Models of Systemic Lupus Erythematosus: Development of Autoimmunity Following Peptide Immunizations of Noninbred Pedigreed Rabbits

Geeta Rai, Satyajit Ray, Robyn E. Shaw, Paula F. DeGrange, Rose G. Mage and Barbara A. Newman

J Immunol 2006; 176:660-667; doi: 10.4049/jimmunol.176.1.660
http://www.jimmunol.org/content/176/1/660

Supplementary Material
http://www.jimmunol.org/content/suppl/2006/01/04/176.1.660.DC1

References
This article cites 70 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/176/1/660.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Models of Systemic Lupus Erythematous: Development of Autoimmunity Following Peptide Immunizations of Noninbred Pedigreed Rabbits

Geeta Rai,* Satyajit Ray,* Robyn E. Shaw,† Paula F. DeGrange,‡ Rose G. Mage,* and Barbara A. Newman*

Reported in this study are the initial results from studies to develop rabbit models of systemic lupus erythematosus (SLE) by immunizations using two distinct peptides on branched polylysine backbones (multiple Ag peptide)-peptides. Eleven rabbits received a peptide from the Sm B/B spliceosomal complex previously shown to be immunogenic in rabbits, and 13 rabbits received a peptide from the rabbit N-methyl-D-aspartate receptor NR2b. All 24 animals in different generations of pedigreed, noninbred rabbits produced peptide-specific responses. Anti-nuclear autoantibody responses, including anti-dsDNA, were seen in 17 of 24 rabbits. To date, two rabbits have been observed to have seizure-like events and a third nystagmus. A model for eliciting development of SLE in genetically related yet heterogeneous rabbits may more closely resemble development of human SLE than do some models in inbred mice. Through selective breeding, it may also ultimately provide additional information about the genetics and etiology of SLE and serve as a model for assessing new treatment options. The Journal of Immunology, 2006, 176: 660–667.

Systemic lupus erythematous (SLE) is a complex, chronic autoimmune disorder that is etiologically multifactorial, with both genetic and environmental factors contributing to disease initiation and progression (1). Clinical presentation, which may manifest as rash, inflammatory arthritis, glomerulonephritis, and generalized cognitive decline, is accompanied by the presence of autoantibodies to various nuclear Ags including dsDNA. A subset of patients diagnosed with neuropsychiatric lupus (NL) exhibit severe and sudden onsets of psychosis or other acute neurological impairment, including seizure disorders, and carry a poorer prognosis. Autoantibody-Ag immune complex formation accompanied by complement-mediated inflammatory processes is believed to contribute to the pathological kidney damage (2, 3); however, the basis for autoimmune damage in NL is largely unexplored (4–6).

Rabbit polyclonal Abs produced after immunization are popular contemporary research reagents. Recent studies (7, 8) have defined mechanisms that result in the rabbit’s unique ability to produce some highly specific high-affinity Abs that are difficult to obtain from other species. Our current understanding of rabbit Ig genes, their allelic forms (allotypes) and the mechanisms of diversification of rearranged Ab H and L chain sequences during development of the primary preimmune repertoire by somatic hypermutation and gene conversion as well as during specific responses after immunization (8), has laid the groundwork that now allows better understanding of rabbit models of autoimmune diseases, including those having neurological components (9–14). Data from rabbit models will complement knowledge derived from the more frequently used mouse models of autoimmune diseases that have revealed roles for B- and T cell-mediated immunity (15–17), cytokines (18, 19), dendritic cells (20, 21), apoptosis (22, 23), and, in particular, begin to decipher the genetic basis for susceptibility to development of SLE (24, 25). Studies of spontaneously SLE-prone inbred mouse strains as well as mutant, transgenic, and knockout murine models (26–31) have revealed that a variety of genetic defects can contribute to development of SLE in general but have not pinpointed those that lead to NL. Human genome-wide studies to date have linked multiple genetic loci to SLE (32–35); the extent to which the findings using inbred or homogeneous artificially mutated strains will apply to heterogeneous outbred human populations remains to be determined. It is our hope that in the future, treatment targets may be identified through genetic studies. A rabbit model may contribute and complement the information obtained in other species and serve as a useful tool in designing therapeutic interventions.

