Lifespan Is Prolonged in Autoimmune-Prone (NZB/NZW) F1 Mice Fed a Diet Supplemented with Indole-3-Carbinol

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ABSTRACT Dietary modulation has the potential to prevent or ameliorate systemic lupus erythematosus (SLE). Indole-3-carbinol (I3C), which is abundant in cruciferous vegetables, was evaluated in a murine model of SLE because of its antiestrogenic activities. Female (NZB × NZW) F1 mice, which develop SLE, were fed an AIN76A diet without or with 0.2 g/kg I3C, starting soon after weaning or at 5 mo of age. At 12 mo of age, 80% of mice fed the I3C-supplemented diet soon after weaning were alive compared with only 10% of controls. When experimental diets were initiated at 5 mo of age, 100% of I3C fed mice and 30% of controls were alive at 12 mo of age. Anti-double-stranded DNA (dsDNA) antibodies developed in all groups, although at several time points, the levels produced in I3C-fed mice were significantly lower. Renal disease (proteinuria, histologic changes, IgG immune complex deposition, subepithelial deposits and diffuse epithelial cell foot process effacement) was more severe in controls with both protocols. The estrogen urinary metabolite ratio of 2- to 16α-hydroxyestrone was increased in I3C-fed mice. These findings demonstrate a profound effect of dietary I3C in experimental SLE.


KEY WORDS: • autoimmunity • systemic lupus erythematosus • indole-3-carbinol • anti-DNA antibodies • glomerulonephritis

Autoimmune disorders, which include systemic lupus erythematosus (SLE), affect as much as 20% of the population in Western countries (1). Treatment requires high doses of immunosuppressive agents, given over prolonged periods of time. SLE and the side effects of therapeutic drugs place heavy burdens on patients (2). Nutritional modulation offers the prospect of delaying or suppressing the effects of SLE, potentially decreasing the need for or amount of medication. Dietary fish oil rich in (n-3) PUFA (3) or food restriction (4) ameliorate autoimmune diseases in mice. Human studies support the efficacy of fish oil (5). A food-restricted diet combined with fish oil extends lifespan even more in mice (6). Some data suggest that diets high in animal fat exacerbate SLE (7), whereas vegetarian diets may ameliorate some symptoms (8).

A disproportionate number of women develop SLE (9). Abnormal estrogen metabolism, which prolongs estrogenic effects, is found in women with SLE (10). In the (NZB/NZW) F1 mice used in the present study, females die of lupus at 8–12 mo of age, whereas males die at 15–20 mo (11). Administration of estradiol to mice expressing a transgene for the heavy chain of an anti-DNA antibody induces a lupus-like phenotype (12). Because indole-3-carbinol (I3C), which is abundant in cruciferous vegetables, is an antiestrogen, we hypothesized that it might prevent, delay or even represent an adjunct treatment for SLE. I3C shifts estrogen metabolism toward less estrogenic metabolites (13) and is a negative regulator of signaling that is dependent on the estrogen receptor (14). Many genes activated by estrogen are abrogated by diindolylmethane, a condensation product of I3C (15). We evaluated the effect of a supplement of I3C, at a level obtainable from diet (16), on the outcome of SLE in lupus-prone (NZB × NZW) F1 mice.

MATERIALS AND METHODS

Mice and diets. Female NZB/NZW F1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). AIN76A diets (17) ± 0.2 g/kg I3C (Sigma, St. Louis, MO) were prepared by Ziegler (Gardner, PA). The AIN76A diet supplies a total of 18.5 MJ/kg with 22% of energy from protein, 11% from fat and 67% from carbohydrates. The nonpurified diet was Harlan Teklad Rodent Chow (Madison, WI). Two feeding protocols were evaluated in parallel with identical sample collections. Early start mice (n = 20) were fed AIN76A diet ± I3C from 5 mo and killed at 12–13 mo for kidney evaluation. Late start mice (n = 20) were fed the nonpurified diet for 5 mo and then fed the AIN76A diet ± I3C, to determine whether I3C ameliorated SLE. Mice consumed their diets ad libitum. Consumption was monitored to ensure comparable intake. Serum, obtained monthly by orbital bleeding, was evaluated for anti-double-stranded DNA (dsDNA) antibodies. Urine samples were collected and evaluated for proteinuria each month early in life and more frequently later. Urine collected at 7 mo was used to measure estrogen metabolites. A subset of mice was killed at 12.5 mo of age, and kidneys removed for evaluation. Lifespan was determined by excessive proteinuria (≥5 g/L); mice were killed by CO2 overdose or unanticipated death. A follow-up study was done with mice (n = 18) fed the AIN76A diets ± I3C from 5 mo and killed at 12–13 mo for kidney evaluation. The study was approved by the Institutional Animal Care and Use Committee at North Shore University Hospital.

