Relationship between Deoxyribonucleoprotein and Deoxyribonucleic Acid Antibodies in Systemic Lupus Erythematosus

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ABSTRACT A soluble preparation of nucleoprotein (sNP), a complex of native deoxyribonucleic acid (DNA) and histones, was isolated from calf thymus nuclei and labeled with $[^{35}S]$iodide. Isotope-labeled antigen ($[^{35}S]$sNP) was used in a primary binding radioimmunoassay method to detect antibodies to both sNP and native DNA. Sera with antibody to native DNA reacted with the DNA moiety of sNP and bound $[^{35}S]$sNP, but this binding was completely inhibited by addition of unlabeled native DNA. Antibody to sNP which reacted with DNA-histone complex was not inhibited in the radioimmunoassay by addition of unlabeled DNA. Thus, antibodies to sNP and native DNA could be detected and differentiated by use of a single isotopically labeled antigen. In systemic lupus erythematosus (SLE), sera with binding to $[^{35}S]$sNP was present in 21/36 (58%) patients. The majority (18/21) had antibodies to sNP and native DNA present simultaneously, one had antibody only to sNP and two had antibody only to DNA. In contrast, patients with other connective tissue diseases rarely showed binding to $[^{35}S]$sNP. Serial studies on SLE patients showed that high serum binding to $[^{35}S]$sNP paralleled renal disease activity as reflected by the degree of proteinuria. A fall in binding was observed with subsidence of renal disease and reappearance of increased binding coincided with exacerbation. In these patients, antibodies to sNP and DNA appeared or disappeared pari passu suggesting that in addition to the previously demonstrated role of antibody to native DNA, antibody to sNP might also be implicated in the pathogenesis of immunologically-mediated tissue lesions such as SLE nephritis.

INTRODUCTION

Antibodies directed against a variety of nuclear components are of common occurrence in the sera of patients with systemic lupus erythematosus (SLE) and related connective tissue disorders (1–6). Nucleoprotein (NP), a complex of native deoxyribonucleic acid (N-DNA) and protein, has been recognized as one of these reactive nuclear antigens (7, 8). A fibrous preparation of NP was used in early studies and found to be a specific inhibitor of serum LE factor (8, 9). Recently, NP was extracted from calf thymus nuclei in soluble form (sNP) and shown to participate in immunologic precipitin reactions with sera from patients with SLE (10).

The present work describes a highly sensitive primary binding radioimmunoassay enabling detection of antibodies to both sNP and N-DNA. Sera from patients with various connective tissue disorders were examined by this method and the distribution of antinuclear antibodies of these two specificities are reported. In addition, sera obtained from sequential bleedings of patients with SLE were tested to determine the relationship between antibody and disease activity.

1 Abbreviations used in this paper: ABC, antigen-binding capacity; DSC, dilute sodium chloride-citrate; N-DNA, native DNA; NP, nucleoprotein; PBS, phosphate-buffered saline; RNP, ribonucleoprotein; sNP, soluble nucleoprotein; SS-DNA, single strand DNA; Sm, a nuclear antigen devoid of nucleic acids.
METHODS

Serum specimens. Venous blood was drawn from patients with various connective tissue diseases. Samples from each disease group were taken at different levels of disease activity. Normal blood was taken from laboratory personnel. Blood was allowed to clot at room temperature for 2 h and serum separated after centrifugation at 4°C. Serum specimens were stored in small portions at -20°C. Serum containing antibody to heat-denatured single-strand DNA (SS-DNA) was obtained from a rabbit immunized with SS-DNA which was complexed to methylated bovine serum albumin according to the method of Plescia, Braun, and Palczuk (11).

