Differential effect of neonatal thymectomy on systemic and organ-specific autoimmune disease

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Abstract

Thymectomy on day 3 after birth (d3tx) depletes CD4\(^+\)CD25\(^+\) regulatory T cells leading to multiple independent organ-specific autoimmune diseases. However, systemic autoimmune disease such as systemic lupus erythematosus has not been reported in d3tx mice. Herein, we investigate the effect of d3tx on spontaneous autoantibody response and immune complex glomerulonephritis (GN) in the lupus-prone (SWR \(\times\) NZB)\(^F_1\) (SNF1) mice. The d3tx SNF1 mice developed accelerated antibody responses to double-stranded DNA and DNA–histone complexes, and an increased frequency of activated CD4\(^+\) T cells. Unexpectedly, the renal histopathology and mortality from GN were significantly ameliorated in d3tx SNF1 mice, which concomitantly exhibited a Th2-biased antibody response. By 16 weeks, the d3tx mice had higher levels of total and autoantigen-specific IgG1, and at 12 months, the autoantigen-specific IgG2a was significantly below that of the sham thymectomized mice. These differences corresponded with reduction in total and autoantigen-specific IgG2a in the renal eluates of the d3tx mice. In addition, while all the mice had immune complex deposition in the mesangium and peripheral capillary loops of the glomeruli, IgG2a and C3 deposits restricted to the mesangial regions alone were more frequent in d3tx mice. D3tx SNF1 mice, protected from lupus-like GN, developed organ-specific autoimmune responses and diseases including prostatitis, orchitis and oophoritis, and antibodies to prostate, cardiac and skeletal muscle antigens. Therefore, d3tx paradoxically protects SNF1 mice from genetically prone lupus-like GN, yet promotes de novo organ-specific autoimmunity.

Introduction

Regulatory or suppressor T cells are known to play an important role in the prevention of spontaneous autoimmune disease in normal rodents (1–3). A murine model of neonatal thymectomy (tx) has been used extensively for the study of regulatory T cells in vivo (4–7). Kojima and Prehn (8) demonstrated that tx on day 3 (d3tx) causes organ-specific autoimmune disease and antibody, which, depending on the mouse strain, may affect the stomach, thyroid, ovary, testes or prostate. In addition, Asano et al. (9) showed that d3tx-induced autoimmune disease results from lack of a unique thymus-derived regulatory CD4\(^+\) T cell subset that constitutively expresses the IL-2 receptor \(\alpha\) chain (CD25). The CD4\(^+\)CD25\(^+\) T cells appear in the peripheral immune system on day 3 and are preceded by CD4\(^+\)CD25\(^-\) T cells that can be detected from birth. Thus, d3tx creates a repertoire depleted of regulatory CD4\(^+\)CD25\(^+\) T cells and enriched for autoreactive CD4\(^+\)CD25\(^-\) T cells. The autoreactive T cells, stimulated by endogenous antigens, then cause severe and progressive autoimmune disease (5,6). In this study, we have examined the effect of d3tx on systemic lupus erythematosus (SLE) that develops spontaneously in (SWR \(\times\) NZB)\(^F_1\) (SNF1) mice.

Spontaneous SLE in SNF1 mice is characterized by activation of autoreactive T cells specific for nucleoproteins, production of antibodies to nuclear antigens, including...
double-stranded (ds) DNA and DNA-histone complex, immune complex deposition in the kidney, and fatal glomerulonephritis (GN) (10,11). CD4+ T cells from the SNF1 mice respond to histone peptides, are of the Th1 type producing IFN-γ upon activation and promote antibody of the IgG2a isotype (12). In this study, we compared d3tx and sham tx (stx) SNF1 mice for their capacity to develop SLE and organ-specific autoimmune disease. Our expectation was that the loss of regulatory function in d3tx mice would exacerbate SLE and elicit organ-specific disease. Surprisingly, d3tx protected the SNF1 mice from fatal renal disease, but promoted organ-specific autoimmune disease development.

