

MECHANISMS OF AUTOANTIBODY PRODUCTION IN AUTOIMMUNE MRL MICE*

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Inbred mice developing autoimmune disease have provided an important model for the study of systemic lupus erythematosus (SLE)¹ (1-4). Like patients with this disease, autoimmune mice demonstrate antinuclear antibodies, immune complex deposition, and glomerulonephritis. Although inherited factors undoubtedly play a major role in the etiology of these illnesses, a common genetic abnormality has not as yet been identified among the now well-characterized strains. In the study of these mice, disturbances have been observed in various aspects of B and T cell maturation and function (5-19), although the relationship of these abnormalities to the development of autoreactivity is unclear. These functional studies have been primarily based on the analysis of the response to ordinary antigens on the assumption that an immunoregulatory defect responsible for autoreactivity would have a counterpart in a normal immune response as well. The validity of this approach is dependent on the existence of common immunoregulatory mechanisms for normal and autoimmune responses in these animals. One way to test this premise is to compare the production of different autoantibodies. It can be argued that, if autoantibody responses resulted from a common pathogenetic mechanism, they should demonstrate similarities in qualitative and quantitative aspects of antibody production such as time-course, magnitude, clonality, and maturation.

MRL substrain mice provide a unique model for investigating this aspect of autoimmunity (3, 4, 20). Both MRL-1pr/1pr and MRL-+/+ mice develop immunological illnesses with features of SLE, although in the 1pr/1pr substrain the disease is greatly accelerated and is associated with the development of massive lymphoproliferation. Unique among the murine models for SLE, both MRL substrains produce antibodies to the Sm antigen, a nuclear glycoprotein (21, 22). In human disease, anti-Sm antibodies (anti-Sm) are considered highly specific for SLE (23-26). In addition, it is known that the MRL-1pr/1pr substrain mice produce high titered antibody to native DNA (anti-DNA), another marker for human SLE (27-29).

In the studies presented here, the time-course and quantitative aspects of anti-Sm and anti-DNA antibody production have been assessed in both MRL substrain mice.

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¹ *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBS-Tween, PBS that contained 0.05% Tween 20; PHA, passive hemagglutination; RNP, ribonucleoprotein; RTE, rabbit thymus extract; SLE, systemic lupus erythematosus.

The results indicate that the two responses differ significantly in their pattern of expression and appear to be regulated by independent mechanisms. A single immunoregulatory disturbance for both responses cannot account for the quantitative aspects of autoantibody production presented here.

Materials and Methods

Mice. Male MRL-+/+ and MRL-1pr/1pr mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Beginning at the age of 8–12 w, mice were bled from the retroorbital plexus at regular intervals. After clotting, sera were separated and stored at -20°C until use.

Passive Hemagglutination Assay of Anti-Sm Antibodies. Anti-Sm antibodies were measured by the passive hemagglutination (PHA) method of Nakamura et al. (26) with modifications. The antigen source was a soluble extract of rabbit thymus powder (Pel Freeze Biologicals Inc., Rogers, Ark.) in phosphate-buffered saline (PBS; 0.15M NaCl, 0.01 M phosphate, pH 7.4). This material, rabbit thymus extract (RTE), was used to coat sheep erythrocytes that had been tanned using a concentration of 0.01% tannic acid. For the hemagglutination assay, the starting dilution of all sera was 1:5 in a vol of 25 μl , and twofold dilutions were carried out to 22 wells. Hemagglutination patterns were determined at 2 and 24 h, and results reported in terms of the number of twofold dilutions of serum producing hemagglutination. The specificity of antibodies detected as anti-Sm was established by hemagglutination testing using RTE cells treated with ribonuclease to remove ribonucleoprotein (RNP) antigen whose detection could confuse the interpretation of the results. Such treated cells, however, gave results equivalent to RTE cells that had not been treated with RNase. In addition, for selected sera, immunodiffusion testing using known index sera from patients with SLE was used to establish the identity of antibodies as anti-Sm.

