Purine Levels and Cytokine Release with Diuretic Administration in Rheumatoid Arthritis

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KEY WORDS: purines; adenosine, cytokines, tumor necrosis factor-α; interleukins; Bumetanide; uric acid; adenosine; cytokines; NECA; arthritis

ABSTRACT

Objectives: Treatment with diuretics can increase blood urate levels, and urate is a potent free radical scavenger. Since free radicals are implicated in rheumatoid arthritis (RA), we examined the effects of treating patients with rheumatoid arthritis with bumetanide to try to improve their arthritic control.

Methods: Seventy patients were recruited from routine rheumatology clinics and were randomized to receive bumetanide 4 mg/day or placebo. We measured blood levels of urate, other major purine metabolites, lipid peroxidation products, and the cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6).

Results: Increased levels of urate, but not other purines, were found in the blood of drug-treated patients compared with placebo-treated controls. There were no significant changes in clinical or biochemical measurements of disease activity. There were no overall differences in the blood levels of the cytokines, nor in the basal or stimulated production of cytokines from the blood cultures.

Conclusion: Bumetanide increases blood urate levels but this is not associated with disease improvement.

INTRODUCTION

The pro-inflammatory cytokines such as TNF-α, interleukin-1β (IL-1β) or interleukin-6 (IL-6) are believed to contribute to joint destruction and other tissue dysfunction in rheumatoid arthritis.[1] The levels of these cytokines are elevated in the joints of arthritic patients and anti-TNF-α antibodies can improve their symptoms.[2] Free radicals are also believed to be important in joint destruction. Both tumor necrosis factor-α (TNF-α) and interferon-γ increase the secretion of hydrogen peroxide by rabbit chondrocytes,[3] leading to the formation of the highly injurious hydroxyl radical.

Plasma levels of TNF-α in rheumatoid arthritis patients correlate with the ability of phagocytes to generate superoxide.[4,5]

Uric acid is known to contribute significantly to the antioxidant status of blood[6,7] and to stabilize vitamin C in blood.[8] Agudelo et al.[9] proposed that persistent hyperuricemia might protect against rheumatoid inflammation, consistent with the fact that patients with hyperuricemic gout rarely develop arthritis. It has been suggested that uric acid is the most important antioxidant compound in patients with rheumatoid arthritis,[10] although our own study on this subject did not confirm this suggestion.[11] The use of diuretics is often associated with an increase in the blood levels of uric acid,[12-16] so the present study was initiated to determine whether treatment with the potent oral diuretic bumetanide could raise serum uric acid levels in patients with rheumatoid arthritis to an extent that would ameliorate inflammation.

Adenosine is known to act on its surface-bound A1, A2, and A3 receptors to modulate the release of pro-inflammatory cytokines from activated macrophages and neutrophils, especially TNF-α.[17,18] There have been few studies to determine whether diuretic therapy directly increases activity along the purine metabolic pathway leading to raised urate levels. Since urate is one of the end products of purine metabolism, it is possible that increased urate levels induced by diuretics could secondarily induce changes in the plasma levels of its precursors including adenosine. We have, therefore, also measured the levels of these purines in addition to urate itself in patients with rheumatoid arthritis treated with bumetanide.

METHODS

Patients and recruitment

Seventy patients, aged 18 to 75 years, were recruited from routine rheumatology clinics by a consultant rheumatologist, and rheumatoid arthritis was diagnosed using the American Rheumatism Association revised criteria.[19] On entry to the study patients were clinically and/or serologically uncontrolled on their medication. Patients were excluded from the study if they were taking any drug known to affect uric acid levels in the blood. On entering the study patients were randomized to receive either bumetanide 4 mg/day or to a placebo treatment group of identical placebo pills. All patients gave written, informed consent to participation in the study. Both groups continued to receive their usual anti-rheumatic therapy and remained on their usual diet. Full clinical assessment was undertaken using the internationally agreed core set of disease activity measures from the American College of Rheumatology. These included tender joint count, swollen joint count, assessment of joint movement, patient's assessment of pain, patient's and physician's global assessment of disease activity, and a modified Health Assessment Questionnaire (HAQ).

Since pain varies with time of day[20] samples were always taken in the morning. Samples were obtained at baseline before treatment and at monthly intervals during treatment for 6 months.