Methods

Mice

Parental strains (NZB males and SWR females) were purchased from the Jackson Laboratories (Bar Harbor, ME) and (NZB × SWR)F1 (SNF1) progeny were produced at the animal facility at the University of Virginia. All animal housing and care was in accordance to NIH guidelines.

Surgery and autopsy study

The tx was performed on male and female mice on days 1–3 after birth as previously described by Sjökin et al. (13). Briefly, the mice were anesthetized by hypothermia. Both lobes of the thymus were aspirated through a mid-sternal incision using a glass pipette. The wound was closed with surgical glue. At the time of sacrifice, tissue from the superior mediastinum was collected and evaluated for histological evidence of residual thymus. Mice with residual thymus were excluded from the study. For the stx (control) group, the chest was opened but the thymic lobes were not aspirated. Mice were sacrificed when moribund or at 1 year of age. A complete autopsy was performed and tissues, specifically including organs that are susceptible to d3tx-induced disease in other mouse strains (kidney, salivary glands, lacrimal glands, gonads, prostate, stomach, heart, lung, liver, small and large intestine, and spleen), were collected in Bouin’s fixative and processed for histopathology. One kidney was frozen in liquid nitrogen for immunofluorescence and antibody elution studies.

Histopathology and GN severity index

Kidneys were collected at sacrifice in Bouin’s fixative for 24 h and embedded in paraffin. Four-micron sections stained with hematoxylin & eosin were examined as coded specimens by an independent observer. Severity of acute GN was graded based on percent glomeruli involved, severity of inflammatory cell infiltration, and proliferative changes in mesangial and or peripheral regions. Chronic GN was scored based on glomerular fibrosis, interstitial fibrosis and tubular atrophy. Each of the above-mentioned changes were scored from 0 to 4, with 0 indicating no pathology and 4 being maximum pathology. The severity index represents a cumulative score. Kidney tissue was also fixed in 2% glutaraldehyde in cacodylate buffer, embedded in plastic and sections stained with toluidine blue.

Immunofluorescence study

Immune complexes in renal glomeruli were detected by direct immunofluorescence. Five-micron sections of snap-frozen kidney were fixed in ice-cold acetone for 10 min. The sections were rinsed in PBS and blocked with normal goat serum in 3% BSA/PBS (1:10 v:v) for 20 min. The sections were then incubated with FITC-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b or C3 (Southern Biotechnology Associates, Birmingham, AL) (1:50 dilution) for 45 min. After two rinses in PBS, the tissue sections were mounted with Vectashield mounting fluid (Vector, Burlingame, CA) and fluorescence intensity was graded from 0 to 4 in a blinded fashion. In addition, the relative distribution of immune complexes in the mesangial regions and/or the peripheral capillary loops was determined.

Serum antibodies to mouse prostate and striated muscle were detected by indirect immunofluorescence. Frozen sections from normal prostate and striated muscle (skeletal and cardiac) were fixed in ice-cold acetone and rinsed with PBS. After blocking with normal goat serum, the sections were incubated with serum (1:50 dilution) from d3tx or stx mice in 3% BSA/PBS for 45 min. After rinsing with PBS, the sections were incubated with fluorescein-conjugated anti-mouse IgG as described above.

Quantitation of prostate-specific antibody

Prostate-specific antibody was detected by ELISA. The mouse prostate extract was prepared as previously described by Kontani et al. (14). Anterior prostates (coagulation glands) were collected from normal B6AF1 mice, homogenized in 0.3 M sucrose in PBS with 1 mM PMSF (Sigma, St Louis, MO) and centrifuged at 600 °C for 10 min to remove nuclei, followed by high-speed centrifugation at 8000 °C to remove the mitochondrial fraction. The supernatant was dialyzed extensively against PBS and the protein content estimated. This preparation was coated overnight on 96-well ELISA plates (Corning, Corning, NY) (10 μg/ml) in 0.1 M bicarbonate buffer, pH 9.6, at 37°C. The plates were washed with PBS containing 0.05% Tween 20 (PBST), blocked with 3% BSA in PBS. After washing, different dilutions of test serum were added and incubated for 2 h at room temperature. This was followed by incubation with an optimal dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Color reaction was developed by o-phenylenediamine (0.5 mg/ml) (Sigma) with freshly added H2O2 in 0.1 M citrate buffer, pH 5.0. The color intensity was determined as optical density at 490 nM by an ELISA reader (Molecular Devices, Menlo Park, CA). Results are expressed as absorbance at 490 nM at serum dilution of 1:250 minus the absorbance of blank wells without serum.