The National Institute of Allergy and Infectious Diseases (NIAID)-pedigreed rabbits are Ig allotype-defined noninbred animals. The genetic heterogeneity of these animals is closer to the human situation. Therefore, they constitute a valuable resource for breeding, selection, and development of models to study Ag-driven production of autoantibodies similar to those seen in SLE, and especially to aid in identification of those that may be relevant to NL pathology. We chose rabbits from a pedigreed group for immunization with two different peptides on multiple Ag-branched polylysine backbones (BB) (multiple Ag peptide (MAP)-peptides). One was a peptide derived from the spliceosomal Smith (Sm) Ag studied previously (11) (called in this study peptide SM), and the
other was a peptide sequence from the neuronal postsynaptic glutamate receptor N-methyl-D-aspartate (NMDA) NR2b chain (peptide GR), based on previous reports that a pathogenic mouse anti-dsDNA Ab that bound kidney tissue recognized a consensus peptide sequence DWEYS found within NMDA NR2 receptor itself (36–38). BALB/c mice immunized with MAP-DWEYS developed anti-dsDNA, anti-cardiolipin, and anti-histone autoantibodies, with glomerular deposition of immune complexes (39). Recently, anti-NR2 Abs have also been suggested to cause brain pathology and cognitive dysfunction (40). Finally, in some SLE patients showing signs of NL, serum Abs that may bind NR2a and NR2b receptors were found (41, 42).

The results reported in this study show that immunization of rabbits with the SM- or GR-MAP peptide led to development of anti-nuclear autoantibodies, anti-dsDNA, as well as neurological symptoms in some rabbits. It is notable that with both SM- and GR-peptide immunizations, autoantibodies specific for Ags very different from the sequence of the immunizing peptide were produced, providing the basis for exploring a potential model of SLE with Ag-driven production of autoantibodies and subsequently arising clinical symptoms associated with NL.

Materials and Methods

Animals

The animal studies described in this study were reviewed and approved by the animal care and use committees of NIAID/National Institutes of Health (Bethesda, MD; animal study protocol L1b) and of the Spring Valley Laboratories (Woodbine, MD), where the NIAID allotype-defined rabbit colony is housed. The three immunization groups studied comprised a total of 31- to 2-year-old rabbits maintained in a room separate from the main colony. Rabbits of Groups 2 or 3 were descendants of Groups 1 or 2 rabbits and/or their siblings. The animals’ designations, sexes, and allotypes at the V_{H}a H chain and C_{b} allotype loci are summarized in Table I. There were 10 males and 14 females among the immunized rabbits. All rabbits were homozygous for the Ig H chain V_{H}a1 allotype but varied in their C_{b} allotype.

Antigens

Two peptide immunogens were each synthesized on branched lysine MAP-8 and MAP-4 BB (AnaSpec) (43). The “SM” peptide sequence PPPGRMPPP corresponds to major antigenic regions at positions 191–198, 216–223, and 231–238 of the nuclear protein Sm B/B, as reported previously (11). The “GR” peptide sequence DEWDYGLP corresponds to a known rabbit sequence of an extracellular epitope of the 2b subunit of the neuronal postsynaptic NMDA receptors. It is related but not identical with the peptide DWEYS reported earlier (36–42).

MAP-8 and MAP-4 BB without peptide were synthesized to serve as control Abs, and all synthesized structures were analyzed by HPLC and MALDI-TOF mass spectrometry (National Institutes of Health Peptide Synthesis and Analysis Unit, Research Technologies Branch, Rockville, MD).

Immunization

Each rabbit received initial s.c. injections of either MAP-8-peptide (Groups 1 and 3), MAP-4-peptide (Group 2), or control BB (in each group) (0.5 mg/0.5 ml; borate-buffered saline (pH 8.0)) emulsified with 0.5 ml of CFA. Boosts were given s.c. at 3-wk intervals with the same Ag concentration emulsified with IFA. Sera were collected immediately before immunization (preimmune) and 1 wk after each boost (postboost) and were aliquoted and stored at −20°C for assays. Group 1 received a total of nine boosts before sacrifice. Groups 2 and 3 received 10 boosts before two rabbits were sacrificed, and the remainder kept for observation without additional immunization.