Blood sampling and cell culture

For purine, neopterin, and lipid peroxidation measurements, blood was collected from patients into plain bottles without anticoagulant, allowed to clot, and the serum then frozen and stored at $-70^{\circ}C$ until required for subsequent assay.

Blood for cytokine analysis was collected from patients into tubes containing heparin to prevent coagulation. The heparinized whole blood was then diluted 1/10 with RPMI 1640 culture medium with L-glutamine and 25 mM HEPES (Invitrogen) containing 1% gentamycin solution (Invitrogen). The diluted blood was aliquoted into 1-mL fractions in 24-well tissue culture plates. Lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma) was added at a final concentration

of 100 ng / ml to stimulate cytokine production. Basal cytokine release was measured in diluted whole blood cultures with no LPS added. All cultures were covered with lids, mixed gently and incubated for 40 hours at 37°C in a CO$_2$ incubator at 5% CO$_2$. Following incubation, the contents of each well were decanted into an eppendorf tube and centrifuged at 3,000 rpm for 5 minutes. The supernatant was then transferred to clean eppendorf tubes and stored at –70°C until required for cytokine analysis.

Standard clinical assays

Erythrocyte sedimentation rate (ESR) was measured by a Starrsed Automated ESR machine (R&R Mechatronics) that utilizes the method recommended by the International Council for Standardization in Haematology,[21] based on the method of Westergren.[22] C-reactive protein (CRP) was measured using a Behring Turbitimer.

Neopterin assays

Neopterin levels were measured in 10-μL aliquots of serum using an immunoassay kit (Immunobiological Laboratories, Germany). All samples were analyzed in duplicate. The assay was a competitive ELISA in which a peroxidase-conjugated and a non-conjugated antigen competed for a fixed number of antibody binding sites. Following incubation with substrate solution, color developed and the optical density was read at 450 nm, unknowns being quantified by reference to known standards.

Lipid peroxidation products

An aliquot of 100 μL of serum was used for quantifying the concentrations of the lipid peroxidation products malondialdehyde and 4-hydroxynonenal measured using a Bioxytech LPO-586 colorimetric assay (R&D systems). The analysis involved the reaction of N-methyl-2-phenylindole with these peroxidation products to form a stable chromogenic indolic dimer, which was estimated spectrophotometrically at 586 nm. All samples were tested in duplicate.

Purine assays

A 500-μL sample of the serum was used for the assay, to which 50 μL of 3.3 M perchloric acid was added, the sample vortex mixed and centrifuged at 13,000 g for 10 minutes. The supernatant was injected onto a Gilson gradient high-performance liquid chromatography (HPLC) system, using a Kingsorb C18 5-μm reverse phase column and a mobile phase of the following composition: solvent A: 0.0025M ammonium dihydrogen phosphate buffered to pH 3.5 using 10% phosphoric acid; solvent B: 940 mL of solvent A plus 60 mL acetonitrile buffered to pH 3.5 with 10% phosphoric acid. The mobile phase gradient was initiated as A:B 80:20, rising to 100% of solvent B in 10 minutes and remaining as such for a further 10 minutes. The purines were detected using a UV detector at 254 nm.

Cytokine analysis

TNF-α was measured in 200-μL aliquots of culture supernatant using a commercial quantitative sandwich enzyme immunoassay kit (R&D Systems). IL-1β and IL-6 were each measured in 200-μL aliquots of culture supernatant using commercial solid phase enzyme amplified sensitivity immunoassay kits (Biosource). All assays were performed in duplicate.

Drugs

LPS from *Salmonella typhimurium* and 5'N-ethylcarboxamidoadenosine (NECA)were obtained from Sigma Chemical Company. 1-deoxy-1-[6-[[(3-iodophenyl)methyl] amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA) was obtained from Tocris Chemicals.

