Molecular Analysis of the Autoantibody Response in Peptide-Induced Autoimmunity

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Immunization of nonautoimmune BALB/c mice with multimeric DWEYSVWLSN, a peptide mimotope of DNA, induces anti-DNA and other lupus-associated Abs. To further investigate the pathogenesis of the autoantibody response induced by peptide immunization, we generated hybridomas from peptide-immunized mice that bound peptide, dsDNA, cardiolipin, Sm/ribonucleoprotein (RNP), or some combination of these Ags. Analysis of 24 IgM Abs led to the identification of three groups of Abs: 1) Abs reactive with peptide alone, 2) anti-peptide Abs cross-reactive with one or more autoantigens, and 3) autoantibodies that do not bind to peptide. The gene families and particular V_H-V_L combinations used in those hybridomas binding DNA were similar to those used in the anti-DNA response in spontaneous murine lupus. Another similarity to the spontaneous anti-DNA response was the generation of arginines in the complementarity-determining region-3 of DNA-binding hybridomas. Interestingly, one Ab had the V_H-V_L combination present in the original R4A anti-DNA Ab used to select the DWEYSVWLSN peptide from a phage display library. Many of the heavy and light chains displayed evidence of somatic mutation, suggesting that they were made by Ag-activated B cells. Analysis of the Ab repertoire in peptide-induced autoimmunity may provide insights into the generation of anti-DNA Abs following exposure to foreign Ag. Furthermore, the recovery of an Ab with the heavy and light chain combination of the Ab originally used to isolate the immunizing peptide confirms the utility of phage display peptide libraries in generating true molecular mimics. The Journal of Immunology, 2000, 164: 2542–2549.

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease, characterized by autoreactivity against a variety of nuclear Ags and immune-mediated damage in several organs, particularly the kidney. The most characteristic serologic abnormality found in patients with SLE is anti-dsDNA Abs (2–4). Anti-dsDNA Abs are considered instrumental in the pathogenesis of the immune glomerulonephritis in SLE (5–8).

The anti-dsDNA Abs found in patients with SLE and in the murine models of the disease display characteristics of Abs arising in an Ag-driven response (9–12). However, the antigenic trigger for the production of anti-dsDNA Abs has yet to be conclusively identified. Bacterial (but not mammalian) DNA, complexes of DNA and DNA binding proteins, and bacterial polysaccharides can induce anti-dsDNA Abs in various experimental models (13–17).

We previously screened a phage peptide display library with the murine IgG2b Ab R4A to identify a peptide mimotope for autoantigen. R4A binds to dsDNA and fibronectin and deposits in glomeruli of nonautoimmune mice. The 5-mer peptide DWEYS inhibited binding of R4A to dsDNA as well as binding of R4A to renal tissue (18). We have recently demonstrated that nonautoimmune BALB/c mice develop lupus-like autoimmunity when immunized with the peptide DWEYSVWLSN (19) (containing the DWEYS sequence) attached to a polylysine backbone. Peptide-immunized mice develop anti-dsDNA Abs as well as other autoantibodies characteristic of lupus, including anti-histone, anticardiolipin, and anti-Sm/ribonucleoprotein (RNP) Abs. At 3 mo of age, immunohistochemical studies demonstrate the presence of IgM and IgG deposits in renal glomeruli of immunized mice.

To further understand the molecular basis for the generation of autoreactivity in peptide-immunized BALB/c mice, we analyzed 24 monoclonal IgM Abs derived from BALB/c mice immunized with multimeric DWEYSVWLSN, and reactive with peptide and/or autoantigen. We focussed on IgM Abs initially to try to identify Abs that may be in their germline configuration as well as somatically mutated Abs. Two important observations can be made from an analysis of these hybridomas. Using peptide libraries, it is possible to derive true peptide molecular mimics for dsDNA. Furthermore, because V_H and V_L genes and V_H-V_L combinations used in the autoantibody response in peptide-immunized BALB/c mice were similar to those used in the anti-dsDNA response in (NZB × NZW)F_1 mice, peptide-induced autoimmunity may be a useful model to study the autoimmunity that arises in response to foreign Ag.

Materials and Methods

R4A

R4A is a murine IgG2b anti-dsDNA Ab that deposits in glomeruli of nonautoimmune BALB/c mice. This Ab is encoded by S107 V_H-11-unknown D-1,4 and V_L-1-unknown genes (20). The immunizing peptide DWEYSVWLSN was derived as previously described (18) by screening a phage peptide-display library with R4A. DWEYSVWLSN inhibits the binding of R4A to dsDNA (18) and induces anti-dsDNA and other autoantibodies by immunization (19).
Peptides and immunization

BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The immunizations were performed as described previously (19). Briefly, DWEYSVWLSN was prepared for immunization on an eight-branched polylysine backbone (multiple antigenic peptide (MAP) from ANA SPEC, San Jose, CA); peptide on MAP from Research Genetics, Huntsville, AL. Six-week-old female mice were immunized s.c. with 100 μg of MAP-peptide in CFA H37 Ra (Difco, Detroit, MI), followed by a boost of 100 μg of MAP-peptide in IFA s.c. 7 and 14 days later. Mice were housed in the Albert Einstein College of Medicine specific-pathogen free animal facility.

