DNA:Anti-DNA Complexes: Their Detection in Systemic Lupus Erythematosus Sera

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ABSTRACT Antibody to DNA was measured before and after treatment of systemic lupus erythematosus (SLE) sera with bovine pancreatic deoxyribonuclease (DNase I). In 11 of 15 cases of SLE with active renal disease there was a significant increase in DNA-binding after DNase digestion, while no such increase was noted in inactive SLE, normal controls or in patients with non-lupus renal disease. The significant rise in DNA-binding after digestion indicated that DNA had bound in vivo to the anti-DNA in these sera. A striking correlation between the occurrence of these complexes and disease activity was shown. In eight cases of SLE nephritis where serial blood samples were obtained, the greatest increase in DNA-binding after DNase digestion occurred at the time of the severest renal disease. In addition, serum from a case of SLE with acute cerebritis but without evidence of renal disease also had a significant rise in binding during the acute phase. This assay provides proof of the existence of circulating DNA: anti-DNA complexes in some cases of SLE and can also be used to measure an apparently critical parameter of disease activity.

INTRODUCTION

The role of DNA: anti-DNA complexes in the pathogenesis of certain cases of systemic lupus erythematosus (SLE)[1] nephritis has been previously suggested and considerable evidence supporting this possibility has accumulated. DNA antibodies frequently occur in association with acute exacerbations of the disease, and DNA has been demonstrated occasionally in the sera of patients with acute lupus nephritis after disappearance of detectable anti-DNA (1–4). In addition, DNA has been shown along the glomerular basement membrane by immunofluorescence and antibodies with antinuclear specificities, including anti-DNA, have been eluted from post-mortem SLE kidneys (3, 4). Recently it was demonstrated that circulating UV-DNA-anti-UV-DNA complexes can be nephrotoxic, supporting the likelihood that circulating complexes of anti-DNA with unaltered DNA may also be nephritogenic (5).

Although it has been assumed that in SLE DNA: anti-DNA complexes are formed in the circulation before entrapment in the kidneys, only indirect evidence of circulating immune complexes in patients with this disease has been previously reported (6–9).

This study presents direct evidence of DNA: anti-DNA complexes in certain SLE sera. The complexes of DNA and antibody to DNA were detected by measuring the binding by SLE sera of ^{125}I-labeled native DNA (^{125}I-DNA) before and after treatment of the sera with deoxyribonuclease (DNase I). If DNase digestion resulted in increased capacity of a serum to bind ^{125}I-DNA, the DNA-binding sites in the untreated serum must have been blocked in vivo by DNA, and therefore, the serum contained DNA: anti-DNA complexes. A significant increase in DNA-binding after such treatment of certain SLE sera occurred, and the relative increase showed a correlation with the disease activity of these patients.

METHODS

Subjects. Sera were obtained from 15 patients with active SLE nephritis, 13 SLE patients with no evidence of

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[1] *Abbreviations used in this paper:* ANA, antinuclear antibody; BGG, bovine gamma globulin; % P corr, mean per cent of 0.1 μg ^{125}I-DNA corrected for the normal control; PBS, phosphate-buffered saline.

nephritis, 1 patient with SLE cerebritis without evidence of renal disease, 18 patients with active non-SLE nephritis, and 45 normal individuals. The 29 patients with SLE all had typical multisystem involvement and were classified as to activity of the disease according to the following criteria: (*a*) Active nephritis—abnormal urinalysis (proteinuria, hematuria, casts). Some patients also had an abnormal serum creatinine, creatinine clearance, and/or blood urea nitrogen (BUN). (*b*) No evidence of nephritis—normal urinalysis, serum creatinine, creatinine clearance, and BUN. In addition serial bleedings from nine of the SLE patients during exacerbation of the disease were studied.

Collection of sera. The blood was collected in sterile polypropylene disposable syringes (Monoject, Sherwood Industries, Deland, Fla.), allowed to clot at room temperature, and as soon as the clot formed, the cellular elements were removed by centrifugation. Sodium azide was added to give a final concentration of 0.1% and the serum was stored at a −20°C.