Statistics

Where multiple comparisons were being made comparing a series of data points with the basal level, an analysis of variance (ANOVA) was performed followed by Dunnett's test. Comparisons between the placebo and treatment values at individual time points were made using a two-sided t test. In all cases a significance threshold of 5% ($P < 0.05$) was employed.
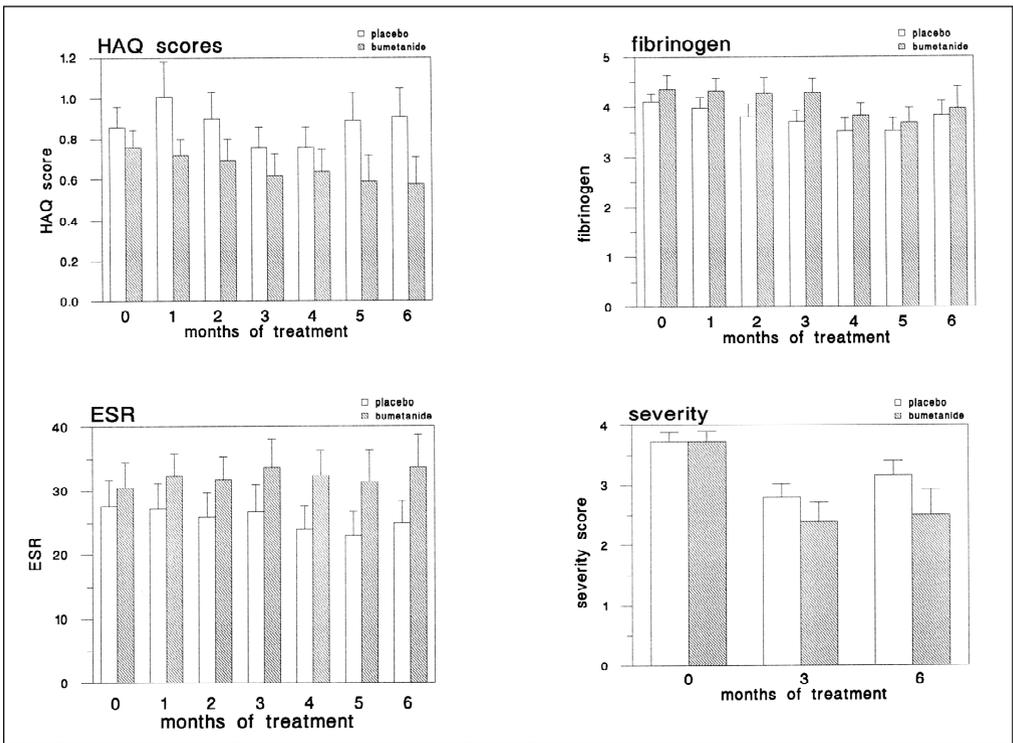
Figure 1. Symptomatic and clinical laboratory measures of disease activity, illustrating the Health Assessment Questionnaire (HAQ) scores, fibrinogen levels, erythrocyte sedimentation rate (ESR), and overall severity score. There were no significant differences between patients treated with placebo (open columns) or bumetanide (shaded columns).

Study size was calculated so that the two-sided tests would have an 80% power to detect a clinically important difference at the 5% level. The calculations were performed using a Query Advisor Release 2.0 software from Statistical Solutions.

RESULTS

Clinical assessments

Despite electing to perform a rigorous quantitative assessment of disease activity in all patients in this study, none of the parameters examined showed a significant change over the time course of the study, either when compared at successive monthly time points relative to the pre-treatment basal levels, or when placebo and treatment groups were compared at each time point. This is illustrated for two of the symptomatic assessments (severity score and HAQ score), and

for two of the laboratory measurements (ESR and fibrinogen) in Figure 1.

Measurements of oxidative stress and inflammation

There were no changes in levels of fibrinogen, ESR, overall disease severity (Figure 1), neopterin, CRP, or lipid peroxidation products during the 6 months of treatment with bumetanide, either when compared at successive monthly time points relative to the pre-treatment basal levels, or when placebo and treatment groups were compared at each time point.