As described previously (19), only mice immunized with MAP-peptide in adjuvant developed significant autoantibody titers. Mice immunized with 1) MAP backbone alone in CFA and boosted twice with IFA (19), or 2) MAP conjugated to an irrelevant peptide using the same adjuvant schedule (data not shown) failed to generate significant increases in serum autoantibody levels.

Spleen cell fusions

One week (fusion 1) and 2 mo (fusion 2) after the final boost, mice were sacrificed, and the spleen cells were fused to the NSO myeloma cell line at a spleenocyte to myeloma cell ratio of 1:1 using standard hybridoma technology (21). Hybrids were screened for binding to peptide, dsDNA, Sm/RNP, and cardiolipin by ELISA (see below). Positive wells were cloned by limiting dilution.

ELISAs

ELISAs for peptide and autoantigen reactivity were performed as previously described (19). Salmon sperm dsDNA (Calbiochem Novabiochem, La Jolla, CA) was purified by filtration with a 0.45-μm pore size filter (Millipore, Bedford, MA), adsorbed to ImmuNo II 96-well microtiter plates (Dynatech, Chantilly, VA) at a concentration of 100 μg/ml in PBS, and dried overnight at 37°C. Cardiolipin (Fluka, Ronkonkoma, NY) at a concentration of 75 μg/ml in ethanol was adsorbed to ImmuNo II 96-well microtiter plates at room temperature overnight. MAP-DWEYSVWLSN (Research Genetics, Huntsville, AL) at 20 μg/ml in PBS and Sm/RNP (Immunovision, Springfield, AR) at 10 μg/ml in PBS were adsorbed to ImmuNo II 96-well microtiter plates at 4°C overnight. Plates (for all Ags) were then blocked with 5% nonfat milk in PBS for 1 h at room temperature and washed twice in PBS/0.05% Tween 20. The ELISA was then continued as described above.

Inhibition ELISAs

Salmon sperm dsDNA (Calbiochem Novabiochem, La Jolla, CA) was adsorbed to ImmuNo II 96-well microtiter plates (Dynatech) at a concentration of 100 μg/ml in PBS and dried overnight at 37°C. The plates were blocked with 3% FCS/PBS for 1 h at 37°C. Abs at 20 μg/ml in PBS were incubated with serial dilutions of the immunizing peptide MAP-DWEYSVWLSN for 1 h at room temperature and transferred to the DSaDoated plates for 2 h at 37°C (final Ab concentration, 10 μg/ml). The ELISA was then continued as described above.

Results

Antigenic specificities of mAbs

To understand the generation of autoimmune following immunization of BALB/c mice with the dsDNA surrogate DWEYSVWLSN, we analyzed IgM mAbs derived from these mice. Two fusions were performed, and IgM-producing B cells were isolated. Abs 23-7, 36-3, 20-1, and 12-16 were isolated from fusion 1, while the rest of the Abs were isolated from fusion 2. As there was no major differences in the gene usage and somatic mutation in Abs from each of these fusions (see below), the Abs from both fusions have been considered together as a group.

Three groups of Abs are discernible based on their antigenic specificity (Table I): 1) Abs reactive with peptide alone (26-20, 23-7, 32-13, 8-3, 39-9), 2) anti-peptide Abs cross-reactive with one or more autoantigens (dsDNA, Sm/RNP, cardiolipin, fibronectin; 15-16, 18-1, 5-1, 25-30, 29-3, 5-16, 2-4, 16-19, 18-18, 16-9, 19-43, 31-26, 29-5, 37g), and 3) autoantibodies (almost all reactive with dsDNA, Sm/RNP, cardiolipin, fibronectin, Sm/RNP and cardiolipin by ELISA (see below). Positive wells were cloned by limiting dilution.

RNA isolation and PCR

Total RNA was extracted from 2 × 10^7 cells using the Ultraspec RNA isolation system and the manufacturer’s protocol (Biotexc, Houston, TX) or the cesium chloride extraction method. Two micrograms of total RNA was reversely transcribed using the Superscript Pre-Amplification system (Life Technologies, Gaithersburg, MD), and an oligo(dT) or a 3’ gene-specific oligonucleotide primer (κ constant region TGGATGGCAGGAGGAGAA-TAGT and IgM constant region GCAGGGAGACCGAGGAGG). This was followed by PCR amplification using the κ constant region primer and a set of seven 5’ degenerate primers for the κ light chain, and the IgM heavy chain primer and a set of 10 degenerate 5’ primers for the IgM heavy chain (22). The PCR reactions were performed in a Perkin-Elmer 9700 thermal cycler (Perkin-Elmer, Palo Alto, CA) for 35 cycles, with denaturation at 94°C for 50 s, annealing at 50°C for 50 s, and extension at 72°C for 40 s. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA).

