

Urinary eicosanoid and tyrosine derivative concentrations in patients with vasculitides

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Background: Vasculitides are classified on the basis of the type of cell involved, namely, eosinophilic vasculitides such as Churg-Strauss syndrome (CSS) and noneosinophilic vasculitides. However, knowledge on inflammatory mediators and oxidative tissue damage associated with vasculitides is insufficient.

Objective: We measured the urinary concentrations of inflammatory mediators and tyrosine derivatives to assess biomarkers associated with the pathophysiology of vasculitides. **Methods:** Urine was collected from 9 patients with CSS during acute exacerbation and during clinical remission, 24 patients with rheumatoid arthritis in stable condition, and 8 patients with vasculitis diseases (VDs) during acute exacerbation. Leukotriene E₄ (LTE₄), 9 α ,11 β prostaglandin F₂, and eosinophil-derived neurotoxin (EDN) concentrations were determined by enzyme immunoassay. 3-Bromotyrosine (BrY) and 3-chlorotyrosine (CIY) concentrations were determined by gas chromatography-mass spectrometry.

Results: The urinary LTE₄, EDN, BrY, and CIY concentrations were significantly higher in the patients with CSS during acute exacerbation than in healthy control subjects and, except for urinary CIY concentration, significantly decreased during clinical remission. The urinary EDN and BrY concentrations were significantly higher in patients with CSS during acute exacerbation than in patients with VD during acute exacerbation. Only urinary LTE₄ concentration was significantly different between the patients with rheumatoid arthritis in stable condition and the patients with VD during acute exacerbation.

Conclusion: Oxidative tissue damage caused by eosinophil peroxidase is a pathophysiological characteristic of eosinophil-associated diseases such as CSS. Urinary LTE₄ concentration may reflect a pathophysiological event involved in eosinophilic and noneosinophilic vasculitides. Cysteinyl-leukotriene pathways are potential therapeutic targets for small-vessel vasculitides. (*J Allergy Clin Immunol* 2004;114:1353-8.)

Key words: Churg-Strauss syndrome, vasculitides, 3-bromotyrosine, 3-chlorotyrosine, leukotriene E₄

Abbreviations used

ANCA: Antineutrophil cytoplasmic autoantibody
BrY: 3-Bromotyrosine
CIY: 3-Chlorotyrosine
cr: Creatinine
CSS: Churg-Strauss syndrome
cysLT: Cysteinyl-leukotriene
EDN: Eosinophil-derived neurotoxin
EPO: Eosinophil peroxidase
HC: Healthy control
HOBr: Hypobromous acid
LT: Leukotriene
MPA: Microscopic polyangiitis
PG: Prostaglandin
RA: Rheumatoid arthritis
TA: Temporal arteritis
VD: Vasculitis disease
WG: Wegener granulomatosis

Eosinophils possess a wide range of biological properties. Namely, eosinophils release proteins, inflammatory cytokines, and mediators, such as eicosanoids and platelet-activating factors, and can cause tissue injury by releasing a spectrum of toxic products. Eosinophil peroxidase (EPO)¹⁻³ also resides in a matrix of cytoplasmic granules and is one of the most abundant proteins in eosinophils.⁴ EPO plays a role in mediating the host-defense mechanism, such as the destruction of invading parasites and the pathological damage of host tissue by oxidizing intermediates. Briefly, activated eosinophils generate superoxide (O₂⁻) by using a membrane-associated nicotinamide adenine dinucleotide phosphate oxidase,⁵ and its dismutation product, H₂O₂.¹ By using H₂O₂ as a cosubstrate, EPO in eosinophils generates a halogenating oxidant, which is a potent reactive, cytotoxic, and diffusible species. Despite the fact that the plasma chloride (Cl⁻) concentration is 1000-fold higher than that of bromide (Br⁻), interestingly, the major product of the EPO-H₂O₂ system is hypobromous acid (HOBr): Br⁻ + H₂O₂ + H⁺ → HOBr + H₂O.⁶ *In vitro*, HOBr reacts with primary amines to form N-mono-bromamines, and it converts tyrosine to 3-bromotyrosine (BrY).^{7,8} Similarly, myeloperoxidase, a structurally and functionally distinct enzyme produced by neutrophils, monocytes, and certain tissue macrophages,⁹ also contributes to inflammatory tissue injury. Neutrophils selectively use Cl⁻ in plasma to

