Immunization of rabbits with purified RNA polymerase I induces a distinct population of antibodies against nucleic acids as well as anti-RNA polymerase I antibodies, both characteristic of systemic lupus erythematosus

[anti-DNA antibodies/autoimmunogenicity/5'-terminal phosphate/antipolyonucleotide antibodies/poly(A) polymerase]

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ABSTRACT Rabbits were immunized with either RNA polymerase I or poly(A) polymerase that had been purified to apparent homogeneity and was devoid of nucleic acids. Sera from rabbits thus immunized were screened for antibodies against nucleic acids. All seven rabbits injected with RNA polymerase I but none of the four rabbits immunized with poly(A) polymerase produced anti-nucleic acid antibodies. Anti-RNA polymerase I antibodies were induced after a single injection of the enzyme. Anti-polynucleotide antibodies were not detectable until after the second immunization. Anti-RNA polymerase I antibodies could be detected with as little as 100 pg of purified RNA polymerase I in the radioimmunoassay. At least 50 ng of poly(A) or 200 ng of DNA was required to detect anti-nucleic acid antibodies. The immunoreactivity of anti-RNA polymerase I antiserum was greater with synthetic polynucleotides than with DNA, particularly early in the immunization schedule. Alkaline phosphatase treatment of poly(A) to remove 5' phosphates nearly abolished its antigenicity with respect to the early sera and decreased antibody binding of later sera by 60%. These results indicate that the anti-nucleic acid antibodies produced early were primarily directed against determinants including the 5'-terminal phosphates while antibodies produced later were directed against other sites. The antinucleic acid antibodies and anti-RNA polymerase I antibodies formed two distinct populations that were not immunologically crossreactive. We suggest that after injection, RNA polymerase I becomes associated with the nucleic acids present in blood plasma which renders them immunogenic; thus, association of nucleic acids with autoimmunogenic RNA polymerase I may be one of the mechanisms by which anti-DNA antibodies are induced in systemic lupus erythematosus.

Sera from patients with systemic lupus erythematosus (SLE) and other rheumatic autoimmune diseases contain antibodies against a variety of proteins and nucleic acids in the cell nucleus (reviewed in ref. 1). It has been postulated that phosphodiester bonds of nucleic acids are autoantigenic determinants in both human SLE (2) and the murine lupus-like syndrome (3). Recently, we identified RNA polymerase I (4) and protein kinase NII (5) as target nucleic antigens in SLE, rheumatoid arthritis, and mixed connective tissue disease and demonstrated (6) that phosphorylation of the polymerase can dramatically increase its antigenicity with respect to SLE patient autoantibodies. The importance of phosphate groups in the autoantigenicity of both RNA polymerase I and DNA and the recent reports that anti-DNA autoantibodies were capable of reacting with phosphoplipids (2, 3) and proteins (7) suggested that anti-RNA polymerase I antibodies might crossreact with DNA. In addition, since RNA polymerase I can interact with DNA as part of its biological function, it is plausible that association of the immunogenic enzyme with DNA, which is present in blood plasma (8), might induce production of anti-DNA antibodies. These possibilities were tested in the present study by analysis of serum from rabbits immunized with purified RNA polymerase I for anti-nucleic acid antibodies.

METHODS

Enzyme Purification. RNA polymerase I (9) and poly(A) polymerase (10–12) were purified to apparent homogeneity from isolated nuclei of the serially transplanted rat tumor, Morris hepatoma 3924A. Purified RNA polymerase I had a specific activity of 125–170 units/mg of protein and exhibited a single Coomassie blue stained band on polyacrylamide gels electrophoresed under nondenaturing conditions (data not shown), which is consistent with our earlier data (9). Purified poly(A) polymerase had a specific activity of greater than 4000 units/mg of protein and exhibited a single band on polyacrylamide gels stained with Coomassie blue after electrophoresis under denaturing conditions (data not shown). Enzyme units and assays were as described (9, 10). Neither the RNA polymerase I nor the poly(A) polymerase used for immunization contained nucleic acids even after concentration as judged by analysis of the most sensitive assays.

