Immunoglobulin Epitope Spreading and Autoimmune Disease After Peptide Immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP Induce Spliceosome Autoimmunity

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Summary

Autoantibodies from many patients with systemic lupus erythematosus bind the Sm autoantigen B/B' polypeptide. The binding of serial serum specimens to the 233 overlapping octapeptides of Sm B/B' have shown that of the B/B'-derived octapeptides, PPPGMRPP and PPPGIRGP are early targets of the autoimmune response in some lupus patients. Rabbits immunized with PPPGMRPP and PPPGIRGP develop antibodies which not only bind these octapeptides, but also subsequently bind many other octapeptides of Sm B/B'. Eventually, the rabbits immunized with one octapeptide develop autoantibodies that bind other spliceosomal proteins including D, 70K, A, and C. Any mechanisms that operate to maintain tolerance or anergy for the spliceosome are thus overcome. Features considered typical of human systemic lupus erythematosus are also found in these peptide-immunized animals, such as antinuclear antibodies, anti-Sm precipitins, anti-double-stranded DNA, thrombocytopenia, seizures, and proteinuria. This disease model provides access to a mechanism for the development of humoral autoimmunity and may provide a basis to explain the immunopathogenesis of lupus in humans.

SLE is a multisystem rheumatic disorder. Autoantibodies are always present though their specificities may vary considerably among patients. The anti-Sm precipitating autoantibody is useful for the diagnosis of SLE (1), and in lupus patients, as much as 20% of the entire immunoglobulin repertoire may bind Sm (2). The dynamic nature of SLE humoral autoimmunity is demonstrated by the progression of anti-Sm responses to also involve the structurally associated nuclear ribonucleoprotein (nRNP)[1] autoantigen (3).

Proteins of the spliceosomal complex are targets of both the anti-Sm and anti-nRNP SLE autoimmune responses. Anti-nRNP antibodies are directed against the 70K, A, and C proteins, whereas the B/B' and D proteins are bound by anti-Sm (4). The spliceosomal proteins of the Sm and nRNP autoantigens are found in complexes with U RNAs. Anti-nRNP is specific for the complex containing U1 RNA, whereas anti-Sm binds complexes containing U1, U2, U4/U6, or U5 RNA. Why the spliceosome is a major target of SLE autoimmunity is not known.

A recent analysis of SLE patient sera which contain mature anti-Sm and anti-nRNP precipitin responses has identified 11 antigenic regions of B/B' (5). One of the major regions of reactivity is a peptide sequence, PPPGMRPP, which is repeated three times in the Sm B protein, and PPPGIRGP which is closely related and present only once in Sm B. Antibodies in human SLE patient sera cross-react between these two octapeptides. All SLE sera tested to date with both Sm and nRNP precipitins bind both octapeptides (5).

Rabbits have been immunized with PPPGMRPP or PPPGIRGP and, not surprisingly, they develop high titers of antibodies against these immunogens. In addition, these animals variably develop an autoimmune response against many different structures of the spliceosome and other findings consistent with an autoimmune disorder.

Materials and Methods

Sera. Human SLE patients were selected on the basis of satisfying the classification criteria for SLE (1), having an anti-Sm precipitin, binding to the Sm B/B' protein in Western blot, and having stored sera available from more than 2 yr. Other patient sera, control SLE patient sera (which do not bind to Sm B/B' and do not have an anti-Sm precipitin), and control normal sera were available from our stored serum collection.

Immunizations. PPPGMRPP and PPPGIRGP were prepared in quantity on a lysine backbone (MAP™; Applied Biosystems, Foster

[1] *Abbreviation used in this paper:* nRNP, nuclear ribonucleoprotein.

City, CA), as suggested (6). CEYRKKMDI (Ro 480), LQEMPLTALLRNLGKMTC (Ro 274), VAFSDEMVPCPVTTDMC (Ro 413), GMTSNGFTIADPDDRGML (Ro 500), and PTQYPPGRGTPPPV (Sm B/B' 168) were also prepared as MAP™ peptides. Six different immunization experiments have been initiated in male and female New Zealand White rabbits upon achieving a weight of at least 2.72 kg (about 8 wk old) after delivery from Schachtele Brothers (Salisbury, MO).

Immunizations were performed with a number of immunogens including PPPGMRPP (13 rabbits, 9 male, 4 female), PPPGIRGP (2 male), Ro 274 (2 male), Ro 413 (2 male), Ro 480 (3 male), Ro 500 (2 male), Sm B/B' 168 (1 male), MAP™ backbone alone (2 male), and Freund's adjuvant with no peptide (3 male, 1 female).

One of two protocols were followed for all immunizations. For immunization schedule A on day 1, CFA was emulsified with an equal volume (1 ml:1 ml) of PBS containing 0.5 mg of immunogen (peptide or control, as indicated) and injected subcutaneously and intraperitoneally. Boosting with 0.5 mg immunogen in IFA into the subcutaneous tissue and intraperitoneal space was done on days 26, 53, and 99. The final boost (0.5 mg immunogen or control) was given intravenously on day 152 without adjuvant.

Five rabbits were immunized under immunization schedule B with PPPGMRPP (4 male) or as a Freund's Adjuvant control (1 male) which was modified from the schedule presented above after initial immunization on day 1. Subcutaneous and intraperitoneal boosting was performed on days 8, 22, and 67. Intraveneous boosts were administered on days 151, 275, and 317.

