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ANTI-RNA POLYMERASE I ANTIBODIES IN SERA OF MRL *lpr/lpr* AND MRL +/+ AUTOIMMUNE MICE

Correlation of Antibody Production with Delayed Onset of Lupus-like Disease in MRL +/+ Mice

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Individuals with systemic lupus erythematosus (SLE)[1] or one of several other rheumatic autoimmune diseases exhibit a number of immunological abnormalities, including the production of antibodies directed against components of cell nuclei (1). The specificities of these antinuclear antibodies have been extensively investigated for the purpose of understanding the pathogenesis of the diseases as well as for use as diagnostic aids. Antibodies against DNA, histones, and a growing list of nonhistone nuclear proteins have been identified in the sera of SLE patients (2). However, at the present time, RNA polymerase I (3) and an associated enzyme, protein kinase NII (4), are the only two nonhistone nuclear protein target antigens of SLE autoantibodies for which enzymatic activities are known. Antibodies against RNA polymerase I have been demonstrated (3) in the sera of all SLE and mixed connective tissue disease patients tested, as well as in the sera of 78% of individuals with rheumatoid arthritis. Because the structure and function of RNA polymerase I are well documented (reviewed in 5), this enzyme provides a unique tool for investigating the mechanism of rheumatic disease autoimmunity.

RNA polymerase I is a complex enzyme composed of eight different subunits (S1–S8) (5). The specificities of the autoantibodies for RNA polymerase I subunits appear to be characteristic of the particular rheumatic disease (3). Thus, only anti-S3 was found in the sera of rheumatoid arthritis patients, whereas sera from SLE patients contained anti-S3 plus anti-S2 and/or anti-S5, and sera from individuals with mixed connective tissue disease contained anti-S4 alone or in combination with anti-S3 and anti-S5. This data demonstrated the nonrandom nature of the rheumatic disease autoimmune response and raised the question of why S2, S3, S4, and S5 were more autoimmunogenic than the other four polymerase polypeptides. Subsequent studies demonstrated (6, 7) that S2, S3, and S5 were by far the most highly phosphorylated subunits, and that S4 and S5

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[1] *Abbreviations used in this paper:* SLE, systemic lupus erythematosus; ssDNA, single-stranded DNA.

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were related to protein kinase NII (4–7). Further, the immunoreactivity of polymerase polypeptides with SLE patient antibodies was shown (8) to be greatly enhanced by prior phosphorylation of the enzyme with protein kinase NII. Conversely, in vitro dephosphorylation of RNA polymerase I with alkaline phosphatase reduced the reaction of the autoantibodies with the polymerase by 40–50%. These data indicated that a significant fraction of the SLE autoantibodies against RNA polymerase I are directed against phosphorylated sites on the enzyme, and suggested that protein phosphorylation may have a major role in the production of autoimmunogenic polypeptides in the rheumatic diseases.

To study the mechanism(s) of RNA polymerase I autoimmunogenicity, we have chosen an animal model. Although several murine strains have been developed that spontaneously develop syndromes resembling human SLE (9–12), anti-Sm antibodies are found almost uniquely in the related MRL *lpr/lpr* (MRL/*lpr*) and MRL +/+ (MRL/++) strains (13). Because anti-Sm is considered a marker for human SLE (2), these strains appeared to be the ones most likely to produce anti–RNA polymerase I autoantibodies. The MRL/*lpr* and MRL/++ strains, developed by Murphy and Roths (10, 11) of The Jackson Laboratory, Bar Harbor, ME, and their coworkers (12), share 90% of their genome. Both sexes of either strain produce anti-DNA and anti-nuclear antibodies, and develop chronic immune complex glomerulonephritis and necrotizing arteritis. MRL/*lpr* mice also exhibit massive generalized lymph node enlargement and die of the disease at an average age of ~20 wk. In contrast, MRL/++ mice do not develop generalized lymphoproliferation, and the onset of lupus-like disease is delayed so that death occurs much later, at an average age of 20 mo.

As the first step in using the MRL/*lpr* and MRL/++ mice as models of human RNA polymerase I autoimmunogenicity, we have screened the sera from these animals over a period of ~30 wk for the presence of anti–RNA polymerase I antibodies. We report here that anti–RNA polymerase I antibodies are produced by all MRL/*lpr* and MRL/++ mice, and that, like the onset of lupus-like disease, production of these antibodies is delayed in the MRL/++ mice. The presence of anti-RNA polymerase I antibodies thus appears to be a better indicator of the onset of the disease, at least in these animals, than either the presence of anti-DNA or anti-Sm antibodies.