Deproteinization of DNA. Native calf thymus DNA (Worthington Biochemical Corp., Freehold, N. J.) was deproteinized by a modification of a previously described procedure (10). A 250 μg/ml solution of DNA in 0.05 M Tris (pH, 8.0), 0.001 M ethylenediaminetetraacetic acid (EDTA), was extracted at room temperature for 1 h with an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline. The aqueous phase was separated from the phenol phase by centrifugation at 1000 × *g* for 5 min and re-extracted as above. The twice extracted aqueous phase was dialyzed against 0.05 M Tris (pH 8.0), 0.001 M EDTA to remove the residual phenol. The DNA solution was adjusted to a density of 1.70 g/cm³ by the addition of cesium chloride crystals (CsCl, optical grade, Harshaw Chemical Co., Solon, Ohio) and centrifuged at 33,000 rpm in a Spinco 40.3 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) for 72 h at 4°C. The purified DNA band was dialyzed against 0.1 M acetate buffer, pH 5.0.

Labeling of DNA with ^{125}I. The deproteinized DNA was labeled with ^{125}I essentially as previously described (11). To 1 mg DNA in 1 ml 0.1 M acetate buffer, pH 5.0, the following reagents were added: 1 mg thallic trichloride (K and K Laboratories, Inc., Plainview, N. Y.), 16 μg KI, and 1 mCi radioactive iodine (carrier-free ^{125}I, sodium salt in 0.1 M NaOH, Cambridge Nuclear Corp., Cambridge, Mass.). The reagents were incubated together at 60°C for 1 h and dialyzed at 4°C against 1% KI in 0.05 M phosphate-buffered physiological saline (PBS), pH 7.2, for 24 h. Finally, the DNA was dialyzed against PBS for an additional 24 h. The radioactive DNA had a specific activity exceeding 3×10^4 cpm/μg.

Measurement of DNA antibody. DNA antibody was measured using an ammonium sulfate precipitation assay previously described (12). The results are expressed as the mean per cent of 0.1 μg ^{125}I-DNA which was bound by antibody and thus precipitable by half saturated ammonium sulfate corrected for the radioactivity in the precipitate of a normal serum (% P corr) (13).

Preparation of soluble DNA: anti-DNA complexes in vitro. Quantitative precipitin reactions were performed with a serum from a patient with SLE to determine the amount of native DNA necessary for equivalence. Four times this amount was added to a sample of the serum to form soluble complexes in the zone of antigen excess. After 3 days incubation at 4°C, the serum was centrifuged at 2000 rpm for 1 h at 4°C to eliminate any precipitate which might have formed (none was visible). The supernatant serum containing soluble DNA: anti-DNA complexes was treated with DNase to determine the efficacy of the enzyme digestion in freeing blocked anti-DNA.

DNase digestion of serum. Two 0.3-ml samples of each serum to be assayed were taken. An equal volume of PBS containing 60 μg DNase I (Worthington Biochemical Corp.) and 1.8 μmol $MgCl_2$ was added to the serum sample to be digested. To the undigested control sample, an equal volume of PBS was added. Both samples were mixed and incubated at 37°C for 1 h. After an overnight incubation at 4°C, the samples were incubated at 37°C for an additional 4 h. Then 0.03 ml of 1 M tetrasodium ethylene diamine tetraacetic acid in distilled water was added to all samples. To obtain a final serum dilution of 1 : 10 in the DNase digested sample, 2.4 ml borate buffer (0.01 M borate, 0.15 M NaCl, pH 8.3) were added. To the undigested control sample 2.4 ml borate buffer containing 60 μg DNase I and 1.8 μmol $MgCl_2$ were added to obtain the same final serum dilution, DNase I and $MgCl_2$ concentration as in the digested sample. The EDTA inhibited further DNase activity in the digested sample, prevented any digestion in the control, and prevented digestion of the ^{125}I-DNA test antigen subsequently added. DNA antibody was then measured in five 0.5-ml portions of the diluted digested and undigested serum. If a 1 : 10 dilution of the undigested serum bound more than 50% of the added antigen, the serum was diluted to bind 50% or less. The results were analyzed using the *t* test for independent samples (14) to determine if there was a significant increase in binding after DNase digestion.

