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PAPER

Antioxidants suppress mortality in the female NZB × NZW F1 mouse model of systemic lupus erythematosus (SLE)

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Inflammation produces reactive oxygen intermediates (ROI) that cause vascular damage and activate T lymphocytes. Conversely, antioxidants not only protect tissue from oxidative damage but also suppress immune reactivity. The objective of this study was to examine immunomodulatory effects of the non-enzymatic antioxidants, *N*-acetylcysteine (NAC) and cysteamine (CYST), on autoimmune disease, glomerulonephritis, and mortality in the female B/W mouse model of human systemic lupus erythematosus (SLE). The development of murine lupus was assessed during the lifespan of female B/W mice given NAC or CYST. Morbidity and mortality were assessed daily. At 6 week intervals mice were examined for weight change, albuminuria, serum BUN, antibodies to DNA, and IgG immunoglobulin levels. Serum prolactin, estrogen and progesterone were measured at 18 weeks of age. In a parallel study, NAC- and CYST-treated and control B/W mice were examined at 24 weeks of age for interval renal histopathology, lymphocyte adhesion molecule expression, and antibody titers and *in vitro* cytokine production in response to immunization with DNP-KLH. CYST significantly suppressed development of albuminuria and azotemia at 36 and 42 weeks of age compared to control and NAC-treated mice. NAC significantly suppressed anti-DNA antibody levels at 24 weeks. In contrast CYST significantly increased anti-DNA antibody levels at 18 weeks of age ($P < 0.001$ CYST *vs* control and NAC-treated mice). Kidneys of CYST-treated mice also had accelerated inflammatory histologic changes despite their lower incidence of albuminuria and azotemia. Mean ($\pm$ s.e.m.) survival of control mice was 33 ± 2 weeks compared to 38 ± 2 weeks in NAC-treated mice ($P < 0.05$ *vs* control), and 48 ± 2 weeks in the CYST-treated group ($P < 0.01$ *vs* control mice). The antioxidants, NAC and CYST, significantly improved mortality in the female B/W mouse model of SLE. NAC suppressed autoantibody formation and modestly prolonged survival. CYST, despite its augmentation of anti-DNA levels and renal inflammatory changes, inhibited the development of renal insufficiency and markedly improved survival. These findings suggest that ROIs play a role in the pathogenesis of lupus nephritis and that antioxidants reduce the damage causing renal insufficiency. Antioxidants may be a beneficial adjunctive therapy in the treatment of human SLE. *Lupus* (2001) **10,** 258–265.

Keywords: lupus; antioxidants; cysteamine; *N*-acetylcysteine; therapy

Introduction

Reactive oxygen intermediates (ROIs)—the superoxide anion, hydroxyl radicals and hydrogen peroxide—are generated during immune processes associated with phagocytic neutrophil and macrophage activity. ROIs not only directly damage endothelium, leading to vascular permeability and edema, but also oxidize cell membrane lipids and induce apoptosis.[1] ROIs also activate immunocompetent cells through effects on intracellular message systems such as protein kinases and transcription factors.[2,3] In contrast, antioxidants not only reduce the damaging effects of ROIs, but are also immunosuppressive properties through inhibition of cellular activation.[3–5]

Systemic lupus erythematosus (SLE) is an inflammatory disease characterized by anti-DNA antibody formation and immune-complex mediated endothelial damage that is manifest by cutaneous disease, arthritis, serositis, cerebritis and glomerulonephritis. Glomerulonephritis is a frequent cause of significant morbidity and mortality.[6] Parameters of oxidative

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damage are elevated in patients with lupus and low levels of endogenous antioxidants may predispose to the development of autoimmune disease.[7–10] ROIs also appear to make DNA more antigenic[11] and lead to the excessive production of potentially immunogenic low molecular weight DNA (LMW-DNA) in SLE patients. ROI scavengers, specifically superoxide dismutase (SOD) and cysteamine (CYST), suppress *in vitro* LMW-DNA formation in peripheral blood mononuclear cells from SLE patients.[12] SOD and associated proteins enzymatically reduce ROIs whereas sulfhydryl-bearing compounds, like CYST, *N*-acetylcysteine (NAC) or glutathione, nonenzymatically reduce ROIs through their sulfhydryl moiety.[1,3]

Given the potential beneficial effects of free radical protection and the immunosuppression offered by antioxidants, we hypothesized that antioxidants would inhibit the development of autoimmune disease and glomerulonephritis in the NZB × NZW F1 (B/W) murine model of SLE.