Preparation of sNP antigen. Fibrous NP was isolated from fresh calf thymus nuclei according to methods previously described (10). sNP was extracted and purified from a solution of NP in 1 M NaCl by addition of distilled water to a NaCl molarity of 0.12. A precipitate containing NP formed and was pelleted by centrifugation at 10,000 g for 30 min. This pellet was redisolved in 1 M NaCl. This cycle was repeated twice and the final 1 M NaCl solution was dialyzed against decreasing ionic strength buffers to is isotonicity with 0.015 M NaCl, 0.0015 M sodium citrate (dilute sodium chloride-citrate, DSC) according to the procedure of Huang, Bonner, and Murray (12). This solution was cleared by centrifugation at 35,000 g for 60 min and the supernate contained purified sNP antigen. The antigen could be lyophilized and kept indefinitely at -20°C. Small amounts were resolubilized in DSC periodically for use in these studies. The protein content of the solution was determined by the Folin method (13) using calf thymus total histones as standards, and the DNA concentration was measured by a modification of the diphenylamine reaction (14) using commercially available calf thymus DNA as standards. By these methods, the average protein to DNA ratio in sNP was between 0.9 and 1.0.

[3H]sNP was prepared by the chloramine-T method (15). 1 mg of sNP in 1 ml of DSC was added to 5 mCi of carrier-free [3H]Na (ICN Corp., Chemical & Radio-isotopes Div., Irvine, Calif.) contained in 1 ml 0.15 M phosphate buffer pH 7.0. While the antigen-iodine mixture was being stirred gently at 0°C, 20 μg of chloramine-T freshly dissolved in phosphate (0.01 M) buffer saline (0.15 M) (PBS) was added dropwise. 10 min after the addition of chloramine-T, an equal amount of freshly dissolved sodium metabisulfite in the same buffer was added to stop the reaction. Nonprotein-bound iodide was removed by overnight dialysis at 4°C against two changes of DSC. The mean isotope uptake varied between 20-25% and specific activity averaged 1 μCi/μg of protein.

Sucrose density gradient ultracentrifugation. This was performed according to a procedure previously described (16). Sucrose was dissolved in DSC and a continuous gradient of 10-40% was used. Approximately 100 μg of dialyzed radiolabeled antigen in a volume of 100 μl was separated on the gradient as a purification step before being used in the radioimmunoassay. Gradients were centrifuged at 35,000 rpm for 16 h in a Beckman SW 50.1 swinging bucket rotor at 4°C (Beckman Instruments, Fullerton, Calif.). Fractions were collected dropwise from the bottom of the tubes.

Radioimmunoassay with [3H]sNP. This assay was developed according to the ammonium sulfate method described by Minden and Farr (17). Antibody-bound sNP can be separated from free sNP by this technique since sNP is soluble in 50% saturated ammonium sulfate solutions, whereas sNP-antibody complexes are insoluble. Sera were depleted of IgM antibody complexes by incubation at 50°C for 30 min and diluted 1:10 in DSC, pH 7.2. Subsequent dilutions were done with pooled normal human sera diluted 1:10 in DSC. A volume of 200 μl of serum dilution was used per tube. The sucrose density ultracentrifugation fractions containing labeled sNP antigen which showed the best reactivity were pooled, dialyzed free of sucrose, and added to an equal volume of pooled decomplemented normal human serum diluted 1:10 in DSC. The radiolabeled antigen kept at 4°C was stable and gave reproducible results for at least 6 wk. [3H]sNP was diluted in DSC to a concentration of 50 ng/ml and 200 μl or 10 ng giving between 5,000 and 10,000 cpm, were added to each tube. The final reaction volume was adjusted to 500 μl by addition of 100 μl of DSC. From this point on, the entire procedure was carried out at 4°C. After an 18 h incubation, 500 μl of saturated ammonium sulfate in DSC were added. Samples were mixed and left at 4°C for 30 min then centrifuged at 2000 g for 30 min. The precipitates were washed with 2 ml of 50% saturated ammonium sulfate in DSC. The final precipitate was resuspended in 0.5 N NaCl solution and residual antigen was determined in a gamma spectrometer (Baird-Atomic Spectrometer model 530, Baird-Atomic, Inc., Bedford, Mass.). In order to differentiate between antibodies specific for sNP and those reacting only with the DNA moiety of sNP, tests were also run in presence of unlabeled N-DNA (2.5 μg) in 100 μl of DSC. Complete inhibition of [3H]sNP binding in presence of unlabeled N-DNA indicated presence of antibody to N-DNA only, lack of inhibition indicated presence of sNP antibody only and partial inhibition presence of antibodies of both specificities.