RNase digestion. Digestion with bovine pancreatic ribonuclease (RNase) (Worthington Biochemical Corp.) was carried out by adding 60 μg of RNase to 0.3 ml of a SLE serum known to show increased binding of ^{125}I after digestion with DNase. The enzyme, while degrading yeast transfer RNA (Miles Laboratories Inc., Kankakee, Ill.) in a standard assay system, had no effect on native calf thymus ^{125}I-DNA under the same conditions.

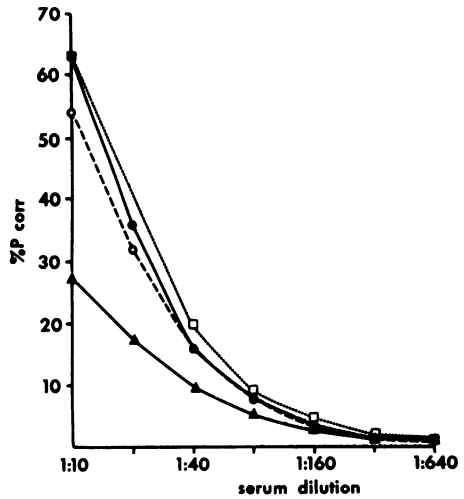

FIGURE 1 The effect of DNase digestion on the ^{125}I-DNA-binding of a SLE serum containing antibody to DNA as well as DNA: anti-DNA formed in vitro. SLE serum LT (□ - - - □); SLE serum LT after digestion with DNase (● —— ●); SLE serum LT containing soluble DNA: anti-DNA complexes formed in vitro (▲ —— ▲); SLE serum LT containing soluble DNA: anti-DNA complexes after digestion with DNase (○ — — ○).

Detection of DNA in the serum. DNA in the serum was determined both immunologically and by the diphenylamine test as described previously (1).

RESULTS

Effect of DNase digestion on a serum containing DNA: anti-DNA complexes formed in vitro. As shown in Fig. 1, serum L.T. diluted 1:10 bound 63% of the ^{125}I-DNA. The same dilution after digestion with DNase still bound 63%. However, when sufficient DNA was added to the serum to form soluble DNA:anti-DNA complexes, the 1:10 dilution bound only 28%. After this serum-DNA solution was digested with DNase, a 1:10 dilution bound 53%. Thus in this "artificial" situation, DNase digestion raised the ^{125}I-DNA-binding capacity back to 87% of that shown by the serum before addition of the DNA. The full titration curves show a similar effect (i.e., raising the binding level of the "blocked" serum back to that of the original serum).

Effect of RNase digestion on an SLE serum containing DNA: anti-DNA complexes formed in vivo. As shown in Table I, RNase digestion had no significant effect on the ^{125}I-DNA-binding by a serum from a patient with SLE. However, DNase digestion showed this serum contained DNA:anti-DNA complexes formed in vivo since the binding increased from 20% to 32%.

Effect of DNase digestion on sera from patients with SLE, non-SLE nephritis, and normal controls. The results are summarized in Fig. 2 and representative results are given in Tables II-V.

As can be seen in Fig. 2, the % P corr for 11 of 15 sera from patients with active SLE showed statistically significant absolute increases ($P < 0.05$) after DNase digestion of 4%–29%. As shown in Table II, the relative increases for these sera, based on the binding by the sera before digestion, varied from 14% to 189%. The other four sera had no significant change in binding.

There was also no significant change in any of the sera from the patients with inactive SLE (Table III and Fig. 2), the 18 with non-SLE nephritis (Table IV and Fig. 2) or the 45 normals (Table V and Fig. 2).

TABLE I

Comparison of the Effect of DNase and RNase Digestion on ^{125}I-DNA-Binding Ability of an SLE Serum

% P Corr			
Untreated* serum	Undigested control‡	RNase digested	DNase digested
20.0	21.0	20.7	31.9

* No enzyme added.

‡ EDTA added before addition of DNase to inactivate enzyme.