Assay of Anti-DNA Antibodies. Antibodies to native DNA were assayed by an enzyme-linked immunosorbent assay (ELISA) that has recently been developed.² A solid phase support for the assay was prepared by coating wells of a polystyrene microtiter plate with native salmon sperm DNA preparations (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 1 $\mu\text{g}/\text{ml}$, and by washing to remove unbound DNA. Individual wells then were exposed for 1 h at room temperature to a 1:100 dilution of serum in PBS that contained 0.05% Tween 20 (PBS-Tween). After additional washing with PBS-Tween buffer, a 1:500 dilution of peroxidase-coupled goat anti-mouse IgG (immunoglobulin heavy and light chain specific; Litton Bionetics, Kensington, Md.) was added for 1 h. After additional washes, the substrate ABTS (2,2'-Azino-di [3-ethyl-benzthiazoline-sulfonate]; Sigma Chemical Co.) was added and incubated for 30 min. The contents of each well were then diluted with 0.75 ml of 0.1 M citrate buffer, pH 4, and the optical density at 417 nm read immediately. All determinations were performed in duplicate. For each serum, the reaction with uncoated wells was also measured. Values are reported as the difference of the average of the colorimetric yield of wells coated with DNA and uncoated wells. Results presented have not been corrected for serum dilution and represent the actual measured differences in optical densities. Evidence that this reaction is specific for anti-native DNA is considered in detail elsewhere.

Results

Kinetics of the Anti-Sm Response in MRL-1pr/1pr and MRL-+/+. To ascertain the pattern of the specific autoantibody responses of individual animals, sequential bleeds from a large number of MRL mice of both substrains were tested for anti-Sm and anti-DNA antibodies. MRL-1pr/1pr mice were followed until at least 6 mo of age, when significant mortality among the study group had already occurred, whereas MRL-+/+ mice were followed until at least the age of 8 mo.

A total of 23 1pr/1pr and 16 +/+ mice were studied and sequential sera analyzed

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for the production of anti-Sm antibody. All but two of the 1pr/1pr animals produced anti-Sm in at least one bleeding. In contrast, 10/16 +/+ animals showed demonstrable anti-Sm production on at least one occasion. The kinetics of the responses in individual animals showed marked variation among individual animals as demonstrated in Figs. 1 and 2, which illustrate the anti-Sm and anti-DNA responses of representative MRL animals.

Comparison of the peak anti-Sm antibodies of both substrains (Fig. 3) also demonstrated large variation in the level of responsiveness; several animals failed to demonstrate detectable anti-Sm antibody, and responders showed a broad distribution in the peak antibody levels. Although the highest titers of anti-Sm antibodies were encountered among the 1pr/1pr animals, the differences in the peak titers among the responding animals of both strains did not achieve statistical significance ($P = 0.08$, Mann-Whitney U test).