Materials and Methods

Mice. MRL/*lpr* and MRL/++ mice were obtained at the age of 5 wk from The Jackson Laboratory. Each individual mouse was bled from the tail, weekly for the first 14 wk of age, then less frequently for the next 16 wk. Sera were stored at −20°C and thawed only once before assay.

Antigens. RNA polymerase I was purified from isolated nuclei of the rat tumor, Morris hepatoma 3924A (7). After phenol extraction of calf thymus DNA (Sigma Chemical Co., St. Louis, MO), single-stranded (ss) DNA was prepared by boiling for 10 min and rapidly cooling on ice.

Radioimmunoassay. Radioimmunoassays for the detection of anti–RNA polymerase I and anti-DNA antibodies have been described in detail previously (3, 14). Briefly, either 100 μl of purified RNA polymerase I (2 μg) or ssDNA (50 μg) was adsorbed to 400 μl capacity, flat-bottomed polystyrene microtiter wells. Sera (100 μl of 1:10-diluted) were then incubated in the antigen-containing wells, followed by quantitation of antibody binding with ^{125}I-labeled (50 μCi/μg) protein A (50,000 cpm/well) and gamma counting.

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The use of ^{125}I-protein A was superior to ^{125}I-labeled anti-mouse IgG in terms of sensitivity and nonspecific binding (data not shown). Each determination was performed in duplicate. Sera from three 18-mo-old female BALB/c mice were pooled and used as the control. Backgrounds of 238 ± 19 and 617 ± 16 (cpm of ^{125}I-protein A bound ± SEM) were obtained with this pool in the radioimmunoassays for anti-RNA polymerase I and anti-ssDNA antibodies, respectively. Sera from five 5-wk-old MRL/++ mice produced a mean value of 191 ± 24 with RNA polymerase I as antigen. Similar background values were obtained with sera from a number of BALB/c mice of both sexes, ranging in age from 6 wk to 2 yr (data not shown). None of the BALB/c control mice produced elevated values with RNA polymerase I as antigen. However, an occasional BALB/c serum sample contained significant anti-ssDNA antibodies (up to 2,500 cpm after subtraction of background), as has been observed by others (12). MRL/*lpr* and MRL/++ mice were considered positive for anti-RNA polymerase I and anti-DNA antibodies when values (cpm) at least twice those of the BALB/c control pool were obtained with sera from two consecutive bleedings. Results with MRL mouse sera are expressed as the quantity (cpm) of ^{125}I-protein A remaining bound to the wells, less the background.

Results

Anti-RNA Polymerase I Antibodies in MRL/lpr Mice. Sera from 15 female and 13 male MRL/*lpr* mice were screened for anti-RNA polymerase I antibodies. Anti-RNA polymerase I antibodies were initially detected in the sera of 1 of the 15 female MRL/*lpr* mice at the age of 5 wk (Fig. 1). The antibodies were not detected in any of the sera from male MRL/*lpr* mice until the age of 8 wk (Fig. 1). External signs of the disease process, such as lymph node enlargement or alopecic dermatitis, were not evident until ~14 wk of age. There was considerable variability among the individual mice with regard to the relative concentration of anti-RNA polymerase I antibodies (Fig. 2; data for individual female mice