Solid-phase Peptide Synthesis and Antibody Assay. The 1,202 possible overlapping octapeptides of the spliceosome (Sm B/B', Sm D, nRNP 70K, nRNP A, and nRNP C) and the 531 octapeptides of 60-kD Ro were prepared using solid-phase peptide chemistry as described in detail for Sm B/B' and 60-kD Ro previously (5, 7). Wash steps and incubations were carried out in sealed plastic containers. Other assay steps were performed by lowering the pins into microtiter plate wells. First, pins were blocked with 3% low-fat milk in PBS for 1 h at room temperature. Pins were then incubated in 1:100 dilutions of sera (rabbit or human) in 3% milk/PBS with 0.05% Tween (PBST) overnight at 4°C in humidified and sealed containers. The pin blocks were then washed four times with PBST for 10 min each with vigorous agitation. Next, each pin was incubated with anti-rabbit or anti-human gamma chain-specific IgG raised in a goat, affinity purified and conjugated to alkaline phosphatase (Jackson Immuno Research Laboratories, West Grove, PA) at a 1:10,000 dilution. Para-nitrophenyl phosphate disodium was used as a substrate for alkaline phosphatase and plates were read at 405 nm with a microelisa reader (Dynatech, Alexandria, VA). Results for each plate were then standardized by comparison with positive control pins. The same peptide sequences were used as controls for all plates and were allowed to develop to a specific absorbance with a known concentration of a standard control serum.

After completion of an assay, pins were sonicated for 2 h in sonication buffer (40 *g* SDS, 4 ml β-ME, and 62.4 *g* sodium phosphate in 4 liters) to remove antibodies, conjugate, and substrate. After sonication, pins were washed twice in hot water and boiled in methanol for 2 min, pins were then allowed to air dry for a minimum of 10 min and were stored with desiccant or used for another assay.

ELISAs. Standard solid-phase assays were used to measure the antibody reactivity in human and rabbit sera. 1 μg of antigen (Sm, nRNP, PPPGMRPP [as a MAP™ peptide], or PPPGIRGP [as a MAP™ peptide] was coated per well in each of 96 polystyrene wells in 125 μl of coating buffer (34.98 g Na_2CO_3, 64.46 g $NaHCO_3$ to 2,200 ml distilled water, pH 9.6) for 2 h at room temperature or at 4°C overnight. Wells were then washed one time with 250 μl of PBST (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 1.5 M NaCl, and 0.05% Tween, pH 7.4) per well. Wells were blocked with 150 μl of 0.1% BSA in PBS per well for 2 h at room temperature. After washing the plates, sera (human control, SLE patient, preimmune rabbit, or immunized rabbit) at varying dilutions were added to each well and plates were allowed to incubate for 2 h at room temperature or overnight at 4°C. After incubation, plates were washed four times with 250 μl of PBST per well. Next, each well was incubated for either 2 h at room temperature or overnight at 4°C with anti-human gamma chain-specific IgG (or anti-rabbit gamma chain-specific IgG) raised in a goat, affinity purified, and conjugated to alkaline phosphatase (Jackson Immuno Research Laboratories) at a 1:10,000 dilution. Para-nitrophenyl phosphate disodium was used as a substrate for alkaline phosphatase and plates were read at 405 nm with a microelisa reader.

To assure that the PPPGMRPP and PPPGIRGP MAP™ peptides were binding to the dish, the assay presented above was compared with the results obtained by cross-linking to the solid phase with glutaraldehyde, as described (8). In outline, bovine IgG was allowed to passively absorb to the plate. Then each well was treated with glutaraldehyde and incubated with peptide. This assay was somewhat more sensitive than was the assay presented above which has no bovine IgG step and allows the MAP™ peptides to directly absorb to the well. The relative results produced by the two assays were very similar, however. As a consequence of synthesis on a MAP™ backbone, we have not, therefore, found it necessary to conjugate these synthetic peptides to any carrier protein. Our experience has shown that these MAP™ peptides sufficiently absorb to polystyrene ELISA plates and provide a sensitive and specific assay.

Other Autoantibody Assays. Characteristic U RNAs were detected by immunoprecipitation following the protocol described by Foreman et al. (9). Precipitating levels of Ro, La, Sm, and/or nRNP autoantibodies were detected by double immunodiffusion (10). Rabbit and human sera were tested for antinuclear antibodies by a standard ANA test (INOVA Diagnostics, Inc., San Diego, CA) and for autoantibodies binding to native DNA by an anti-nDNA test (Protrac Industries, Kerrville, TX) using previously published protocols (11, 12).

Western Blot. Affinity-purified Sm and nRNP antigens and HeLa cell extract were subjected to electrophoresis in 12.5% polyacrylamide gels containing 1% SDS (in 0.15 M Tris HCl, pH 8.8) using a protocol described previously (13). These proteins were transferred to nitrocellulose and various concentrations (1:100 and 1:1000) of rabbit and human sera were reacted with the proteins to assess for binding (13).

Anti-PPPGMRPP Absorption. The MAP™ PPPGMRPP peptide was covalently bound to cyanogen preactivated Sepharose 4B by the method recommended by the manufacturer (Sigma Chemical Co., St. Louis, MO) (14, 15). 15 mg of the PPPGMRPP peptide was added per each gram of Sepharose 4B used, sufficient to prepare a 2-ml column. 1 ml of rabbit serum was circulated through this column for a total of four passes at 4°C overnight and constituted the absorbed serum. More than 95% of anti-MAP™PPPGMRPP reactivity was removed as measured in ELISA.

Results

Multiple longitudinal sera were available from two patients with anti-Sm precipitins. One of these patients had precipitating levels of Sm and nRNP autoantibodies from the initial serum sample. Her precipitin specificities remained constant over an 8-yr period; however, her autoimmune response