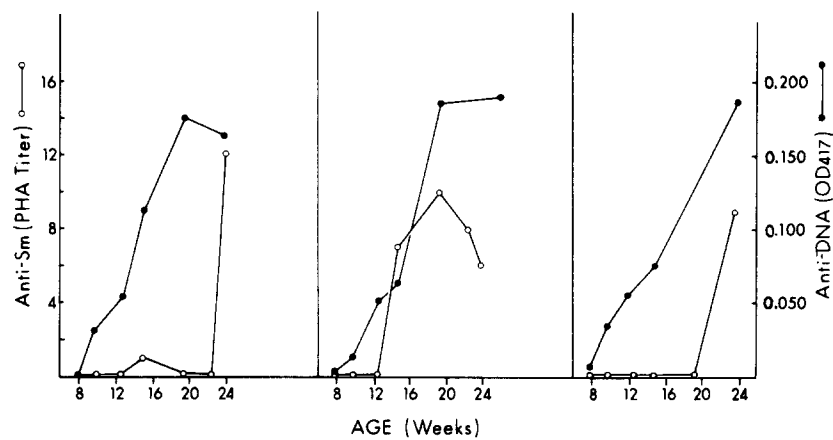


FIG. 1. Anti-DNA and anti-Sm antibody expression in MRL-1pr/1pr mice. Serial determinations of anti-DNA and anti-Sm antibody levels are presented for three representative MRL-1pr/1pr mice that expressed both anti-DNA and anti-Sm antibodies. Results for anti-Sm antibody are presented in terms of the PHA titer using RTE cells, whereas anti-DNA antibody levels are presented in terms of the OD417 produced in an ELISA assay.

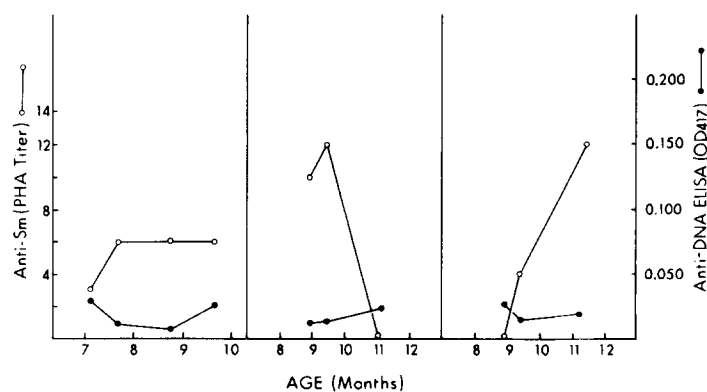


FIG. 2. Anti-DNA and anti-Sm antibody expression in MRL-+/+ Mice. Determinations of anti-DNA and anti-Sm antibody levels were performed on serial bleedings on three of the +/+ animals studied that produced significant anti-Sm antibodies. Results are presented as described in Fig. 1.

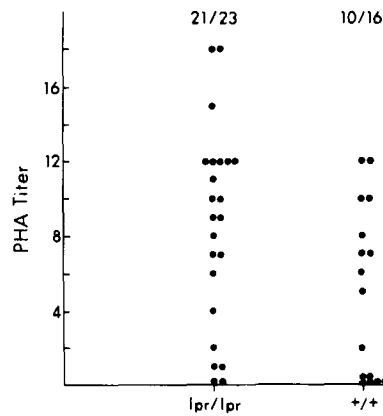


FIG. 3. Comparison of peak anti-Sm antibody responses of MRL mice. The peak anti-Sm antibody titers by the PHA assay have been plotted for the individual *lpr/lpr* and *+/+* mice studied.

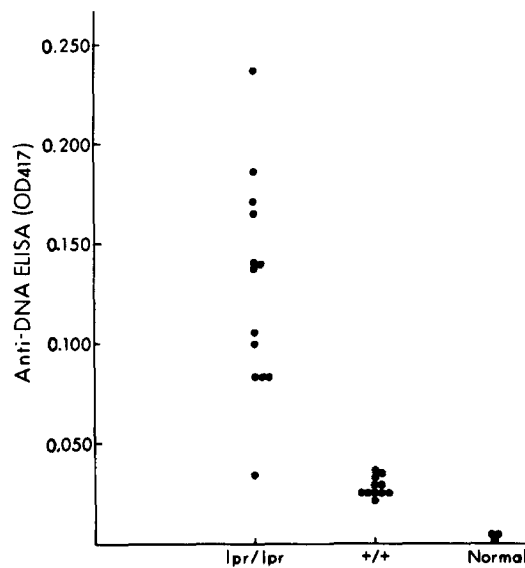


FIG. 4. Comparison of peak anti-DNA antibody responses of MRL mice. Peak anti-DNA antibody levels for individual MRL mice of both substrains are plotted in terms of the OD417 obtained by an ELISA assay.