Clinical assessment

Rabbits were observed continually by video surveillance cameras equipped for color vision (Bullet Multicam color IR; Central Alarm Systems) and recorded onto DVD so that abnormal behavior, particularly seizure activity or other evidence of neurological dysfunction, could be detected. Whole blood and sera collected after boosts were sent to a veterinary diagnostic laboratory (Antech Diagnostics) for complete blood counts and chemistry panels.

Assay for anti-peptide Abs

Serum Abs to MAP-peptide immunogens were assayed by standard solid-phase ELISA. Polystyrene 96-well plates (Corning; catalog no. 3590) were coated with 50 μg/ml of either SM, GR, or BB (MAP-8 or MAP-4) at 10 μg/ml in bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed three times with PBS (pH 7.2) containing 0.1% Tween 20 and blocked with 100 μl of blocking solution for 1 h at 37°C (Quality Biologicals). Wells were then incubated 1 h at 37°C with 50 μg/ml sera titrated by 4-fold dilutions in blocking solution, washed five times, incubated for 1 h at 37°C with 50 μl of a 1/2000 dilution (0.4 ng/μl) of affinity-purified HRP-conjugated goat anti-rabbit IgG (H + L) secondary Ab (Jackson ImmunoResearch Laboratories), developed with 3,3'5,5' tetramethylbenzidine (TMB) (INNOVA Diagnostics), and the resulting OD read at 450 nm.

Detection of anti-nuclear Abs (ANA) by indirect immunofluorescence

Commercially available slides coated with fixed Hep-2 cells (Antibodies, Inc.) were incubated with rabbit antisera diluted 1/10 in 5% goat serum (Jackson ImmunolResearch Laboratories) for 30 min. at room temperature (RT). ANA binding was detected by fluorescence microscopy following 30-min incubation at RT with 12.5 ng/μl FITC-goat anti-rabbit IgG (Southern Biotechnology Associates). Fluorescent binding patterns were compared with reference pictures provided by Antibodies, Inc.

Table 1. Immunized rabbits, sexes, and Ig allotypes

<table>
<thead>
<tr>
<th>Group</th>
<th>Rabbit Code</th>
<th>Rabbit no.</th>
<th>Sex</th>
<th>Allotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SM-1</td>
<td>XX129–3</td>
<td>M</td>
<td>a1/1 b4/5</td>
</tr>
<tr>
<td>2</td>
<td>SM-14</td>
<td>LL191–1</td>
<td>M</td>
<td>a1/1 b4/5</td>
</tr>
<tr>
<td>3</td>
<td>SM-15</td>
<td>2LL179–1</td>
<td>M</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>4</td>
<td>SM-16</td>
<td>ILL163–3</td>
<td>F</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>5</td>
<td>SM-17</td>
<td>ILL164–4</td>
<td>F</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>6</td>
<td>GR-18</td>
<td>ILL178–2</td>
<td>M</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>7</td>
<td>GR-19</td>
<td>ILL178–3</td>
<td>F</td>
<td>a1/1 b4/4</td>
</tr>
<tr>
<td>8</td>
<td>GR-20</td>
<td>ILL178–4</td>
<td>F</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>9</td>
<td>GR-21</td>
<td>ILL178–5</td>
<td>M</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>10</td>
<td>GR-22</td>
<td>ILL178–6</td>
<td>F</td>
<td>a1/1 b4/9</td>
</tr>
<tr>
<td>11</td>
<td>BB-24</td>
<td>ILL164–1</td>
<td>M</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>12</td>
<td>BB-25</td>
<td>ILL164–3</td>
<td>F</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>13</td>
<td>BB-26</td>
<td>2LL179–3</td>
<td>M</td>
<td>a1/1 b9/9</td>
</tr>
<tr>
<td>14</td>
<td>BB-27</td>
<td>ILL163–4</td>
<td>F</td>
<td>a1/1 b5/9</td>
</tr>
<tr>
<td>15</td>
<td>BB-31</td>
<td>2LL179–2</td>
<td>M</td>
<td>a1/1 b9/9</td>
</tr>
</tbody>
</table>

a Rabbits of Groups 1 and 3 received peptides (SM or GR) synthesized on MAP-8 BB, and rabbits from Group 2 received the peptides on MAP-4 BB. BB indicates control rabbits that received BB alone. M, Male; F, Female.