Purine levels

The serum concentrations of adenosine and its metabolites are summarized in Figure 2. No change was noted in the levels of adenosine, inosine, hypoxanthine, or xanthine at any point during the 6 months of treatment with either placebo or bumetanide. Uric acid
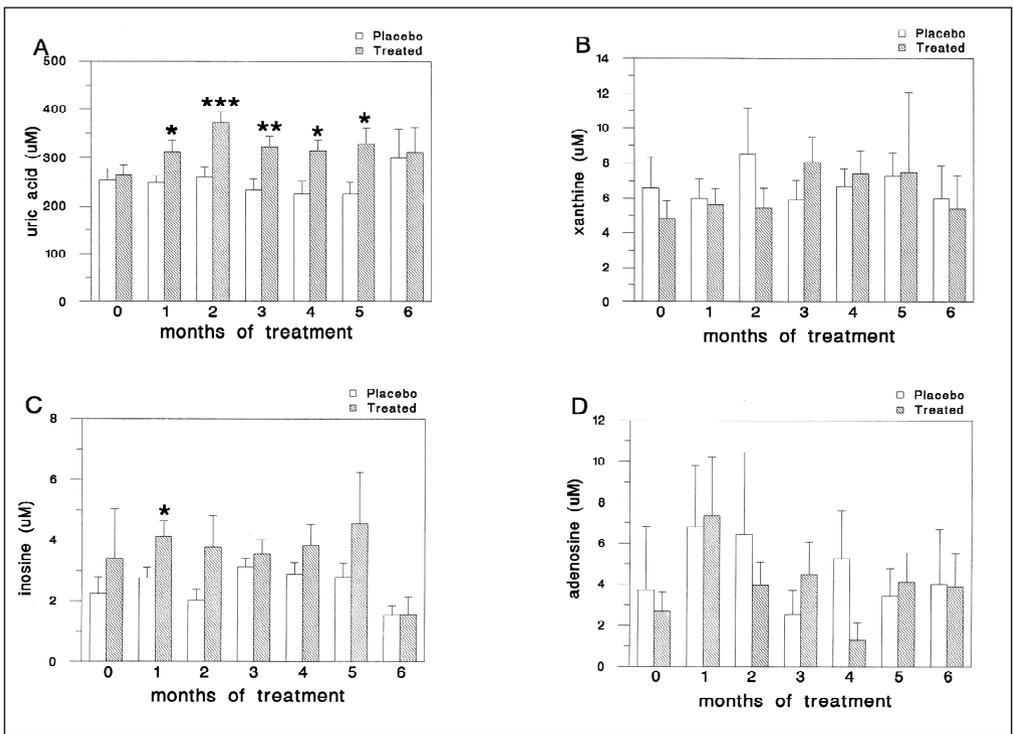
Figure 2. Purine levels in the blood, showing the levels of (A) uric acid, (B) xanthine, (C) inosine, and (D) adenosine during the 6 months of treatment using placebo (open columns) or bumetanide (shaded columns). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for drug-treated patients compared with the corresponding placebo control group.

levels were significantly higher in the drug-treated patients compared with placebo controls at each monthly assessment except the last, although large standard errors occurred in the final samples caused by the loss of patients defaulting from the study.
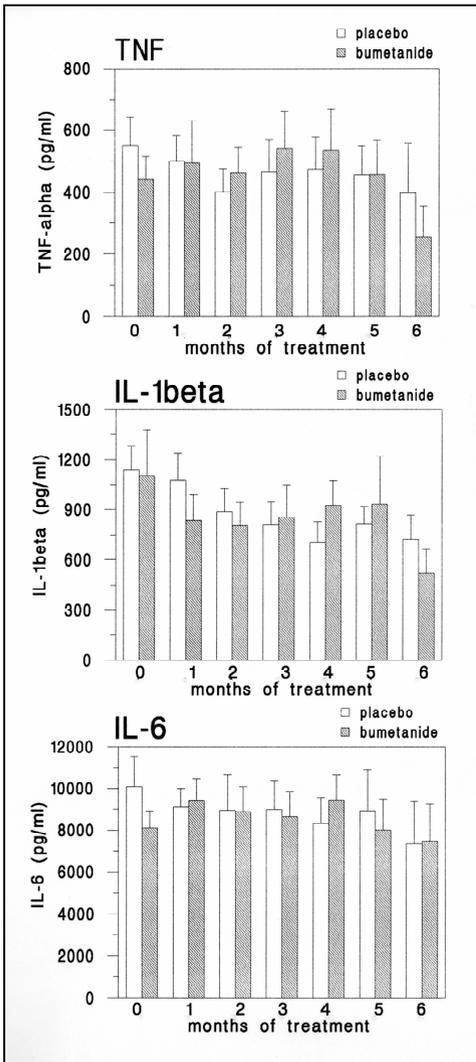
Cytokine levels

The serum levels of TNF-α, IL-1β, and IL-6 did not change throughout the period of study, and showed no differences between bumetanide or placebo-treated patients (Figure 3).