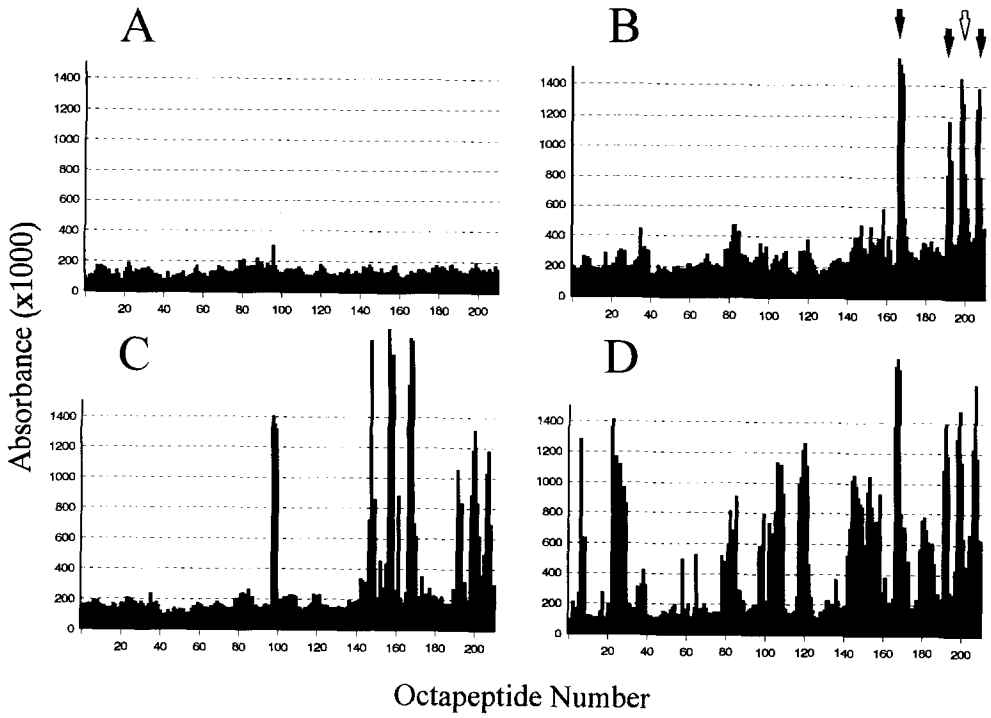

Figure 1. Development of an anti-Sm B/B′ response in one anti-Sm and anti-nRNP precipitin positive patient. Binding to 211 octapeptides of Sm B/B′ is recorded as absorbance (×*1000*) at 405 nm relative to controls. An example of the background binding found in a normal human serum is presented in *A*. The anti-Sm and anti-nRNP precipitin positive patient shows an increase in the number of antigenic regions over time, as shown in *B* (4/1/86), *C* (7/7/87), and *D* (12/26/88) that spans 2.5 yr. Six intervening sera were also analyzed that show the step-wise accumulation of new specificities as shown above (data not presented). The initial four groups of octapeptides represent PPPGMRPP (↓), which is repeated three times, and PPPGIRGP (⇩).

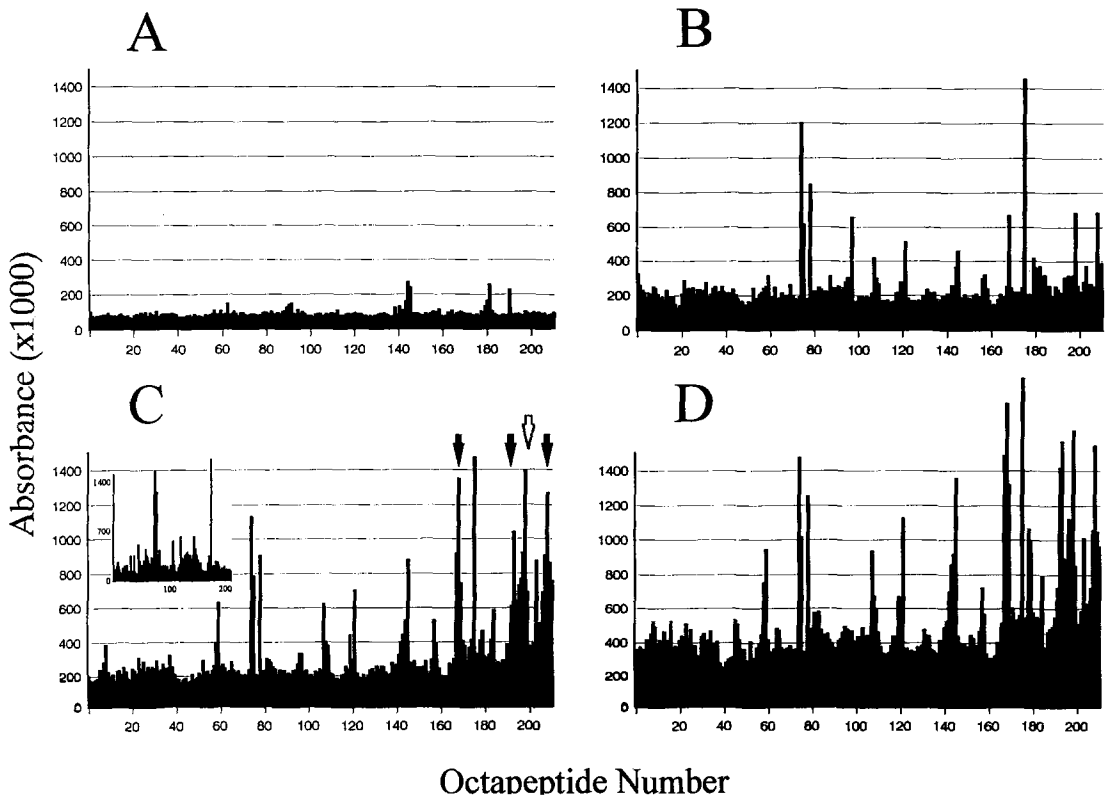

Figure 2. Development of rabbit antibody binding to 211 octapeptides of Sm B/B′ in one PPPGMRPP immunized rabbit (rabbit #463). (*A*) Binding of the preimmune serum of rabbit #463. (*B–D*) Binding of sera from collection dates 3, 8, and 10 wk after primary immunization. (*Inset*, *C*) Binding of this serum after absorption over a PPPGMRPP peptide column. Antibodies to the PPPGMRPP repeated peptides and the PPPGIRGP peptide have been removed as indicated (↓) and (⇩), respectively.

expanded from initially being directed against 70K and Sm B/B′ proteins to subsequently binding 70K, nRNP A, nRNP C, Sm B/B′, and Sm D proteins. The other patient initially had only an anti-Sm precipitin binding Sm B/B′ and Sm D in Western blot. She eventually developed anti-nRNP precipitins. Her serum then also bound to 70K and nRNP C in Western blot. The PPPGMRPP and PPPGIRGP peptides were the only B/B′ octapeptides found to be antigenic in the earliest available serum from each of these patients. The binding of this earliest serum from the former patient to B/B′ octapeptides is presented in Fig. 1 *B* with the maturation of the response shown in Fig. 1, *C* and *D*.