Immunoization. Male New Zealand White rabbits were injected subcutaneously every 2 weeks with either purified RNA polymerase I (50 µg) or purified poly(A) polymerase (25 µg). The injected enzymes (0.25–0.50 ml) were emulsified with an equal volume of Freund’s complete adjuvant. Sera were prepared (13) from arterial blood collected every 2 weeks and stored at −20°C.

Radioimmunoassay. The standardized radioimmunoassay (4) was utilized for the detection of specific antibodies. Briefly, the antigen of interest was adsorbed to polystyrene microtiter wells. Wells were washed to remove unadsorbed antigen, and the remaining adsorption sites were saturated with bovine serum albumin. Sera were then incubated in the wells, and the unbound antibodies were removed by washing. Immune complexes were detected with 125I-labeled protein A, and radioactivity was quantitated with a gamma counter. All reactions were performed in triplicate, and results are expressed as the mean cpm ± SEM.

Endonuclease Treatment of DNA. Phenol-extracted (14) calf thymus DNA (Sigma) was incubated for 6 hr at 37°C in...

Abbreviation: SLE, systemic lupus erythematosus,
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the presence of \textit{Hae} III restriction endonuclease (Bethesda Research Laboratories; 4 units/mg of DNA; 4 units/ml, final concentration) in 6 mM Tris-HCl (pH 7.5)/6 mM MgCl$_2$/6 mM 2-mercaptoethanol. The reaction was terminated by addition of 0.5 M EDTA (adjusted to pH of 8.0 with NaOH) to a final concentration of 20 mM. The DNA was then extracted twice with phenol and was concentrated by ethanol precipitation. That endoribonuclease cleavage had indeed occurred was confirmed (data not shown) by subjecting a sample of the treated DNA to agarose (0.5%) gel electrophoresis and examining bands of DNA stained with ethidium bromide (15).

Alkaline Phosphatase Treatment of Poly(A). Poly(A) (Miles) was incubated for 1 hr at 68°C in the absence or presence of bacterial alkaline phosphatase (Bethesda Research Laboratories; 0.7 unit of Poly(A)/\(\mu\)g; 2 units/\(\mu\)l). Poly(A) was extracted twice with phenol/chloroform/isoamyl alcohol 25:24:1 (vol/vol), then was extracted twice with chloroform/isoamyl alcohol, 24:1 (vol/vol), and was concentrated by ethanol precipitation. That dephosphorylation had indeed occurred was confirmed when the treated DNA could not be ligated (15). \textit{EcoRI}-cleaved \(\lambda\) DNA incubated with poly(A) in the presence of phosphatase was not ligated, indicating that 5' phosphates had been removed, while \textit{EcoRI}-cleaved \(\lambda\) DNA incubated alone was ligated (data not shown).

**RESULTS**

Reaction of Sera from Rabbits Immunized Against RNA Polymerase I or Poly(A) Polymerase with DNA. Sera from rabbits that had been immunized with either purified poly(A) polymerase or RNA polymerase I were tested by radioimmunoassay for the presence of anti-DNA antibodies. Each rabbit was immunized with enzyme from a separate purification. Antibody binding to DNA immobilized on polystyrene microtiter wells was detected with \textit{125I}-labeled protein A. A background of 290 \pm 20 (cpm \pm SEM) was obtained with Preimmune serum (data not shown). Sera from all seven rabbits immunized with poly(A) polymerase produced results equivalent to background (Table 1). In contrast, sera from all seven rabbits immunized with RNA polymerase I contained a significant quantity of antibodies against DNA (Table 1). Thus, immunization of rabbits with RNA polymerase I, but not with poly(A) polymerase, seemed to induce the production of anti-DNA antibodies.

