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Research Article

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In Vitro Effect of Antibodies to DNA on the Template Activity of DNA

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ABSTRACT Blood samples containing antibodies to DNA were obtained from patients with systemic lupus erythematosus (SLE) and rabbits immunized with denatured DNA complexed to methylated bovine serum albumin. The immunoglobulin fractions from these sources did not decrease the over-all template activity of single-stranded DNA with DNA polymerase or DNA-dependent RNA polymerase. In competition studies, both DNA polymerase and DNA-dependent RNA polymerase inhibited the binding of DNA antibodies to single-stranded DNA, as evidenced by inhibition of micro-complement fixation. These findings suggest that antibodies to DNA fail to decrease denatured DNA template activity because the enzymes which use a single-stranded DNA template can displace or block the antibodies from the denatured DNA as a result of greater binding affinity to the denatured DNA. The anti-DNA antibodies associated with SLE, therefore, may not be involved in the pathogenesis of the intracellular abnormalities associated with the disease.

INTRODUCTION

A number of unusual antibodies can be detected in the sera of patients with systemic lupus erythematosus (SLE). Most of these unique antibodies are directed against various nuclear and cytoplasmic constituents of the cell and are species and organ nonspecific. Components of the nucleus against which antibodies have been demonstrated include DNA (1–4), deoxynucleoprotein (5, 6), histone (7), nuclear materials extracted with low ionic strength buffers (7–9), and a ribonuclease sensitive constituent of the nucleolus (10).

The serum from a patient with SLE usually contains antibodies directed against more than one of these nuclear components (7, 9). The types, titers, and specificities of these antibodies change during the course of the disease. The most commonly observed correlation between serological findings and disease activity is an increase in titer of the antibody against DNA during an exacerbation of the disease (2, 11–15).

Because of the direct temporal relationship between the appearance of antibodies to DNA and exacerbation of SLE, these antibodies have been suspected of being one of the primary causes of the cellular derangement in SLE. Since the antigen DNA, against which the antibodies are directed, is of major importance in cellular physiology, it is conceivable that antibodies to DNA might interfere with the functions controlled by DNA. The question posed is the following: could antibodies to DNA, by binding with DNA, alter the template activity of DNA for self-replication and RNA transcription and, in so doing, provoke pathological changes in subcellular activity? To answer this question the effect of antibodies to DNA on the *in vitro* template activity of DNA was studied.

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METHODS

DNA. Calf thymus and salmon sperm DNA were commercially obtained [1] and used without further purification. The concentration of DNA in solution was determined on the basis of OD measurements with the references of $E^{1\%}_{260} = 221$, pH 12, for calf thymus DNA E and $^{1\%}_{260} = 190$, pH 12, for salmon sperm DNA. DNA was thermally denatured by boiling for 10 min followed by rapid cooling in ice.

Sources of antibodies to DNA. Blood samples containing naturally occurring antinuclear factors were obtained from nine patients with SLE (Table I). Control blood samples were taken from nine persons, six with diseases other than SLE and three without overt disease (Table II). Antibodies to DNA were produced by immunizing New Zealand white rabbits with intravenous injections of denatured calf thymus DNA complexed to methylated bovine serum albumin (MBSA) according to the method of Plescia, Braun, and Palczuk (16). Blood from unimmunized rabbits was also taken.

The serum prepared from each blood sample was divided into two parts. One part was stored in aliquots at − 25°C. From the second part, the immunoglobulin (Ig) fraction was collected by precipitation with 33⅓% $(NH_4)_2SO_4$ (17). The Ig was dissolved in 0.15 M NaCl containing Tris buffer, pH 8.4, 5×10^{-3} mole/liter, and was then dialyzed for 18 hr at 4°C against 700 volumes of the same buffer. At the end of the dialysis period the protein concentration of the Ig fractions was determined, with BSA as the standard (18). The protein concentration was adjusted to 10 mg/ml with 0.15 M NaCl containing Tris buffer, pH 8.4, 5×10^{-3} mole/liter, and each sample was stored in aliquots at − 25°C.