Anti-dsDNA antibodies. The ELISA assay for dsDNA antibody was performed as described previously (18). Aliquots (100 μL) of 1:100 diluted serum were incubated overnight at 4°C on high binding
For the early start mice, survival was longer for those fed I3C. I3C-fed mice were alive. Survival of mice in the early start and was evaluated by multiple methods. Using formalin-

Microsoft Excel software.

proteinuria and kidney morphology scores). Data were analyzed using the addition of 100

/H9262

/H11003

/OHE) and 16α-hydroxyestrone (16α-OHE) were measured as de-

scribed previously (19) using the Estramet ELISA kit (Immunacare, Lehigh, PA). The assay is a competitive enzyme immunoassay in which binding of the antigen-enzyme conjugate to immobilized ant

body is inhibited by the addition of free antigen.

Renal pathology. Protein in urine was evaluated using Chem-

strips (Boehringer Mannheim, Indianapolis, IN). Glomerular disease was evaluated by multiple methods. Using formalin-fixed 5-μm kid-

ney sections stained with hematoxylin and eosin, glomerular nephri-
tis (glomerular enlargement, hypercellularity/proliferation, crescents, sclerosis) and intratrabecular nephritis (infiltrates, necrosis, fibrosis) were measured semiquantitatively (0–4+) using a standard score for se-

verity (20). Immunofluorescence was detected in 8-μm sections, which had been frozen in optimal cutting temperature solution (Sakura Finetek, Torrance, CA), using fluorescein-5-isothiocyanate-labeled anti-mouse IgG. For electron microscopy (EM), the renal cortex was fixed in 200 mmol/L glutaraldehyde, postfixed in 39

mmol/L osmium tetroxide in 100mmol/L sodium cacodylate (pH 7.3), stained in bloc with 118 mmol/L uranyl acetate in 50 mmol/L sodium acetate, 26 mmol/L sodium barbital buffer (pH 6.8), dehydrated in graded ethanol and embedded in Eppapoy resin (EM Sciences, Ft.

Washington, PA). Sections (50–100 nm) were examined on a JEM 100CXII transmission EM (JEOL, Tokyo, Japan). The severity of epithe-

lial foot effacement was evaluated semiquantitatively (0–4+) and the presence and site of electron-dense deposits due to immune complex deposition were determined.

Statistics. Survival for each cohort was analyzed monthly using the product-limit method and compared using the log-rank test (SAS Institute, Cary, NC). Other analyses included independent t-test (anti-dsDNA antibodies, estrogen metabolites) and χ² testing (pro

teinuria and kidney morphology scores). Data were analyzed using Microsoft Excel software.

RESULTS

Life-span. Dietary I3C extended the life span of NZB × NZW F1 mice (Fig. 1). Control mice began to die at 8 mo. For the early start mice, survival was longer for those fed I3C (P < 0.0008). At 12 mo, 10% of controls and 80% of I3C-fed mice were alive. Survival for late start I3C-fed mice was also longer (P < 0.0011). At 12 mo, 30% of controls and 100% of I3C-fed mice were alive. Survival of mice in the early start and late start protocols did not differ. A set of I3C-fed mice lived to 20.5 mo of age.

Anti-dsDNA antibody levels. I3C treatment did not affect the development of anti-dsDNA antibodies (Fig. 2), which were detectable at 5 mo and continued to rise over time. Lower titers were present in I3C-treated mice at 7 mo of age (P < 0.05) in both early and late start feeding protocols with the greatest difference at 8 mo. This trend did not continue, and levels of antibodies became indistinguishable. This discrepancy may have been due to increased deaths in controls; analyses of later times included only surviving mice.

Renal pathology. I3C delayed pathological deterioration of kidneys. Proteinuria was lower in I3C-treated mice, regard-

less of the time treatment was initiated. An estimated 300

mg/L of protein was detected in urine in almost all mice as early as 7 mo of age (not shown). However, the number of mice with 5 g/L of proteinuria rose more quickly in the controls than either I3C-fed group (Fig. 3). Morphologically, there was less severe renal damage between 12 and 13 mo of age in mice fed I3C (Fig. 4). Glomeruli of the I3C groups were generally similar to those of normal nonlupus-prone BALB/c mice. In the untreated (NZB × NZW) F1 mice, histological changes included thickening of glomerular capillary loops, nodular expansion of the mesangium and tubular casts. IgG staining was barely evident in normal BALB/c mouse kidneys, weak in I3C-treated mice, but glomerular staining for IgG was

FIGURE 1 Dietary indole-3-carbinol (I3C) extends lifespan in (NZB × NZW) F1 mice fed an AIN76A diet without or with 0.2 g/kg I3C, starting soon after weaning (left panel) or at 5 mo of age (right panel). Values represent numbers of surviving mice. Each censored (+) is a mouse killed for additional analysis. Early and late starts of diets were done in parallel.