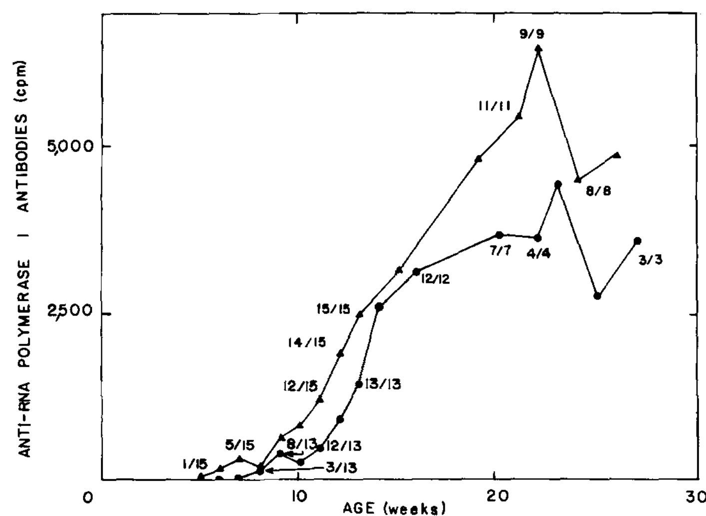

FIGURE 1. Anti-RNA Polymerase I antibodies in sera of male and female MRL/*lpr* mice. Serum samples from male (●) and female (▲) MRL/*lpr* mice, collected over a period of 27 wk, were analyzed for the presence of anti-RNA polymerase I antibodies as described in Materials and Methods. Results shown are the mean values of all determinations with male (n = 13, initially) or female (n = 15, initially) animal sera. Numbers in the graph indicate the number of male or female mice with anti-RNA polymerase I antibodies, over the number of surviving mice.

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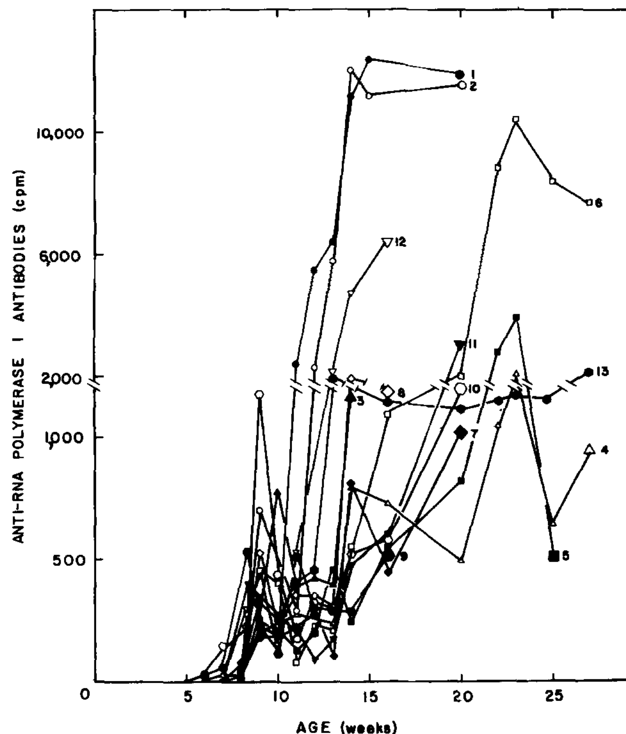

FIGURE 2. Anti-RNA polymerase I antibodies in sera of individual male MRL/*lpr* mice. Serum samples taken from male MRL/*lpr* mice at the ages indicated were analyzed for anti-RNA polymerase I antibodies as described in Materials and Methods. Results shown are the average of duplicate determinations with sera from each individual animal. Each symbol represents a different mouse. Slightly larger symbols indicate that the particular mouse died before the next bleeding. Numbers in the graph are those assigned randomly to the animal at the beginning of the experiment.

not shown). Nevertheless, all MRL/*lpr* mice had detectable anti-RNA polymerase I antibodies in their sera before death. The antibodies were present in the sera of 100% of female and male mice by the age of 13 wk. In general, females produced the antibodies earlier (mean, 9.5 wk) as compared to males (mean, 9.7 wk), and at higher relative concentrations than males (Fig. 1). One-third of females had the antibodies before they were detected in any of the males. Neither the time of appearance nor the relative concentration of anti-RNA polymerase I antibodies correlated with age at time of death (see Fig. 2).

Anti-ssDNA Antibodies in MRL/lpr Mice. Antibodies against ssDNA were demonstrable in the sera of all female and 11 of the 13 male MRL/*lpr* mice at the age of 5 wk (Fig. 3). The antibodies were absent only in the sera of male mice numbers 1 and 5 at this time (not shown). However, by 6 wk of age, these animals also had anti-ssDNA antibodies in their sera. As was observed with anti-RNA polymerase I antibodies, females produced slightly higher serum concentrations of anti-ssDNA antibodies than did the males (Fig. 3). There was less variability among individual animals with respect to relative concentration of anti-DNA antibodies (not shown) than was observed with anti-RNA polymerase I. How-