Reaction of Anti-Poly(A) Polymerase and Anti-RNA Polymerase I Antibodies with Polynucleotides. Anti-DNA antibodies found in human (2) and murine (3) lupus and in animals immunized with cardiolipin (16) are capable of reacting with a variety of single- and double-stranded nucleic acids. The serum from the rabbit with the highest titer of anti-DNA antibodies was tested by radioimmunoassay to determine whether antibodies with broad polynucleotide reactivity were also produced by a rabbit immunized with RNA polymerase I (Table 1). Control serum came from a rabbit that had been immunized with poly(A) polymerase but did not contain anti-nucleic acid antibodies. While the serum from the rabbit immunized with RNA polymerase I reacted strongly with RNA polymerase I, it also contained antibodies capable of binding every polynucleotide tested to essentially the same extent.

The relative concentrations of antibodies in the anti-RNA polymerase I serum capable of reacting with the polymerase and poly(A) were compared by titrating the serum (data not shown). The reaction with both antigens (1 \(\mu\)g of RNA polymerase I; 50 \(\mu\)g of poly(A)) was within the antibody-limiting, log-linear range beginning at serum dilutions of 1:20-1:40. Anti-RNA polymerase I antibodies were still detectable at a dilution of 1:1280 while 1:320 was the last dilution at which a reaction with poly(A) was detectable.

**Table 1.** Immunoreactivity of rabbit anti-RNA polymerase I or anti-poly(A) polymerase antisera

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Anti-RNA polymerase I</th>
<th>Anti-poly(A) polymerase</th>
</tr>
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<tbody>
<tr>
<td>DNA</td>
<td>1,158 \pm 291</td>
<td>221 \pm 57</td>
</tr>
<tr>
<td>Poly(dA)poly(dT)</td>
<td>10,132 \pm 329</td>
<td>256 \pm 8</td>
</tr>
<tr>
<td>Poly(A)poly(U)</td>
<td>11,965 \pm 416</td>
<td>194 \pm 58</td>
</tr>
<tr>
<td>Poly(G)poly(C)</td>
<td>10,306 \pm 216</td>
<td>166 \pm 30</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>13,387 \pm 610</td>
<td>115 \pm 35</td>
</tr>
<tr>
<td>Poly(G)</td>
<td>11,252 \pm 201</td>
<td>141 \pm 37</td>
</tr>
<tr>
<td>Poly(C)</td>
<td>12,838 \pm 218</td>
<td>198 \pm 18</td>
</tr>
<tr>
<td>Poly(U)</td>
<td>10,915 \pm 810</td>
<td>175 \pm 20</td>
</tr>
<tr>
<td>RNA polymerase I</td>
<td>25,472 \pm 816</td>
<td>281 \pm 48</td>
</tr>
<tr>
<td>Poly(A) polymerase</td>
<td>188 \pm 37</td>
<td>33,624 \pm 1022</td>
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Nucleic acids (75 \(\mu\)g), RNA polymerase I (2 \(\mu\)g), or poly(A) polymerase (0.6 \(\mu\)g) were adsorbed onto microtiter wells and incubated with sera (diluted 1:10) from rabbits that had received five injections of either RNA polymerase I or poly(A) polymerase.

*Results with DNA are the mean of determinations with sera from seven individual rabbits immunized with RNA polymerase I (range: 695 \pm 19 cpm to 1555 \pm 74 cpm) or from four rabbits immunized with poly(A) polymerase (range: 162 \pm 8 cpm to 314 \pm 19 cpm). Each rabbit received enzyme from a separate purification. The serum from the rabbit immunized with either enzyme that gave the greatest reaction with DNA was then tested with the other antigens.

Hence, the relative concentration of anti-poly(A) antibodies appeared to be one-third to one-fourth that of anti-RNA polymerase I antibodies but was nevertheless significant. At a serum dilution of 1:5, anti-RNA polymerase I antibodies were detectable with as little as 100 pg of the enzyme in the radioimmunoassay, but 50 ng of poly(A) or 200 ng of DNA were required for the detection of anti-nucleic acid antibodies. These data indicate that, compared to the nucleic acids, RNA polymerase I contained 2000 times as many antigenic determinants per unit weight.