Acid elution of Ig from kidney

Ig were eluted from the kidney as previously described by Woodruff and Wilson (15). Briefly, each individual frozen kidney was weighed and homogenized in cold PBS on ice. The homogenate was centrifuged at 1200 g for 10 min at 4°C. The pellets were washed by resuspending in chilled PBS, followed by vortexing and centrifugation to remove serum proteins. The washing was repeated until the OD280 of the supernatant was...
The pellet was then suspended in 0.1 M glycine with 1% BSA, pH 2.8 (10 ml/g kidney) and mixed gently at 4°C for 20 min. The suspension was centrifuged and supernatant immediately neutralized with 1 M Tris base.

Detection of IgG, dsDNA and DNA–histone-specific antibody, and their isotypes in serum and renal acid eluate

IgG, IgG1, IgG2a and IgG2b were quantitated by an ELISA. Briefly, 96-well plates were coated with goat anti-mouse IgG, IgG1, IgG2a or IgG2b (0.5 µg/ml) (Southern Biotechnology Associates) in 0.1 M phosphate buffer, pH 6.0, overnight at 4°C. The plates were blocked with 3% BSA in PBS. After washing with PBST, the plates were incubated with serial dilutions of serum (or kidney eluates). This was followed by an optimal dilution of a horseradish peroxidase-conjugated antibody (goat anti-mouse IgG, IgG1, IgG2a or IgG2b) (Southern Biotechnology Associates). The color reaction was developed as described above. To establish a standard curve, known concentrations (from 0.3 to 80 ng/ml) of purified IgG, IgG1, IgG2a or IgG2b (Southern Biotechnology Associates), instead of the sample, were added to the wells. The amount of isotype in the serum and kidney eluate was estimated based on a standard curve included in each ELISA plate.

Antibody to dsDNA was determined by ELISA as previously described by Hylkema et al. (16). Ninety-six-well plates were coated with streptavidin in water followed by biotinylated plasmid (pGEM-z) DNA in PBS. After washing with PBST, the plates were blocked with 3% BSA/PBS, followed by incubation with different dilutions of serum (or kidney eluates). This was followed by an optimal dilution of a horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a or IgG2b (Southern Biotechnology Associates). The color reaction was developed as described above. To establish a standard curve, known concentrations (from 0.3 to 80 ng/ml) of purified IgG, IgG1, IgG2a or IgG2b (Southern Biotechnology Associates), instead of the sample, were added to the wells. The amount of isotype in the serum and kidney eluate was estimated based on a standard curve included in each ELISA plate.

Flow cytometry

Splenocytes from d3tx and stx SNF1 female mice were collected and red blood cells lysed with Tris–ammonium chloride. The cells were stained with antibodies purchased from BD Biosciences (San Diego, CA), directly conjugated with FITC–anti-CD8 (53-6.7), –anti-CD62L (MEL-14) and –anti-CD44 (1M7); phycoerythrin–anti-CD69 (H1.2F3) and –anti-CD25 (PC61), and CyChrome–anti-CD4 (RM4-5). Isotype-matched reagents were used as controls. The cells were analyzed on a Becton Dickinson FACScan using CellQuest software.

Statistical analysis

Methods for statistical analysis include the Student’s t-test, ANOVA, Mann–Whitney and χ² analyses using Graph Pad Prism version 3.02 (Graph Pad Software, San Diego, CA).
Results

D3tx SNF1 mice have accelerated antibody production and a higher frequency of activated T cells.

