All assays were performed in duplicate. The extent of cytochrome c reduction was measured as the change in absorbance at 550 nm (Hitachi spectrophotometer, model U-2000) against the respective reagent blank.

Total NO_2–/NO_3– determination. Total NO_2–/NO_3– concentrations in the SF and serum samples were determined by the colorimetric Griess reaction for nitrite[24], following nitrate reductase-mediated nitrate ion reduction[25]. The absorbance of the chromophore was read at 504 nm; under our conditions, the method had a sensitivity of 30 pmol for each anion.

Determination of NT-containing proteins. The NT-proteins present in SF or serum samples were analyzed by Western blotting, as described[26]. Briefly, total proteins (1 mg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), 10% polyacrylamide[27], and electrophoretically transferred to a nitrocellulose membrane. After blocking non-specific sites with 0.2% casein, the membranes were incubated overnight at 4˚C with the primary murine monoclonal antibodies raised against NT-modified KLH (500 ng/ml; Upstate, Biotechnology, Lake Placid, NY, USA). Membranes were washed with Tris-buffered saline containing 0.2% Tween 20 and incubated with an alkaline phosphatase-conjugated rabbit anti-mouse antibody. A chemiluminiscent assay (Immun-Star; Bio-Rad, Hercules, CA, USA) was used to detect immunoreactive NT-containing proteins. The images were captured with a ChemiImager system (Alpha Innotech Corp., San Leandro, CA, USA), and the band intensities were estimated by densitometric analysis employing the software that accompanies the equipment.

MPO activity assay. The MPO activity present in SF samples was measured by the spectrophotometric method described by Bradley, *et al*[28], and it is based on the increase in absorbance (at 460 nm) due to the MPO-catalyzed reaction between hydrogen peroxide (H_2O_2) and o-dianisidine (1 unit MPO splits 1 mmol H_2O_2/min).

Interleukin 6 (IL-6) determination. Serum and SF IL-6 concentrations were determined by an ELISA method (microplates R&D Systems, Minneapolis, MN, USA), with a detection limit of 0.1 pg/ml.

Statistical analysis. Results are expressed as mean ± SEM. Results were analyzed using non-parametric tests. Intergroup differences were analyzed by the Wilcoxon signed rank test. Spearman's rank correlation coefficient was calculated for SF and serum NO_2–/NO_3–. For other analyzed variables, p values less than 0.05 were considered significant.

RESULTS

Superoxide anion production by SF cells. Results of superoxide anion production by cells collected from inflamed

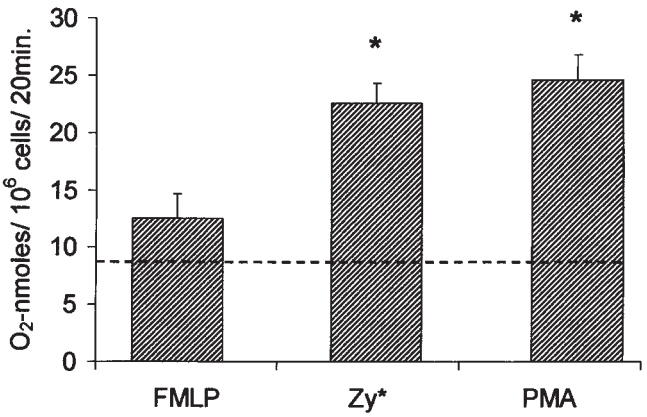

Figure 1. Superoxide anion (O_2–) production by 10^6 leukocytes collected from aspirated knee joints of patients with JIA. The columns represent the mean ± SEM of the assays performed with cells collected from 35 patients stimulated with either FMLP (10^{-7} M), PMA (50 ng/ml), or opsonized zymosan (1 mg/ml). The dotted line represents the basal O^2– production (without stimulation). *p < 0.05 in comparison with O^2– basal production