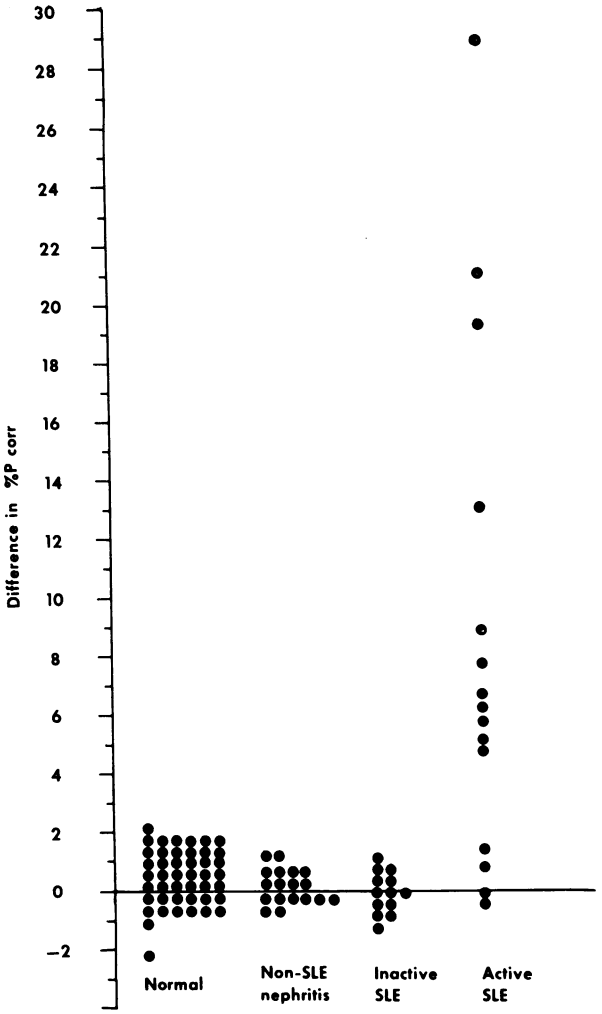

FIGURE 2 Change in binding to ^{125}I-DNA after DNase digestion of all sera from normal, non-SLE nephritis, inactive SLE nephritis, and active SLE nephritis patients.

In Table VI the mean change for each group is shown. Statistical examination of the four groups using the Kolmogorov-Smirnov one tail, two sample test (15) showed there was a significant difference in the increase in binding for the group with active disease compared with the inactive disease, non-SLE nephritis and normal groups ($P < 0.01$). There was no difference among the latter three groups ($P > 0.05$).

Serial studies on sera from nine SLE patients were performed. Fig. 3 depicts the course of a 24-yr-old female with a 3 yr history of SLE during an acute nephritis episode. Initially her serum exhibited considerable ^{125}I-DNA-binding. Then, even though her apparent ability to bind ^{125}I-DNA fell, she developed proteinuria and a high fever. However, DNA was detectable in her serum at this time, and digestion with DNase markedly raised the DNA-binding. The greatest increase coin-

TABLE II

Representative Changes in Binding of ^{125}I-DNA by Sera from Patients with Active SLE Nephritis after DNase Digestion

Patient	Laboratory findings						^{125}I-DNA-binding				
	Urine protein	Casts*	24 h urine protein	Serum creat.	BUN	C'3‡	% P§ undigested	% P digested	Diff. in % P	Relative change in P	*P*
			g	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*					
1	4+	2+	3.0	NA‖	18	NA	15.7±0.5¶	44.6±0.7¶	28.9	189%	<0.001
2	3+	3+	0.7	0.9	60	45	42.9±0.6	62.2±0.7	19.3	45%	<0.001
3	3+	1+	0.4	0.8	19	58	27.2±0.8	39.9±2.0	12.7	47%	<0.001
4	3+	3+	NA	1.8	50	20	34.6±0.4	43.5±0.4	8.9	20%	<0.001
5	2+	1+	0.9	0.8	16	198	38.0±1.0	45.7±1.0	7.7	21%	<0.05
6	2+	1+	2.2	2.0	106	45	35.2±0.1	41.9±1.0	6.7	19%	<0.01
7	4+	—	0.7	0.8	14	115	6.2±0.4	12.5±0.2	6.3	101%	<0.001
8	3+	1+	NA	NA	25	119	38.7±0.4	43.9±0.4	5.2	14%	<0.001
9	1+	—	0.4	1.3	23	105	47.4±0.3	47.0±0.8	−0.4	−0.8%	>0.05
10	3+	±	2.7	0.8	9	150	22.4±0.5	22.3±0.6	−0.1	−0.5%	>0.05