Anti-DNA Responses in MRL Substrain Mice. The anti-DNA antibody levels in these serial bleedings were determined by an ELISA assay specific for anti-native DNA antibodies. For *lpr/lpr* mice, a very consistent pattern of responsiveness was observed, with individual animals producing serum anti-DNA antibody according to a similar time course (Fig. 1). Individual *+/+* mice, on the other hand, displayed uniformly low levels of anti-DNA antibodies until as long as a year of age (Fig. 2). In terms of the peak antibody levels, *lpr/lpr* responses varied by a factor of 4, whereas for the *+/+* mice, with much lower responses, the variation for individual animals was less than twofold (Fig. 4). Unlike the antibody response to Sm, the peak anti-DNA levels

of the animals of the two substrains differed in a highly statistically significant fashion ($P < 0.001$, Fisher exact test).

Time-Course of the Response of Anti-Sm and Anti-DNA Antibody. As demonstrated in Figs. 1 and 2, anti-DNA and anti-Sm antibody showed evidence of independent expression, with each response showing a distinct time-course in individual animals. Although not shown in these figures, there were 1pr/1pr animals that failed to demonstrate anti-Sm antibodies over the time period studied; the kinetics and magnitude of the anti-DNA response of these animals did not differ from other 1pr/1pr mice.

Discussion

The results presented here suggest the existence of distinct immunoregulatory disturbances involved in the expression of anti-DNA and anti-Sm antibodies. These two autoantibody systems have both been considered highly specific markers of human SLE, and their production by MRL animals has been cited as evidence for the existence of common pathogenetic mechanisms for murine and human disease. Nevertheless, by a variety of criteria, these two autoantibodies appear to be separately controlled. Thus, 1pr/1pr and +/+ mice showed comparable anti-Sm responses while demonstrating significantly different anti-DNA responses. In 1pr/1pr animals, where both antibodies were found, anti-Sm and anti-DNA demonstrated different kinetics, substantiating further their independent expression.

Anti-DNA and anti-Sm antibodies also differed in their quantitative production by individual animals. Whereas serum anti-DNA antibody varied over less than an order of magnitude in individual animals of both substrains, anti-Sm antibody levels varied enormously. Comparison in levels of autoantibody necessarily involves certain assumptions. Because autoantibodies can participate in immune complex formation, becoming unmeasurable when bound in serum or deposited in the tissues, serum levels may not accurately reflect production. Analysis of anti-Sm antibody production at the cellular level, however, would resolve ambiguities relating to measurement of antibodies hidden by immune complex formation, and is being initiated. The second assumption concerns comparison between strains of animals that develop disease according to different time-courses. MRL-1pr/1pr succumb to fatal autoimmune disease by 6–8 mo, whereas MRL-+/+ live as long as two years. It is possible, therefore, that at an older age +/+ animals would in fact produce as much anti-DNA antibody as did the 1pr/1pr animals, and older +/+ animals are now being analyzed to investigate this possibility. The occurrence of late anti-DNA production in +/+ animals would suggest that the action of the 1pr gene is more accurately defined in terms of its effect on the time-course of anti-DNA production rather than its magnitude, as these studies would suggest. Whatever the precise mechanism of 1pr gene action, these experiments demonstrate it influences anti-DNA much more profoundly than the anti-Sm response.

What are the mechanisms allowing different autoantibodies to be produced? For the anti-DNA response, the production of these antibodies appears to be related to polyclonal B cell activation with a time-course similar to the increase in spontaneous Ig-producing cells in the MRL-1pr/1pr mice (15, 30–32). This temporal increase in splenic Ig-producing cells has been considered a reflection of an abnormal state of B cell activation. Because NZB/W mice, which also have B cell hyperactivity, express

anti-DNA antibodies, an abnormal state of B cell activity may be a predominant mechanism for the production of these antibodies. The correlation of anti-DNA production with generalized B cell hyperactivity is further substantiated by the failure of MRL-+/+ mice to produce significant quantities of anti-DNA antibody when their level of spontaneous B cell activation is comparable with many normal mice strains.