We immunized rabbits with PPPGMRPP or PPPGIRGP peptides to develop antipeptide positive control reagents. Antisera were produced that bound these peptides and purified Sm antigen at titers exceeding 1:10^6. Unexpectedly, from the later sera the immunizing peptide was a relatively poor inhibitor of antibody binding to Sm antigen, though the immunizing peptide in solution effectively inhibited binding to the same peptide on the solid phase. In the absence of extensive absorption, these antisera could not be used, therefore, for their original purpose. This observation led us to suspect that an autoimmune response may have been generated against components of Sm B/B′ in addition to the antigenic epitopes defined by the PPPGMRPP and PPPGIRGP peptides.

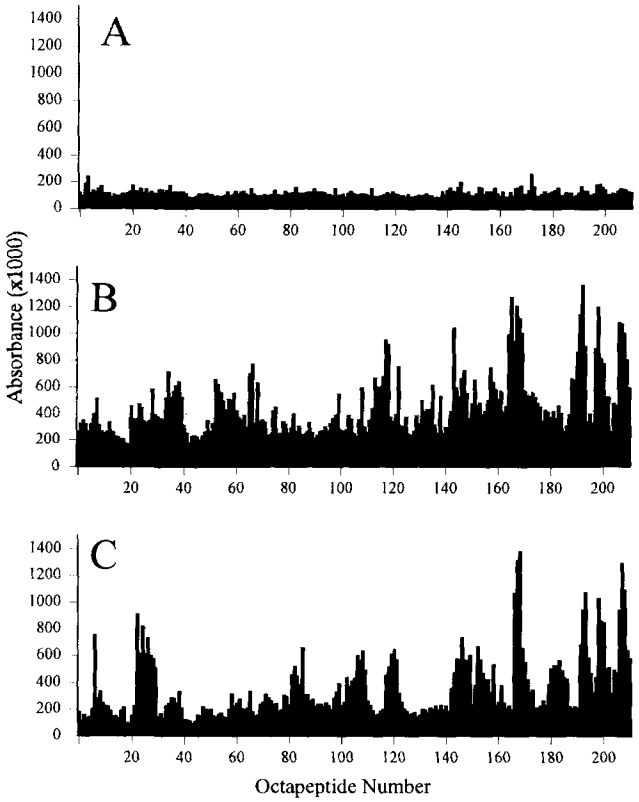

Figure 3. Sm B/B′ octapeptides bound by rabbits immunized with the PPPGMRPP peptide on a MAP™ lysine$_7$ backbone (5). (*A*) Average binding of 10 controls, none of which significantly bind any of these peptides above background. These negative controls include the preimmune sera from the PPPGMRPP and PPPGIRGP immunized rabbits as well as other rabbits immunized with CEYRKKMDI (Ro 480), LQEMPLTALLRNLGKMTC (Ro 274), VAFSDEMVPCPVTTDMC (Ro 413), and GMTSNGFTIADPDDRGML (Ro 500) derived from the 60-kD Ro sequence or with Freund's adjuvant alone. The sera from eight rabbits immunized with PPPGMRPP which have been immunized with protocol A and followed for at least 160 d are presented as an average in *C*, whereas *D* is the average binding of 14 human SLE sera with anti-Sm precipitins.

Assessment of binding using the 233 octapeptides of Sm B/B′ has shown that in addition to binding PPPGMRPP and PPPGIRGP, sera from rabbits immunized with these peptides had expanded their immune responses to bind other octapeptides of Sm B/B′. An example of the maturation of the binding to Sm B/B′ octapeptides is shown in Fig. 2 from rabbit #463 which was immunized with PPPGMRPP.

The serum used to produce the binding shown in Fig. 2 *C* was absorbed to a column with a MAP™ PPPGMRPP ligand. This absorption removed the binding activity to the three PPPGMRPP and the one PPPGIRGP octapeptides found in Sm B/B′ whereas the binding remained essentially unaltered to the other seven of the 11 groups of octapeptides with the greatest binding by this serum (refer to *inset*, Fig. 2 *C*). Absorption of anti-PPPGMRPP binding antibodies specifically reduces the anti-Sm activity (per milligram IgG) in this rabbit serum by 34%, as measured in an ELISA using purified Sm antigen.

Many of the same octapeptides of Sm B/B′ are bound by serum from the rabbit shown in Fig. 2 are also bound by serum from the human serum with an anti-Sm B/B′ responses shown in Fig. 1. This result is consistent with the hypothesis that PPPGMRPP immunization had induced Sm B/B′ autoimmunity. Subsequent experiments, including characterization of the autoimmune response of animals similarly immunized with these and control immunogens, were performed to test the validity of this particular interpretation of the unexpected initial results.

Other rabbits were immunized with PPPGMRPP. In addition, control peptide, MAP™ backbone and sham immunizations were performed to explore the specificity of the observed response. A control peptide was chosen from a region of Sm B/B′ which was known to be less commonly autoantigenic in human SLE sera with anti-Sm precipitins (Sm B/B′ 168) and from nonautoantigenic and autoantigenic regions of human 60-kD Ro (Ro 274, Ro 413, Ro 480, and Ro 500). All 15 rabbits immunized with PPPGMRPP or PPPGIRGP bound the MAP™ PPPGMRGP peptide in the solid phase, whereas none of the 14 control immunized rabbits bound this peptide.