* −, none; ±, trace; 1+, occasional; 2+, moderate; 3+, many.
‡ Normal range 90–175 mg/100 ml.
‖ NA, not available.
¶ Mean of five samples ±SE.
§ % P corrected.
Sera one and three had DNA detectable by ouchterlony and diphenylamine analysis.

cided with the peak of the renal disease. At this time she was put on 80 mg prednisone per day, the change in binding decreased, and the nephritis subsided.

Four other patients showed a similar course with acute renal disease developing concomitantly with a significant rise in ^{125}I-DNA-binding after DNase digestion. As the difference between the digested and undigested sera decreased, the nephritis subsided and normal urinalysis returned. Three patients had chronic lupus nephritis, and although they also exhibited a similar rise in binding after DNase digestion, at the time of acute exacerbations of their renal disease, urinalysis did not return to normal when the change in binding fell. In the ninth patient, a 15-yr-old female, there was a general disease exacerbation (Fig. 4) characterized by a drop in the hematocrit to 34%, an increase in the erythrocyte sedimentation rate to 60, a low C4, a marked rise in ANA titer, and a fever of 40°C. In addition, the patient became extremely lethargic, developed a severe headache, and marked nuchal rigidity. The opening pressure on a lumbar puncture at this time was 55 cm water, and the cerebrospinal fluid contained 344 white blood cells (84% polymorphs). Glucose and protein were normal. A diagnosis of SLE cerebritis was made and was subsequently supported when all cultures were negative. She was put on 60 mg prednisone daily. Within 12 h there was considerable improvement in her central nervous system symptoms, and after 36 h there was no remaining neurologic abnormality. At this time there was an opening pressure of 30 cm of water on lumbar puncture; only 3 lymphocytes and protein and glucose were normal. As can be seen in Fig. 4, again there was

TABLE III

Representative Changes in Binding of ^{125}I-DNA by Sera from Patients with Inactive SLE after DNAse Digestion

Patient	Laboratory findings						^{125}I-DNA-binding				
	Urine protein	Casts	24 h urine protein	Serum creat.	BUN	C'3	% P undigested	% P digested	Diff. in % P	Relative change in P	*P*
			g	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*					
1	Neg	—	Neg	0.7	9	170	14.3±0.2	14.9±0.1	0.6	4.2%	>0.05
2	Neg	—	Neg	1.0	14	102	4.7±0.4	4.6±0.7	−0.1	−2.1%	>0.05
3	Neg	—	Neg	1.1	NA	145	49.8±1.2	48.9±0.8	−0.9	−1.8%	>0.05
4	Neg	—	Neg	1.1	18	180	22.3±0.4	22.4±0.5	0.1	0.4%	>0.05
5	Neg	—	Neg	NA	NA	115	15.6±0.4	16.2±0.1	0.6	3.8%	>0.05

TABLE IV

Representative Changes in Binding of ^{125}I-DNA by Sera from Patients with Non-SLE Nephritis

		^{125}I-DNA-binding			
		% P corr undigested	% P corr digested	Difference in % P corr	*P*
1	Membrano-Prolif GN*	3.1±0.2	3.8±0.3	0.7	>0.05
2	Chronic GN	3.6±0.1	4.5±0.3	0.9	>0.05
3	Chronic GN	2.2±0.4	2.7±0.2	0.5	>0.05
4	Acute GN	4.8±0.3	4.5±0.3	−0.3	>0.05
5	Subacute GN	4.3±0.4	5.0±0.3	0.7	>0.05

* Glomerulonephritis.

an apparent decrease in DNA-binding during this acute exacerbation, but after DNase digestion the anti-DNA level did go up during the acute phase. In addition, the greatest rise in binding after digestion occurred during the severest stage of the exacerbation. Although permission for a renal biopsy could not be obtained, this patient had no clinical evidence of renal abnormality as judged by normal urinalysis, creatinine clearance, BUN, and serum creatinine.