In whole blood cultures, the levels of TNF-α in the supernatant after 40 hours in culture from patients treated with bumetanide or placebo are summarized in Figure 4. Basal levels were readily measurable in both groups of patients, and incubation with LPS (100 ng/mL) did not significantly increase this further. In the case of IL-1β and IL-6, LPS activation

induced an approximately 250% increase of interleukin release, but this was not different between patients and control subjects.

DISCUSSION

The autoimmune process in rheumatoid arthritis includes the penetration of macrophages and neutrophils into synovial fluid. These cells can liberate reactive oxygen species, which contribute to joint damage.[11,23] In adult patients, stimulated phagocytes produce larger amounts of superoxide when compared with controls and with subjects with non-rheumatic disorders. Hydroxyl radicals have been shown to be generated in the synovial fluid of arthritic subjects.[24] Hydroxyl radicals, in particular, cause catabolism of hyaluronic acid[25,26] but can also disrupt proteoglycans,[27] collagens[28] and tissue and fluid proteinase inhibitors such as α-antiproteinase.[29] They may also

Figure 3. Cytokine levels in the blood, showing the levels of tumor necrosis factor (TNF)-α, interleuken (IL)-1β, and IL-6 during the 6 months of treatment. There were no significant differences between patients treated with placebo (open columns) or bumetanide (shaded columns).

induce covalent cross-linking of immune complexes.[30] Direct confirmation that hydrogen peroxide can produce severe tissue damage and arthritis has come from injections of a peroxide generating system (glucose oxidase) into the joints of mice.[31,32] Uric acid inhibits oxidation of ascorbic acid, is an effective erythrocyte membrane lipid antioxidant, protects against oxidative dam-

age to deoxyribonucleic acid (DNA), protects against hydroxyradical-induced oxidation of certain proteins, and scavenges oxygen free radicals and free radical intermediates. The fact that uric acid is a major contributor to the antioxidant activity of blood[7,10,33] was one of the factors that led us to study this metabolite.

Xanthine oxidase is a major producer of reactive oxygen species (ROS), which have a role in killing bacteria. Uric acid is increased in active rheumatoid joints and it has been suggested that "classical" antioxidants are "used" first and, when these are exhausted, the serum uric acid level assumes importance, possibly by maintaining the level of uric acid in the joint to promote reversal of xanthine oxidase activity, thereby increasing levels of xanthine, hypoxanthine and, possibly, adenosine.