Sequencing and data analysis

Automated sequencing of the PCR products was performed at the DNA sequencing facility of the Albert Einstein College of Medicine using the ABI 377 sequencer (Applied Biosystems, Foster City, CA). Analysis was performed comparing the obtained sequences to the GenBank database using the Advanced Blast search program from the National Center for Biotechnology Information, National Institutes of Health.

SDS-PAGE and Western blotting

Protein separation and Western blotting were performed as previously described (23). Briefly, —250 ng of Ig from the hybridoma supernatants was boiled for 5 min in sample buffer with and without the presence of DTT (Calbiochem, La Jolla, CA). The samples were loaded in a 4–15% Tris-HCl Precast SDS-PAGE minigel (Bio-Rad, Hercules, CA) and run at 100 V for 10 min for the stacking gel and 150 V for 40 min for the running gel. Polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA) were presoaked in methanol for 15 s, washed in water for 2 min, and soaked in transfer buffer (25 mM Tris, 192 mM glycine, and 10% methanol) for 5 min at room temperature. The proteins were transferred to the polyvinylidene difluoride membrane at 200 mA for 1 h at room temperature using a minigel transfer apparatus from Bio-Rad. The membrane was blocked with 5% nonfat milk in PBS for 1 h at room temperature and washed twice in PBS/0.05% Tween 20. The membrane was incubated with HRP-labeled goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) at a dilution of 1/5000 in 5% milk/PBS for 30 min at room temperature and washed twice with PBS/0.05% Tween-20. The chemiluminescence assay was developed using the ECL™ Western blotting detection system (Amersham, Aylesbury, U.K.) and was photographed with ECL Hyperfilm (Amersham) after about 60 s.

Inhibition ELISAs

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Ab polyreactivity

To study the nature of the polyreactivity of some of the elicited autoantibodies, we studied whether polyreactive Abs (defined arbitrarily as binding to three or more of the peptide and autoantigen panels) would also bind to irrelevant Ags (Table II). Of the 10 tested autoantibodies meeting the definition for polyreactivity, only 16-9 and 19-43 reacted strongly with all of the tested Ags; 29-3 bound weakly to BSA, cytochrome c, and lysozyme, and 18-18 bound weakly to phosphorylcholine-keyhole limpet hemocyanin; other Abs in this panel (5-1, 29-5, 15-16, 5-16, 32-13, 37g)
The repertoire present in the anti-dsDNA response in spontaneous as well as induced lupus, with representation of multiple heavy and light chain gene families. Eight Abs used a J558 heavy chain, with two Abs (38-11, 18-18) exhibiting the highest degree of homology to the J558 BW-16 V_H gene closely associated with the anti-DNA response in (NZB × NZW)F1 mice (24). While 16-19 is more closely homologous to a different J558 family member, this Ab did not bind to any of the irrelevant self or foreign Ags. Thus, the polyreactivity detected is not pan-reactivity but, rather, probably reflects the presence of a common antigenic epitope among a variety of autoantigens.

To determine whether the interaction of 16-9 and 19-43 with the tested Ags was specific, we performed competitive inhibition studies. As shown in Fig. 1, the immunizing peptide inhibited 90% of the binding of 16-9 and 19-43 to dsDNA, supporting a specific binding of these Abs to the tested Ag mediated through an identical or contiguous Ag binding site. Furthermore, Sm/RNP and cardiolipin showed a similarly high degree of inhibition of the 16-9 and 19-43 Abs binding to dsDNA (data not shown).

To determine whether high Ab avidity contributed to Ab polyreactivity, we studied whether the hybridomas we selected were secreting monomeric or polymeric IgM Abs by SDS-PAGE and Western blotting. Of the 24 hybridomas described above, only the 15-16 cell line was secreting monomeric IgM Abs (data not shown). Thus, the differences in antigenic specificities described above were not, for the most part, a function of Ab valency.

**V_H and V_L gene usage**

The nucleotide sequences of the heavy and light chain variable regions are available through GenBank (accession nos. AF178584–178607 for the heavy chains and AF178608–178631 for the light chains). The V_H and V_L gene usage of the monoclonal IgM Abs is given in Table III. The V_H and V_L gene families used in the autoantibody response in immunized mice are similar to the repertoire present in the anti-dsDNA response in spontaneous as well as induced lupus, with representation of multiple heavy and light chain gene families. Eight Abs used a J558 heavy chain, with two Abs (38-11, 18-18) exhibiting the highest degree of homology to the J558 BW-16 V_H gene closely associated with the anti-DNA response in (NZB × NZW)F1 mice (24). While 16-19 is more closely homologous to a different J558 family member, this Ab did not bind to any of the irrelevant self or foreign Ags. Thus, the polyreactivity detected is not pan-reactivity but, rather, probably reflects the presence of a common antigenic epitope among a variety of autoantigens.