b SM animals received MAP- peptide derived from the Sm Ag spliceosomal B/B complex. GR animals received MAP-peptide derived from the NMDA glutamate receptor.
c Ig heavy chain V_{H}a allotypes and κ light chain C_{b} allotypes.
Jo-1 were assayed by ELISA using adaptations of commercially available human diagnostic kits (INOVA Diagnostics). Assays for autoantibodies to calf thymus dsDNA were adapted similarly using two different commercially available kits (Vidia, Vestec (Kit A); Zeus Scientific (Kit B)). Manufacturers’ instructions were followed. Briefly, 100 μl of rabbit sera diluted 1/100 in the proprietary sample diluents were added to Ag-coated wells and incubated for 60 min at 37°C (Kit A) or 30 min at RT (Kit B). Wells were then washed, incubated for 60 min at 37°C (Kit A) or 30 min at RT (Kit B) with secondary Ab HRP-goat anti-rabbit IgG Fc (Jackson ImmunoResearch Laboratories), and developed with TMB for reading OD at 450 nm. All results are presented as absorbance of the postboost sera OD minus the preimmune sera OD at 450 nm. An OD difference ≥ 0.1 above preimmune OD was considered positive.

Results

Serum response to MAP-peptide immunization

All 24 rabbits that were immunized with SM or GR peptides, on either the MAP-8 or MAP-4 BB, produced antisera that bound the immunizing peptide in ELISA at titers exceeding 10⁵ by the 5th boost (Fig. 1). Ab response patterns among all SM peptide-immunized rabbits showed only slight variations, with the response peaking as early as 49 days after the primary immunization (post-2nd boost) and persisting through the 9th boost (240 days) (Fig. 1A); responses of GR peptide-immunized rabbits were more variable (Fig. 1B). Antisera from neither peptide group ever exhibited cross-reactivity with the alternate peptide; similarly, there was no detectable reactivity with the MAP-4 or MAP-8 BB. Control rabbits immunized with only MAP-4 or MAP-8 BB showed negligible or no Ab response to the BB, and no reactivity in ELISA with either of the MAP-peptides.

Rabbit pedigree

The pedigree of the rabbits used in this study is shown in Fig. 2. Within each symbol of the peptide-immunized rabbits are representations of anti-dsDNA, anti-Sm, anti-Rnp, and other autoantibody (anti-ENA, anti-SS-A, and anti-SS-B) responses that may be traced through descendants.
representations of anti-dsDNA, anti-Sm, anti-Rnp, and other autoantibody responses (anti-ENA, anti-SS-A, and anti-SS-B) that may be traced through descendants. Lupus-like serology was elicited among Group 1 rabbits and among their relatives and descendants in Groups 2 and 3 (Fig. 2 and Table I). Autoantibody responses in the members of the pedigree immunized thus far reveal no preference in reactivity of females over males and also no obvious correlation with the L chain allotype.

Autoantibody responses measured by ANA immunofluorescence

Among the 24 immunized rabbits, six developed anti-nuclear Abs detected by immunofluorescence assays on Hep-2 cells using

![FIGURE 3. ANA immunofluorescence. A, Preimmune serum of SM-13 at a dilution 1/10 shows negligible immunofluorescence on Hep-2 cells as compared with 3rd postboost (B), 5th postboost (C), and 7th postboost sera (D). The speckled pattern suggests the presence of Abs against Sm and/or Rnp. Maximum fluorescence was seen 3rd postboost. Both preimmune (E) and postboost (F–H) sera of control BB-26 exhibited very faint immunofluorescence.](http://www.jimmunol.org/)

![FIGURE 4. Anti-nuclear Ag autoantibody responses and their persistence following peptide immunization. Solid lines indicate SM, and dashed lines indicate GR rabbits. Group 1 rabbits are denoted by unshaded symbols, Group 2 by shaded symbols, and Group 3 by symbols containing crossed lines. Results of analyses of sera diluted 1/100 are presented as absorbance of the postboost sera OD minus the preimmune sera OD (450 nm) as measured by ELISA for anti-Sm (A), anti-Rnp (B), anti-SS-A (C), anti-SS-B (D), and anti-ENA (E).](http://www.jimmunol.org/)
FITC-labeled secondary Ab specific for Fc of rabbit IgG (SM-1, GR-9, SM-13, SM-15, GR-19, GR-20, GR-30). The Abs’ staining patterns appeared nuclear and speckled, suggesting the presence of either anti-Sm or anti-RNP. Fig. 3 shows an example of staining by serum from rabbit SM-13 compared with a control (BB-26). Pre-immune sera from these six rabbits and postboost sera from rabbits immunized with control MAP-4/8 BB were all negative for ANA.