Also, the average binding of the serum from eight rabbits, followed for at least 160 d of the immunization Schedule A, shows that they also tend to bind the same octapeptides from Sm B/B′ as bound by a cohort of 14 patients with anti-Sm and whose serum bind Sm B/B′ in Western blot (Fig. 3). 16 of the 18 Sm B/B′ octapeptide groups bound by the PPPGMRPP or PPPGIRGP immunized rabbit sera are also bound by the human anti-Sm sera evaluated (odds ratio =13.3, 95% confidence interval 11.6 to 15.0, χ^2 = 11.27, degrees of freedom = 1, p <0.0001).

That rabbits immunized with PPPGMRPP or PPPGIRGP

develop a specific autoimmune response is also supported by other observations. 14 of the 15 rabbits immunized with PPPGMRPP or PPPGIRGP bound the purified nRNP and Sm antigens in solid-phase assays whereas none of the control immunized animals developed this specificity. The same 14 of 15 PPPGMRPP or PPPGIRGP immunized animals developed antibodies against other groups of octapeptides from Sm B/B'. One of these patterns of binding to Sm B/B' was less complex than the example of rabbit #463 shown (Fig. 2), whereas others were more complicated, thereby revealing the variation that would be expected from an Sm autoantigen-driven response. Most of the 11 Sm B/B' regions antigenic in humans were also bound by serum from each rabbit with an autoimmune response that spreads among the Sm B/B' octapeptides.

If autoimmunity directed against Sm B/B' had been generated in these animals and if the immunization protocols generate an animal model with some of the features of immune dysregulation found in human SLE, then spliceosomal autoimmunity beyond Sm B/B' would be expected. Therefore, a subset of the experimental and control animals was evaluated for binding to the 1,202 overlapping octapeptides from the autoantigenic proteins of the spliceosome, which include Sm B/B', Sm D, nRNP 70K, nRNP A, and nRNP C. None of these octapeptides was bound by sera from the three rabbits immunized with Ro 480, the four Freund's adjuvant sham-immunized control animals, nor the eight available preimmune PPPGMRPP or PPPGIRGP animals. In contrast, all 13 of the PPPGMRPP immunized animals bound octapeptides from at least one other spliceosomal protein beyond Sm B/B'. 12 of the 13 sera bound octapeptides from Sm D and from nRNP 70K. All 13 bound octapeptides from Sm C. 11 of the 13 bound octapeptides from nRNP A. Variation within limits is found between animals with regard to the particular octapeptides bound, analogous to findings in humans (5, 16). Between 19 and 87 groups of octapeptides from the spliceosome were bound by sera from the 13 animals immunized with PPPGMRPP and assayed for binding to the 1,202 octapeptides from the spliceosome.

As an additional control, four of the PPPGMRPP or PPPGIRGP immunized animals were evaluated for binding to the 531 octapeptides from 60-kD Ro. None of the 60-kD Ro octapeptides was bound by sera from these animals, despite their extensive binding to the spliceosomal octapeptides.

Two of the animals immunized with PPPGMRPP or PPPGIRGP were followed for over a year, respectively, rabbits #462 and #465. Both of these long-term survivors have developed such high titers of anti-Sm antibodies that their later sera form precipitins in Ouchterlony immunodiffusion with Sm antigen (Fig. 4). This finding was absent in sera from control rabbits immunized with Freund's adjuvant alone, in rabbits immunized with peptides from the Ro autoantigen (Ro 274, Ro 500, Ro 413, and Ro 480), or in a rabbit immunized with a less antigenic octapeptide of Sm B/B' (Sm B/B' 168).

Rabbits immunized with the PPPGMRPP and PPPGIRGP peptides also immunoprecipitate the human U RNAs associated with the Sm and nRNP proteins (Fig. 5). U1, U2, U4, U5, and U6 form significant bands with the rabbit antibodies. Rabbit preimmune sera and a rabbit sham immunized with Freund's adjuvant alone do not immunoprecipitate any of the U RNAs.

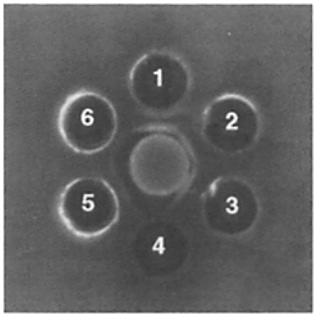

Figure 4. Oüchterlony immunodiffusion of PPPGMRPP, PPPGIRGP, and control peptide immunized rabbits. Antigen (calf thymus extract) is in the center well. An anti-Ro, anti-Sm, and anti-nRNP precipitin positive human SLE serum (*1*) is in the top well. Clockwise from this position is a postimmunization serum from rabbit #462 (*2*), an anti-nRNP precipitin positive SLE serum (*3*), a preimmune serum from rabbit #462 (*4*), an anti-Sm precipitin positive SLE serum (*5*), and finally another sample of the postimmunization serum from rabbit #462 (*6*). Immunized rabbit sera was collected 16 wk into the immunization protocol. Lines of identity with anti-Sm and anti-nRNP have developed in the serum of rabbit #462 during immunization.