Source of other nuclear antigens. SS-DNA was prepared by immersing a solution of calf thymus N-DNA at a concentration of 500 μg/ml in PBS in a boiling water bath for 10 min followed by rapid chilling in ice water. A saline extract of calf thymus nuclei containing both sNP antigen (a nuclear antigen devoid of nucleic acids) and a ribonucleoprotein (RNP) antigen were prepared from fresh calf thymus according to procedures previously described (18, 19). Commercially available yeast RNA was used.

Other immunologic techniques. Double diffusion in agarose was used to demonstrate precipitin reactions between patient's N-DNA and sera of normal human. Other techniques, the method previously described (18). Anti-N-DNA antibody was isolated from the serum of a patient with SLE by addition of N-DNA at the early equivalence zone and subsequent DNAse digestion of the washed precipitate (20).

RESULTS

General characteristics of the radioimmunoassay. Fig. 1 illustrates the distribution of [3H]sNP in a 10-40% sucrose density gradient. Components associated with radioactivity sedimented in a polydisperse fashion but peaked in fraction 5, showing a faster sedimentation velocity than the 19S marker. The chloramine-T labeling procedure was responsible for the appearance of non-dialyzable, low molecular weight products which remained in the top fractions of the sucrose gradient. A serum containing strong anti-sNP activity, obtained.
from a patient with SLE, was used in the radioimmunoassay to detect fractions with reactive sNP antigen. As indicated by the broken line in Fig. 1, the radiolabel of fraction 4 through 12 showed at least 90% precipitability with this specific antibody. In contrast, only 12% of the lighter sedimenting material was precipitable in presence of identical amounts of this serum. Thus, material contained in fractions 4-12 was pooled and used in the radioimmunoassay. In presence of normal human sera, between 12 and 15% of the antigen was precipitated in 50% saturated ammonium sulfate. This nonspecific precipitation did not vary significantly from values obtained after addition of saturated ammonium sulfate in absence of serum (10-12%). The percent binding of normal human sera could not be decreased by addition of unlabeled N-DNA. At least 95% of the radioactivity could be precipitated by addition of trichloroacetic acid to a final concentration of 10%.

The immunochemical specificity of the labeled antigen was demonstrated by inhibition experiments using a variety of unlabeled nuclear antigens. A lupus serum capable of binding 95% of the labeled antigen was added in an amount sufficient to bind only 50% of the radiolabel. This represented a region where the binding of labeled antigen was most readily inhibited by addition of unlabeled homologous antigen. Addition of unlabeled yeast RNA, Sm, and RNP antigens, SS-DNA or an unrelated polyanion such as heparin in amounts up to 20 µg failed to produce any inhibition of binding. In contrast, addition of only 5 µg of unlabeled sNP inhibited binding completely and 10% inhibition could be produced by as little as 100 ng of this antigen. In addition, sera with precipitating antibodies to other nuclear antigens such as Sm and RNP obtained from patients with SLE and a rabbit serum containing antibody specific for SS-DNA failed to bind more radiolabeled sNP than normal controls.

The reproducibility of the radioimmunoassay proved to be very good when freshly labeled antigen was used (no more than 6 wk old). Upon repeated testing of certain sera using the same or different freshly labeled sNP preparations, minimal variations in percent binding occurred (0-5%); the greatest variations being observed with the highest binding specimens. When two triplicate sets of a given specimen were run simultaneously, the difference between their mean value varied between 0 and 3%. Since specimens were always tested simultaneously in presence and absence of N-DNA, any fall in binding greater than 3% after N-DNA addition was considered significant and reflected the presence of antibodies to N-DNA.