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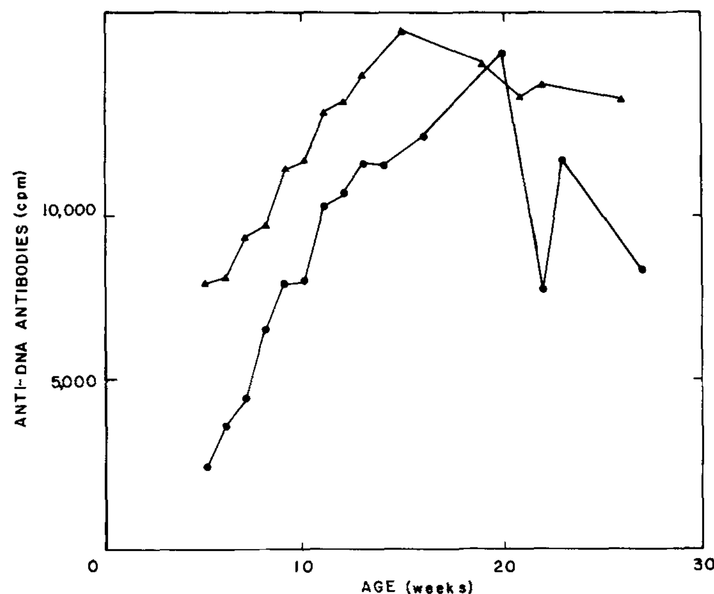

FIGURE 3. Anti-ssDNA antibodies in sera of male and female MRL/*lpr* mice. Sera from male (●) and female (▲) MRL/*lpr* mice were analyzed for anti-ssDNA antibodies as described in Materials and Methods. Results shown are the means of the determinations with male or female animal sera.

ever, there was no correlation between relative concentration of anti-ssDNA antibodies and severity of external disease symptoms or age of death. Similarly, there was no apparent correlation between relative concentration of anti-ssDNA and anti-RNA polymerase I antibodies. In 27 of the 28 mice tested, detection of anti-ssDNA antibodies preceded detection of anti-RNA polymerase I antibodies. Antibodies of both specificities were present at the earliest time tested (5 wk) in one female MRL/*lpr* mouse.

Anti-RNA Polymerase I and Anti-ssDNA Antibodies in MRL/++ Mice. Three of the five MRL/++ mice tested (male) had anti-ssDNA antibodies in their sera at 5 wk of age (Fig. 4*A* and Fig. 5). Like the MRL/*lpr* mice, all the MRL/++ animals had these antibodies by 6 wk. Up to ~11 wk of age, the mean anti-ssDNA antibody concentration of the male MRL/++ animals was very similar to that of the male MRL/*lpr* mice (compare Fig. 5 with Fig. 3) even though onset of lupus-like disease is delayed in MRL/++ (10–12). No external signs of the disease were evident in these mice until 25 wk (see below).

Anti-RNA polymerase I antibodies were not detected in the sera of MRL/++ mice for the first 9 wk of age (Fig. 4*B*). At 10 wk, serum from mouse number 2 had anti-RNA polymerase I antibodies (2.3 times background). However, this reactivity had returned to background by 11 wk, and the animal was thus not considered positive for the antibodies at this time (see Materials and Methods). Similar, short-duration bursts of anti-RNA polymerase I antibodies were observed in sera from animals 1 and 5 at 14 wk of age. Sera from animal number 3 contained the antibodies on wk 13 and 14, and so was scored as positive at 13 wk of age, even though only background levels were observed until 23 wk of

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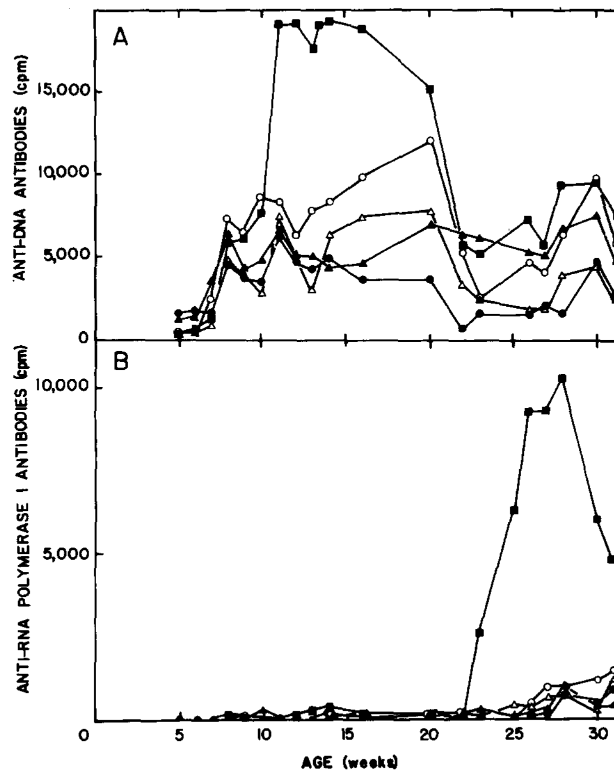