Immunofluorescent studies. Immunofluorescent staining for antinuclear antibodies was done by a modification of the method of Beck (10). Cryostat sections (4 μ) of rat kidney, which served as the source of nuclei, were air dried and fixed in 95% ethanol. 0.15 M NaCl containing 0.01 M phosphate buffer, pH 7.2, was used for all washing steps and for making dilutions of serum. Staining was done with fluoresceinated rabbit antihuman whole Ig [2] which was layered onto the tissue sections previously incubated with serum. Absorption studies with denatured calf thymus DNA (0.5 mg/ml) were performed with selected sera before immunofluorescent staining. In the absorption studies, equal volumes of whole serum and denatured DNA were allowed to stand at 25°C for 30 min. The mixture was centrifuged at 30,000 *g* for 90 min, and the supernate was tested for antinuclear factors as described above. The slides were viewed with a Leitz Labrolux microscope equipped with an ultraviolet light source.

Immunological methods. The quantitative micro-complement (C′) fixation technique (19) was used for the detection of antibodies to DNA. The incubations for these tests were carried out at both 4°C for 18 hr and at 37°C for 1 hr, with 0.01–0.1 μg of denatured DNA as the antigen. A variation of the same method was used to study the inhibition by DNA polymerase and DNA-dependent RNA polymerase of the fixation of C′ by denatured DNA and antibodies to DNA experimentally produced in rabbits. For the inhibition studies, the enzymes were diluted in 0.14 M NaCl containing 0.01% (w/v) bovine serum albumin (BSA, fraction V),[3] 4×10^{-4} M $MgSO_4$, 1.5×10^{-4} M $CaCl_2$, and 10^{-2} M Tris buffer, pH 7.4. Varying amounts of enzyme were mixed at 0 or 37°C with 0.01 μg of denatured DNA before adding the enzyme-denatured DNA solution to the tubes containing diluted antiserum and C′. In the inhibition studies, an incubation period of 1 hr at 37°C was permitted for C′ fixation. These latter conditions were selected in order to provide optimal conditions for binding of the presumably 19S rabbit anti-DNA antibody, even though our results showed that the titers of the positive sera from SLE patients were identical under both conditions of

[1] Calbiochem, Los Angeles, Calif.

[2] Microbiological Associates, Bethesda, Md.

[3] Pentex, Inc., Kankakee, Ill.

TABLE I

*Patients with Systemic Lupus Erythematosus**

No.	Age	Duration of SLE	Disease activity‡	Immunofluorescent nuclear pattern	Antibodies to DNA
		yr			
1	39	9	R	Homogeneous	−
2	68	11	R	Marginal	−
3	30	11	R	Homogeneous	−
4	37	2	R	Homogeneous	−
5	36	0§	E	Homogeneous	−
6	21	0	E	Homogeneous	−
7	38	5	R	Homogeneous	+
8	38	0	E	Marginal	+
9	30	0	E	Marginal	+

* All SLE patients in this series were female Negroes.

‡ R, remission; E, exacerbation.

§ Newly diagnosed cases of SLE.

TABLE II

Control Group of Patients without Systemic Lupus Erythematosus

No.	Age, race, and sex	Primary disease
10	53CM	Herniated nucleus pulposa
11	16CM	Periarteritis nodosa
12	18CF	Infectious monoarthritis
13	55CF	Nutritional cirrhosis
14	49CF	Sarcoidosis
15	25CM	Rheumatic fever
16	26WF	No overt disease
17	30WF	No overt disease
18	26WM	No overt disease

assay. The shorter period, furthermore, was chosen in order to avoid a prolonged exposure of the DNA to any nuclease activity either in the enzyme preparation or the serum being assayed.

The titer of anti-BSA antibodies [4] was measured by passive hemagglutination of tanned erythrocytes coated with crystalline BSA [4] (20).

Purification of DNA polymerase and DNA-dependent RNA polymerase. DNA polymerase was prepared from *Escherichia coli* B [5] according to the method of Richardson (21). Fraction VI of the preparation, which is able to use denatured DNA as a template, was used. DNA-dependent RNA polymerase was purified to the fraction III stage of the procedure of Chamberlin and Berg (22).