**Autoantibody responses to ENA, its component Ags, and to dsDNA**

Assays using commercially available kits designed for human samples were successfully adapted to rabbit serum samples by using HRP-conjugated goat anti-rabbit IgG (Fc) as the secondary Ab. Fig. 4 shows results of ELISA for autoantibody responses to ENA and its components Sm, Rnp, SS-A (Ro), and SS-B (La), and Fig. 5 shows results of assays for Abs to dsDNA. In Figs. 4 and 5, ELISA results presented as postboost OD minus preimmune OD are shown at four time points: preimmune (day 0); postboost 3 (day 70); postboost 5 (day 112); and postboost 7 (day 154). Some rabbits exhibited high preimmune values. In Fig. 6, where results of all assays are summarized, the maximum differences found between the OD at 450 nm in preimmune and postboost sera for each test are given, and the actual preimmune OD is shown in parentheses. A difference of 0.1 OD over the preimmune OD was selected as a cut off. Although only 3 of 24 rabbits tested positive with the commercial ELISA for Ab to total ENA (containing lower concentrations of each component), Abs were detected in more of the rabbits when higher concentrations of the ENA components were assayed individually: 7 of 24 (3 GR and 4 SM) were positive for Sm (Fig. 4A); 7 of 24 (3 GR and 4 SM) were positive for Rnp (Fig. 4B); 3 of 24 (1 GR and 2 SM) were positive for SS-A (Ro 60 and 52 kDa) (Fig. 4C); and 3 of 24 (all GR) were positive for SS-B (La) (Fig. 4D). The variation in Ab response patterns over time after the 3rd boost (day 70) did not correlate with the profiles of the Enb responses to the peptide immunogen itself (Fig. 1). The results show clearly that rabbits can develop autoantibody responses to ENA components when immunized with either of the two unrelated peptides (conjugated to either MAP-4 or MAP-8 BB), whereas no rabbit immunized with either BB alone showed such response.

Overall, 42% (10 of 24) of all the immunized rabbits (7 of 13 of the GR; and 3 of 11 of the SM) developed anti-dsDNA responses (Figs. 5 and 6). All sera from rabbits immunized with the control MAP-4 or MAP-8 BB were negative for anti-dsDNA autoantibodies, as they were for the other Abs to nuclear Ags assayed with the

![FIGURE 5.](image-url) Autoantibodies to dsDNA following peptide immunization. Sera diluted 1/100 were assayed by ELISA. Results are presented as postboost sera OD minus preimmune sera OD.

![FIGURE 6.](image-url) Summary of autoantibody responses following MAP-peptide immunization. Preimmune and postboost sera, 3rd, 5th, and 7th, were tested. Shaded boxes represent responsive rabbits; the numbers in the boxes indicate the maximum difference between postboost and preimmune sera OD; the absolute preimmune OD value at 450 nm is given in the parenthesis. In the ANA column, results have been presented as a semi-quantitative measure of the fluorescence on a scale of 1 to 3: 3+, optimum fluorescence; 2+, dim fluorescence; 1+, very subdued fluorescence as compared with the preimmune sera. The three values represent the fluorescence after 3rd, 5th, and 7th boosts.
exception of one control rabbit BB-31. This rabbit had a high preimmune anti-dsDNA value (0.8) that increased over time (0.9, 1.2, 1.2 at 3rd, 5th, and 7th postboost, respectively) for reasons not yet understood. Two rabbits (SM-I and GR-9) that have thus far had documented seizure-like episodes, described further below, and captured by video⁴ were among those with the highest anti-dsDNA levels.