DISCUSSION

It has been previously shown that DNase would dissolve the precipitate formed when DNA was added in vitro to SLE sera containing DNA antibodies. The resultant solution contained active DNA antibody which had been bound in the precipitate to the added DNA (16). Thus it was known that DNase could release anti-DNA antibody from DNA:anti-DNA complexes. It therefore seemed logical to DNase digest SLE sera suspected of containing such complexes. Evidence of increased DNA antibody after such treatment would indicate that the antibody must have been at least partially blocked by DNA in vivo, i.e., that soluble DNA:anti-DNA complexes were present.

An assay was devised to examine this possibility and control experiments showed that under the conditions used one could free most of the DNA antibody bound in soluble DNA:anti-DNA complexes formed in vitro. Therefore, similar results could be expected with sera containing complexes formed in vivo.

RNase had no effect on an SLE serum which appeared to contain DNA:anti-DNA complexes—evidence of the specificity of this test. Thus the increase in binding, observed with certain SLE sera, must have been specifically due to the release of DNA antibody from DNA:anti-DNA complexes.

The findings described in this paper show that DNA:anti-DNA complexes occur in some SLE sera at certain times, and there is often a correlation between the occurrence and amount of these complexes and renal disease activity. This relationship was most striking in those patients without significant chronic renal involvement, since renal function was normal before the acute exacerbation and returned to normal after it. There was also evidence of increased renal injury in those with an antecedent chronic lupus nephritis. In such patients, when the complexes disappeared from the circulation, renal function remained abnormal, often more abnormal than before the complexes were present. Presumably this was due to the fact that the chronically damaged kidneys had little or no reserve and the additional injury further increased their permanent functional disability.

TABLE V

Representative Changes in Binding of ^{125}I-DNA by Normal Controls

	^{125}I-DNA-binding			
Sera	% P corr undigested	% P corr digested	Difference in % P corr	*P*
1	4.0±0.2	4.5±0.2	0.5	>0.05
2	1.8±0.4	3.1±1.0	1.3	>0.05
3	5.1±0.2	5.6±0.6	0.5	>0.05
4	1.6±0.2	2.5±0.1	0.9	>0.05
5	7.1±0.4	7.1±0.3	0.0	>0.05

TABLE VI

Mean Change in ^{125}I-DNA-Binding for Each Group after DNase Digestion

Group	No. of sera	Mean difference in % P corrected*
Active SLE nephritis	15	8.8±2.2
Inactive SLE	13	0.3±0.2
Non-SLE nephritis	18	0.2±0.2
Normal controls	45	0.2±0.1

* Expressed as mean for all sera in group ±SE.

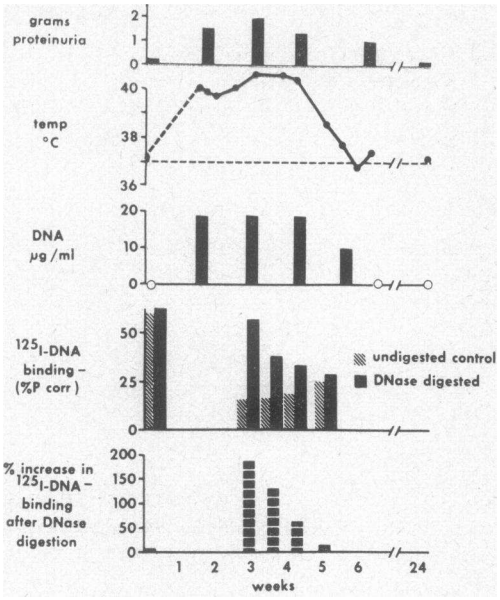

FIGURE 3 Course of a patient with an acute episode of SLE nephritis. The exacerbation was characterized by proteinuria and an elevated temperature. Concomitant with this was an increase in the binding of the serum to ^{125}I-DNA after DNase digestion.