The stx female SNF1 mice had detectable serum IgG antibodies to dsDNA after the age of 16 weeks (Fig. 1). In contrast, increase in anti-dsDNA antibodies was already evident in d3tx females by 8 weeks; and the antibody titers were significantly higher in d3tx than stx mice at the 8-, 16- and 20-week time points (Mann–Whitney test: \( P \) values of 0.003, 0.004 and <0.001 respectively). The serum antibody titers to another lupus autoantigen, DNA–histone, were also significantly higher in d3tx females compared to stx females at 16 weeks \([30.3 \pm 4.1 \text{ (} n = 11) \text{ and } 14.5 \pm 1.9 \text{ (} n = 11) \text{ U/ml respectively; Mann–Whitney test: } P = 0.002\]. After week 28, the anti-dsDNA antibody titers of the two groups were comparable \((P = 0.17)\).

Accelerated autoantibody response was also detected in d3tx male SNF1 mice. The d3tx males \((n = 7–16)\) had significantly higher anti-dsDNA antibody levels at 8, 12, 16, 20 and 24 weeks \((P = 0.004, 0.0015, 0.022, 0.001 \text{ and } 0.0002 \text{ respectively by Student’s } t\text{-test})\) compared to stx males \((n = 12–25)\). The titers between stx and d3tx males were comparable \((P = 0.2)\) at 28 weeks.

Mohan et al. (17) have reported increased levels of gp39+ T cells in 1-month-old SNF1 female mice compared to age-matched parental SWR females. This finding suggests that spontaneous T cell activation occurs in the female SNF1 mice well before detectable autoantibody response or renal disease. We therefore studied the splenocytes of 1-month-old SNF1 mice and determined whether increased T cell activation was associated with the accelerated autoantibody response in the d3tx mice (Table 1). The percentages and absolute numbers of both CD4+ and CD8+ splenic T cells were found to be reduced in d3tx female mice (Table 1). However, the CD4+ T cells of d3tx mice had significantly higher percentages of cells expressing recent activation T cell markers (CD69, CD25) and memory T cell markers (CD62Llow, CD44high) (Table 1). Thus, the accelerated antibody response was associated with T cell activation in the d3tx mice.

D3tx increases survival and ameliorates GN in SNF1 females

Surprisingly, the early appearance of anti-dsDNA antibody in d3tx mice did not result in parallel exacerbation of lupus-like nephritis. On the contrary, the mortality in d3tx SNF1 females was significantly lower, reaching 16% at 12 months (ANOVA: \( P = 0.008\)). In the stx females, however, the anti-dsDNA antibody titers paralleled their mortality due to end-stage renal disease, reaching 67% at 12 months (Fig. 3A).

The protection from renal disease was supported by a reduction in renal histopathology at the time of sacrifice. The d3tx SNF1 females \((n = 18)\) had a lower incidence of acute proliferative GN \((P = 0.002)\) and chronic GN \((P = 0.008)\).
compared to stx females (n = 17) by χ² analyses (Fig. 3B). The characteristic pathologic appearances of acute proliferative GN and chronic GN are illustrated in Fig. 4. The severity for acute GN was also significantly lower in the d3tx mice (by Student’s t-test; P = 0.006). However, in the d3tx mice that developed GN, the disease severity scores were comparable to stx mice (P = 0.14) (Fig. 3B). This was not due to residual thymi undetected on histological examination since the d3tx mice with chronic GN also developed tissue-specific antibody to muscle antigens (see below). Moreover, death in the d3tx females before 1 year of age was due to renal disease and not to other causes.

Male SNF1 mice are significantly less susceptible to fatal GN than female mice. Renal histopathology of stx males (n = 23) at 12 months of age showed changes of acute GN in 30% and chronic GN in 22% of the mice studied. However, d3tx males had a further reduction in GN (acute GN in 17% and chronic GN 8%; n = 12). Thus, d3tx in both male and female SNF1 mice exhibited accelerated serum antibody response but reduced lupus-like GN. Since fatal lupus-like nephritis is seen predominantly in females, additional studies were done only on female SNF1 mice.