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**Table I. Peptide and autoantigenic specificity of mAbs derived from peptide-immunized BALB/c mice**

<table>
<thead>
<tr>
<th></th>
<th>dsDNA</th>
<th>Peptide</th>
<th>Sm/RNP</th>
<th>Cardiolipin</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>26-20</td>
<td>0.132</td>
<td>0.748</td>
<td>0.169</td>
<td>0.307</td>
<td>0.192</td>
</tr>
<tr>
<td>23-7</td>
<td>0.214</td>
<td>0.921</td>
<td>0.244</td>
<td>0.335</td>
<td>0.185</td>
</tr>
<tr>
<td>32-13</td>
<td>0.135</td>
<td>0.743</td>
<td>0.36</td>
<td>0.356</td>
<td>0.38</td>
</tr>
<tr>
<td>8-3A</td>
<td>0.168</td>
<td>0.679</td>
<td>0.155</td>
<td>0.431</td>
<td>0.181</td>
</tr>
<tr>
<td>39-9A</td>
<td>0.156</td>
<td>0.789</td>
<td>0.158</td>
<td>0.455</td>
<td>0.224</td>
</tr>
<tr>
<td>15-16</td>
<td>0.317</td>
<td>0.844</td>
<td>0.556</td>
<td>0.436</td>
<td>0.223</td>
</tr>
<tr>
<td>18-1</td>
<td>0.219</td>
<td>0.509</td>
<td>0.54</td>
<td>0.126</td>
<td>0.255</td>
</tr>
<tr>
<td>5-1B</td>
<td>2.214</td>
<td>0.637</td>
<td>0.212</td>
<td>1.673</td>
<td>0.225</td>
</tr>
<tr>
<td>5-16B</td>
<td>2.354</td>
<td>0.798</td>
<td>0.22</td>
<td>1.42</td>
<td>0.231</td>
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<tr>
<td>25-30</td>
<td>0.274</td>
<td>0.775</td>
<td>0.683</td>
<td>0.43</td>
<td>0.243</td>
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<tr>
<td>29-3</td>
<td>1.646</td>
<td>0.671</td>
<td>2.174</td>
<td>0.476</td>
<td>0.847</td>
</tr>
<tr>
<td>2-4</td>
<td>0.198</td>
<td>0.83</td>
<td>0.247</td>
<td>0.491</td>
<td>0.673</td>
</tr>
<tr>
<td>18-18C</td>
<td>1.827</td>
<td>2.717</td>
<td>3.162</td>
<td>1.841</td>
<td>0.821</td>
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<tr>
<td>16-19C</td>
<td>0.79</td>
<td>0.8</td>
<td>2.42</td>
<td>1.85</td>
<td>ND</td>
</tr>
<tr>
<td>16-9D</td>
<td>3.41</td>
<td>3.418</td>
<td>&gt;3.5</td>
<td>3.452</td>
<td>2.047</td>
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<tr>
<td>19-43D</td>
<td>&gt;3.5</td>
<td>&gt;3.5</td>
<td>&gt;3.5</td>
<td>&gt;3.5</td>
<td>2.874</td>
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<tr>
<td>31-26</td>
<td>0.352</td>
<td>1.361</td>
<td>0.573</td>
<td>0.573</td>
<td>0.356</td>
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<tr>
<td>29-5</td>
<td>3.46</td>
<td>0.907</td>
<td>0.321</td>
<td>0.581</td>
<td>0.356</td>
</tr>
<tr>
<td>37g</td>
<td>0.562</td>
<td>1.747</td>
<td>0.963</td>
<td>0.786</td>
<td>0.517</td>
</tr>
<tr>
<td>19-19</td>
<td>0.637</td>
<td>0.075</td>
<td>0.08</td>
<td>0.16</td>
<td>ND</td>
</tr>
<tr>
<td>36-3</td>
<td>1.056</td>
<td>0.132</td>
<td>0.137</td>
<td>0.139</td>
<td>0.169</td>
</tr>
<tr>
<td>38-11</td>
<td>2.57</td>
<td>0.241</td>
<td>0.737</td>
<td>0.433</td>
<td>0.135</td>
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<tr>
<td>20-1</td>
<td>1.419</td>
<td>0.306</td>
<td>0.164</td>
<td>0.265</td>
<td>0.198</td>
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<tr>
<td>12-16</td>
<td>0.661</td>
<td>0.426</td>
<td>0.146</td>
<td>0.262</td>
<td>0.208</td>
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<tr>
<td>30-6</td>
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<td>0.242</td>
<td>0.116</td>
<td>0.178</td>
<td>0.173</td>
</tr>
<tr>
<td>PC2μ</td>
<td>0.107</td>
<td>0.164</td>
<td>0.12</td>
<td>0.142</td>
<td>0.178</td>
</tr>
<tr>
<td>TEPC 183</td>
<td>0.119</td>
<td>0.204</td>
<td>0.195</td>
<td>0.251</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Normalized supernatants at 10 µg/ml were incubated on Ag-coated microtiter plates for 2 h at 37°C, followed by alkaline phosphatase linked goat anti-mouse IgM at 1:1000 for 1 h at 37°C, and substrate. 30-6, PC2μ, and TEPC 183 are irrelevant isotype-matched Abs. Numbers reflect optical density readings at 405 nm. ND, not done. An Ab is defined as positive for a given specificity if the optical density reading is at least twice that of the negative control; i.e., >0.24 for dsDNA, >0.48 for peptide, >0.39 for Sm/RNP, >0.5 for cardiolipin, and >0.36 for fibronectin. ND, not done. The top group of Abs in the table reacts with peptide alone, the middle group are Abs cross-reactive with peptide and one or more autoantigens, and the bottom group are autoantibodies that do not bind peptide.