While a large number of available antioxidants exist, we considered highly the aminothiol compounds *N*-acetylcysteine (NAC) and cysteamine (CYST), as they have proven clinical efficacy. NAC is a sulfhydryl-containing derivative of cysteine and precursor of glutathione, a potent endogenous antioxidant and free radical scavenger. NAC has been used in the treatment of acetaminophen toxicity,[13] has demonstrated radioprotective effects in humans[14] and has been shown to suppress murine experimental autoimmune encephalomyelitis (EAE).[15] CYST, another sulfhydryl-containing aminothiol compound, also exhibits potent antioxidant and immunosuppressive activities. It has been used in treatment of acetaminophen toxicity[16] as well as nephropathic cysteinuria,[17] is known to induce radioprotection,[18] and suppresses a variety of immune responses *in vitro* and *in vivo*.[19,20] CYST also has endocrinologic properties that include suppression of immunomodulatory pituitary hormones such as prolactin (PRL), follicle stimulating and luteinizing hormones (FSH/LH), and growth hormone.[20–22] This property may also be beneficial, as PRL has been shown to stimulate autoimmunity in the B/W mouse.[23] In this study both NAC and CYST significantly improved survival, but through apparently disparate effects on the development of autoimmunity in this murine model of SLE.

Materials and methods

Mice

Female NZB × NZW F1 (B/W) mice were obtained from our breeding colony at the Jackson VA Animal Research facility. This model of autoimmunity develops anti-DNA antibodies and immune complex glomerulonephritis that closely resemble lupus nephritis. Ninety-five percent of female B/W mice die of glomerulonephritis and renal insufficiency by 60 weeks of age.[24,25] Animals were kept at this AALAC-approved facility with a 12 h light:12 h dark cycle and provided with water and food *ad libitum*. Mice were entered into the study at 10 weeks of age.

Reagents

CYST and NAC (Sigma, St Louis, MO) were administered orally to separate groups of mice at dosages of 250 mg/kg/day in their drinking water. Previous studies in rodents had demonstrated immunomodulatory effects at this dosage.[15,19,20] Control animals received distilled water alone.

Study protocol

Mice were examined daily for morbidity and mortality. At 6 week intervals, mice were weighed and urine was expressed from the bladder and checked for albumin. Anesthetized mice were bled from the orbital plexus for assessment of anti-DNA antibodies, total IgG, blood urea nitrogen (BUN), serum prolactin (PRL), estrogen (E) and progesterone (P). Moribund animals were terminated and necropsied. Death was attributed to lupus glomerulonephritis when urine albumin measurement was > 2+, BUN was > 30 mg/dl, and necropsy demonstrated parenchymal renal disease. Necropsy diagnosis was confirmed histologically. Parallel, identical groups of control, NAC- and CYST-treated mice were terminated at 24 weeks of age and examined for interval renal histopathology and lymphocyte phenotype (see below). Parallel groups of mice were also immunized with dinitrophenol-keyhole limpet hemocyanin (DNP-KLH) and examined for antibody response and *in vitro* production of interleukins in response to KLH and Con A stimulation.

Renal function

Urine expressed from the bladder at 6 week intervals was examined for albumin by Albustix (Bayer Corporation, Elkhart, IN) and ranked on a scale of negative to 4+. BUN was determined by an endpoint colorimetric reaction using the BUN (Endpoint) 50 kit (Sigma Chemical, St Louis, MO) per the manufacturer's instructions.