Assay of DNA polymerase. DNA polymerase was assayed by a modification of the method of Richardson (21). The reaction mixture of 0.30 ml contained 20 μmoles of glycine buffer, pH 9.2; 7 μmoles of $MgCl_2$; 0.3 μmole of 2-mercaptoethanol; 50 mμmoles each of deoxyadenosine-5′-triphosphate (dATP),[6] deoxycytidine-5′-triphosphate (dCTP),[6] deoxyguanosine-5′-triphosphate (dGTP),[1] and thymidine-5′-triphosphate (TTP);[1] 0.05 mμmoles of tritiated TTP,[7] (5×10^7 cpm/μmole); 0.005 ml of DNA polymerase solution containing approximately 20 U of enzyme; and 5 μg of denatured calf thymus DNA. (1 U of DNA polymerase activity is defined as that amount of enzyme which catalyzes the incorporation of 1 mμmole of TTP into an acid insoluble product after a 15 min incubation at 37°C using a template of 20 μg of denatured calf thymus DNA). Each 0.3 ml reaction mixture contained, in addition, 1.0 mg of Ig protein dissolved in 0.1 ml of 0.14 M NaCl-Tris buffer, 5×10^{-3} mole/liter, pH 8.4, or 0.1 ml of the same buffer without Ig protein. Whole Ig fraction was used so that both 7S and 19S antibodies would be included. The Ig solution or buffer alone was mixed with the denatured DNA first, and the remainder of the reactants were added either immediately or after a preincubation period of 30 min at 37°C. After incubation of the entire reaction mixture at 37°C for a defined period of time, 0.05 ml aliquots of the reaction mixture were placed on duplicate filter paper disks (No. 542, Whatman filter paper, 2.4 cm diameter) which were washed according to the procedure of Starr and Goldthwait (23). The disks were then dried, and each was placed in a glass vial containing 15 ml of scintillation fluid (each liter consisting of 100 g of napthalene, 5 g of 2,5-diphenyloxazole (PPO), and 0.3 g of dimethyl 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene). They were counted in a Nuclear-Chicago Liquid Scintillation Spectrometer.

Assay of DNA-dependent RNA polymerase. The procedure of Chamberlin and Berg (22) was basically followed. In the reaction mixture were 10 μmoles of Tris buffer pH 8.3; 2.5 μmoles of $MgCl_2$; 0.25 μmoles of $MnCl_2$; 3 μmoles of 2-mercaptoethanol; 50 mμmoles of spermidine phosphate; 400 mμmoles each of ATP,[6] cytidine-5′-triphosphate (CTP);[6] 1.8 mμmoles of tritiated GTP,[7] and uridine-5′-triphosphate (UTP);[6] 10–30 U RNA polymerase in 0.01 ml; and 2 μg of denatured calf thymus DNA. (1 U of DNA-dependent RNA polymerase activity is defined as that amount of enzyme which catalyzes the incorporation of 1 mμmole of GTP into an acid insoluble product in 15 min of incubation at 37°C using as a template 60 μg of denatured calf thymus DNA). Each of the 0.275 ml reaction mixtures also contained 1.0 mg of Ig protein dissolved in 0.1 ml of 0.15 M NaCl-Tris buffer, 5×10^{-3}, pH 8.4 or 0.1 ml of the same buffer without Ig protein. The method of incubation was identical with that for DNA polymerase except that Whatman glass paper (type GFA cut in 1.5 × 1.5 cm squares) rather than filter paper was used, and, after the cold trichloracetic acid (TCA) washes, a fifth wash with cold absolute ethanol was included.

DNase measurements. In order to measure degradation of DNA to acid soluble oligonucleotides, the method of Heins, Taniuchi, and Anfinsen (24) was followed. Native salmon sperm DNA served as the substrate, and the reaction mixtures were incubated at 37°C for 1 hr. In order to detect digestion to smaller, but still acid insoluble polynucleotides, we performed a variation of the assay as follows: highly polymerized calf thymus DNA, the same DNA used in the C′ fixation assays, was incubated at 37°C in the presence of borate buffer and calcium with either 0.025 ml of DNA polymerase solution (45 μg of enzyme protein) or 0.02 ml of RNA polymerase solution (100 μg of enzyme protein). Aliquots of the mixtures before and after incubation were analyzed by alkaline sucrose gradient centrifugation, using a 5–25% gradient containing 0.001 M EDTA; 0.7 M NaCl, and 0.3 M NaOH. Centrifugation was for 8 hr at 50,000 rpm or 16 hr at 40,000 rpm, using a Spinco SW-50 rotor. Four-drop samples of the gradients were collected by tube puncture for measurement of distribution of OD at 260 mμ. In order to determine whether the DNA lost its ability to fix complement with anti-DNA after incubation with the enzymes, the DNA was recovered from large batch incubation mixtures. This was accomplished by deproteinizing the solutions by repeated extractions with ⅓ volumes of chloroform-octanol 24:1 (v/v). The DNA was harvested by winding on a glass rod the strands which formed at the interface of the aqueous layer over which ethanol had been layered. This postincubation DNA was assayed for its activity as an antigen in the micro-C′ fixation assay, and its sedimentation characteristics were determined by alkaline sucrose gradient centrifugation.