The d3tx alters the distribution of glomerular immune complexes and complement C3

Deposition of immune complexes in the renal glomeruli followed by the ensuing local inflammatory response is considered important in the pathogenesis of lupus GN. For example, the distribution of the immune complexes within the glomeruli, the extent of glomerular involvement, the isotypes of the immune complexes and their ability to fix complement are factors shown to influence GN severity (18,19). We therefore studied the locations of the glomerular immune complexes of different Ig isotypes in a double-blinded study. Granular deposition of IgG, IgG1, IgG2a and IgG2b complexes was noted in both d3tx and stx mice. In addition, complement C3 co-localized with IgG2a and IgG2b. In stx mice, the immune complexes were distributed in the glomerular mesangium as well as the peripheral capillary loops (Fig. 5A and C). In contrast, immune complexes in d3tx mice were detected predominantly in the mesangium (Fig. 5B and D). Significantly, more stx mice had a high incidence of peripheral capillary deposits of IgG2a (100%), IgG2b (75%) and C3 (85%) (n = 8–13) compared to d3tx mice (50, 54 and 53% respectively; n = 13–17) (χ² analyses; P < 0.05). In contrast, there was no significant difference in IgG1 deposits in the peripheral capillary loops between stx (75%) and d3tx mice (69%). These data support the findings in human lupus nephritis where immune complexes restricted to the mesangial regions are associated with mild renal disease (WHO type II lupus GN) and patients with peripheral capillary loop immune deposits (WHO type III or IV lupus GN) have a less favorable prognosis (18).

We next quantitated the amount of Ig eluted from the kidneys of d3tx and stx mice. While the total IgG concentration was comparable between the d3tx and stx mice, IgG2a concentration was reduced in the d3tx mice (Table 2). In addition, the concentration of anti-dsDNA and anti-DNA–histone antibodies of the IgG class and IgG2a isotype was significantly reduced in d3tx mice compared to stx mice. In contrast, the d3tx and stx mice had comparable amounts of total and antigen-specific IgG1 and IgG2b, as well as total IgG3 (P = 0.39). Thus, significantly fewer d3tx mice had peripheral capillary loop deposits of IgG2a, IgG2b and complement C3 compared to stx mice. In addition, d3tx resulted in the reduction of complement activating IgG2a immune deposits in the glomeruli. Studies by other investigators on antibody isotype and clearance of circulating immune complexes suggest that immune complexes of IgG2a isotype are preferentially deposited in renal glomeruli (20,21). To investigate whether the reduced renal IgG2a in d3tx mice is indicative of the systemic reduction of IgG2a antibody response, we determined the isotype of the serum antibody.

Serum antibody responses, like renal immune complexes, are skewed to the T,2 type in d3tx mice

The d3tx and stx females at the time of sacrifice were studied for total serum IgG1 and IgG2a levels, and for the isotypes of serum anti-DNA–histone and anti-dsDNA antibodies. Compared to stx mice, the d3tx mice had increased serum IgG1 but comparable IgG2a levels (Table 3). Likewise, the anti-DNA–histone antibody titers of the IgG1 (but not the IgG2a) isotype were greater in d3tx mice than stx mice. Finally, while the level of anti-dsDNA antibody of the IgG1 isotype was comparable between the two groups, anti-dsDNA antibody of
IgG2a isotype was significantly lowered in the d3tx mice. Consequently, the IgG1/IgG2a ratios of total serum Ig, anti-dsDNA antibody and anti-DNA–histone antibody were significantly higher in d3tx than stx mice. These findings are consistent with a reduced T_h1 and a dominant T_h2 helper T cell response in d3tx SNF1 mice.