Rabbits immunized with the PPPGMRPP or PPPGIRGP peptides develop high titer antinuclear antibodies. Rabbit #462 developed antinuclear antibodies to a titer of 1:1028. This level of autoantibodies remained constant even without additional peptide boosting. Rabbits #463 and #465 also developed antinuclear antibodies with a titer of 1:360. All of these immunized rabbit sera have antibodies with a nuclear speckled pattern, consistent with the presence of either anti-Sm or

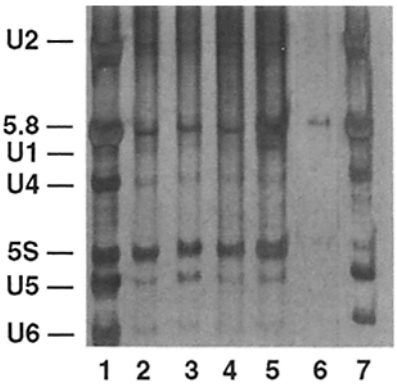

Figure 5. RNA immunoprecipitation from HeLa cell extracts by serum from PPPGMRPP and PPPGIRGP immunized rabbits. (Lane *1*) Immunoprecitation of U1, U2, U4/U6, and U5 RNAs by an anti-Sm and anti-nRNP precipitin positive human serum (also shown in Fig. 1 *D*) and serves as a positive control. (Lane *2*) Immunoprecipitation of U1, U2, U4/U6, and U5 by serum from rabbit #462 160 d after immunization with PPPGMRPP. (Lanes *3* and *4*) Immunoprecipitation of U1, U2, U4/U6, and U5 by rabbits #463 and #464, respectively. These sera were collected 72 d after immunization. Rabbit #465 serum (collected 160 d after immunization) also immunoprecipitates U1, U2, U4/U6, and U5 and is presented in Lane *5*. A control serum, presented in lane *6*, does not bind any of the U RNAs. This serum was collected 183 d after immunization with Freund's adjuvant alone. (Lane 7) Immunoprecipitation of U1, U2, U4/U6, and U5 by another anti-Sm and anti-nRNP precipitin positive human serum is again shown and serves as an additional human control.

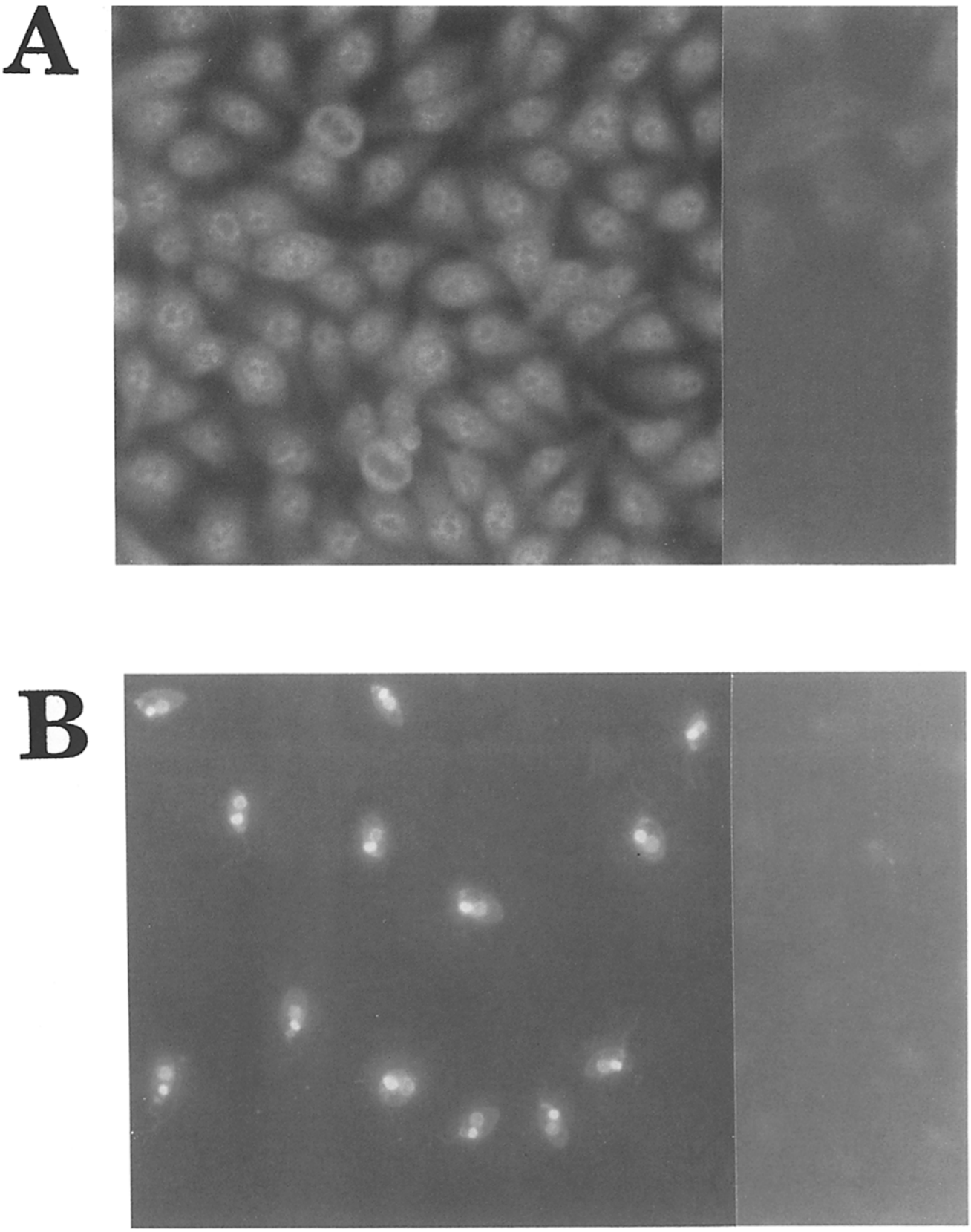

Figure 6. Antinuclear antibodies and anti-dsDNA antibodies found in PPPGMRPP and PPPGIRGP immunized rabbit sera. (*A*) Antinuclear fluorescence pattern on HEp-2 cells by a 1:1080 dilution of serum from rabbit #462 collected 181 d after primary immunization. Background binding of the preimmune serum from this rabbit is shown as an inlay on the right. This preimmune serum is diluted at 1:360 and was exposed six times as

anti-nRNP antibodies (Fig. 6 *A*). Preimmune sera from these animals, serum from an animal immunized with a less antigenic region of Sm B/B′, and sera from the sham-immunized animals were all negative for antinuclear antibodies.