**Time Course of Anti-Poly(A), Anti-DNA, and Anti-RNA Polymerase I Antibodies.** Next, sera from a rabbit immunized with an RNA polymerase I were monitored for over one year for anti-nucleic acid and anti-RNA polymerase I antibodies. The same preparation of purified enzyme was used throughout the injection schedule. As shown in Fig. 1, a significant quantity of anti-RNA polymerase I antibodies was present in the serum two weeks after the first injection, but no anti-poly(A) or anti-DNA antibodies were detected. Two weeks after the second injection of the polymerase, antibodies capable of reacting with nucleic acid were detectable. After three more injections at 2-week intervals, the titer of anti-RNA polymerase I antibodies continued to increase, while those against the nucleic acids remained fairly constant. The titer of anti-DNA antibodies was always much lower than that of anti-poly(A) antibodies. After the sixth injection of RNA polymerase I, the rabbit produced increasing quantities of anti-RNA polymerase I antibodies. Anti-poly(A) antibodies appear to have increased in response to this injection but then returned to the previous level. The quantity of anti-DNA antibodies remained relatively low even after the sixth injection. However, following the seventh injection of purified RNA polymerase I, there was a dramatic increase in the anti-DNA antibodies. The quantity of anti-poly(A) and anti-RNA polymerase I antibodies also rose after this injection, but the increase in these antibodies was not as great as that observed for the anti-DNA antibodies. In fact, the level of anti-DNA antibodies was at least 3-fold higher within two weeks of the final injection of the polymerase. All three types...
of antibodies, then, remained at fairly stable levels for nearly 8 months.

**Competition of Antibody Binding to RNA Polymerase I**

RNA polymerase I is a complex molecule composed of several polypeptide subunits, each of which is phosphorylated in vivo (17). A significant fraction of the autoantibodies against RNA polymerase I in the sera of SLE patients appears to be directed against these phosphorylated sites, but these sites do not appear to be antigenic in our rabbits (6).

Neither dephosphorylation nor further phosphorylation of RNA polymerase I had an effect on the anti-RNA polymerase I antibody reaction. Nevertheless, it was possible that the anti-nucleic acid antibodies in the rabbit anti-RNA polymerase I antisera had actually been produced against phosphorylated sites of the enzyme. Further, although not considered likely, it was possible that a small polynucleotide might have been associated with the purified RNA polymerase I when it was injected into the rabbit. In either case, a polynucleotide would be expected to compete for antibody binding to purified RNA polymerase I in a competition radioimmunoassay. Thus, RNA polymerase I or DNA was adsorbed to microtiter wells and incubated with a limiting amount of anti-RNA polymerase I antisera that had been incubated with various quantities of either the enzyme or DNA. As would be expected, increasing quantities of either soluble RNA polymerase I or DNA resulted in log-linear decreases in the antibody bound to the wells that were coated with the same antigen (Fig. 2). These results showed that the antibody concentration was limited and that conditions were appropriate for competition. However, preincubation of the antisem with soluble DNA had no effect on antibody binding to RNA polymerase I, and preincubation with the polymerase had no effect on antibody binding to DNA (Fig. 2). Similar results were obtained when poly(A) was substituted for DNA, although DNA could compete (but not totally) with poly(A) and vice versa (not shown). Thus, the antisem contained two distinct populations of antibodies; one was directed against RNA polymerase I, and the other was directed against polynucleotides.

**Effect of Endoribonuclease Treatment of DNA on Its Reaction with Anti-RNA Polymerase I Antisera.** Synthetic polynucleotides were more antigenic with respect to the antibodies in anti-RNA polymerase I antisem than a comparable quantity of DNA (Table 1), particularly in sera collected early in the immunization schedule (Fig. 1). Because the synthetic polynucleotides were shorter than the DNA, these results suggested that a significant fraction of the antibodies were directed toward sites at the ends of polynucleotide chains. To investigate this possibility, the effect of endonuclease treatment on the antigenicity of DNA was tested by radioimmunoassay in which 50 μl of DNA was adsorbed to each microtiter well, and antisera were diluted 1:10. Generation of additional 3'-OH and 5'-phosphate termini by treatment with the restriction endonuclease *Hae* III resulted in an increase of nearly 5-fold in the immunoreactivity of DNA with antisem collected (12.5 weeks) early in the immunization schedule (untreated, 689 ± 62 cpm; *Hae* III treated, 3321 ± 158 cpm) but less than a 1.5-fold increase in serum collected later (34 weeks) (untreated, 1191 ± 93 cpm; 1763 ± 109 *Hae* III treated). These results indicated that a significant fraction of the antibodies produced early were directed against antigenic sites located at the ends of polynucleotide chains and that more of the antibodies produced later were directed against other sites.