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TABLE I. Demographic characteristics of patients with CSS

Male/female sex	2/7
Age, y, mean (SD)	51.8 (15.2)
Age at onset, y, mean (SD)	
Asthma onset	42.3 (14.3)
CSS onset	49.8 (14.5)
Blood eosinophil $\times 10^6/L$, median (range)	
During acute exacerbation	6590 (2510-17,880)
During remission	70 (10-700)
IgE-radioimmunosorbent test, IU/mL, median (range)	467 (18-3360)
Cumulative organ involvement, n	
Mononeuritis multiplex	9
Lung	9
Sinus	7
Kidney/urinary tract	7
Heart	4
Skin	2
Mediastinum	1

generate chlorinating oxidants.¹⁰⁻¹² Thus, 3-chlorotyrosine (CIY) is considered to be a selective marker of myeloperoxidase-catalyzed oxidation, whereas BrY is that of EPO-catalyzed oxidation.¹³

The characteristic feature of Churg-Strauss syndrome (CSS),^{14,15} an eosinophilic necrotizing vasculitis,¹⁶ is hypereosinophilia in blood and tissues, such as those of the lungs, gastrointestinal, nerves, and kidneys. The extent of eosinophilia commonly reflects clinical disease activity.^{14,15} Previous studies demonstrated that the seromarkers of the activation of eosinophils, such as eosinophil cationic protein and eosinophil-derived neurotoxin (EDN), can predict a relapse.¹⁷ However, measurement of these seromarkers is not applicable to patients with noneosinophilic small-vessel vasculitides, such as Wegener granulomatosis (WG) and microscopic polyangiitis (MPA). Considering the oxidative reaction-associated myeloperoxidase in the human artery,^{12,18} we hypothesized that measuring both BrY and CIY may serve as a powerful method for estimating oxidative tissue damage and the relative contributions of eosinophils versus neutrophils *in vivo*. Furthermore, the high levels of antibody against myeloperoxidase-specific antineutrophil cytoplasmic autoantibody (ANCA) are observed in patients with myeloperoxidase-ANCA-related vasculitides such as WG and MPA.^{19,20} Because ANCA-activated neutrophils can adhere to and destroy endothelial cells *in vitro*, ANCA is considered to inhibit the inactivation of myeloperoxidase, resulting in tissue damage.^{21,22} In addition, Mayatepek and Lehmann²³ demonstrated a high urinary leukotriene (LT) E₄ concentration in patients with Kawasaki disease, which is the most common childhood vasculitis. However, there has been little experimental evidence to substantiate the close relationship between cysteinyl-leukotriene (cysLT) and vasculitides. According to the National Institutes of Health workshop report,²⁴

there is no objective evidence that CSS is actually caused by LT receptor antagonists. Because there have been no comparative studies of urinary eicosanoid concentrations and clinical characteristics, we aimed to characterize the profiles of eicosanoid, BrY, and CIY concentrations in patients with systemic small-vessel vasculitides, including CSS.