Two PPPGMRPP or PPPGIRGP octapeptide immunized rabbits also developed antibodies to double-stranded DNA. Sera from rabbit #462 reacted strongly with the kinetoplast of *Crithidia luciliae* at a dilution of 1:270 (Fig. 6 *B*) whereas rabbit #465 is positive at 1:30. Preimmune sera from these rabbits, sera from animals immunized with Sm B/B′ 168, Ro 500, and Ro 413, MAP™, and Freund's adjuvant alone were all negative for antibodies to native DNA.

Sera from some of the immunized rabbits were also evaluated for antigen binding by Western blot analysis against HeLa cell extract and Sm/nRNP antigens (Fig. 7). After immunization serum from rabbit #462 binds the appropriate molecular weight proteins in Western blot for nRNP 70K, nRNP A, nRNP C, Sm B/B′, and Sm D antigens. Rabbit #463 (also immunized with PPPGMRPP) sera binds the 70K nRNP, nRNP C, Sm B/B′, and Sm D proteins. Rabbit #465, immunized with PPPGIRGP, binds the 70K nRNP, nRNP A, nRNP C, Sm B/B′, and Sm D proteins and serum from rabbit #464 (which died early in the immunization protocol) bind only the nRNP C, Sm B/B′, and Sm D proteins. Preimmune sera from these animals did not bind any proteins in Western blot. Rabbits immunized with MAP™, Freund's adjuvant, Ro 500, or Ro 413 did not bind these proteins. The sera from rabbits immunized with peptides from autoantigenic regions of 60-kD Ro, however, strongly bound to 60-kD Ro and to octapeptides derived from 60-kD Ro (data not shown; Scofield, R. H., J. A. James, and J. B. Harley, manuscript in preparation).

Anti-Sm precipitins are relatively specific for and useful in the diagnosis of SLE (1). Their presence led us to examine the progression of immune maturation and to evaluate these animals for the presence of other features found in human SLE. Rabbit #462 serves as an example and has had an especially interesting disease course after immunization with PPPGMRPP (Fig. 8). In addition to the antispliceosomal antibodies (detected by several different methods as presented above), serum from this rabbit had anti-Sm precipitins, antinuclear antibodies, and antidouble-stranded DNA antibodies. Rabbit #462 had substantial proteinuria and mild renal insufficiency with a creatinine as high as 1.7 mg/dl. The proteinuria was measured to be 168 mg/kg/d, which is approximately the level found in a human with 8 g proteinuria/d. Not surprisingly, his albumin decreased to 1.3 g/dl. His serum level of creatinine phosphokinase was elevated. The platelet count dropped as low as 27,000/μl. This animal lost hair much more rapidly than controls and had lost 2.49 kg or 39% of its weight. Rabbit #462 clearly satisfies the criteria

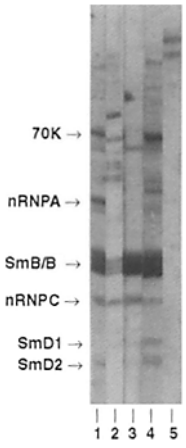

Figure 7. Western blot analysis of PPPGMRPP and PPPGIRGP immunized rabbit sera. (Lane *1*) Reactivity of a postimmune serum from rabbit #462 with the 70K, nRNP A, nRNP C, Sm B/B′, and Sm D proteins. (Lane *2*) Reactivity of rabbit #463, which died early in the immunization protocol, with the 70K nRNP, nRNP C, Sm B/B′, and Sm D proteins. (Lane *3*) Reactivity of rabbit #464 with nRNP C, Sm B/B′, and Sm D. (Lane *4*) Binding of rabbit #465, a long-term survivor immunized with PPPGIRGP, with 70K, nRNP A, nRNP C, Sm B/B′, and Sm D proteins after immunization. (Lane *5*) Background reactivity of a rabbit sham-immunized with Freund's adjuvant alone.

for SLE (1) ordinarily applied to humans by virtue of the presence of a positive ANA, anti-Sm, anti-DNA, proteinuria, and thrombocytopenia.

Rabbit #465 (PPPGIRGP), the other long-term survivor, has had anti-Sm precipitins, a positive antinuclear antibody (1:360), 3+ proteinuria (with hypoalbuminemia), and seizures, all consistent with SLE (1). No seizures and no persistent proteinuria was observed in any of the control animals.

Three animals died early in the immunization protocol. All were immunized with PPPGMRPP or PPPGIRGP. One died of bowel obstruction unrelated to SLE autoimmunity. Two died before being evaluated for SLE or autoimmunity. Both had seizures and among the most complex antispliceosome responses at that point in the immunization protocol, as measured by the 66 and 84 groups of spliceosome octapeptides bound.

Two immunization schedules were applied. Schedule A was uniformly successful in inducing antispliceosomal antibody by a number of measures. Schedule B was used in only one experimental set. These PPPGMRPP immunized animals experienced immunoglobulin spreading much more slowly than the others. Since the number of animals immunized was small and since a simultaneous comparison was not attempted, no firm conclusions can be drawn from these observations.

Discussion

These findings not only have important implications for understanding SLE, but may also be important for explaining the pathogenesis of other humoral autoimmune disorders. We have demonstrated that immunity against a single structure defined by a peptide may initiate an expanded autoimmune response against the molecule from which the peptide has been derived. While the rules defining which peptides are capable of inducing this response are not yet apparent,

long as *A*. (*B*) Serum at a dilution of 1:270 from rabbit #462 collected 155 d after primary immunization which binds strongly to the kinetoplast of *C. luciliae*. Anti-dsDNA antibodies were first found in this rabbit #462 on day 71 after primary immunization. Again, background reactivity of the preimmune serum from this rabbit is presented as an inlay on the right of *B*. This control serum is diluted 1:10 and the photograph has been exposed eight times as long as the image in the remainder of *B*.