**Table II. Specificities for irrelevant Ags in polyreactive Abs**

<table>
<thead>
<tr>
<th></th>
<th>PC-KLH</th>
<th>BSA</th>
<th>Cytochrome c</th>
<th>Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-16</td>
<td>0.103</td>
<td>0.123</td>
<td>0.134</td>
<td>0.143</td>
</tr>
<tr>
<td>5-1</td>
<td>0.105</td>
<td>0.129</td>
<td>0.163</td>
<td>0.135</td>
</tr>
<tr>
<td>5-16</td>
<td>0.099</td>
<td>0.134</td>
<td>0.131</td>
<td>0.159</td>
</tr>
<tr>
<td>29-3</td>
<td>0.191</td>
<td>0.353</td>
<td>0.396</td>
<td>0.473</td>
</tr>
<tr>
<td>18-18</td>
<td>0.258</td>
<td>0.102</td>
<td>0.19</td>
<td>0.214</td>
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<tr>
<td>16-19</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>16-9</td>
<td>0.862</td>
<td>1.008</td>
<td>1.663</td>
<td>1.776</td>
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<td>19-43</td>
<td>1.104</td>
<td>2.12</td>
<td>2.857</td>
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<tr>
<td>31-26</td>
<td>0.129</td>
<td>0.118</td>
<td>0.117</td>
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<tr>
<td>29-5</td>
<td>0.107</td>
<td>0.183</td>
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<tr>
<td>37g</td>
<td>0.161</td>
<td>0.185</td>
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<td>0.172</td>
</tr>
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<td>30-6</td>
<td>0.099</td>
<td>0.115</td>
<td>0.135</td>
<td>0.23</td>
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</table>

* Normalized supernatants at 10 µg/ml were incubated on Ag-coated microtiter plates for 2 h at 37°C, followed by alkaline phosphatase-linked goat anti-mouse IgG at 1:1000 for 1 h at 37°C, and substrate. 30-6 is an irrelevant isotype-matched Ab. Numbers reflect optical density readings at 405 nm. ND, not done. An Ab is defined as positive for a given specificity if the optical density reading is at least twice that of the negative control; i.e., >0.2 for PC-KLH, >0.23 for BSA, >0.28 for cytochrome c, and >0.46 for lysozyme.
appears to be clonally related to 18-18 (see below), and therefore the 16-19 heavy chain may also be encoded by V_{H}11BW-16. Four Abs used a V_{H} derived from the V_{H}606 family. The presumed V_{H}606 germline encoding the heavy chain of these Abs has been reported in the IgM anti-DNA response in (NZB × NZW)F_{1} mice (25). A V_{H} gene from the V_{H}36–60 family used to encode one Ab was homologous to the germline gene used to encode an arsionate Ab, but showed poor homology to a VH 36 – 60 gene encoding for an anti-DNA Ab in an (NZB × NZW)F_{1} mouse (12).

The V_{H}11 S107 gene used in the 20-1 Ab was 96% homologous to the VH 238 V segment reported in the IgM anti-DNA response in (NZB × NZW)F_{1} mouse (12). Five Abs used the VK 1 light chain, four Abs used VK 21E, three Abs each used VK 4 and VK 19, and two Abs each used VK 2, VK 8, and VOX-1. Two Abs (2-4 and 32-13) expressed the V_{H}1558-V_K 8 combination, and one Ab (25-30) expressed a V_{H}1558-V_K 1 combination, which are common among autoantibodies from (NZB × NZW)F_{1} mice (12).

**Somatic mutation**

Somatic mutation was not essential to encode an autoantibody response. The 25-30 Ab, which bound peptide and Sm/RNP, had both heavy and light chains that were germline encoded. Yet, as reported previously for IgM anti-DNA Abs isolated from (NZB × NZW)F_{1} lupus-prone mice (29), most of the IgM Abs isolated displayed somatic mutations throughout the heavy and light chain variable regions. In the heavy chain, the number of mutated bases ranged from 0–20, with most Abs having <10 mutations. Similarly, most of the light chains differed from the putative germline gene by <10 bases.