RESULTS

Serological studies

Immunofluorescent staining. All nine patients with SLE had positive fluorescent antinuclear tests (Table I) while the tests were negative in all those without SLE. Nuclear staining patterns were ho-

[4] Hyland Laboratories, Los Angeles, Calif.

[5] Grain Processing Corporation, Muscatine, Iowa.

[6] Sigma Chemical Company, St. Louis, Mo.

[7] Schwartz Bioresearch, Incorporated, Orangeburg, N. Y.

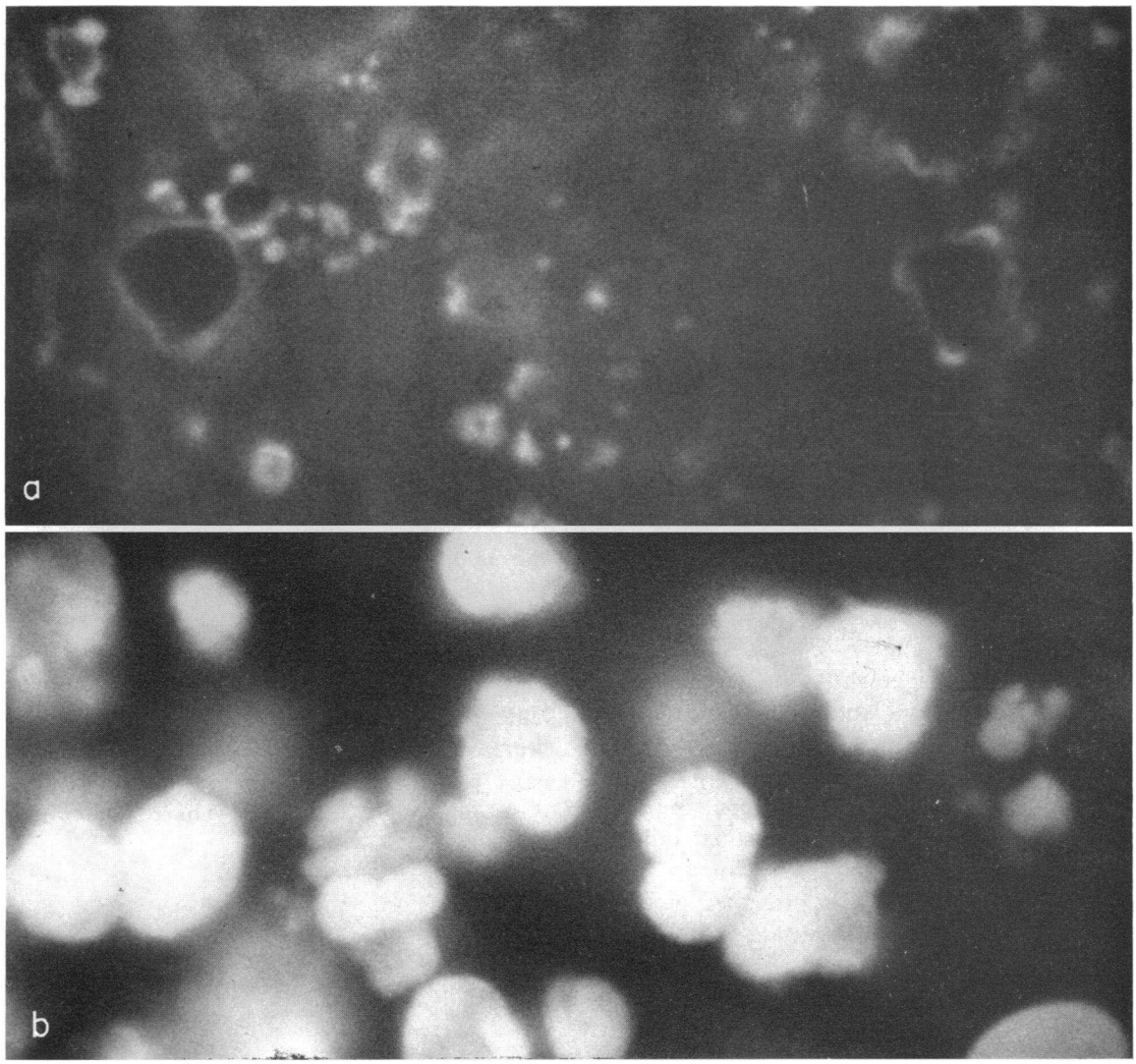

FIGURE 1 *a*, Immunofluorescent nuclear staining of marginal type by serum from patient 8. *b*, Homogeneous staining pattern of same serum previously absorbed with denatured DNA. × 1250.

mogeneous in six of the SLE patients and marginal in the remaining three.

In an effort to determine the relationship of the marginal pattern of nuclear fluorescence and antibodies to denatured DNA, the sera of the patients (Nos. 2, 8, and 9) with the marginal stain were absorbed with denatured calf thymus DNA before immunofluorescent staining. The staining pattern in none of the three samples was abolished, but, rather, was changed from marginal to homogeneous (Fig. 1). To demonstrate that there had not been nonspecific binding of antibody protein by the denatured DNA, the absorption study was repeated with a commercially obtained, unrelated antibody, anti-BSA, added to the mixtures of patient serum and DNA. There was no decrease in titer of anti-BSA after absorption with denatured DNA, thereby ruling out nonspecific absorption.

Antibodies to DNA. Antibodies to denatured DNA were detected by micro-C′ fixation tests in the sera of three patients with SLE (Table I). Six patients with SLE had no detectable antibodies to DNA nor did any of the controls. Stollar and Sandberg (25) have reported that the anti-DNA antibody in SLE patients is predominantly 7S and reacts best in C′ fixation at 4°C for 16–18