FIGURE 2 Effect of indole-3-carbinol (I3C) on anti-double stranded DNA antibody in (NZB × NZW) F1 mice fed an AIN76A diet without or with 0.2 g/kg I3C, starting soon after weaning (left panel) or at 5 mo of age (right panel). Values are means ± SEM of anti-double-stranded DNA antibody titers determined on all surviving mice. Early and late start of diets were done in parallel. *Different from I3C at that time, P < 0.05.
intense in untreated (NZB × NZW) F1 controls. Semiquantitative results (Table 1) indicated that the difference in severity of kidney disease (glomerulonephritis and interstitial nephritis) was significantly less in I3C-fed mice. In EM studies (Fig. 5, Tables 1 and 2), untreated controls had more extensive glomerular lesions with increased immune complex deposition (granular electron dense deposits) compared with I3C groups. Large subepithelial deposits with spikes and diffuse effacement of visceral epithelial cell foot processes were readily evident in untreated groups but only occasionally seen in the I3C-fed mice.

**Estrogen metabolism.** The ratio of 2-OHE to 16α-OHE in urine at 7 mo of age, was greater than in controls in I3C-treated mice given I3C early (P < 0.002) and later (P < 0.04) in life (Fig. 6), consistent with less estrogenic activity in mice fed I3C.

**DISCUSSION**

Our results indicate that I3C, as a dietary supplement, dramatically increases lifespan of lupus-prone (NZB × NZW) F1 mice. I3C is effective when initiated early, before onset of disease, or later when the disease develops. The findings support the view that I3C may benefit persons at risk for SLE as well as those in the early stages of disease. Therefore, I3C may

**TABLE 1**

<table>
<thead>
<tr>
<th>Glomerulonephritis</th>
<th>Interstitial nephritis</th>
<th>Foot process effacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>Score</td>
<td>Score</td>
</tr>
<tr>
<td>Control</td>
<td>I3C</td>
<td>Control</td>
</tr>
<tr>
<td>Control</td>
<td>I3C</td>
<td>Control</td>
</tr>
<tr>
<td>n</td>
<td>n</td>
<td>n</td>
</tr>
</tbody>
</table>

1 Values show numbers of mice devoid of disease (≤1+) to having severe disease (4+). 2 P < 0.05 comparing I3C and control between moderate (1–2+) and severe (3–4+) using χ² analysis.

2 Sections were stained for evaluation by hematoxylin and eosin.

3 Sections were evaluated by electron microscopy.

**TABLE 2**

<table>
<thead>
<tr>
<th>Site of deposits</th>
<th>Control</th>
<th>I3C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangium</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Subepithelial, scattered</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Subepithelial, many, with spikes</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Intramembranous</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Subendothelial</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Values represent the number of mice out of 8 with electron-dense deposits determined by electron microscopy.
allow a decrease in the dose of immunosuppressive drugs required to treat SLE, reduce toxic side effects and be of value for the prevention of recurrences.

Our findings suggest that I3C suppresses an autoimmune inflammatory response, delaying renal failure due to glomerulonephritis. It is difficult to evaluate precisely the effects of I3C on the development of anti-dsDNA antibodies. The mode by which I3C affects the immune system in SLE may be complex and involve actions other than direct antibody suppression. Tamoxifen, like I3C, often behaves as an antiestrogen, decreasing cytokines and suppressing renal damage in autoimmune (NZB × NZW) F1 and MRL-lpr/lpr mice (21,22).

Estrogen metabolism was clearly altered in our I3C-treated mice. The ratio of 2-OHE to 16α-OHE was increased by I3C, a result consistent with less estrogenic activity. 16α-OHE binds covalently to the estrogen receptor (23) and has a prolonged estrogenic effect (24), whereas 2-OHE is not estrogenic (25). Lupus patients have decreased 2-hydroxylation (13), and the relative use of the alternate (10) and thus increased estrogenic activities. I3C increases estrogen-induced genes downregulated by AR agonists in MCF-7 breast cancer cells using suppression subtractive hybridization. Gene 262: 207–214.