Differentiation between sNP and N-DNA antibodies. The antigen-binding capacity (ABC) of two sera containing precipitating antibody to sNP and not to N-DNA (B. T. and J. T.) was compared with that of two sera with precipitating antibody to N-DNA (A. M. and R. M.). For each serum, the binding capacity was determined before and after addition of 2.5 µg of N-DNA. As can be seen in Fig. 2, addition of N-DNA failed to reduce significantly the ABC of the two sera with sNP antibody. With one serum (J. T.) no change in binding occurred, with the other serum (B. T.) only a slight fall was noted. In contrast, a marked decrease in binding was observed with the two sera containing detectable precipitating antibody to N-DNA. In order to rule out the possibility that 2.5 µg N-DNA was insuf-
Table I
Effect of Unlabeled N-DNA, and sNP in Radioimmunoassay

<table>
<thead>
<tr>
<th>![\textsuperscript{\textit{3}I}]sNP binding</th>
<th>A. M.*</th>
<th>J. T.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unabsorbed serum control</td>
<td>79</td>
<td>70</td>
</tr>
<tr>
<td>Unabsorbed serum in presence of 2.5 µg N-DNA</td>
<td>76</td>
<td>51</td>
</tr>
<tr>
<td>Serum absorbed with N-DNA</td>
<td>76</td>
<td>52</td>
</tr>
<tr>
<td>× 3 (total 10 µg N-DNA)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Serum absorbed with N-DNA in presence of 5.0 µg sNP</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

* Two SLE sera which were selected on the basis of detectable precipitating antibodies to N-DNA and sNP.

sufficient to achieve maximum inhibition at a 1:10 serum dilution, the following experiment was performed (Table 1). 5-ml serum aliquots taken from patient J. T. (anti-sNP) and patient A. M. (anti-N-DNA and anti-sNP) were absorbed with 10 µg N-DNA and the supernates tested in radioimmunoassay for residual ![\textsuperscript{\textit{3}I}]sNP binding. With serum J. T., the ABC was not reduced significantly after absorption with N-DNA (3%). In contrast, the ABC of absorbed serum A. M. fell from the control value of 70–52%. This binding inhibition was analogous to that obtained with unabsorbed serum in presence of 2.5 µg N-DNA (51%). Finally, addition of 5 µg of sNP antigen to both of these absorbed sera reduced their ABC to levels observed with normal sera. Furthermore, using isolated anti-N-DNA antibody which bound 66% of the radiolabel, addition of 2.5 µg of N-DNA reduced binding to normal levels confirming that maximum binding inhibition was obtained with only 2.5 µg unlabeled N-DNA.

It was important to establish that the fall in binding observed with certain sera after addition of N-DNA indeed reflected the presence of antibody to native DNA. N-DNA preparations used in these studies contained less than 1% protein. If the protein present were all in the form of DNA-histone complexes, it would have amounted to less than 25 ng DNA-histone in 2.5 µg DNA used for inhibition. This amount of sNP (25 ng) was insufficient to produce inhibition of sera showing significant binding to ![\textsuperscript{\textit{3}I}]sNP. Supporting the fact that addition of N-DNA did not interfere with sNP antibody was the study in Table I, showing that there was no difference in binding of J. T. serum with either 2.5 or 10 µg N-DNA. If trace contamination with DNA-histones were present in N-DNA preparations, decrease in binding of J. T. serum would have been observed when four times the amount of N-DNA (10 µg) was added. These studies have been repeatedly confirmed with other sera.

Incidence of sNP and N-DNA antibodies in various disease states. Sera taken from patients with various rheumatic diseases and normal donors were examined by radioimmunoassay for antibody to sNP and N-DNA. The sera was assayed at a 1:10 dilution with and without addition of N-DNA. Results are illustrated in Fig. 3. The mean precipitation of ![\textsuperscript{\textit{3}I}]sNP ±2 SD obtained for the 20 normal sera was 10.2±4.7% and was taken as the range of nonspecific precipitation. Precipitation of the labeled antigen by normal human serum was not modified by addition of N-DNA. With only a few exceptions, sera showing significant binding of the labeled antigen were from patients with SLE. 21 of the 36 (58%) SLE patients' sera had antibody to sNP and/or N-DNA. The percent binding of only one of these positive sera could not be reduced by addition of N-DNA antigen indicating presence of antibody to sNP only. Two other sera were found to contain antibody to N-DNA alone since addition of this antigen abolished their binding completely. The percent binding of the remaining 18 sera was only partly inhibited in presence of N-DNA indicating simultaneous presence of antibodies to sNP and N-DNA. In the other disease groups, antibody to N-DNA alone was found in only two sera, one from a rheumatoid arthritis (RA), one from a hypertensive patient treated with hydralazine. In the