Anti-DNA antibodies and serum IgG concentrations

Mean anti-DNA antibody levels, median end-point titration, and serum total IgG concentrations were analyzed by ELISA as previously described.[25]

Renal histology

Kidneys from control, NAC- and CYST-treated B/W mice in the interval study were fixed in 10% buffered formalin. Five micron thick sections of all kidneys were sectioned and stained with hematoxylin/eosin and PAS. Sections of renal tissue from individual mice were scored blindly (by AL) for glomerulonephritis as previously described.[25]

Flow microfluorometry

Flow microfluorometry of treated and control splenocytes was performed as previously described.[25] The following anti-murine antibodies were purchased from Pharmingen (San Diego, CA): anti-CD4, anti-CD8, anti-IgD, anti-CD44, anti-VLA-4 and anti-LFA-1. Percentage positive cells were enumerated on a Becton-Dickinson FacScan (San Jose, CA).

DNP-KLH immunization and serum total anti-DNP IgG

Dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH), keyhole limpet hemocyanin (KLH), and DNP-bovine serum albumin (DNP-BSA) were obtained from Calbiochem-Novabiochem Corporation (San Diego, CA). Mice were immunized as previously described and sera were obtained 10 days after immunization. Total IgG in response to the immunization with DNP/KLH was determined as described.[25]

Cytokine analysis

For assessment of splenic cytokine response to Con A or KLH, 1×10^6 splenocytes were dispensed into 24-well plates in RPMI complete media containing 5% fetal calf serum, L-glutamine, sodium pyruvate, MEM vitamins, non-essential amino acids, 2-β-mercaptoethanol, and antibiotic/antimycotic (all purchased from Fisher Scientific, Norcross, GA) and placed in a 5% CO_2 incubator at 37°C. Con A (2 μg/ml, Sigma) stimulated supernatants were collected after a 24 h incubation, KLH (100 μg/ml) stimulated supernatants were collected at 72 h. Cytokine assays for IL-2, IL-4, IL-6 and IFNγ were performed by ELISA using Pharmingen OptEIA Cytokine kits per the manufacturer's instructions. Absorbance was determined at 450 nm and cytokine concentrations were measured by plotting sample absorbance against the standard curve.

Hormonal analysis

Serum prolactin was assayed by radioimmunoassay as previously described.[23] Estrogen and progesterone concentrations were determined by EIA (Cayman Chemical, Ann Arbor, MI) per the manufacturer's instructions.

Measurement of SOD

Superoxide dismutase activity was measured in the kidneys and livers of representative mice from the control, NAC- and CYST-treated mice at 24 weeks of age by enzymatic assay (Oxis Health Products, Portland, OR) per the manufacturer's instructions. SOD activity was corrected for cell lysate protein concentration and expressed as units/per mg protein.

Statistical analysis

One-way analysis of variance (one-way ANOVA), followed by Bonferroni's *post-hoc* test, was used to compare differences of mean values among the three groups of mice. The chi-square test was used to compare the difference of the number of positive findings between groups of mice regarding albuminuria, azotemia and survival. The Mann–Whitney test was used to compare statistically anti-DNA, cytokine supernatant and flow microfluorometry values. Survival curves were estimated using the Kaplan–Meier method and the curves were compared using the Breslow statistic. Data was considered to have a statistically significant difference if $P < 0.05$.

Results

Administration of NAC and CYST

NAC and CYST were well tolerated without apparent side effects. Weight gain was not significantly differ-

ent in the three groups up to 30 weeks of age. At 30 weeks of age weight in the control and NAC groups was significantly higher than in the CYST-treated group (control mean = 35 ± 1.6 g, NAC group mean = 34 ± 1.1 g *vs* CYST group = 30.5 ± 0.8 g; $P < 0.05$). This difference did not persist beyond 30 weeks of age. There was no significant difference in food intake per mouse/body weight as determined by weekly food intake. Similarly there was no clinical or necropsy evidence of infectious or gastrointestinal complications.