Comparison of Ab responses to MAP-4 vs MAP-8 peptide conjugates

MAP-8 peptide conjugates were used for immunization of pedigree Groups 1 and 3; MAP-4 conjugates were used for Group 2 (Table I and Fig. 2). The decision to use MAP-4 was based on the possibility that these could be synthesized more reliably, but the response pattern observed led to a return to MAP-8. Among rabbits immunized with the MAP-4 peptides, 7 of 11 developed autoantibody responses to one or more nuclear autoantigens (ENA, SM, Rnp, SS-A (Ro), or SS-B (La) (Figs. 4 and 6), but only 3 of 11 developed detectable anti-dsDNA (Figs. 5 and 6). In contrast, 7 of 13 of the MAP-8 peptide-immunized rabbits had anti-dsDNA Abs. In Group 3, all three (GR-28, -29, -30) MAP-8-immunized progeny of responders from Group 1 produced detectable autoantibodies to one or more nuclear Ags and two (GR-28, -29) to dsDNA.

Clinical observations and hematological studies

The video recordings have been reviewed thus far for Group 1 only. The extensive monitoring of the rabbits by animal care staff and by video surveillance led to observations of three rabbits in this group that exhibited neurologically related symptoms. GR-8 was diagnosed with nystagmus (involuntary oscillatory movements of the eye) that is found associated with clinical SLE (44–46); GR-9 and SM-I showed seizure-like episodes on day 141 (29 days after the 5th boost) and day 59 (17 days after the 2nd boost), respectively. Rabbit SM-I manifested episodic, possibly seizure-like behavior at least five times in 1 day. The first event occurred at 10:33 a.m. and was characterized by a short period of extreme agitation followed by 4 min of abrupt unconsiousness and then sudden awakening.⁴ The nature of this episode resembles “atonic seizures” in man, which are characterized by sudden loss of postural muscle tone and brief impairment of consciousness (47). The other four behaviors of SM-I were similar with extreme agitation followed by a brief period of abrupt unconsciousness and took place at 1:09 p.m., 2:19 p.m., 4:09 p.m., and 5:19 p.m.

Complete blood counts and serum chemistry panels for >47 parameters including serum creatinine and total protein was done for all rabbits after each boost, but no other significant change or variation was noticed in any of them. Although some fluctuation was seen in the absolute numbers of lymphocytes, no definite pattern was observed.

Discussion

There is no general unifying theory to explain initiating events that lead to SLE in patients. The fact that we do not know the specific pathways by which genetic and immune-modulating environmental factors interact, such that one individual develops SLE whereas others do not, is evidence that additional new approaches are needed. The need for additional specific prognostic indicators and treatment modalities also remains. Rabbits of the NIAID colony have been bred to maintain Ig allotypes, and pedigrees of multiple breeding generations permit us to trace both their genetics and immune responsiveness to chosen Ags. This genetically related yet more heterogeneous background differs substantially from the genetic uniformity of inbred mouse strains and begins to approach the heterogeneity among humans more closely.

To obtain a more in-depth understanding of the etiology and pathogenesis of SLE, several recent investigations in animal models have focused on understanding Ag-driven mechanisms. Puttermann and Diamond (39) identified a peptide mimotope that was bound by a monoclonal anti-dsDNA Ab, R4, as well as by other anti-dsDNA Abs. A related sequence was later shown to occur in the NMDA (NR2b) receptor (37). Our immunization study in rabbits was initiated using the actual peptide sequence from the rabbit NR2b. We found that this MAP- peptide could not only elicit anti-dsDNA responses but also other autoantibodies found in lupus patients. The generation of Abs binding to other than the immunizing peptide, may result from somatic mutation or, in rabbits, gene conversion of rearranged H and L chain sequences in peptide-specific B cells (48). It is known, for example, that anti-dsDNA-producing cells can be generated by somatic mutation of B cells producing anti-phosphorylcholine Abs during in vivo responses (49, 50). Epitope spreading can also be used to explain the generation of novel autoantibody specificities. The spreading can occur between proteins that are known to be physically associated in vivo, such as the spreading from Sm B/B′ immunization to include other regions of the small nuclear ribonucleoprotein particle (11, 51, 52), or it may involve recognition of self Ags that appear to differ in molecular structure. For example, it has been shown that immunization with short peptides from the 60-kDa Ro particles can lead to noncross-reactive intra- and intermolecular epitope spreading (53–56).