**Effect of Phosphatase Treatment on Antigenicity of Poly(A).** Several recent reports have provided evidence that the phosphate groups of polynucleotides are of primary importance in the antigenicity of nucleic acids in both human (2) and murine (3) lupus. In view of these reports, it seemed possible that the 5'-phosphates of the polynucleotides were involved in the antigenic determinant sites for anti-RNA polymerase I antibodies. To test this possibility, 5'-phosphates were removed from poly(A) by treatment with alkaline phosphatase, then 50 μg of treated or untreated poly(A) was adsorbed to microtiter wells. Serum used was diluted 1:80. Alkaline phosphatase treatment nearly abolished the antigenicity of poly(A) with respect to antibodies produced early (12.5 weeks) in the injection schedule (untreated, 5368 ± 58 cpm; phosphatase treated, 390 ± 96 cpm). With serum
collected later (34 weeks), phosphatase treatment decreased the antibody binding only 60% (untreated, 8104 ± 121 cpm; phosphatase treated, 3108 ± 75 cpm). Hence, the 5'-phosphates of polynucleotides appear to be involved in antigenic sites early in the course of antibody production but not particularly to be involved later.

**Reaction of Anti-RNA Polymerase I Antiserum with Nucleotides.** The phosphatase and nuclease treatment experiments indicated that anti-polynucleotide antibodies directed against sites other than 5'-phosphate termini were produced in higher proportion later in the immunization schedule. This was further investigated by testing the sera for antibodies capable of reacting with individual nucleosides and nucleotides (Table 2). Antibody binding to any of these antigens could not be detected with serum collected early, whereas serum collected later contained antibodies capable of reacting with all of those tested. Thus, antibodies against antigenic sites, which include the 5'-phosphate termini of polynucleotides, appear to be produced early while those directed against sites, which include the purine or pyrimidine ring structures, are produced later. The antibody reactions with individual nucleosides and nucleotides were quite weak compared to the reaction with polynucleotides and were not detected with serum dilutions greater than 1:10 (data not shown). This indicates that the majority of the antibodies against polynucleotides in this serum were directed against antigenic determinants formed by the polynucleotide chain.

**DISCUSSION**

The present study has offered a mechanism by which nucleic acids may become autoimmunogenic. Seven out of seven rabbits, each immunized with a separate preparation of purified RNA polymerase I, produced antibodies against the enzyme and against nucleic acids. In the one animal that was monitored over time, anti-nucleic acid antibodies were produced shortly after those against RNA polymerase I were elicited. Because RNA polymerase I binds to polynucleotides as part of its biological function, it seems likely that association of the nucleic acids present in blood plasma (8) with the injected enzyme rendered them immunogenic. Alternately, cell damage, resulting from inflammation caused by immunization, could release the cellular DNA that could then bind to the injected enzyme and, thereby, render itself immunogenic. It is well established that nucleic acids are nonimmunogenic (18–20) unless conjugated with an immunogenic protein (21). It is possible that binding of serum polynucleotides to the autoimmunogenic RNA polymerase I (4) could induce the immune response against nucleic acids that occurs in SLE. Interestingly, tissue injury has been postulated to play a role in autoimmune diseases (1).

**Table 2. Anti-RNA polymerase I antisera reactivity with purines and pyrimidines**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>cpm (mean ± SEM)</th>
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<tbody>
<tr>
<td></td>
<td>Early antisera</td>
</tr>
<tr>
<td>CTP</td>
<td>0 ± 5</td>
</tr>
<tr>
<td>UTP</td>
<td>15 ± 14</td>
</tr>
<tr>
<td>GTP</td>
<td>0 ± 13</td>
</tr>
<tr>
<td>ATP</td>
<td>82 ± 35</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0 ± 21</td>
</tr>
<tr>
<td>Adenine</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>dATP</td>
<td>0 ± 16</td>
</tr>
</tbody>
</table>

*Individual purines and pyrimidines (50 μg) were adsorbed to microtiter wells and the indirect enzyme-linked immunosorbent assay performed.