Renal function

The cumulative incidence of severe albuminuria ($\geq 2+$) in control, NAC and CYST-treated mice is shown in Figure 1 (panel A). Compared with control mice, CYST significantly suppressed cumulative albuminuria at 30, 36 and 42 weeks of age ($P < 0.01$ at 30 weeks, $P < 0.04$ at 36 and 42 weeks of age). The median onset of severe albuminuria in control, NAC- and CYST-treated B/W mice were 30, 33 and 40 weeks respectively ($P < 0.05$ comparing CYST with control or NAC). Cumulative azotemia (BUN > 30 mg/dl) in control, NAC- and CYST-treated B/W mice is shown in Figure 1 (panel B). Elevation of serum BUN concentrations paralleled the development of albuminuria and indicated progressive renal insufficiency due to glomerulonephritis in control and NAC-treated animals and confirmed suppression of renal insufficiency in CYST-treated B/W mice. At 30 weeks of age, 44% of control female B/W mice had azotemia, whereas only 20% of NAC-treated mice had azotemia, whereas no mouse in the CYST-treated group had azotemia. Fewer CYST-treated mice also had azotemia at 36 ($P < 0.03$ *vs* control) and 42 weeks ($P < 0.001$ *vs* control). Although NAC-treated mice had a consistently lower incidence of albuminuria and azotemia compared to control mice at specific time points, differences did not reach statistical significance.

Anti-DNA antibodies and total serum IgG

Serum anti-DNA IgG antibodies were determined in control, NAC- and CYST-treated female B/W mice at 18, 24 and 30 weeks of age. Compared to control B/W mice, NAC significantly suppressed anti-DNA antibody levels at 24 and 30 weeks (Figure 2). End-point titration also demonstrated that NAC suppressed development of anti-DNA antibodies at 24 and 30 weeks (2-fold difference compared to control mice; median end point titer control = 1:1600 and 1:3200 *vs* NAC-treated group = 1:800 and 1:1600, respectively). In contrast, CYST significantly increased anti-DNA antibody levels at 18 weeks of age (Figure 3, $P < 0.05$ CYST *vs* control). End-point titration sera of

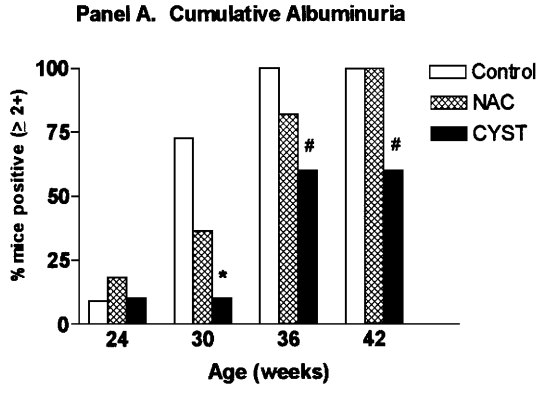

Figure 1 (A) Cumulative incidence of albuminuria ($\geq 2+$) in control, NAC-treated and CYST-treated female B/W mice. *$P < 0.01$; #$P < 0.04$. (B) Cumulative azotemia (BUN > 30 mg/dl) in control, NAC-treated and CYST-treated female B/W mice. *$P < 0.03$ *vs* control; #$P < 0.001$ *vs* control.

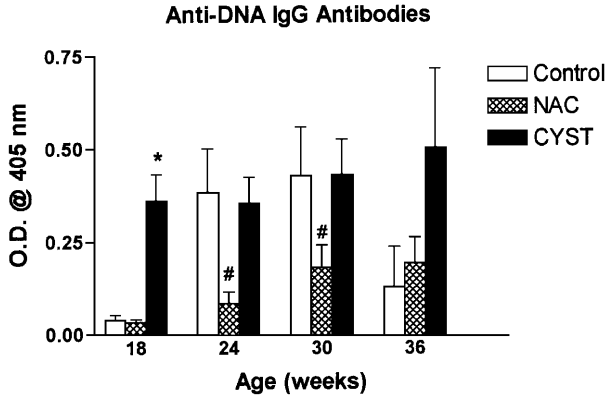

Figure 2 Serum anti-DNA antibodies in control, NAC-treated and CYST-treated female B/W mice at 18, 24 and 30 weeks of age. *$P < 0.05$ *vs* control and NAC-treated mice; #$P < 0.05$ *vs* control.