### Arginines in V_{H} CDR3

Anti-DNA Abs in spontaneous murine lupus are characterized by an increased number of arginine residues in the V_{H} CDR3 (11). This observation had suggested that the acquisition of an arginine through N-terminal additions, D region reading frame shifts, or other mechanisms in spontaneous murine lupus substantially contributes to binding to dsDNA, and that this residue is selected for during affinity maturation of the anti-DNA response. It is thought that arginine contributes to DNA binding through formation of ionic bonds with the negatively charged phosphodiester backbone of DNA. An arginine in V_{H} CDR3 is present in 11 of the 14 mAbs that binds with moderate to high affinity to dsDNA (≥0.5 OD units, Table I), compared with only 3 of 10 Abs that show weak or no dsDNA binding (Table IV). One Ab that binds to dsDNA, 38-11, has two N-nucleotide-encoded arginines in V_{H} CDR3 at positions 95 and 97.

### Comparison of 20-1 to R4A

The 20-1 Ab is encoded by the S107 V_{H}11-1D_{H}Q52-J_{H}12 and VK_{1}-J_{K}1 segments (20). The R4A Ab used to isolate the DWESYS peptide has a very similar structure, encoded by S107 V_{H}11-unknown D_{H}J_{H}4 and VK_{1}-J_{K}1 segments. The heavy chain variable regions of R4A and 20-1 differ only by a conserved glycine to alanine change in FR3 (Fig. 2A). Both D regions have an arginine at position 95, while the J_{H} regions differ by two amino acids (Fig. 2A). The light chains of 20-1 and R4A also have small differences, a single amino acid change in FR3.
in FR1 and two amino acid differences in light chain CDR1 (Fig. 2B). Although we cannot directly compare the antigenic specificities of R4A (IgG2b) to those of 20-1 (IgM) because of the differences in isotype, we assume that the loss of binding to peptide by 20-1 is due to the differences from R4A in the light chain.

Analysis of clonally related Abs

There appear to be four pairs of clonally related Abs, sharing identical V_H, D_H, J_H, V_L, and J_L gene segments. 16-19 and 18-19 differ by five nucleotide substitutions in the heavy chain, leading to three amino acid substitutions in FR1 and a serine to glycine change in CDR2. In the light chain there are eight nucleotide differences, leading to two amino acid substitutions in FR1 and four amino acid substitutions in FR2. Abs 5-1 and 5-16 have five identical substitutions throughout the heavy chain variable region, have an identical V_L,CDR3, and share the same truncated J_H4. Ab 5-1 and 5-16 have a V_K2-encoded light chain with nine nucleotide differences, leading to one substitution each in FR1, CDR2, and FR3, and three replacements in FR3. These light chain differences probably account for the minor differences in binding of 15-16 compared with 5-1 (Table 1). Ab 19-19 has a heavy chain VDJ identical to those of 5-1 and 5-16, but the light chain is encoded by a VK21E light chain. As 19-19 binds DNA with less affinity than Abs 5-1 and 5-16, it is tempting to postulate that this Ab has a receptor-edited light chain in a partially successful attempt to shift away from autospecificity. Abs 16-9 and 19-43 have six nucleotide differences in the heavy chain, leading to a single amino acid difference in FR1 and six nucleotide differences in the light chain leading to four amino acid differences in FR1.

Discussion

Analysis of the expressed Ab repertoire in nonautoimmune BALB/c mice immunized with a peptide surrogate for dsDNA reveals several interesting features. Autoantibodies derived from peptide-immunized mice share several structural similarities with anti-dsDNA Abs from mice with spontaneous lupus, including gene family usage, pairing of particular V_H and V_L gene segments, and generation of arginines in V_L,CDR3. This would support the hypothesis that peptide Ags alone, without nucleic acid, are sufficient to induce an anti-dsDNA Ab response. Furthermore, the structural similarity between induced and spontaneous anti-dsDNA Abs as well as the isolation of an Ab closely resembling the anti-dsDNA Ab used to derive the peptide from a phage display library confirm that peptides can indeed be molecular mimics of dsDNA.