FIGURE 4. Anti-ssDNA and anti-RNA polymerase I antibodies in sera of male MRL/++ mice. Anti-ssDNA and anti-RNA polymerase I antibodies in the sera of five male MRL/++ mice up to the age of 31 wk were quantitated as described in Materials and Methods. Results are from duplicate determinations of anti-ssDNA (*A*) or anti-RNA polymerase I (*B*) antibody content in sera from each individual animal. Results obtained with sera from each mouse (numbered 1-5) are indicated with a different symbol (●, ▲, ■, △, and ○, respectively).

age. At 28 wk of age, sera from all five mice were considered positive for anti-RNA polymerase I antibodies (mean age, 22.8 wk). Hence, unlike anti-ssDNA antibody production, anti-RNA polymerase I antibody production was delayed in MRL/++ male mice more than 3 mo as compared to MRL/*lpr* male mice. Mouse number 3 (Fig. 4*B*) exhibited minor alopecia at age 25 wk, while the remaining four MRL/++ mice appeared normal.

A precipitous decrease in anti-ssDNA antibodies was observed in the MRL/++ mice at the age of about 20 wk (Fig. 4*A* and Fig. 5). This drop was followed by the appearance in the sera of anti-RNA polymerase I antibodies. Interestingly, the decrease at this time in each individual animal's anti-DNA antibodies was directly proportional ($R^2 = 0.964$) to the following initial rise in anti-RNA polymerase I (Fig. 6), suggesting a possible relationship between the two phenomena. Smaller decreases, of shorter duration, in anti-ssDNA antibodies were observed at ~9-10 and 13-14 wk of age (Fig. 4*A* and Fig. 5), which corresponded to short-duration bursts of anti-RNA polymerase I antibodies described in the preceding paragraph. Although the appearance of anti-RNA polymerase I antibodies in several of the MRL/*lpr* mice was accompanied by a

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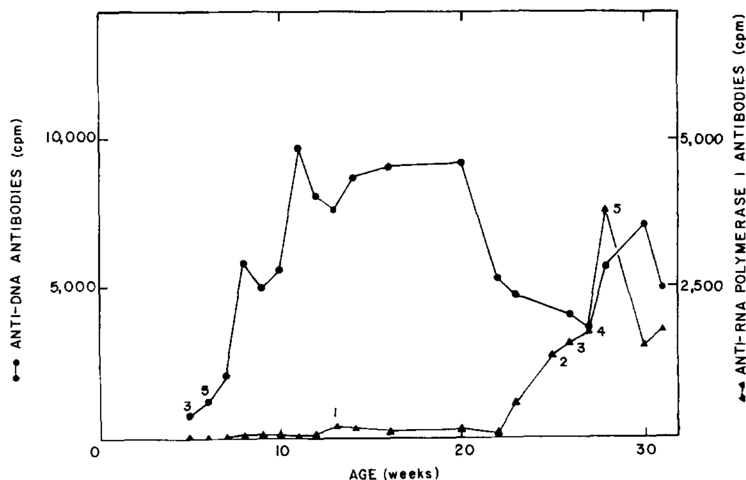

FIGURE 5. Means of serum anti-ssDNA (●) or anti-RNA polymerase I (▲) antibodies of MRL/++ mice as a group. Numbers in the graph indicate the number of mice in which anti-RNA polymerase I was detected at that particular age.