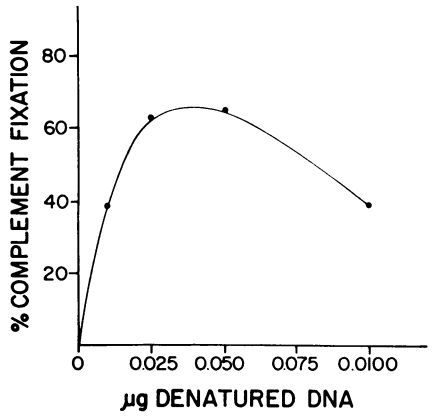

FIGURE 2 Fixation of C′ by denatured DNA reacted with serum from patient 9. Thermally denatured calf thymus DNA was incubated with a 1:50 dilution of serum from patient 9 in the presence of C′ at 37°C for 1 hr. Sensitized sheep erythrocytes were added and the level of hemolysis after incubation at 37°C was quantitated.

hr, whereas the anti-DNA antibody found in rabbits immunized with DNA-MBSA is mainly 19S and is detected in highest titer after an incubation at 37°C for 1 hr. Our studies showed, however, that the sera of the three patients who had anti-DNA antibodies had identical titers when tested under both sets of conditions. No attempt was made to determine whether the reacting antibodies were 7S or 19S. The information from a typical C′ fixation test is graphically presented in Fig. 2. Although this test was performed at 37°C for 1 hr, an identical titer was obtained after assay at 4°C for 18 hr. This curve, similar to those reported by Stollar, Levine, and Marmur (26), shows that maximum fixation of C′ in this antigen-antibody reaction occurs over a narrow range of low concentrations of denatured DNA. As pointed out by Wasserman and Levine (19), the typical micro-C′ fixation curve is similar to the findings of a quantitative precipitin test which include zones of antibody excess, equivalence, and antigen excess.

Of the three patients with antibodies to DNA, two (Nos. 8 and 9) were in an exacerbation of SLE and had marginal nuclear staining patterns. The other patient (No. 7) with antibodies to DNA was in a remission and had a homogeneous staining pattern at all dilutions of serum producing positive immunofluorescent nuclear staining. There was one patient (No. 2) whose serum produced a marginal stain, but which had no antibodies to DNA.