In spontaneous murine SLE, pathogenic autoantibodies are primarily of the IgM and IgG isotypes, and the IgG Abs are clonally related to the IgM Abs. The multiple features of structural similarity of anti-DNA Abs isolated from peptide-immunized mice and described above to spontaneous anti-DNA Abs from lupus mice strongly support the relevance of this model to the study of systemic autoimmunity. We are now isolating cross-reactive IgG Abs from peptide-immunized BALB/c mice with similar specificities to the ones described here.
(NZB × NZW)F₁ mice undergo a spontaneous isotype switch from IgM to IgG in the anti-DNA response at about 6 mo of age. Tillman et al. (12) studied many IgM anti-DNA Abs from (NZB × NZW)F₁ mice and found that IgM anti-DNA Abs showed evidence of somatic mutation with selection for changes in the Ag binding pocket, suggesting an Ag-driven response. Furthermore, in individual mice, IgM and IgG anti-DNA Abs may be clonally related, suggesting that high affinity IgG anti-DNA Abs arise by clonal expansion and differentiation of IgM anti-DNA Abs present earlier in autoimmune mice. Similar results have been reported by Hirose et al., who found that at least some IgG anti-DNA-producing B cells originate from clonally related IgM anti-DNA Abs (29). The model they suggest for the progression of the anti-DNA response in the (NZB × NZW)F₁ lupus mouse is clonal expansion and selection of nonmutated IgM anti-DNA Abs, followed by somatic mutation beginning before isotype switching, isotype switching to IgG, and further accumulation of mutations increasing the affinity for DNA. We isolated here from mice immunized with a peptide mimotope for dsDNA clonally expanded Abs of the IgM isotype that displayed somatic mutations. Because IgM and IgG deposition is present in renal glomeruli of these mice, we believe that the Abs described in this report are themselves potentially pathogenic and are precursors of the pathogenic IgG anti-DNA Abs that are present in peptide-immunized BALB/c mice.

It has been postulated that in lupus, peptides processed from anti-DNA Abs may activate autoreactive T cells, leading to autoup-regulation and T cell help for anti-DNA Ab production. Hahn and coworkers (31) reported spontaneous T cell autoreactivity to idiopeptides derived from certain J558 anti-DNA Abs before the onset of disease in autoimmune mice. Tolerizing young (NZB × NZW)F₁ mice to these peptides had a significant effect on disease, with decreased titers of anti-DNA Abs, improved renal function, and prolonged survival (32). The heavy chains of four of the eight J558 Abs described in this paper have a $\approx 80\%$ amino acid homology to the heavy chain of the A6.1 anti-DNA Ab from which these immunogenic (and tolerogenic) idiopeptides were derived. Interestingly, five of the Abs we describe in this report had a

The IgM Abs described in this paper. Analysis of these IgG auto-

FIGURE 2. Sequence comparison between R4A and 20-1. Amino acid homology between R4A, a murine IgG2b anti-dsDNA Ab used to screen the dsDNA mimotope DWEYSVWLSN, and the 20-1 Ab derived from a peptide-immunized BALB/c mouse is illustrated. A dash denotes identity at that position. Amino acids of CDR1 and CDR2 are in bold and underlined. A, Comparison of R4A and 20-1 heavy chains. B, Comparison of R4A and 20-1 light chains.
>80% amino acid homology (four Abs had >90%) in the CDR2-FR3 region (residues 58–69) from which the p58 peptide was derived. This leads us to hypothesize that one possible mechanism for the autoimmunity seen in peptide-immunized mice is through up-regulation of autoreactive T cells against self-peptide determinants derived from Abs arising in the anti-peptide response. While Hahn and coworkers (31) demonstrated that BALB/c mice had no spontaneous immunity to V<sub>k</sub>-derived peptides, the activation of B cells expressing Abs with a p58-like peptide in their sequence may have converted the mice to responders.

How do autoantibodies that no longer bind peptide arise in response to peptide immunization? As the portion of the DWEYSVWLSN peptide that mimics DNA is DWEYS (18), we have begun to analyze epitope recognition in DWEYSVWLSN, comparing the binding of our mAb panel to MAP-DWEYSVWLSN and MAP-DWEYSVWLSN. While for many Abs the binding was comparable (data not shown), several Abs (18-1, 29-3, 32-13) displayed a much higher binding to MAP-DWEYSVWLSN. Furthermore, the 12-16 Ab, which bound dsDNA but not peptide (MAP-DWEYSVWLSN), bound well to MAP-DWEYSVWLSN. This suggests that some anti-DNA Abs from MAP-DWEYSVWLSN-immunized mice that do not recognize the complete immunizing peptide may still recognize the DWEYS peptide motif that mimics DNA.

What are the implications of the use of peptide on a polylsine backbone to induce an autoimmune response to understanding a possible role of foreign mimotopes in breaking tolerance? Although Ags with highly repetitive epitopes are usually T independent, peptide-induced autoimmunity is a T cell-dependent response, as indicated by the presence of somatic mutations in the elicited autoantibodies and data not shown (M. Khalil and B. Diamond, manuscript in preparation). The nature of the T cell epitope in peptide-induced autoimmunity is of obvious interest and is being actively pursued at this time. Preliminary data suggest that the MAP backbone may be part of the T cell epitope, or alternatively, may play a role in targeting the Ag to a specific intracellular compartment for processing (M. Khalil and B. Diamond, manuscript in preparation).

Although the anti-dsDNA specificity is closely associated with nephritis, lupus nephritis can occur in patients with SLE and in mouse models for the disease without high titer of anti-dsDNA Abs. The etiology of glomerulonephritis in these instances is not clear (2). We isolated anti-peptide Abs that did not bind or bind weakly to DNA. Several of these Abs bound fibronectin (Table I). Although we do not yet have evidence that such Abs are pathogenic, we demonstrate here that Abs arising in an anti-fibogen response (peptide) can acquire a pathogenic, potentially nephritogenic specificity (fibronectin) without binding to dsDNA. We postulate that similar scenario may contribute to lupus nephritis presenting without the serological hallmark of anti-dsDNA Abs.