Several previous studies have demonstrated an increase in urate levels during treatment of patients with diuretics, at least temporarily[34] and especially with the thiazide and loop diuretics such as bumetanide[13-16] although others have failed to demonstrate significant change unless diuretics were used in combination with drugs such as angiotensin converting enzyme inhibitors,[35] and results may vary from patient to patient.[36] In the present study, in patients with active rheumatoid arthritis, urate levels were consistently and significantly higher in those treated with bumetanide when compared with placebo-treated controls. This result supports the concept that diuretics can increase urate levels, and the increased urate could then contribute to the scavenging of free radicals involved in the destruction of arthritic joints.[3,10] The absence of changes in any of the urate precursors in the purine metabolic pathway (adenosine, inosine, xanthine) suggests that the increase of urate is more likely to result from renal actions of the diuretics than from a direct increase in purine catabolism. There were no significant changes in the levels of the lipid peroxidation products 4-hydroxynonenal and malondialdehyde in
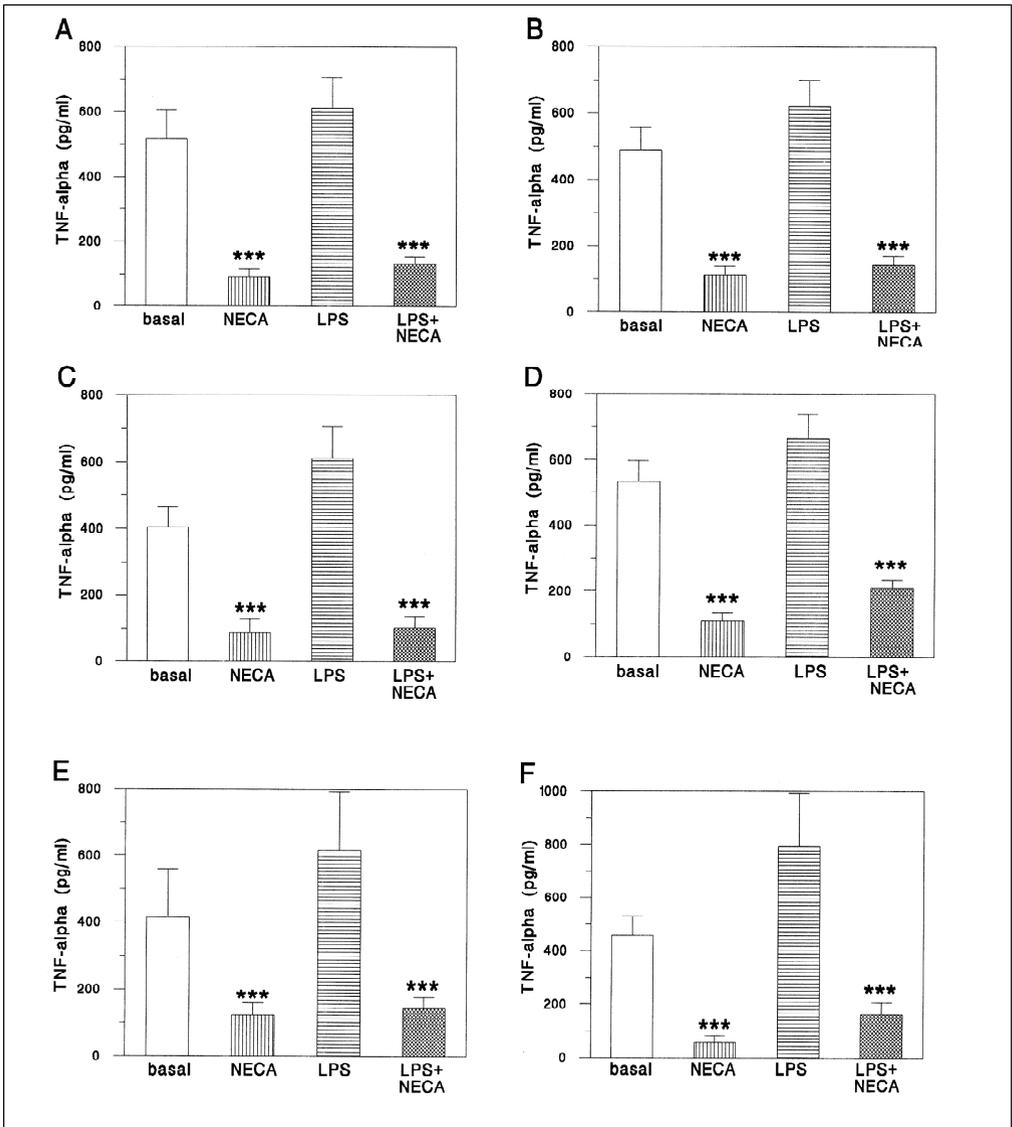
Figure 4. Levels of TNF-α in the supernatant of whole blood cultures from patients treated with placebo (A, B, C) or bumetanide (D, E, F). Results are shown at the start of the study (week 0, before the initiation of placebo or bumetanide treatment (panels A, D)), and after 3 months (B, E) and 6 months (C, F) of treatment. Each panel summarizes the basal level of TNF-α released into the supernatant without experimental manipulation, the effect of 5'N-ethylcarboxam-ideadenosine (NECA) (2 μM) on basal release, the effect of lipopolysaccharide (LPS) (100 ng / ml), and the combined effect of LPS and NECA. *** $P < 0.001$ for NECA compared with basal levels, or LPS + NECA compared with LPS alone.

this study--though this is perhaps not surprising given the small and variable changes in uric acid levels. The examination of cytokine release revealed that there was no difference between the secretion of TNF-α, IL‑1β, or IL-6 from blood cells taken from placebo and drug-treated patients.

In conclusion, 6 months of treatment with the diuretic bumetanide does raise the levels of urate in the blood of patients with rheumatoid arthritis, but this apparently is not associated with a change in disease

activity, oxidative stress, purine metabolism, or cytokine levels.

ACKNOWLEDGMENTS

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