In an earlier study that used nonpedigreed rabbits, James et al. (11) provided support for the molecular mimicry theory of autoimmune disease. They were able to initiate autoimmune responses in some normal rabbits immunized with an Sm B/B′-derived epitope PPPGMRPP, which is very similar to PPPGRRP from the EBV-encoded nuclear Ag (EBNA 1), The fact that this work was not reproduced by another group (57) is possibly explained by structural variation among MAP-8-peptide Ag synthesized in different batches, undetected infections of some rabbits, and other unknown environmental and genetic factors. Given that only a few of the 15 rabbits studied by the James group (11) exhibited lupus-like responses to immunization, an important difference may simply have been the small sample size of six rabbits in the second study. The present study included 24 allotype-defined, pedigreed, noninbred rabbits maintained in a controlled but not specific pathogen-free environment. Seventeen of these 24 rabbits developed some lupus-like autoantibody responses, and at least three rabbits manifested neurological symptoms in the form of seizure-like episodes or nystagmus; mild lymphopenia may also have occurred in some rabbits. In this and the previous report (11), not all rabbits developed lupus-like symptoms, which is similar to the human scenario.

Reviewing the Ab responses we observed, sera from all rabbits immunized with SM or GR peptide bound to the immunizing MAP peptide on the ELISA plates, and no response to the MAP BB was detected at the limit of sensitivity of MAP-peptide ELISA. Although SM peptide is derived from a sequence within the Sm Ag, only 5 of 11 SM peptide-immunized rabbits’ sera bound when assayed against the commercially available Sm ELISA kit. Perhaps the Sm Ag coated on the plate did not expose the immunizing peptide epitope sufficiently to bind all anti-peptide Abs. It is also possible that rabbit sera that were positive in the Sm ELISA recognized other epitopes; epitope spreading reported in both earlier studies (11, 57) may have occurred to different extents in different rabbits. Similar technical issues may also account for our finding

---

⁴ The online version of this article contains supplemental material.
that although all rabbit sera that were positive for ENA were posi-
tive for at least one of the component Ags, ENA-negative sera
from eight rabbits including both SM and GR peptide-immunized
rabbits (SM-6, GR-9, SM-14, SM-15, GR-20, GR-28, GR-29, GR-
30) bound to one or more of the component Ags of the ENA in
ELISA for Sm, Rnp, Ro, and La. In addition to epitope availability
discussed above, the likelihood that the epitopes necessary for
binding were present at sufficient density is greater with wells
coated with a single Ag. Furthermore, the assay kits were origin-
ally intended and optimized for use with human samples and
were adapted for use with our rabbit samples. It is noteworthy
that although the GR peptide is unrelated to Sm Ag, three rabbits
(GR-9, GR-29, and GR-30) exhibited Ab response to Sm, three
(GR-20, GR-28, and GR-30) responded to Rnp, three (GR-19, GR-
28, GR-30) responded to SS-B (La), and one (GR-30) responded to
SS-A (Ro) (Figs. 4 and 6).

It has recently been documented that NMDA receptors, known
to be essential for multiple brain functions and classically consid-
ered to be localized within the CNS, are also present on tissues of
peripheral organs, including bone osteocytes, osteoblasts and os-
teoclasts (58, 59), testis (60), pancreas (61), skin keratinocytes,
(62), heart (63), and adrenal glands (64). Specific anti-GR peptide
or anti-NMDA receptor Abs may have bound peripheral NMDA
receptors, caused excessive cell death, resultant release of nuclear
Ags, including spliceosomal Ags and dsDNA, and hence triggered
Ab responses to these autoantigens.

In all autoantibody assays, initial screenings done using Abs
specific for rabbit H + L chains gave higher background values in
some preimmune sera. For this reason, IgG Fc-specific secondary
Ab was used in ELISA for autoantibodies. High-affinity anti-
dsDNA IgG Ab and the resulting immune complex formation is
considered a key component in the development of pathology seen
clinically in SLE (65–68). Assays for anti-dsDNA are known to
have substantial variability (69, 70). Configuration and density of
dsDNA on coated wells, the presence of ssDNA, and details of
assay protocols may influence charge-sensitive anti-dsDNA Ab
binding. We used two different assay kits with standard calf thym-
us DNA-coated wells, both developed for human diagnosis, to
increase the probability of detecting anti-dsDNA responses. Inter-
estingly, most rabbits that made higher anti-dsDNA responses had
lower or no response to autoantigens Sm, Rnp, Ro, or La (Sm-1,
GR-8, GR-9, GR-10, GR-23), and conversely most of those with
low or no anti-dsDNA response showed greater reactivity with the
other nuclear autoantigens assayed (SM-3, SM-6, SM-14, SM-15,
GR-19, GR-20). Exceptions to this were GR-29 and SM-13, which
showed moderate response to both dsDNA and others autoantigens
like ENA, Sm, and Rnp (Fig. 6). Whether these observations of
response correlations are just coincidental or reflect selective
breeding will require further investigation. Analysis of the evolu-
tion of autoantibodies to dsDNA, ENA, Sm, and Rnp in SM-13
over time showed that they were all present by day 49, the earliest
time point we sampled (data not shown).