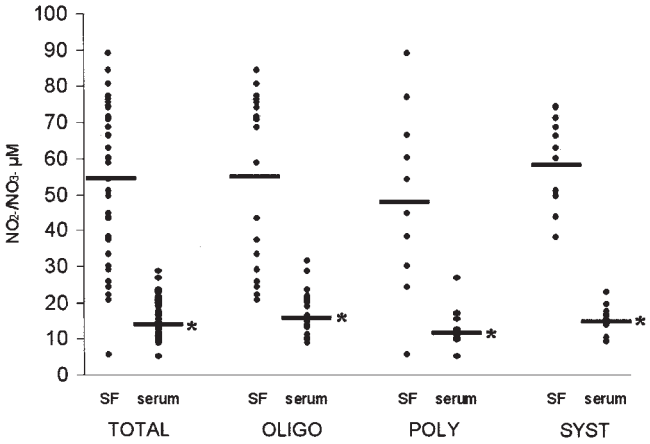

Figure 2. Total nitrite/nitrate (NO_2–/NO_3–; in µM) concentrations measured in SF and serum samples obtained from 40 patients with JIA: 19 with oligoarticular (OLIGO), 10 with polyarticular (POLY), and 11 with systemic (SYST) disease onset. Bars represent the mean value obtained for each group. *: p < 0.0001 in comparison with SF concentrations.

joints of patients with JIA are presented in Figure 1. Both PMA and ZY enhanced superoxide production by SF cells when compared to the basal level (shown by the dotted line). In contrast, FMLP failed to potentiate the superoxide production by non-stimulated cells. Superoxide anion production did not correlate with either clinical or laboratory variables of disease activity (Table 1), and was not affected by any patient treatment. No difference was observed between cells collected from patients with oligoarticular, polyarticular, or systemic type of disease regarding superoxide production.

Total NO_2–/NO_3– concentrations. Figure 2 shows individual and mean (for each group with oligoarticular, polyarticular, or systemic disease onset) NO end product concentrations measured in the SF and serum samples obtained from 40 patients with JIA. There was no significant difference among the different groups. As shown, NO_2–/NO_3– concentrations measured in the SF samples were significantly higher than those measured in serum samples from either patients or 10 sex- and age-matched healthy subjects. In addition, a significant positive correlation exists between SF nitrite/nitrate levels and the number of lymphomononuclear cells in the joint (r = 0.41; p = 0.02) (Figure 3).

Similar to superoxide anion production, there was no significant correlation between SF or serum NO_2–/NO_3– levels and the evaluated clinical or laboratory variables of disease activity (Table 1). Interestingly, the group of 18 patients with hip involvement with a higher radiological score (p = 0.02) and disease duration (p = 0.0001) compared with 22 patients without hip involvement also had significantly higher concentrations of NO_2–/NO_3– in SF (64.2 ± 3.5 vs 48.8 ± 5.0 mM, p = 0.02, respectively).

Expression of NT-containing proteins in SF and serum of patients with JIA. Both serum and SF samples from 12 representative patients (4 of each onset type) with different degrees of disease activity were analyzed for the presence of NT-containing proteins. This group of selected patients had different radiological scores (3 patients in each radiological class I-IV), mean CRP values of 35.33 ± 15.32 (> 5-145 mg/l), and mean IL-6 serum levels of 39.67 ± 9.52 (28-110 pg/ml).

Western blotting assay revealed the expression of 2 main bands of approximate molecular weights of 50 and 30 kDa, in addition to the 66 kDa band corresponding to serum albumin (Figure 4). Densitometric analysis of the bands showed no differences between serum and SF in terms of NT-protein expression. The mean densitometric values obtained for NT-proteins present in serum samples from the

Table 1. Correlations between nitrite/nitrate (NO_2-/NO_3-), superoxide anion (O_2-), myeloperoxidase activity (MPO) and nitrotyrosine (NT) containing proteins in the SF of 12 patients with JIA with clinical and laboratory variables evaluated.