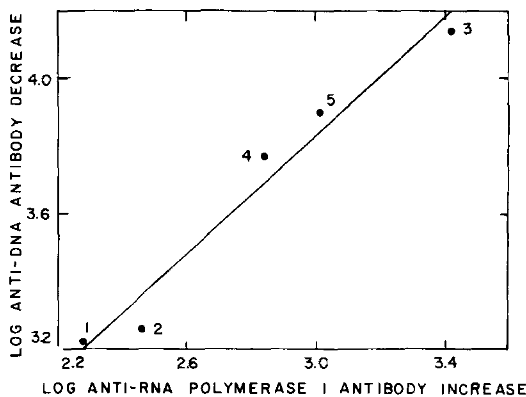

FIGURE 6. Correlation of decrease in anti-ssDNA antibodies with appearance of anti-RNA polymerase I antibodies in sera of MRL/++ mice. The log of the decrease in anti-ssDNA antibodies from each MRL/++ mouse serum was plotted against the log of the increase in anti-RNA polymerase I antibodies. The decrease in anti-ssDNA antibodies was figured from the time the quantity of these antibodies began to decline precipitously (16 wk for mouse 3, 20 wk for the other four mice) to the time the quantity of these antibodies began to increase or anti-RNA polymerase I antibodies appeared in the sera (23 wk for mouse 3, 27 wk for the other four mice). The quantities of anti-RNA polymerase I antibodies at 23 wk for mouse 3 and at 27 wk for the other four mice were used in the plot. The line was fitted by linear regression analysis (R^2 = 0.964). Numbers in the graph refer to the identification number assigned to each animal.

drop in anti-DNA antibodies (not shown), a direct proportionality was not evident. The accelerated disease process in the MRL/*lpr* animals may necessitate more frequent serum samples to observe such a relationship.

Discussion

All mice of the autoimmune strains MRL/*lpr* (n = 28) and MRL/++ (n = 5) were found to produce anti-RNA polymerase I antibodies. The presence of

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these antibodies in the sera is yet another characteristic of human SLE that is shared by these two murine strains. Eisenberg et al. (13) reported earlier that MRL mice also produce anti-Sm antibodies, a specificity found almost exclusively in human SLE. However, unlike anti–RNA polymerase I antibodies, which are found in 100% of both human (3) and MRL-murine lupus, anti-Sm has been demonstrated in only 30–40% of human (2) and MRL-murine (13) lupus populations. Further, anti-Sm antibodies were not detected in the sera of MRL/*lpr* mice until the age of 4 mo (13) when the disease is already quite advanced (10, 11), and many of the animals were moribund (13). In contrast, anti–RNA polymerase I antibodies were detected in the sera of >50% of MRL/*lpr* mice at the early age of 10 wk, and in 100% by 13 wk of age. This time of appearance of anti–RNA polymerase I antibodies (mean, 9.6 wk of age for males and females combined) corresponds to the reported (10–12) onset of massive generalized lymph node enlargement in these animals at ~8 wk of age. It should be pointed out that anti-Sm antibodies were detected by use of the double immunodiffusion technique (13) while anti–RNA polymerase I antibodies were quantitated here by use of radioimmunoassay. Whether use of a radioimmunoassay would allow earlier detection of anti-Sm or detection in a larger proportion of MRL/*lpr* animals, is not certain.

We recently reported (14) that immunization of rabbits with purified RNA polymerase I resulted in the production of anti-DNA antibodies shortly after production of antibodies against the enzyme. RNA polymerase I has a high affinity for DNA, inherent to its functional role in transcription. We therefore proposed that the injected, immunogenic RNA polymerase I had become associated with the DNA present in blood plasma (15), which led to induction of an immune response against the normally nonimmuogenic (16) nucleic acid component of the complex. The immunogenic property of nucleic acids when they are complexed with protein has been well established (17). Because antibodies against RNA polymerase I and DNA are both characteristic of SLE (1–3), we suggested (14) that a similar mechanism may be involved in the induction of anti-DNA antibody formation in rheumatic disease. In this study, significant quantities of anti-DNA antibodies were present in the sera of all but one of the MRL/*lpr* and MRL/++ mice before anti–RNA polymerase I antibodies could be found. At least two factors could explain why anti-DNA antibodies were detected earlier in these mice. First, a relatively small quantity of immunogenic RNA polymerase I complexed with DNA in the sera of the mice could conceivably induce an immune response against the nucleic acid, which then would develop more rapidly than the response against the enzyme itself, due to a higher level of DNA in blood plasma. Antibodies against RNA polymerase I were detected before those against nucleic acids in rabbits immunized with the enzyme (14), most likely because of the availability of excess quantities of injected enzyme. Second, it is possible that anti–RNA polymerase I antibodies were produced at an earlier time, but that they were complexed with antigen and thus not detectable as free serum antibodies. The fact that anti–RNA polymerase I antibodies decreased in relative concentration in the sera of MRL/++ mice whenever anti-ssDNA antibodies increased, and vice versa, suggests that anti–RNA polymerase I antibodies may have been produced at an earlier time than 5 wk, but either became bound