METHODS

Subjects

The subjects of this study were 9 patients with CSS (age range, 27-75 years; mean age, 51.8 years; 2 male and 7 female), 8 patients with vasculitis diseases (VDs) during acute exacerbation (age range, 50-77 years; mean age, 66.0 years; 2 male and 6 female), 24 patients with rheumatoid arthritis (RA) in a clinically stable condition (age range, 39-77 years; mean age, 60.1 years; 6 male and 18 female), and 8 healthy control (HC) subjects (age range, 27-55 years; mean age, 37.3 years; 4 male and 4 female). CSS was diagnosed according to the 1990 American College of Rheumatology criteria and the 1992 Chapel Hill definition.^{25,26} Three patients had recurrent CSS. The mean age at onset of CSS was 49.8 (Table I). Histopathological confirmations, such as necrotizing vasculitis, extravascular necrotizing granulomas, and/or hypereosinophilia in extravascular tissues, were present in all 9 patients with CSS. Clinical examinations and staging included the lung function test, chest radiography, bronchoalveolar lavage test, echocardiography, radioisotope scintigraphy, otorhinolaryngologic and neurologic examinations, and laboratory screening for ANCA.²⁷ During the acute exacerbation of CSS, all patients with CSS showed vasculitis symptoms involving multiple organs, such as eosinophilic pneumonia and cardiopathy, in addition to peripheral hypereosinophilia (mean %, 58.1%) and mononeuritis multiplex, as shown in Table I. After intensive immunosuppressive therapies with drugs including systemic corticosteroids (n = 5 with intravenous administration of 1000 mg/d methylprednisolone for 3 days; n = 9 with 30-40 mg/d prednisolone), cyclosporine (n = 5, 50-100 mg/d), and/or intravenous immunoglobulin²⁷ (n = 7), all 9 patients with CSS were clinically in a disease remission phase, maintained at a dose of 5 to 25 mg/d prednisolone (n = 7) in addition to cyclosporine (n = 4, 50-100 mg/d) at the time of follow-up examination. The mean percentage of peripheral eosinophils was 2.3%. The duration between acute exacerbation and remission was 6.1 ± 2.5 months.

The patients with VD had an acute exacerbation of vasculitis accompanied by autoimmune diseases, such as MPA,²⁶ WG,^{26,28} temporal arteritis (TA, giant-cell arteritis),²⁹ and RA³⁰ (Table II). The patients with VD were diagnosed on the basis of clinical and laboratory examination findings, such as the presence of ANCA (n = 4) and immunocomplex C1q. Pathological vasculitis was confirmed in 5 of 8 patients. In contrast, the stable RA group, composed of 24 patients with RA in a stable condition, was a comparative control for patients with an acute exacerbation of VD. Nine patients received systemic corticosteroid (mean prednisolone dose, 4.2 mg/d), whereas 12 patients received methotrexate therapy. None of the patients had an upper respiratory tract infection in the 4 weeks preceding the study. Permission to conduct the study was obtained from the Ethics Committee of the National Sagami Hospital, and all of the patients who participated gave their informed consent.

Measurements

Spot urine was collected between 9:00 and 11:00 AM from patients with CSS during acute exacerbation and during clinical remission, patients with VD during acute exacerbation, patients with stable RA,

TABLE II. Demographic characteristics of patients with VD

Male/female sex	2/6
Age, y, mean (SD)	66.0 (8.4)
White blood cells $\times 10^6/L$	9453 (3024)
Neutrophil %, mean (SD)	81.0 (8.7)
Eosinophil %, mean (SD)	2.1 (1.9)
C-reactive protein, mg/dL, mean (SD)	9.7 (6.6)
CIq, mg/mL, median (range)*	5.0 (1.6-29.6)
Underlying diseases, n	
RA	3
MPA	1
MPA, PSS, and Sjogren syndrome	1
WG	1
Mixed-connective tissue disease and RA	1
TA and Behcet disease	1
Cumulative vasculitis-associated symptoms, n	
Mononeuritis multiplex	5
Skin ulcer	2
Scleritis	2
RA nodule/TA nodule	3/1
Pathological findings, n	5
Obstructive vasculitis/necrotizing vasculitis	3/2

*n = 7.

and HC subjects. In particular, in the cases of acute exacerbations of CSS and VD, urine was collected before intensive immunosuppressive therapy. We determined the urinary concentrations of LTE_4 (Cayman, Ann Arbor, Mich), $9\alpha,11\beta$ prostaglandin (PG) F_2 (Cayman), which corresponds to the PGD_2 metabolite, and EDN (MBL, Nagoya, Japan) by enzyme immunoassay as previously reported.³¹ The urinary concentrations of BrY and CIY, the selective markers of EPO-catalyzed and myeloperoxidase-catalyzed oxidations, respectively, were determined by gas chromatography-mass spectrometry by using ^{13}C -labeled compounds as internal standards, as reported elsewhere.³² Briefly, after the addition of $^{13}C_6$ -BrY (50 ng) and $^{13}C_6$ -CIY (30 ng) to 2 mL urine, BrY and CIY were extracted with 25% methanol by using a reverse-phase column and then converted to the corresponding heptafluorobutyl *tert*-butyldimethylsilyl derivatives.^{33,34} BrY and CIY concentrations were determined by using Shimadzu gas chromatography-mass spectrometry QP2010 (Kyoto, Japan) equipped with a SPD-5 capillary column (15 m; 0.25-mm internal diameter; 0.25- μ m film thickness; Supelco, Bellefonte, Pa) in the negative ion chemical ionization mode with methane as the reagent gas. BrY and CIY concentrations were determined by measuring the fragment ions at mass-to-charge ratio (*m/z*) 489.10 for endogenous compounds and *m/z* 495.15 for the internal standards. Urinary LTE_4 , $9\alpha,11\beta$ PGF₂, EDN, BrY, and CIY concentrations were normalized to urinary creatinine (cr) concentration.