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hydralazine-treated patients, 17/46 (37%) had antinuclear antibodies by the indirect immunofluorescent method. Antibody to sNP associated with antibody to N-DNA was found in three patients (one each with scleroderma, dermatomyositis, and hydralazine-induced lupus syndrome). One patient with RA had antibody to sNP alone. By double diffusion in agarose, only seven of the SLE sera formed precipitin reactions with either sNP or N-DNA. Four sera showed reactivity to sNP and three to N-DNA. Sera which demonstrated precipitating antibody bound more than 50% of the labeled antigen in the radioimmunoassay and generally represented those with higher binding titers.

Correlations between presence of antibody to sNP and N-DNA, disease activity, and response to therapy. Titers of sNP and N-DNA antibody were determined by radioimmunoassay on serial bleedings taken from four patients with clinically active SLE before and during therapy. These patients have now been followed for periods of 4–34 mo. All four patients had high serum antibody titers to both sNP and N-DNA before the onset of treatment. Elevated antibody titers coincided with substantial proteinuria and low serum C3 and CH50 values. Therapy consisted of prednisone alone or in association with Imuran. Serum antibody of both specificities disappeared rapidly with treatment. As illustrated in Fig. 4 for one of these patients, this coincided with a gradual decrease in proteinuria and a progressive increase in serum C3 and CH50 values towards normal levels. In another patient (Fig. 5), after a remission period of 24 mo serum antibody to sNP and N-DNA reappeared concomitant with an exacerbation of disease associated with increased proteinuria. All serum samples from these four patients were examined for presence of sNP and N-DNA antigens by double diffusion and found to be negative.

**DISCUSSION**

A primary binding radioimmunoassay method enabling detection of antibody to both sNP and N-DNA has been described. The general characteristics of this method are the following: (a) insolubility of free labeled antigen in 50% ammonium sulfate varies between 10 and 12%; (b) the mean nonspecific precipitation of antigen in presence of normal human serum does not exceed 15%; (c) between 95 and 100% of radioactive counts can be precipitated in 10% trichloroacetic acid; (d) as much as 95% of the labeled antigen is bound by a serum with high antibody activity.

Despite the modest specific activity of the labeled antigen, this assay proved to be extremely sensitive. Certain
sera with precipitating antibody to sNP could be diluted out to 1: 5120 and still bound significantly more radiolabel than normal human sera at a 1/4 dilution (Fig. 2). Sera with precipitating antibody to N-DNA had titers between 1/320 and 1/1280 by radioimmunoassay. Specific activities up to 4 mCi/mg of protein increased the sensitivity of the assay by twofold without affecting the stability of the labeled antigen. In this respect, iodination of sNP presents a definite advantage over DNA internal labeling procedures using [3H] or [14C] thymidine where relatively low incorporation of radioisotope into thymidine is a limiting factor. In addition, this radioimmunoassay presents a major advantage over radioimmunoassays for N-DNA alone since it allows detection of antibodies to both sNP and N-DNA.

The assay proved to be specific for sNP and N-DNA antibody. The labeled antigen was shown to be free of other nuclear antigens such as Sm, RNP, and SS-DNA since sera with precipitating antibody to these antigens failed to bind more radiolabel than normal controls. Furthermore, the binding capacity of serum with specific anti-sNP antibody and that of isolated anti-N-DNA antibody could be completely abolished by addition of unlabeled sNP and N-DNA respectively. Differentiation between anti-sNP and anti-N-DNA antibody was performed by adding unlabeled N-DNA which specifically inhibited the binding of anti-N-DNA antibody. Thus for sera containing a mixture of sNP and N-DNA antibody, two different antigen binding curves were obtained before and after addition of unlabeled excess N-DNA.