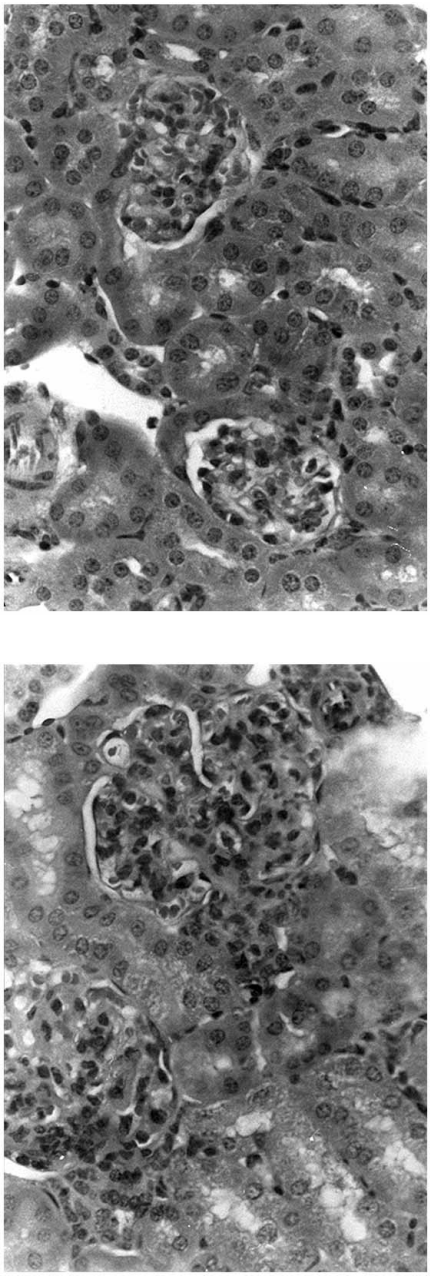

Figure 3 Representative histologic sections of kidneys from control (4A) and CYST-treated (4B) female B/W mice. CYST-treated mice (4B) had increased mesangioproliferative glomerulonephritis and interstitial mononuclear cell infiltrate compared to control (4A) and NAC treated mice (not shown). Section magnifications are 100×; stained with hematoxylin and eosin (H and E).

control and CYST-treated B/W mouse sera at 18 weeks of age revealed a 16-fold difference in anti-DNA antibodies (median end point titer: CYST-treated mice = 1:3200 *vs* control mice = 1:200). Serum total IgG concentrations were not different among the three groups at any time point examined (data not shown).

Splenocyte phenotype and cytokine analysis

There were no significant differences at 24 weeks of age between percentages of CD4+ or CD8+ T cells or IgD+ B cells in the spleens of control, NAC- or CYST-treated mice. Likewise, differences in lymphocyte expression of VLA-4, LFA-1 or CD44 were not observed (data not shown). Splenocyte proliferation in response to Con A or KLH (100 μg/ml) was also not significantly different between treatment groups. *In vivo* anti-DNP serum IgG antibody responses to DNP-KLH immunization were higher in both NAC- and CYST-treated mice, but the difference did not achieve statistical significance (data not shown). There were no significant differences in serum IFN-γ or IL-6 concentrations or *in vitro* splenocyte production of IL-2, IL-4, IL-6 or IFNγ in response to Con A or KLH.

Renal histopathology

Four mice from each group were randomly selected, sacrificed, and kidneys were examined in a blinded fashion. Histopathologic findings are recorded in Table 1. One of the control mice had glomerular pathology at 24 weeks of age. In the treatment groups, one of four NAC-treated mice had significant glomerular infiltrate whereas three of four CYST-treated mice had glomerulonephritis by histopathologic examination. This occurred in the absence of severe albuminuria and azotemia (Figures 1A and 2B). Representative histologic sections are shown in Figure 3A and B.