For anti-Sm antibody, it is not possible to correlate its expression with evidence of B cell activation. Thus, 1pr/1pr animals with B cell hyperactivity frequently fail to express anti-Sm antibodies, whereas +/+ mice with apparently normal levels of B cell activation produce anti-Sm antibodies to a similar extent as 1pr/1pr animals. This result may suggest that B cells producing anti-Sm antibodies belong to a subset whose activity is not mirrored by spontaneous Ig production and is presumably different from the subset of cells producing anti-DNA antibody. Whereas the activation of this population may be responsible for anti-Sm production, these cells may represent a minority population and may therefore be difficult to detect. Alternatively, anti-Sm production may differ entirely from that of anti-DNA and may not require an abnormal population of B cells. Rather, immunoregulatory defects consequent to disturbances among T cells may initiate anti-Sm production.

Whatever the mechanism responsible for anti-Sm production, it is necessary to account for the large variability of this response. This pattern could be explained in part by the existence of a limited number of precursors for anti-Sm production. If the activation of these precursors (by whatever mechanism) occurred randomly, then the distribution of responses would be expected to follow a Poisson distribution; the number of animals that express anti-Sm antibody would be determined by the number of responding units or precursors present in each animal. A limiting number of such precursors in contrast to a much larger number for anti-DNA precursors could account for highly variable anti-Sm levels in face of more consistent anti-DNA responses. The number of precursors for these two antibodies may in turn reflect the number of distinct specificities for anti-DNA and anti-Sm responses in the antibody repertoire. From hybridoma antibody studies, it is now known that for MRL mice there exist at least several distinct types of anti-DNA antibodies of unique binding specificity (33). With diversity among anti-DNA antibodies, there exists the potential for a large B cell population committed to this type of autoantibody production. The activation of even a limited number or subset of such precursor cells would result in anti-DNA antibody in every animal of the strain. The existence of germ line genes for anti-DNA antibodies would assure representation of such specificities in the repertoire (and B cell precursor population), and provide a mechanism for their invariant expression when B cells are activated. In contrast, the repertoire of unique anti-Sm specificities in MRL mice may be very limited, relating perhaps to the paucity or absence of germ line genes for such antibodies. Without genetically determined specificities, somatic mutation would be required for the generation of a repertoire of anti-Sm antibodies. The magnitude of the response of individual animals would therefore reflect the total number of appropriate precursors generated by mutational events, with the variation among individual animals demonstrating the random quality of this process. If such mutational events occurred at various time points throughout the life of an animal, the highly variable time course of the anti-Sm response would be explained. It is hoped that the study of autoantibodies from the

perspective of idiotype will define more fully the contribution of these various mechanisms for the generation of autoantibodies.

Summary

The quantitative expression of anti-DNA and anti-Sm antibodies has been investigated in autoimmune MRL-1pr/1pr and MRL-+/+ mice. Anti-Sm antibodies were detected in sera from 21/23 1pr/1pr and 10/16 +/+ mice, with individual animals showing striking variation in the time-course and magnitude of this autoantibody response. The peak antibody levels of the responding animals of each substrain did not differ significantly. For anti-DNA antibody, a different pattern of responsiveness was observed. Individual animals of each substrain produced very similar responses in terms of the magnitude and time-course of serum anti-DNA antibody. The differences in the peak levels of the two substrains were highly significant, with 1pr/1pr mice demonstrating a much greater anti-DNA antibody response than +/+ mice. In 1 pr/1pr mice tested for both autoantibody systems, serum anti-DNA and anti-Sm antibodies showed distinct time-courses. These studies indicate that anti-DNA and anti-Sm antibodies are expressed independently in MRL mice, with the expression of anti-DNA, but not anti-Sm antibody markedly influenced by the presence of the 1pr gene. A fundamental difference in the mechanisms involved in the generation of anti-DNA and anti-Sm antibodies is suggested by the quantitative pattern of the two responses.

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