Analysis of data

Demographic characteristics are expressed as means $\pm$ SDs. The urinary eicosanoid, EDN, BrY, and CIY concentrations are expressed on a log scale in the figures. These urinary concentrations in the 4 groups (CSS during acute exacerbation, VD during acute exacerbation, stable RA, and HC groups) were first compared by using the Kruskal-Wallis test. When a significant difference was found, the Mann-Whitney *U* test with the Bonferroni correction for comparison between groups was performed. The urinary concentrations of the 5 biomarkers in CSS patients during acute exacerbation and clinical remission were compared by using the Wilcoxon *t* test. Relationships were analyzed by using the Spearman rank correlation test. *P* values of less than .05 were regarded as statistically significant.

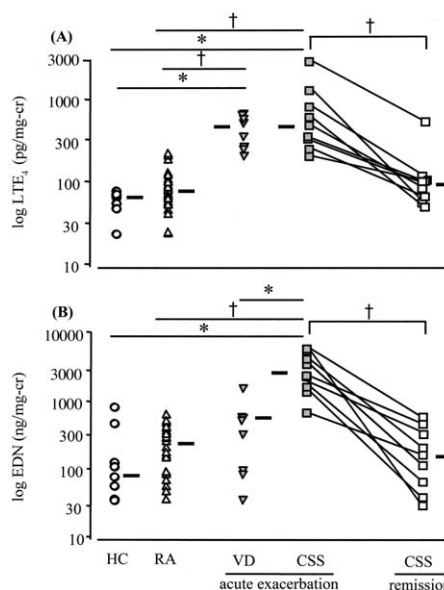


FIG 1. Urinary LTE_4 (A) and EDN (B) concentrations in each group. Urinary concentrations are expressed by using the log scale. Patients with CSS, VD, and RA and HC subjects are denoted by closed squares, closed triangles, open triangles, and open circles, respectively. Horizontal bars indicate medians. **P* < .05; †*P* < .01.

RESULTS

As shown in Fig 1, the urinary LTE_4 concentration was significantly higher in the patients with CSS during acute exacerbation (median, 449.6 pg/mg-cr) than in the patients with stable RA (median, 79.3 pg/mg-cr; *P* < .01) and the HC subjects (67.5 pg/mg-cr; *P* < .05). A significantly higher urinary EDN concentration was observed in the patients with CSS during acute exacerbation (2404.0 ng/mg-cr) than in the patients with VD (432.3 ng/mg-cr; *P* < .05), the patients with stable RA (296.7 ng/mg-cr; *P* < .01), and the HC subjects (94.9 ng/mg-cr; *P* < .05), respectively. Fig 2 shows the urinary BrY and CIY concentrations in each group. The urinary BrY concentration was significantly higher in the patients with CSS during acute exacerbation (182.6 ng/mg-cr) than in the patients with stable RA (36.8 ng/mg-cr; *P* < .01) and the HC subjects (25.2 ng/mg-cr; *P* < .05). The urinary CIY concentration was significantly higher in the patients with CSS during acute exacerbation (6.1 ng/mg-cr; *P* < .05), the patients with VD during acute exacerbation (9.2 ng/mg-cr; *P* < .05) and the patients with stable RA (4.7 ng/mg-cr; *P* < .05) than in the HC subjects (1.2 ng/mg-cr). No significant difference in urinary $9\alpha,11\beta$ PGF₂ concentration was observed among the 4 groups (data was not shown). Next, we examined the correlation between these urinary parameters and the involvement of vasculitis. As shown in Figs 1 and 2, there were significant differences in urinary LTE_4 , EDN, and BrY concentrations in the patients with CSS during acute exacerbation and clinical remission (median, for LTE_4 , 449.6 pg/mg-cr vs 91.2 pg/mg-cr;