Survival

Survival curves for control, NAC- and CYST-treated female B/W mice are shown in Figure 4. Analysis of

Table 1 Renal histopathology in control, NAC-treated and CYST-treated female B/W mice

Group	*Number with GMN*[a]	*Histopathologic findings*
Control	1/4	Mild mesangioproliferative GMN with few interstitial mononuclear infiltrates
NAC-treated	1/4	One sample with crescentic GMN and moderate interstitial mononuclear infiltrate
CYST-treated	3/4	Mild to moderate mesangioproliferative GMN with moderate to large interstitial mononuclear infiltrate

[a]GMN = glomerulonephritis.

survival curves revealed that both NAC and CYST prolonged survival in B/W mice compared to control animals ($P = 0.035$, control *vs* NAC; $P < 0.0001$ control *vs* CYST). Mean survival of control and NAC-treated mice ($\pm$ s.e.m.) were 33 ± 2 and 38 ± 2 weeks ($P < 0.05$ *vs* control), respectively, compared to 48 ± 2 weeks in the CYST-treated group ($P < 0.01$ *vs* control mice). At 43 and 48 weeks of age, respectively, none of the control mice ($n = 11$) or NAC-treated mice ($n = 11$) were alive; all had died from murine lupus nephritis. In contrast, survival was 50% for CYST-treated female B/W mice ($n = 10$) at 54 weeks of age.

Measurements of SOD

There were no significant differences in kidney or liver SOD activity between the control and treatment groups (data not shown).

Serum hormone concentrations

Serum prolactin, estrogen and progesterone concentrations were assessed in randomly selected 18-week-old control, NAC, and CYST-treated mice ($n = 6-8$ mice/group). CYST-treated mice had the lowest serum prolactin concentrations compared to control and NAC-treated groups, but there were no statistically significant differences. Prolactin (ng/ml $\pm$ s.e.m.) was 122 ± 68 in control mice, 228 ± 104 in NAC-treated mice, and 71 ± 38 in CYST-treated mice. Estrogen and progesterone concentrations, likewise, were not significantly different between the three groups (data not shown).

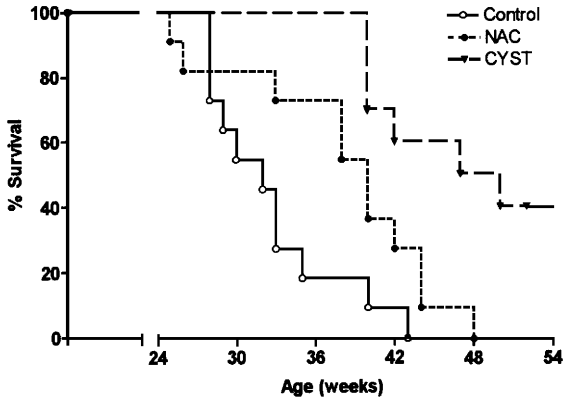

Figure 4 Survival curves for control, NAC-treated and CYST-treated female B/W mice. Survival curves of NAC- and CYST-treated mice were significantly different from control mice ($P = 0.035$ and $P = 0.0001$, respectively).

Discussion

In this study both aminothiol antioxidant compounds, NAC and CYST, successfully prolonged survival in the female B/W mouse model of SLE. We entertained the possibility that CYST-treated mice had alterations of pituitary function as a mechanism of immunomodulation in this study. Prolactin is known to modulate phenotypic and immunologic development in mice[26,27] and stimulate autoimmune disease activity in murine lupus,[23] and in human SLE.[28] Although prolactin was lowest in CYST-treated mice, it was not significantly different between groups at 18 weeks of age; moreover, estrogen and progesterone were not different between groups, suggesting normal pituitary function. While not completely excluded, endocrinologic actions of CYST administration do not appear to be the explanation for the beneficial mechanism of action on B/W autoimmune disease in this study.