We next determined whether the changes in the Ig and antibody isotypes of the d3tx mice were observable earlier in the antibody response, when the d3tx and stx mice had comparable serum IgG levels \((P = 0.45)\), but different antibody titers. At 16 weeks, the IgG1 antibodies to dsDNA and DNA–histone were already higher in the d3tx mice (Fig. 6A and C). This finding suggests that the accelerated anti-dsDNA and anti-DNA–histone antibody responses in the d3tx mice, shown in Fig. 1, were due largely to a preferential increase in antibodies of the IgG1 isotype. In contrast, antibodies of the IgG2a isotype were not significantly different between d3tx and stx mice at 16 weeks (Fig. 6B and D). In addition to the IgG1-dominant antibody responses, the total serum IgG1 level was also elevated in the d3tx mice at 16 week (Fig. 6E). Therefore, d3tx in SNF1 mice is associated with a T_h2 shift early in their autoimmune response.

### Fig. 5. Localization of immune complexes in SNF1 kidney by direct immunofluorescence, detecting IgG (A and B) and complement C3 (C and D). (A and C) Depositions in both the mesangium and the peripheral capillary loops, typically found in stx mice. (B and D) Predominant mesangial deposits (m) and less peripheral capillary deposits (arrows), typical for d3tx mice.

### Table 2. Total Ig and autoantibody of different isotypes eluted from d3tx and stx female SNF1 kidneys

<table>
<thead>
<tr>
<th>IgG (µg/g of kidney)</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx mice</td>
<td>0.57 ± 0.13(^a)</td>
<td>0.04 ± 0.02</td>
<td>0.19 ± 0.04</td>
<td>0.03 ± 0.01</td>
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<tr>
<td>d3tx mice</td>
<td>0.35 ± 0.1</td>
<td>0.06 ± 0.02</td>
<td>0.08 ± 0.03</td>
<td>0.02 ± 0.001</td>
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<tr>
<td>(P) value</td>
<td>0.17</td>
<td>0.47</td>
<td><strong>0.013</strong></td>
<td>0.08</td>
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</table>

<table>
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<tr>
<th>Anti-dsDNA antibody (µg of kidney)</th>
<th>stx mice</th>
<th>d3tx mice</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.88 ± 0.2</td>
<td>0.11 ± 0.1</td>
<td>1.37 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>0.19 ± 0.08</td>
<td>0.07 ± 0.03</td>
<td>0.42 ± 0.2</td>
</tr>
<tr>
<td>(P) value</td>
<td><strong>0.0008</strong></td>
<td>0.64</td>
<td><strong>0.014</strong></td>
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<table>
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<tr>
<th>Anti-DNA–histone antibody (µg of kidney)</th>
<th>stx mice</th>
<th>d3tx mice</th>
<th>(P) value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0.91 ± 0.13</td>
<td>0.21 ± 0.07</td>
<td>0.51 ± 0.06</td>
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<tr>
<td></td>
<td>0.36 ± 0.11</td>
<td>0.13 ± 0.05</td>
<td>0.31 ± 0.06</td>
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<tr>
<td>(P) value</td>
<td><strong>0.004</strong></td>
<td>0.34</td>
<td><strong>0.034</strong></td>
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</tbody>
</table>

\(^a\)Mean ± SEM. \(P\) values were determined by Student’s \(t\)-test based on 19 d3tx mice and 13 stx mice, and those showing statistical significance are in bold.
Table 3. Total serum Ig and autoantibody isotypes and IgG1:IgG2a ratio in female SNF1 mice at 12 months or when moribund