Both rabbits immunized with denatured DNA-MBSA developed antibodies to DNA during the 5th wk after the initiation of the immunization. The titer of antibodies to DNA rose and fell rapidly after i.v. booster immunization with denatured DNA-MBSA. Similar observations have been reported by others (27). In order to obtain sera with a high level of antibodies to DNA, it was necessary to obtain blood from the animals 5–7 days after i.v. booster immunization. The whole serum and serum Ig fractions from only one of the rabbits, RB2, were used for further testing.

Enzyme assays

Characterization of the systems for enzyme assay. In order to study the effects of antibodies to DNA on DNA template activity, the conditions for enzyme assay had to be determined to satisfy two demands: (*a*) DNA must be limiting; and (*b*) nonspecific inhibition by the additional protein must be minimal. With DNA limiting in the reaction, any reduction of available template, such as binding of antibody, should be accompanied by a decrease in template activity. Nonspecific inhibition must be eliminated in order to avoid artifactual variation in template activity which would obscure the effects of antibodies.

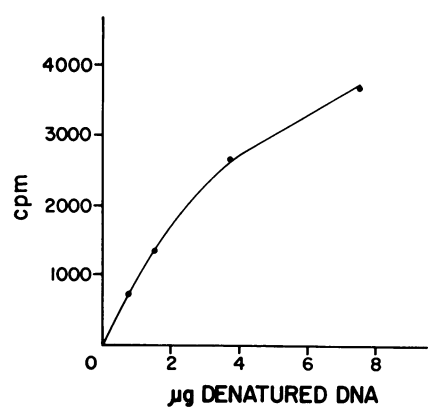

FIGURE 3 Assay of DNA polymerase with varying amounts of denatured DNA. The incubation mixture (0.30 ml) consisted of 20 μmoles of glycine buffer, pH 9.2; 7 μmoles of $MgCl_2$; 0.3 μmole of 2-mercaptoethanol; 50 mμmoles each of dATP, dCTP, dGTP, and TTP; 0.05 mμmoles of tritiated TTP (5×10^7 cpm/μmole); 20 U of DNA polymerase; 0.1 ml of 0.14 M NaCl-Tris buffer, 5×10^{-3} mole/liter, pH 8.4; and varying amounts (0.8–7.5 μg) of single stranded calf thymus DNA. The incubation was for 15 min at 37°C.

DNA polymerase was assayed in the presence of varying amounts of denatured DNA (Fig. 3). There was a steadily increasing level of incorporation through 7.5 μg of denatured DNA, and 5 μg of denatured DNA was selected as the amount to use in the assay system. Using 5 μg of denatured DNA and the standard concentration of reactants for assaying DNA polymerase (21), we observed a nonspecific inhibitory effect of 80% when we performed the assay in the presence of 1.0 mg of Ig protein. This inhibition could be reduced to about 15% by increasing Mg^{++} concentration twofold and the concentration of deoxyribonucleoside triphosphates fivefold (Table III). The increased levels of Mg^{++} and nucleotides did not change the template activity of DNA when Ig protein was not present. An incubation time curve for DNA polymerase with 2 μg of denatured DNA is shown in Fig. 4. The template activity reached a maximum after 15 min of incubation. By increasing the amount of denatured DNA present from 2 to 5 μg a plateau was reached after the same length of incubation, but the number of counts per minute at 15 min of incubation increased from 1180 to 3280. On the basis of this information, a 15 min incubation period was selected for further assays of 5 μg of denatured DNA for template activity with DNA polymerase.

A similar series of experiments determined the optimum assay conditions for DNA-dependent RNA polymerase. 2 μg was selected as the limiting level of DNA (Fig. 5). Nonspecific inhibition had also been observed when DNA-dependent RNA polymerase was assayed in the presence of Ig protein. This inhibition was reduced by increasing the concentration of Mg^{++} and ribonucleoside triphosphates and by using thermally denatured rather than double stranded DNA (Table IV). As reported by others (28), the template activity of denatured DNA was less than native DNA with DNA-dependent RNA polymerase. A time curve of the template activity of denatured DNA with RNA polymerase failed to reach a plateau after 1 hr (Fig. 6). Therefore, two incubation times, 10 and 20 min, were used for the assay without preincubation. In tests where DNA was preincu-

TABLE III

*Incorporation of TMP-^{3}H into DNA in the Presence and Absence of Ig Protein**

Mg^{++}	Deoxytriphosphates	Without Ig protein	With Ig protein
mμmoles	*mμmoles*	*cpm*	*cpm*
3.5	10	3795	813
7.0	50	3558	3174

* 5 μg of denatured DNA was assayed with DNA polymerase at 37°C for 15 min with and without 1.0 mg of Ig protein. Ig protein was derived from a serum without antinuclear or anti-DNA activity.