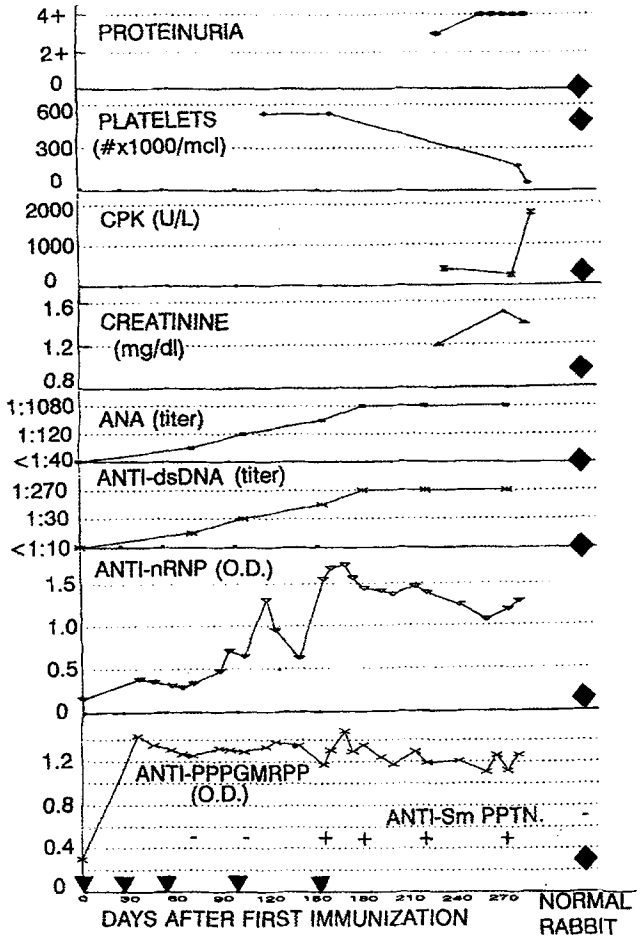

Figure 8. The development of autoimmunity in rabbit #462 after immunization with PPPGMRPP. Immunizations are indicated (▼) in *A*. Dilutions of sera (1:100) were tested in ELISAs for antibodies binding to PPPGMRPP and bovine nuclear ribonucleoprotein (nRNP). Serum titers of antibody binding to fixed HEp-2 cells (ANA) and *C. luciliae* kinetoplasts (anti-dsDNA) are determined by immunofluorescence assay. The level of creatinine, creative phosphokinase, and platelets in the peripheral blood and protein in the urine is presented. Proteinuria is measured on a semiquantitative scale of 0 to 4+. Anti-Sm precipitins by Ouchterlony immunodiffusion are present (+) in later sera, but absent in earlier sera (−). The average results of normal rabbits are presented as a point of reference (◆).

it is probably important that an antigenic peptide of the spliceosome was chosen. Immunization with peptides from other autoantigens or from an area antigenic in only a few human sera from Sm B/B′ (Sm B/B′ 168) failed to induce the cascade of events leading to spliceosomal autoimmunity. Also, analogy to the recently demonstrated spreading of T cell autoimmune epitopes (17) is obvious.

An autoimmune response progressing from one or a few structures to a complicated humoral autoimmune response is consistent with molecular mimicry theories of autoimmune disease (7, 18, 19). Of the known protein sequences, the immunogen used here, PPPGMRPP, is most similar to PPPGRRP from Epstein-Barr nuclear antigen 1 (EBNA-1) of EBV which is also bound by human sera containing anti-Sm autoantibodies (4).

If some of the other experimentally induced models of SLE (20–24) are related to the new model presented here, then they must have common features. Perhaps SLE autoimmunity is the result of dysregulation of a paratope-idiotype–anti-idiotype network of immune maturation via any one of a number of initial immune responses. Whether the paratope, idiotype or anti-idiotype acts to trigger the response may be relatively immaterial.

While the immunological details and subsequent disease characteristics may differ, autoimmunity may be a characteristic and common consequence of a number of different kinds of initiating events. Perhaps most, if not all, optimally challenged immunologically competent mammalian immune systems have the potential to develop SLE autoimmunity. For example, BALB/c mice have been immunized with PPPGMRPP or PPPGIRGP. Each of the 10 mice available for analysis have developed an antispliceosome autoimmune response (some with concurrent proteinuria) that has been absent from control immunized animals (James, J. A., and J. B. Harley, unpublished observations).

Particular antibodies, here anti-PPPGMRPP and anti-PPPGIRGP as well as the B cells that produce them, are therefore likely to be important in the induction of SLE autoimmunity. Indeed, these findings strongly support a recently advocated concept postulating a central role for B cells in generating autoimmune responses (25, 26). In our experiments, the B cell that binds both PPPGMRPP or PPPGIRGP and endogenous spliceosome of the rabbit (which also would contain the peptide immunogen) is postulated to have the capacity to not only bind the spliceosome, but also to process and present this and other spliceosome-derived peptides to T cells. With the appropriate immune stimulus, immunoglobulin epitope spreading and autoimmunity evidently follow.

Our findings support the possibility that human SLE is accompanied or induced by immune responses to relatively simple antigenic structures. The development of autoimmune disease would appear to be the consequence of immunoregulatory events that, once initiated, progress automatically and culminate in a systemic autoimmune disorder. These results also demonstrate the potential for individual autoantibody specificities, as well as, for the SLE RNA-protein autoantigens (Sm, nRNP, Ro, and La) to contribute toward pathogenesis of disease.

The extent to which clinical manifestations observed in our PPPGMRPP or PPPGIRGP immunized animals are directly attributable to and specific for a SLE autoimmune process is not known. The PPPGMRPP or PPPGIRGP immunized rabbits have variably had seizures, proteinuria, thrombocytopenia, and alopecia, which are all known to be part of human SLE. Of course, immunization with Freund's adjuvant is a substantial immune challenge and may account for some of these findings. Concluding that these particular findings bear more than a superficial resemblance to SLE autoimmunity must await a direct and rigorous description of the mechanisms involved. In any case, a new method of generating autoimmunity and for observing the subsequent maturation of the autoimmune response has been described.

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