	Radiological Score	Disease Duration	CRP	ESR	IL-6
NO_2-/NO_3-	NS	NS	NS	NS	NS
O_2-	NS	NS	NS	NS	NS
MPO	NS	NS	NS	NS	NS
NT~50kDa	r = 0.649, p = 0.02	r = 0.776, p = 0.004	r = 0.591, p = 0.04	NS	r = 0.793, p = 0.001
NT~30kDa	NS	NS	r = 0.804, p = 0.002	NS	r = 0.669, p = 0.02

NS: not significant.

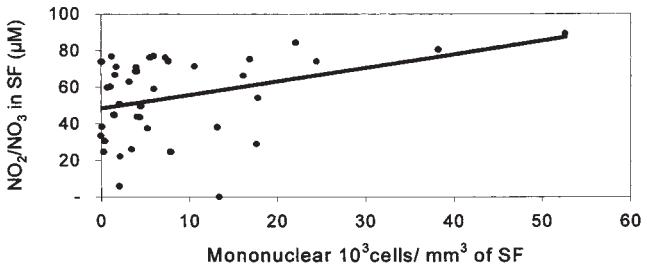

Figure 3. Correlation between SF nitrite/nitrate concentrations (NO_2–/NO_3–; in µM) and the number of lymphomononuclear cells present in the articular cavity (r = 0.042, p = 0.02).

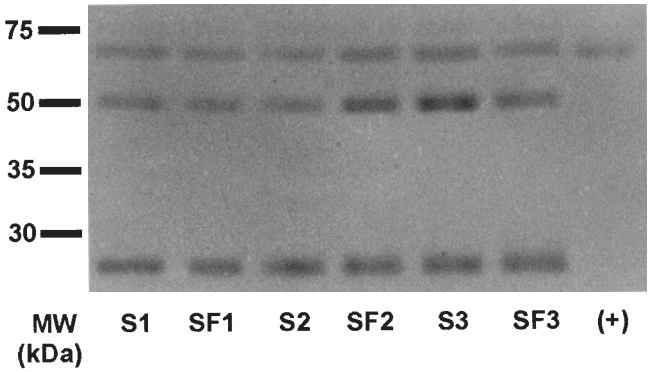

Figure 4. Representative Western blot for nitrotyrosine containing proteins (NT) in SF and serum (S) samples obtained from 3 patients with JIA (1 mg/lane) using monoclonal anti-nitrotyrosine antibody (500 ng/ml). Bovine serum albumin (2 mg) was nitrated *in vitro* and used as a positive control [lane (+)].

patients with JIA did not differ from those measured in serum samples obtained from 10 sex and age-matched healthy subjects. The intensity of the 30 kDa NT band present in the joint cavity was positively correlated with both CRP and IL-6 levels (Table 1). As expected, a positive correlation between CRP and IL-6 levels (r = 0.776, p = 0.04) was also observed . There was a highly positive correlation between the 50 kDa NT band and CRP, IL-6, radiological score, or disease duration.

MPO activity. Other tyrosine nitration pathways can occur during inflammatory oxidative reactions. Neutrophil-derived MPO might be responsible for nitration reactions. Our results showed that MPO activity in SF samples presented a high variation between values (2.5 to 39.7 U/mg protein). However, there was no correlation between these values and clinical and laboratory variables evaluated (Table 1).

DISCUSSION

This is the first report of the presence of high amounts of the NO end products NO_2–/NO_3– in SF samples obtained from patients with JIA. These were found at higher concentrations than those observed in the corresponding sera. These findings suggest an increased local production of NO in the inflamed joint. Farrel, *et al*[29] found that in 22 out of 25 evaluated adult patients with RA, total nitrite concentrations in SF were higher than those found in serum, an observation subsequently confirmed by ourselves and others[4,30-32]. In agreement with the increased production of NO in the inflamed joint, we also found that the number of infiltrating lymphomononuclear cells is positively correlated with the SF NO_2–/NO_3– concentrations. Borderie, *et al*[31] showed that iNOS is highly expressed in the infiltrating mononuclear cells and is responsible for the consequent increase in the local production of NO. The higher SF NO end product concentrations found in JIA patients with hip involvement, which carries the worst disease prognosis, is additional evidence of the *in situ* production of NO that could be implicated in the joint damage process.