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG1:IgG2a ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Ig (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx mice</td>
<td>249 ± 51a</td>
<td>706 ± 128</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>d3tx mice</td>
<td>1383 ± 290</td>
<td>1406 ± 295</td>
<td>0.99 ± 0.08</td>
</tr>
<tr>
<td>*P value</td>
<td>0.005</td>
<td>0.08</td>
<td>&gt;10^-6</td>
</tr>
<tr>
<td>Anti-dsDNA antibody (U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx mice</td>
<td>14.4 ± 8.3</td>
<td>5.5 ± 1.7</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>d3tx mice</td>
<td>39.7 ± 19.6</td>
<td>1.7 ± 0.52</td>
<td>37 ± 14</td>
</tr>
<tr>
<td>*P value</td>
<td>0.31</td>
<td>0.016</td>
<td>0.02</td>
</tr>
<tr>
<td>Anti-DNA-histone antibody (U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx mice</td>
<td>23.1 ± 4.3</td>
<td>69.1 ± 8.7</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td>d3tx mice</td>
<td>50.2 ± 6.9</td>
<td>53.8 ± 6.7</td>
<td>1.05 ± 0.16</td>
</tr>
<tr>
<td>*P value</td>
<td>0.006</td>
<td>0.142</td>
<td>0.0004</td>
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*Mean ± SEM. *P values were determined by Student’s t-test based on 19 d3tx mice and 13 stx mice, and those showing statistical significance are in bold.

Fig. 6. Comparison of serum anti-dsDNA antibody and anti-DNA-histone antibody levels between stx (open circles) and d3tx (filled circles) SNF1 mice at 16 weeks (A–D). Statistical analyses by Student’s t-test showed a significant increase in the antibodies of the IgG1 isotype, but not of the IgG2a isotype in d3tx mice. A comparison of IgG1 (E) and IgG2a levels (F) shows a preferential increase in IgG1 in d3tx SNF1.

D3tx induces organ-specific inflammation and diseases in SNF1 mice.

The d3tx male SNF1 mice developed a high incidence of prostatitis (eight of 13) (Fig. 7A) (Student’s t-test: *P = 0.003) and antibody to prostate-specific antigens (11 of 15) (Fig. 7B and C). Some d3tx mice also developed autoimmune orchitis (three of 16), antibody to sperm (seven of 15) and autoimmune oophoritis (three of 17). Interestingly, high incidences of serum
antibody to striated muscles were also detected in the d3tx male (13 of 17) and female (16 of 19) mice (Fig. 7D). These muscle antibodies, which have not been described in d3tx mice, recognized either skeletal muscle and/or cardiac muscle, suggesting antibody response to multiple antigenic epitopes. No significant histopathology was seen in the other organs studied. Specifically, they were free of inflammatory cellular infiltration. The stx male or female SNF1 mice (n = 10–24) were free of organ-specific inflammation or tissue-specific serum antibodies.

Discussion

It is well known that the loss of regulatory T cells in d3tx mice and the ensuing activation of the autoreactive neonatal T cell repertoire can result in organ-specific autoimmune disease (5,6,9,22). In d3tx SNF1 mice, we now documented autoimmune disease affecting the prostate, testis and ovaries, and the production of autoantibodies against prostate and the unusual skeletal and cardiac muscle striation antigens. In addition, d3tx also resulted in accelerated anti-dsDNA and anti-DNA–histone antibody production in the lupus-prone SNF1 mice. This is consistent with the report of enhanced activation of anergic dsDNA-specific transgenic B cells and antibody production in the absence of regulatory T cells (23). However, the protection of d3tx SNF1 mice from fatal GN despite the accelerated autoimmune response was unexpected. Therefore, in a paradoxical way, d3tx in SNF1 mice ameliorated their spontaneous lupus-like GN, but at the same time induced de novo organ-specific autoimmune diseases and autoantibody responses. In addition, the sera and renal eluates of the d3tx SNF1 mice were found to have significantly more IgG1 and less complement-fixing IgG2a antibodies when compared with the stx SNF1 mice. Although a causal relationship is not yet established between reduction of lupus-like GN and the T_{h}2-deviated antibody response, it is supported by circumstantial evidence.

While earlier reports proposed a T_{h}2 response (or a mixed T_{h}1 and T_{h}2 response) as the relevant pathogenic response in lupus-like GN (24,25), recent studies utilizing a genetic approach have documented the importance of a Th1 response in lupus-like GN. Thus, lupus-prone mice deficient in IFN-γ or IFN-γ receptor were protected from GN (26,27). Moreover, Kono et al. (28) showed that lupus mice deficient in IL-4 developed GN despite a reduction in serum IgG1 levels. The histone-specific T cells of nephritic SNF1 mice have been found to preferentially produce IFN-γ and their dominant anti-dsDNA autoantibodies were of the IgG2a isotype (12). Therefore, the deviation of the autoantibody response to a T_{h}2 phenotype in the d3tx SNF1 mice is consistent with an expected amelioration of lupus-like GN in these mice.