NAC inhibited anti-DNA antibody formation, possibly through a direct or indirect action on T cells,[2–5] whereas CYST augmented anti-DNA antibody development. A disparity between the immunomodulatory effects of NAC and CYST on T cells has been previously reported,[5] but remains unexplained.[2–5] The relative potency in the prolongation of B/W survival may have been due not only to different mechanisms of action, but also to the requirement for the conversion of NAC to glutathione for maximal antioxidant potency.[3]

CYST administration in this study was associated with accelerated renal inflammatory cell infiltration, yet suppressed glomerular damage as assessed by albuminuria and azotemia. This finding is internally consistent with the finding of CYST elevation of anti-DNA antibodies, the presumed pathogenesis of lupus nephritis in this model,[24] and the reduction of glomerular damage. It is possible that aminothiol compounds have multiple potential beneficial actions requiring elucidation in this model. The actions include, but are not limited to, suppression of nitric oxide synthase activity,[29] elevation of nitric oxide levels,[30] suppression of vasoconstricting F2-isoprostanes and thromboxanes,[30] inhibition of pro-inflammatory cytokines,[30,31] and stimulation of antioxidant enzymes.[30] Previous work in our laboratory has shown that the development of albuminuria in the B/W mouse coincides closely with the infiltration of activated CD4+ T cells (manuscript in preparation). Cellular infiltration of the kidney is also consistent with the generally accepted pathogenic scheme of

lupus nephritis: deposition of autoantibodies and immune complexes preceding and inducing mononuclear cell inflammation of the kidney.[32] We did not anticipate that, despite increased anti-DNA antibodies and kidney cellular infiltration, CYST would reduce glomerular damage and improve survival. As traditional measures of autoimmune dysfunction in this model did not differ between groups in this study, suppression of glomerular damage in the CYST-group most likely occurred either by scavenging of locally produced ROIs or inhibition of effector cell activation and inflammatory mediators in the glomeruli. This postulation requires verification and investigations beyond the focus of this initial characterization.

Markers of lipid peroxidation (ie ROI damage) are elevated in SLE patients[8,30] and have been correlated with the presence of active glomerular disease[10] and degree of proteinuria.[9] Mohan and Das have previously proposed that ameliorative mechanisms of antioxidants in autoimmune disease occurs through increases in nitric oxide and antioxidant enzymes, reduction of ROIs and pro-inflammatory cytokines, or both.[30] Antioxidants may also prevent renal dysfunction independent of their ability to inhibit lipid peroxidation or thromboxane synthesis.[33] Both NAC and CYST stimulate basal levels of lipid peroxidation, but only CYST produces net suppression of induced lipid peroxidation.[34] Previous studies have suggested that production of ROIs and injurious prostaglandins can be modulated by dietary intervention with antioxidant eicosopentanoic acid (fish oil).[30,35–38] Increasing levels of antioxidants have been shown to decrease pro-inflammatory cytokines, increase antioxidant enzymes, and suppress inflammatory damage in murine autoimmune disease.[38–40] High dietary omega-3 fatty acids have been shown decrease anti-dsDNA and anticardiolipin antibodies and reduce kidney damage in murine lupus.[41] In a limited clinical study, improvement in SLE disease activity induced by omega-3 fatty acids was characterized by reductions of lipid peroxidation markers and increases in antioxidant enzyme levels.[30] Similarly, captopril, a sulfhydryl-containing antihypertensive, has also been shown to significantly reduce proteinuria and prolong survival in murine SLE, possibly due to its antioxidant effects.[42,43] Beneficial effects of captopril have been anecdotally recorded in human lupus nephritis.[44,45] CYST treatment has been examined in a small, open-label clinical trial of severe, long-standing lupus with little beneficial effect.[12] This result may have occurred because of a selection bias towards severe long-standing lupus that precluded a beneficial response, the lack of understanding of ROIs and antioxidant status in the pathogenesis of lupus, or the varied effects of CYST on DNA as a possible autoantigen.[46]

In summary, the current study demonstrates significant immunoregulatory effects of nonenzymatic antioxidants on the pathogenesis of murine autoimmune disease and suggests that ROIs play a critical role in the pathogenesis of lupus nephritis. Further study of ROIs in the pathogenesis of lupus nephritis are warranted in order to provide a better understanding of the pathogenesis of lupus nephritis and to identify potential therapeutic benefits and toxicities of antioxidants.

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