Significant binding values were observed almost exclusively with sera from SLE patients. Nearly all of these sera contained sNP and N-DNA antibody simultaneously (18/21 or 86%), confirming the high degree of association previously found by precipitin analysis (10). Aside from being a very sensitive technique, this radioimmunoassay may be a more reliable diagnostic tool than the LE cell test since it has been reported that antinuclear antibody of the IgM class do not produce the LE cell phenomenon (23). It is of interest that only a very low degree of significant binding was observed with only 2 of 46 sera from patients taking hydralazine, a drug often associated with the induction of a lupus-like syndrome (24, 25). Antinuclear antibodies found in this drug-induced disease would appear to have a specificity that differs from that of antibody found in spontaneously occurring lupus. In vitro experiments have shown that hydralazine complexes with sNP and causes physicochemical alterations of this nuclear antigen (26). Should this mechanism occur in vivo, drug-altered nuclear antigens could possibly lead to production of antibody of a different specificity. The very low incidence of sNP antibody found in the other rheumatic diseases confirms previously reported results obtained by a less sensitive method (10). The incidence of serum antibody to N-DNA by our radioimmunoassay for an unselected group of SLE patients is in the range reported by others, using radioimmunoassays (27-29). 1 out of 31 RA sera had weak anti-N-DNA antibody activity. This low incidence is in agreement with results published by others (27, 29, 30). 1 of 24 scleroderma sera showed anti-N-DNA activity. This same serum had very high sNP binding and the possibility that accompanying SLE was present cannot be excluded. A similar very low incidence of N-DNA antibody in adult scleroderma sera has been reported by others using various techniques (30, 31). Using a Farr-type assay, some investigators have claimed that low levels of anti-N-DNA antibody may also be present in the sera of normal subjects (32). Our results obtained with sera of 20 normals do not support that possibility since the amount of labeled antigen precipitated by these sera did not differ significantly from that brought down by saturated ammonium sulfate in absence of serum. In addition, no reduction of binding could be observed when unlabeled N-DNA was added to the same sera.

Presence of serum antibody to N-DNA accompanying low circulating complement levels are generally associated with active lupus nephritis (33). The appearance of free N-DNA antigen in serum previously containing anti-N-DNA antibody was found to correlate with an exacerbation of disease activity probably as a result of circulating immune complex formation (14). Evidence drawn from experimental models (34, 35), antibody elution experiments (20, 36), and the identification of N-DNA antigen along the glomerular basement membrane in SLE nephritis (20) provide strong arguments linking DNA-anti-DNA complexes with tissue injury in this disease. In our study, a good correlation existed between presence of N-DNA antibody and activity of disease as reflected by the degree of proteinuria. The new finding was that such a correlation also existed for sNP antibody since the latter was so closely associated with DNA antibody in incidence, disappearance with remission and reappearance with exacerbation of disease activity. This observation is of particular interest since antinuclear antibody of this specificity could be eluted from the glomeruli of several patients with SLE nephritis (20, 36), and raises the possibility that complexes of sNP and antibody may also be implicated in renal tissue injury. Certain technical problems might be encountered in experiments designed to identify sNP antigen deposits along the glomerular basement membrane, as has been shown for DNA (20). These experiments usually require dissociation of antibody from

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antigen by the use of high salt or low pH buffers, both of which would also dissociate histones from DNA thereby destroying antigenic determinant sites reacting with sNP antibody. Another approach would be through examination of serum to determine if sNP antigen itself might be present in circulation at some stage of disease activity, since this would create situations conducive to soluble immune complex formation. The current study focuses attention on another immune system which might be of pathogenetic significance in SLE and studies are in progress to elucidate its role in disease activity.

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