In addition to the reduction in the amount of complement fixing IgG2a antibody, there is also a shift in the distribution of glomerular IgG2a and C3 in the d3tx SNF1 mice. The glomerular IgG2a and C3 in the d3tx SNF1 mice were localized mainly in the mesangial region, whereas the deposits were equally distributed in the mesangia and the peripheral capillaries of the stx SNF1 mice. Our understanding of the mechanisms responsible for immune complex localization in renal glomeruli is currently incomplete, although the antibody specificity and immune complex size are considered potentially important factors (19). Our findings that d3tx altered the
antibody isotype in SNF1 mice suggests that antibody isotype may also influence the localization of the immune complex and therefore the extent of glomerular injury. Regardless of the mechanism, the preferential mesangial immune complex deposition is also consistent with reduction in severity of lupus-like GN observed in the d3tx SNF1 mice.

Following d3tx, the lymphopenic state and the depletion of regulatory T cells causes an early expansion and activation of the peripheral T cells (5,29). In addition, it is known that neonatal murine T cells have a propensity to mount a Th2 response upon activation (30,31). This may explain the Th2-deviated response in the d3tx SNF1 mice. In d3tx mice with organ-specific autoimmune diseases, a Th2-dominant response has also been documented. In autoimmune ovarian disease of the d3tx (C57BL/6 × A/J)F1 (B6AF1) mice, the Th2 response may be pathogenetically relevant (32). The polyclonally activated T cells of these d3tx mice had more IL-4-producing cells compared with the dominant IFN-γ-producing cells of age-matched stx mice. In addition, treatment with IL-12 protected the d3tx B6AF1 mice from autoimmune ovarian disease and this was accompanied by an increase in IFN-γ-producing T cells. In a model of SJögren’s disease, d3tx NFS/sd mice had an early increase in IL-4-producing T cells at 2 weeks, followed by an increase in IFN-γ-producing T cells at 8 weeks and salivary gland inflammation. Treatment with IL-4 antibody was found to block early autoreactive T cell response (33). Finally, an increased frequency of IL-4-producing cells was also reported in d3tx BALB/c mice (34). However, a Th2 pathogenic basis for d3tx-induced autoimmune disease was not supported by the study of Barrett et al. (35) who documented an increased IFN-γ production by d3tx splenocytes and prevention of autoimmune gastritis by IFN-γ antibody. Until this controversy is resolved, it may be hypothesized that d3tx has resulted in an accelerated T h2-type neonatal T cell response and concomitant reduction of a T h1 response, and this may explain the paradoxical finding between lupus-like GN and organ-specific autoimmune diseases in the SNF1 mice.

It should be emphasized that d3tx of different lupus mouse strains have also yielded variable results that are in part strain-dependent. For example, the fatality of (NZB × NZW)F1 mice was not altered by d3tx (36). In the BXSB mice, fatal GN was observed to be unchanged in one study (37) and enhanced in another study (36). In the MRL mice, d3tx was associated with enhanced survival; however, this was observed only in mice with the lpr mutation (36). The variable responses of the lupus mice suggest that many aspects of the immune response are altered in d3tx mice besides CD4+CD25+ regulatory T cell depletion. Some changes that impact on spontaneous lupus-like GN may vary among lupus strains. Alternatively, the mechanism of lupus-like GN may vary among different lupus mouse strains. In clinical terms, the findings further emphasize the complexity of the systemic lupus syndrome.

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Abbreviations

d3tx thymectomy on day 3 after birth
ds double stranded
GN glomerulonephritis
stx sham thymectomy on day 3
lx thymectomy

References