Using a peptide bound by the anti-DNA Ab R4A, we elicited the 20-1 Ab, which shows close structural homology to R4A and also binds DNA. Thus, the DWEYSVWLSN peptide was also an immunogenic mimic of DNA and elicits an Ab structurally homologous to the R4A Ab used to initially select the peptide. Other laboratories have produced similar results. Demangel et al. recently reported that a phage clone bound by an anti-malaria Ab elicited an anti-peptide response that also bound malaria (33). Comparing two malaria-binding mAbs from the immunized mice to the parental Ab initially used to derive the peptide immunogen, similarities were found in the heavy and light chain variable region sequences. Similarly, Valadon et al. found that a peptide that inhibits binding of capsular polysaccharide of Cryptococcus neoformans to a protective anti-polysaccharide Ab elicits anti-peptide mAbs (albeit that do not bind polysaccharide) with a high V<sub>k</sub>-J<sub>k</sub> homology to the parent mAb (34). The similarity in DNA binding and structure between R4A and 20-1 described here confirms the utility of the phage peptide display technology in defining peptide antigenic mimics for nonprotein Ags. Nevertheless, while 20-1 is similar to R4A, by peptide immunization we elicited cross-reactive Abs encoded by several different heavy and light chain gene families, indicating that a structurally similar binding site can be encoded for by more than one heavy and light chain combination.

The V<sub>H</sub> BW-16 heavy chain variable region gene is closely associated with the anti-dsDNA response in lupus mice in both the (NZB × NZW)F<sub>1</sub> and MRL/lpr models (24). Moreover, Abs with a heavy chain encoded by this J558 family member have been demonstrated to be pathogenic. Interestingly, V<sub>H</sub> BW-16 encoded heavy chains have also been recently identified in several models of experimental anti-dsDNA responses in nonautoimmune mice (35, 36). Eilat et al. studied the characteristics of Abs using V<sub>H</sub> BW-16-encoded heavy chains in nonautoimmune mice with an induced anti-dsDNA response and in diseased (NZB × NZW)F<sub>1</sub> mice (24). In comparison to mice with spontaneous lupus, V<sub>H</sub> BW-16-positive Abs from mice with induced lupus were clonally restricted, had a lower frequency of class switching from IgM to IgG, and had decreased affinity for DNA. Although we did not measure the affinity of the Abs encoded by the V<sub>H</sub> BW-16 heavy chain to DNA, these Abs were IgM, and two of the three were clonally related. Our data are consistent with the observations by Eilat et al. and confirm that anti-dsDNA Ab responses in nonautoimmune mice induced by a variety of stimuli may be genetically similar to the anti-dsDNA Ab response in spontaneous lupus.

It is of interest to compare the autoantibodies derived from peptide-immunized mice to those reported in other well-described models of induced autoimmunity. Gilkeson et al. (37) analyzed a panel of 10 anti-DNA Abs derived from three BALB/c mice immunized with bacterial DNA. There was a predominant utilization of J558 family genes (9 of 10) and the J<sub>k</sub> heavy chain gene segment (6 of 10), with a variety of D region genes. In the light chain V<sub>k</sub> C<sub>21</sub> (5 of 10) and V<sub>k</sub> C<sub>23</sub> (2 of 10) predominated, with a variety of J<sub>k</sub> gene segments. In the analysis of Ab V<sub>H</sub> CDR3, arginines were present in four Abs, but no induced anti-DNA Abs contained more than one arginine in CDR3 or an arginine at position 100 or 102a. These anti-DNA Abs from normal mice displayed a broad range of reactivity with polynucleotides, cardiolipin, and lupus-associated autoantigens (Sm, Ro, and La) (38). Marion et al. (35) described a panel of six anti-DNA Abs (four IgG and two IgM) derived from a single BALB/c mouse following immunization with a DNA binding peptide (Fus 1) in complex with native mammalian DNA. V<sub>H</sub> region structures and DNA specificities of induced anti-DNA Abs were similar to those of spontaneous anti-DNA Abs from (NZB × NZW)F<sub>1</sub> mice. Many of the induced anti-DNA Abs described by Gilkeson et al. (37) and Krishnam and Marion (35), like the induced Abs we now describe, are highly homologous to Abs arising spontaneously in murine lupus. These results in the peptide-induced autoimmunity model suggest that nucleic acid is not essential to trigger the production of anti-DNA Abs similar in structure and antigenic specificity to those produced spontaneously in autoimmune lupus mice or following exposure to an immunogenic form of DNA. Induction of anti-DNA Abs by peptide immunization that are structurally homologous to anti-DNA Abs in spontaneous lupus would support the idea that protein Ags may be responsible for loss of tolerance to dsDNA in systemic lupus.
References