It is important to emphasize that the concentrations of NO_2–/NO_3– in SF obtained from patients with JIA were much higher than those measured in SF samples from adults employing the same analytical method (54.6 ± 3.2 µM vs 24.6 ± 6.5 µM for JIA and adult RA patients, respectively; p = 0.015) (results not shown). Recent studies in normal donors showed enhanced levels of both serum NO metabolite levels[33] and urinary nitrite/nitrate excretion in the youngest children[34]. These levels decreased in an age dependent manner. Furthermore, the endothelium-dependent vasodilation is impaired in the elderly. *In vitro* studies with chondrocytes from young and old rheumatoid patients by Min, *et al*[35] showed that basal NO production was not altered by aging, although IL-1ß-induced NO formation decreases with age. These results corroborate the observed increase in SF NO_2–/NO_3– detected in JIA when compared with adult RA patients.

As a whole, our results suggest the presence of an enhanced oxidative stress activity in the inflamed joints of children with JIA. In addition to the previously discussed local overproduction of NO, we also studied the production of superoxide anion by cells collected from the articular cavity. Bender, *et al*[36] have already shown that polymorphonuclear cells collected from the inflamed joints of RA patients release higher amounts of O_2– than the corresponding circulating cells. It is very probable that the SF cells collected from patients with JIA in this study were also over-stimulated, considering that *in vitro* basal O_2– production by these cells was not significantly increased by the addition of the potent stimulant, FMLP.

Macrophages present in human SF express iNOS and can produce large amounts of NO[37]. On the other hand, activated macrophages can produce peroxynitrite[38], strongly suggesting that ONOO– may have a key role in inflammatory processes, such as RA. Peroxynitrite formation from the reaction between NO and O_2– is one of the major nitrating sources *in vivo*; indeed, NT detection (either free or as protein residues) is routinely used as a marker of peroxynitrite production.

It has been shown that both serum and SF samples from adults with RA contain significant amounts of NT, while undetectable NT levels were found in healthy subjects and in patients with osteoarthritis[39]. Furthermore, in the inflamed synovium of adults with RA, 3-NT expression was found in the macrophages[17]. Our results seem to confirm the hypothesis that in the inflamed joints of JIA patients increased NO production leads to peroxynitrite formation and further nitration of protein tyrosine residues. The positive correlation observed between the expression of NT-containing protein, accepted markers of disease activity, and articular damage (such as radiological score, duration of the disease, CRP, and IL-6 levels) indicate that NT-modified proteins are possibly linked to articular damage and could be an additional and useful marker of the disease.

Neutrophils and other immune cells can also produce nitrated species through the reaction between MPO-derived hypochlorous acid and nitrite[12], including tyrosine nitration[38]. However, our results indicate that MPO activity present in the SF samples of the JIA patients did not correlate with the detected NT-proteins or with the evaluated disease activity and joint damage variables. These observations suggest that, at least in this group of patients, MPO-mediated tyrosine nitration does not participate in this process.

In addition to tyrosine nitration, peroxynitrite also mediates the formation of other oxidation products such as oxidized thiols, oxidized iron-sulfur centers and 8-hydroxyguanidine, which could in turn act as (or be part of) transduction mechanisms that up-regulate the inflammatory process. For example, a recent study shows that exposure of rheumatoid synovial cells to peroxynitrite results in up-regulation of both COX-2 protein and mRNA expression[40]. These results suggest that peroxynitrite, apart from its own deleterious effects on tissue integrity, could also act as an amplifier of the prostanoid-dependent component of the inflammatory response during the rheumatoid process[40]. The effect of peroxynitrite participation on the pathophysiology of joint damage, as well as the search for therapeutic approaches to control peroxynitrite production to ameliorate the disease, deserve further investigation.

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