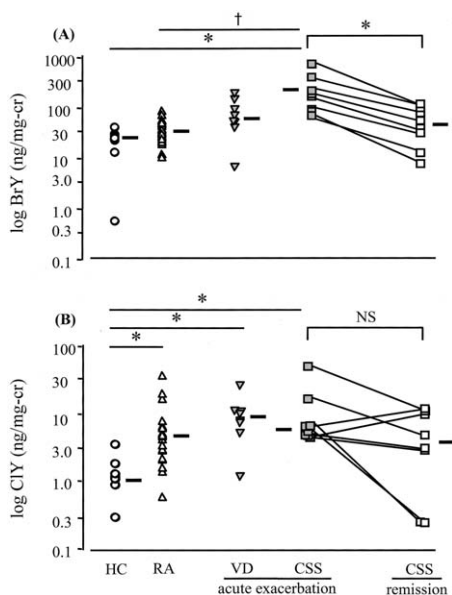


FIG 2. Urinary BrY (A) and CIY (B) concentrations in each group. Results are expressed as in Fig 1. * $P < .05$; † $P < .01$.

$P < .01$; for EDN, 2404.0 ng/mg-cr vs 151.7 ng/mg-cr; $P < .01$; for BrY, 182.6 ng/mg-cr vs 44.4 ng/mg-cr; $P < .05$). Only urinary LTE₄ concentration was significantly different between the patients with stable RA and the patients with VD during acute exacerbation (median, 79.3 pg/mg-cr vs 434.0 pg/mg-cr; $P < .01$). No correlation was found among these 5 urinary markers in any of the 4 groups.

DISCUSSION

Leukotriene E₄ has been identified as a major metabolite of LTC₄, and urinary LTE₄ concentration is now considered the most appropriate analytical parameter for monitoring the endogenous synthesis of cysLTs.³⁵ In this study, we demonstrated for the first time that urinary LTE₄ concentration was significantly higher in patients with CSS during acute exacerbation than in HC subjects, and significantly decreased during clinical remission. It is most interesting to note that the increased urinary LTE₄ concentration in patients with VD during acute exacerbation was observed despite relatively low EDN concentrations. Recent studies have demonstrated a close relationship between cysLTs and vascular events. Sjöström et al³⁶ demonstrated that microsomal glutathione S-transferase 2, a distant homologue of LTC₄ synthase, is a critical enzyme present in vascular walls for LTC₄ biosynthesis, originating from the transfer of LTA₄ from granulocytes to endothelial cells. In addition, an increased urinary LTE₄ concentration was observed in patients with ischemic heart diseases.³⁷⁻³⁹ Taking these findings together, transcellular biosynthesis among mononuclear cells and endothelial cells plays an important role in the cysLT overproduction in vasculitides.

Aspirin intolerance is also characterized by a cysLT overproduction profile.⁴⁰⁻⁴² In particular, the clinical features of CSS are quite similar to those of the aspirin intolerance phenotype—namely, bronchial asthma, eosinophilic sinusitis, and hypereosinophilia. We previously demonstrated that basal urinary LTE₄ concentration in patients with asthma is higher than that in HC subjects, and that basal urinary LTE₄ concentration in asthmatic patients with eosinophilic sinusitis is higher than that in asthmatic patients without eosinophilic sinusitis.⁴⁰ In addition, we preliminarily confirmed in this study that markedly high urinary LTE₄ concentrations (167.6, 188.3, and 199.0 pg/mg-cr) were observed in 3 patients with nonvasculitic eosinophil diseases (acute eosinophilic pneumonia, episodic eosinophilic angioedema, and bronchial asthma with hypereosinophilia). However, despite markedly high percentages of blood eosinophils (mean, 34.6%), the extents of increase in urinary LTE₄ concentrations in these 3 patients with nonvasculitic eosinophil diseases were relatively smaller than in patients with CSS. Thus, particularly in CSS, eosinophilic vasculitides may be involved in cysLT overproduction in addition to eosinophilic pneumonia and sinusitis. Transcellular biosynthesis among endothelial cells and LTC₄ synthase-positive cells, including eosinophils, plays a key role in the mechanism underlying cysLT production in CSS. The vicious cycle, in which cysLTs promote the progenitor effect of LTC₄-producing cells,⁴³ possibly contributes to the further increased production of cysLTs in patients with an acute exacerbation of vasculitides. At least, this study demonstrated that urinary LTE₄ concentration as a new biomarker determined by a non-invasive methodology possibly contributes to the early diagnosis of small-vessel vasculitides.