Studies in other laboratories have shown that, like the rabbits immunized with RNA polymerase I, animals immunized with cardiolipin produce anti-DNA antibodies (16). However, in the case of RNA polymerase I immunization, two noncrossreacting populations of antibody were induced. The anti-nucleic acid antibody molecules did not react with RNA polymerase I as evidenced by the failure of polynucleotide to compete for antibody binding to the enzyme. In contrast, the antibodies capable of reacting with DNA in sera from animals immunized with cardiolipin are most likely directed against antigenic sites on DNA and RNA. Thus, the antibodies produced against nucleic acids and phospholipids, namely the phosphodiester groups (2, 3). Although RNA polymerase I is a phosphoprotein (9, 16) and the phosphorylated state of the enzyme affects its reactivity with SLE patient autoantibodies (6), the lack of immunological crossreactivity of the enzyme and nucleic acids indicates that the phosphoryl groups of RNA polymerase I were not involved in the induction of anti-nucleic acid antibodies. Further, the failure of the RNA polymerase to compete with nucleic acids for antibody binding and vice versa demonstrated that the injected RNA polymerase I preparation was not contaminated with nucleic acid.

Anti-RNA polymerase I antiserum has been previously used (9, 21–23) in the study of the structure of the enzyme and its relationship to other nucleic acid metabolizing enzymes. The antiserum used in such experiments is generally collected after only a few injections when the relative concentration of anti-DNA antibodies is quite low compared to that of anti-RNA polymerase I antibodies (Fig. 1). Further, compared to DNA on a unit weight basis, RNA polymerase I binds approximately 2000 times more antibody molecules. Hence, because small amounts of enzyme (μg quantities) and excess DNA (μg quantities) are present in most experimental systems, the anti-DNA antibodies are not likely to be of consequence. This is illustrated by the fact that anti-RNA polymerase I antiserum inhibits in vitro RNA polymerase I transcription of calf thymus DNA template but not transcription catalyzed by Escherichia coli RNA polymerase or RNA polymerase II (22). Clearly, if the binding of anti-DNA antibodies to the DNA template had been a factor in these experiments, inhibition of all three enzymes would have been observed. In another study utilizing RNA polymerase I antiserum in an indirect immunofluorescence technique with rat liver cells, fluorescence was observed almost exclusively in nucleoli (23), the site of RNA polymerase I action. If binding of anti-polynucleotide antibodies had been significant in this study, fluorescence would have been observed throughout the nucleus.

RNA polymerase I is not the only protein capable of binding nucleic acids. Polyclonal antiserum also has affinity for nucleic acids as part of its biological function. However, none of the four rabbits injected with polyclonal antiserum produced antibodies against nucleic acids (Table 1). The nucleic acid binding site of polyclonal antiserum might be more labile following injection than that of RNA polymerase I. Regardless, the induction of anti-DNA antibodies does not appear to be a general phenomenon of immunization with enzymes involved in nucleic acid metabolism. Further, autoantibodies against RNA polymerase I and DNA are found in the sera of 100% (4) and 67% (see ref. 1) of SLE patients, respectively. Antibodies against other SLE target antigens known to be associated with nucleic acids, such as Sm, La, and Ro ribonucleoproteins (RNP), are present in the sera of a relatively small percentage of patients (see ref. 1). Hence, if the mechanism by which nucleic acids become immunogenic in SLE involves their association with an autoimmunogenic protein, RNA polymerase I appears to be one of the most probable candidates. The mechanism by which RNA polymerase I becomes autoimmunogenic is not fully understood. Since treatment of the enzyme with protein
phosphatase or kinase drastically alters the immunoreactivity of the polymerase with patient antibodies (6), protein phosphorylation appears to be involved.

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