An unanticipated finding was that in contrast to anti-peptide
responses, autoantibody responses might differ depending on the
MAP conjugate used (MAP-4 or MAP-8); the MAP component
was previously thought to augment the Ab response but not evoke
an Ab response itself. However, 5 of 10 Group 1 and 2 of 3 Group
3 rabbits receiving MAP-8 peptide produced elevated anti-dsDNA
levels compared with only 3 of 11 of the Group 2 recipients of
MAP-4 peptides. Either this observation is coincidental, or the
MAP carrier BBs do indeed influence elicited responses. Addi-
tional evidence suggesting that MAP carrier influences the elicited
response comes from preliminary DNA microarray analyses
(G. Rei, S. Ray, R. Mage, B. Newman, R. Lempicki, and J. Yang,
unpublished observations), where analyses in progress show that
MAP-4- and MAP-8-immunized rabbits have some distinct pat-
terns of clustering.

The rabbit model introduced in this study presents numerous
opportunities for increasing the understanding of the autoimmune
responses associated with human SLE. Genetic and immune re-
response heterogeneity clearly exists within patient populations,
and the appearance of autoantibodies well before the onset of clinical
symptoms and pathology has been documented (71–74). The pres-
ence of autoantibodies in the absence of abnormal laboratory val-
tues in the rabbits documented in this study could reflect an early
stage of development of lupus-like disease; hence, some members
of Groups 2 and 3 are being monitored as they age, before being
euthanized for immunohistochemical studies.

Although no animal model can exactly replicate the complexity
of human disease, great potential exists for a noninbred rabbit
model contributing significantly to information gathered previ-
ously from other species. Ongoing protein microarray analyses are
further characterizing the full repertoire of autoantibodies pro-
duced by the rabbits described here. Furthermore, Western blotting
and immunohistochemical studies in progress will further elucidate
reactivity with potential neuroimmune targets. Finally, ongoing anal-
yses of gene expression in peripheral blood leukocytes following pep-
tide immunization will hopefully serve to enhance understanding of
NL and provide avenues for therapeutic intervention.

Acknowledgments

We are grateful to Dr. Hai Qi for his help with formatting the seizure video.
We appreciate the valuable suggestions about the manuscript from Drs.
Mike Mage, Richard Siegel, and Ronald Wilder, and all the members of
the Molecular Immunogenetics Section, Laboratory of Immunology,
National Institute of Allergy and Infectious Diseases, and thank Shirley
Starnes for outstanding editorial assistance. We dedicate this paper to the
memory of the late Dr. Timothy O’Neill, who provided enthusiastic vet-
erinary support and advice during the initial phase of this study.

Disclosures

The authors have no financial conflict of interest.

References

autoantibodies form the glomerular immune deposits in patients with systemic
manifestations in systemic lupus erythematosus: a disease in search of autoanti-
obodies, or autoantibodies in search of a disease? J. Rheumatol. 31: 2093–2098.
development in rabbits: the requirement of gut-associated lymphoid tissues.
9. Pender, M. P., and T. A. Sears. 1984. The pathophysiology of acute experi-
10. Thomas, F. P., W. Trojaborg, C. Nagy, M. Santoro, S. A. Sadiq, N. Latov, and
A. P. Hays. 1991. Experimental autoimmune neuropathy with anti-GM1 anti-
obodies and immunoglobulin deposits at the nodes of Ranvier. Acta Neuropath.
epitope spreading and autoimmune disease after peptide immunization: Sm BI/B-
derived PPPGRMRPP and PPPGIRGP induce splicesome autoimmunity. J. Exp.