It is also of considerable interest that one patient without clinical evidence of renal disease had complexes in her circulation at the time she developed acute cerebritis. Unfortunately, this was a retrospective study and cerebrospinal fluid was not examined for complexes. There may be several reasons for the apparent lack of renal disease. It appears that considerable complex deposition can occur in the kidneys before clinical abnormalities in renal function. Immunoglobulins and complement have been found in the glomerular mesangium without alteration in glomerular function (17). The mesangium may generally be the initial site of localization of complexes, with an accumulative increase until an overflow along the basement membrane occurs resulting in clinical renal disease. This patient's first symptoms of SLE occurred only 5 mo before the acute episode described. Significant lupus nephritis often does not occur until one or more years after the initial symptoms of the disease (17, 18), possibly because the deposition of complexes in the kidneys has been insufficient to cause abnormal function. Another possibility is that the nature of the complexes plays an important role in their deposition. That is, certain antigen-antibody complexes, because of the characteristics of the antigen, the antibody, and/or the combination tend to deposit in some sites more readily than in others. Perhaps most likely, a number of factors all contribute to how soon and how severely renal disease occurs. It would have been of great interest to examine her renal tissue, but permission for a biopsy could not be obtained.

It is important to note several features of the results obtained. First is the observation that the DNA-binding level was higher in sera from certain patients with inactive disease than it was in sera from some patients with active disease (Tables II and III). Although this finding might initially seem contradictory, it may simply show the necessity of immune complex formation for the development of pathologic changes, since none of the inactive patients had demonstrable DNA : anti-DNA complexes. Second, even though a correlation was found between the increased DNA-binding after DNase digestion and renal disease activity in eight of the nine serial studies performed, sera from certain patients with active nephritis showed no evidence of DNA : anti-DNA complexes (e.g., active disease sera 9 and 10 in Table II). These two patients had normal C3 levels, which might suggest their renal disease was due to a non-complement-dependent mechanism or was a chronic rather than acute condition without added injury occurring at the time of our study. Furthermore, other antigen-antibody complexes are almost certainly involved in the pathogenesis of lupus nephritis. The assay described was capable of detecting only DNA : anti-DNA complexes. Although DNA has been the only extrinsic antigen identified in the renal basement membrane thus

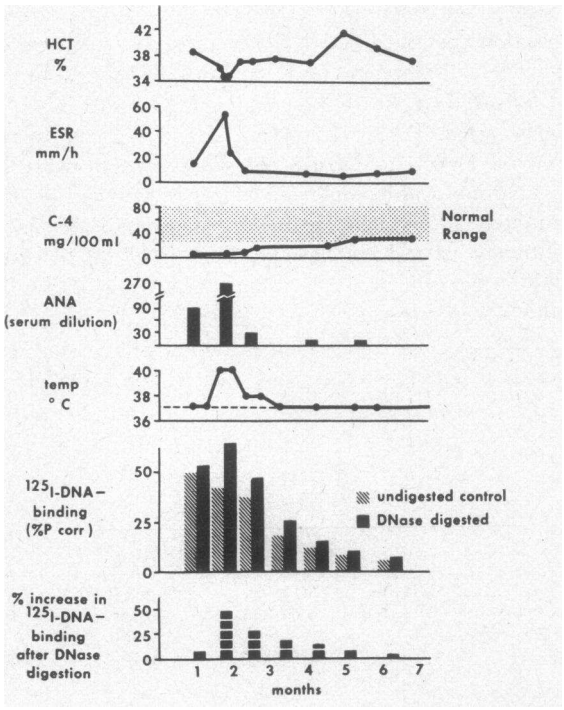

FIGURE 4 Serial study on a patient with SLE cerebritis. DNA : anti-DNA complexes were evident during the disease exacerbation.

far, antinucleoprotein and antiribonucleoprotein antibodies have been eluted from SLE glomeruli (3, 19). In addition, of interest in this regard is the recent description of anti-BGG and anti-bovine serum albumin antibodies in the sera of some patients with SLE and the occurrence of a BGG-like material in the sera of certain patients (20).

Of prime interest in regard to the DNA:anti-DNA complexes is the origin of the DNA. Although it has been suggested that the DNA may be of endogenous origin (1), the same authors stated that without full characterization of the DNA, the possibility that it might be of exogenous origin could not be excluded. Recently, preliminary studies have suggested that in at least some cases the DNA is in fact of exogenous origin since protein-free DNA isolated from five SLE sera was found to have a different buoyant density from that of normal human DNA (21).

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