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to serum antigen or decreased to undetectable levels when anti-ssDNA antibodies began to increase rapidly. The important issue here is that a relationship does seem to exist between anti-DNA and anti–RNA polymerase I antibodies.

Perhaps the most significant finding of this investigation is that, like the onset of lupus-like disease (10–12), the appearance of serum anti–RNA polymerase I antibodies was delayed ~3 mo in MRL/++ compared to MRL/*lpr* mice. The age of appearance of anti-Sm antibodies in the sera appears to be about the same in the two strains (13). Similarly, the age of first appearance and relative concentration of anti-DNA antibodies in the sera of the MRL/++ mice was not much different than that observed with the MRL/*lpr* mice. These results suggest that anti-DNA antibodies by themselves are neither reliable indicators of lupus-like disease, nor solely responsible for disease symptoms. The relatively common occurence of anti-DNA antibodies in the sera of immunologically normal mice (12) and humans with no apparent autoimmune disease (18) supports this contention. Thus, if anti-DNA antibodies are involved in the disease process, some other factor(s) must also be present to elicit disease symptoms. Anti–RNA polymerase I antibodies were present in the sera of all MRL/*lpr* mice before or at the time of onset of external disease symptoms such as gross lymph node enlargement and alopecic dermatitis. These symptoms, as well as detectable anti–RNA polymerase I antibodies were absent in MRL/++ animals for more than 5 mo. Interestingly, one of the MRL/++ mice (mouse number 3) began exhibiting minor alopecia at an age of ~24 wk, just after the appearance and sharp increase of anti–RNA polymerase I serum antibodies. The other four MRL/++ mice exhibited no external disease symptoms at this time, and had little or no anti–RNA polymerase I antibodies in their sera. Even more intriguing was the apparent direct relationship between the decrease in anti-DNA antibody concentration in the sera of the MRL/++ mice and the appearance of anti–RNA polymerase I antibodies. This decrease in free anti-DNA antibodies in the sera may indicate that these antibodies are forming complexes with antigen and are being cleared from the sera. How RNA polymerase I antigen and antibody could be involved in this process is only conjecture at this point. However, the direct proportionality of the anti-DNA antibody decrease with the appearance and increase in anti–RNA polymerase I antibodies seems to be too close ($R^2 = 0.964$) for a fortuitous relationship. Clearly, the relationship of anti-DNA and anti–RNA polymerase I antibodies, and the interaction of the two antigens in the rheumatic disease process deserves further study. The MRL/*lpr* and MRL/++ autoimmune murine strains will be invaluable to this endeavor.

Summary

Sera from individual MRL/*lpr* and MRL/++ mice, which develop an autoimmune disease similar to human systemic lupus erythematosus (SLE), were screened over a period of ~30 wk for the presence of anti–RNA polymerase I and anti-ssDNA antibodies. Even though onset of the disease is delayed in MRL/++ as compared to MRL/*lpr* mice, anti-ssDNA antibodies were present in comparable concentrations in the sera of all mice by the age of 6 wk. As observed in sera of human SLE patients, anti–RNA polymerase I antibodies were detected in the sera of all MRL mice. However, unlike the anti-ssDNA antibodies, anti–

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RNA polymerase I antibodies were detected much later in MRL/++ mice (mean age, 22.8 wk) as compared to MRL/*lpr* mice (mean age, 9.6 wk). The presence of anti–RNA polymerase I antibodies in sera of MRL mice was thus a much better indicator of disease status than the presence of anti-ssDNA antibodies. The appearance and increase in anti–RNA polymerase I antibodies in the sera of MRL/++ mice correlated ($R^2 = 0.964$) with a precipitous decrease in anti-ssDNA antibodies, starting at about 20 wk of age. These results suggest a possible relationship between the RNA polymerase I and DNA autoimmune reactions.

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