In this study, we determined the urinary concentrations of 2 halogenated oxidation products—that is, BrY and CIY. BrY is considered a candidate marker of eosinophil activation,^{13,32} CIY of neutrophil and monocyte activation. BrY and CIY concentrations in biological samples such as bronchoalveolar lavage fluid^{13,44} and sputum⁴⁵ have been determined. Thus, we hypothesized that the adaptation of this methodology is expected to identify oxidative tissue damage and the involvement of specific inflammatory cells in vasculitides and hypereosinophilia.^{46,47} In the patients with CSS, the urinary BrY concentration significantly increased during acute exacerbation and decreased during clinical remission. We previously demonstrated the significantly higher urinary BrY and CIY concentrations in patients with asthma than in the HC subjects.³² Similarly, 3 patients with nonvasculitic eosinophil diseases described previously also showed high urinary BrY concentrations (97.4, 122.9, and 62.0 ng/mg-cr). Thus, these findings suggest that the oxidative tissue damage caused by activated eosinophils is a pathophysiological characteristic of eosinophil-associated diseases such as bronchial asthma and CSS. In contrast, we also analyzed the time course of the concentrations of urinary tyrosine derivatives in 2 patients with severe anaphylactic shock. This additional analysis showed normal urinary tyrosine derivative concentrations despite

marked increases in urinary $9\alpha,11\beta$ PGF₂ and LTE₄ concentrations during severe anaphylactic shock, suggesting that the effect of activated mast cells on urinary tyrosine derivative concentration (data not shown) is negligible or limited. Moreover, these results strongly suggest that BrY and CIY are preferentially produced by activated eosinophils and neutrophils/monocytes, respectively. Interestingly, 2 deceased patients with VD showed the highest concentration of urinary BrY (145.6 and 196.2 ng/mg-cr), and 1 of them also had methotrexate-induced pneumonitis.⁴⁸ There is clear evidence linking eosinophils and methotrexate-induced lung injury. The blood and tissue eosinophilia are often found in patients with methotrexate-induced pneumonitis.⁴⁹ Considering that the patients with VD in our study are heterogeneous, further investigations of a large number of patients with VD are required to determine whether urinary BrY concentration may be a useful prognostic predictor of vasculitides and methotrexate-induced pneumonitis. There was no significant difference in urinary CIY concentration among the patients with RA, VD, and CSS, suggesting that urinary CIY concentration may be a less sensitive biochemical indicator of noneosinophilic oxidative tissue damage. Urinary BrY and CIY concentrations may not be directly associated with the pathogenesis of vasculitides. However, we must consider that the stable and major metabolites of BrY and CIY in urine have not yet been elucidated. Other candidate biomarkers for oxidative tissue damage in vasculitides may be total nitrite and nitrate ($\text{NO}_2^- + \text{NO}_3^-$) concentration⁵⁰ and 3-nitro-4-hydroxyphenylacetic acid concentration⁵¹ in urine.

In conclusion, although urinary LTE₄ concentration failed to discriminate these 2 eosinophilic and noneosinophilic vasculitides, urinary LTE₄ concentration may be used as a sensitive biomarker for monitoring the pathophysiological events involved in vasculitides. These data suggest that cysLT pathways may be new therapeutic targets for small-vessel vasculitides. In addition, we demonstrated for the first time that oxidative tissue damage caused by activated eosinophils is a pathophysiological characteristic of eosinophil-associated allergic diseases such as bronchial asthma and CSS.

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