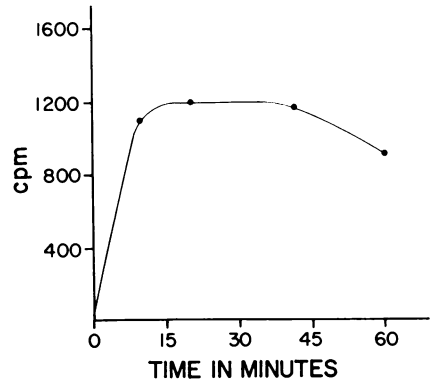

FIGURE 4 Assay of DNA polymerase with 2 μg of denatured DNA for varying periods of time. Conditions were identical with those for Fig. 3 except that only 2 μg of denatured DNA was used and incubation period was varied from 10 to 60 min.

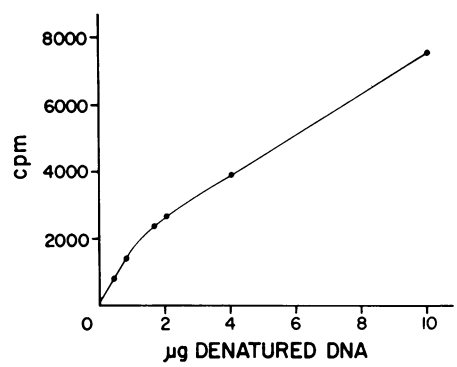

FIGURE 5 Assay of DNA-dependent RNA polymerase with varying amounts of denatured DNA. The incubation mixture (0.275 ml) consisted of 10 μmoles of Tris buffer, pH 8.3; 2.5 μmoles of $MgCl_2$; 0.25 μmoles of $MnCl_2$; 3 μmoles of 2-mercaptoethanol; 50 mμmoles of spermidine phosphate; 400 mμmoles each of ATP, CTP, GTP, and UTP; 1.8 mμmoles of tritiated GTP (2.5×10^7 cpm/μmole); 15 U of DNA-dependent RNA polymerase; 0.1 ml of 0.14 M NaCl-Tris buffer, 5×10^{-3} mole/liter, pH 8.4; and varying amounts (0.4–10.0 μg) of single stranded calf thymus DNA. The incubation was for 15 min at 37°C.

TABLE IV

*Effect of the State of DNA on GMP-^{3}H Incorporation into RNA in the Presence and Absence of Ig Protein**

State of DNA	Without Ig protein	With Ig protein
	cpm	*cpm*
Native	3517	2596
Denatured	3000	2822

* 2 μg of DNA was incubated with DNA-dependent RNA polymerase at 37°C for 30 min in the presence or absence of 1.0 mg of Ig protein. Ig protein was from same source as that in Fig. 3.

bated with Ig protein, only a 15 min incubation for template activity was done.

Effects of anti-DNA antibodies on DNA template activity. To provide a basis for comparison, the sources of the Ig fractions were classified into four groups as follows: group I, six patients with SLE, but without antibodies to DNA; group II, three patients and one rabbit with antibodies to DNA; group III, six hospitalized patients with diseases other than SLE; and group IV, three normal human beings and one unimmunized rabbit. The results of the enzyme assay with Ig protein (in which there was no preincubation of Ig protein with denatured DNA) from each source are summarized in Table V. The decimals represent the ratios of the number of mμmoles of radioactive nucleoside monophosphate incorporated in the presence of Ig protein divided by the amount incorporated in the absence of Ig protein. The numbers were in the range of 9 mμmoles of thymidine monophosphate-^{3}H (TMP) incorporation in DNA synthesis and 2.5 mμmoles of guanosine monophosphate-^{3}H (GMP) incorporation in the case of RNA synthesis. The averages of the ratios in each group revealed no significant differences among the groups (Table VI). The four Ig protein fractions containing antibodies to DNA and two Ig protein fractions without antibodies to

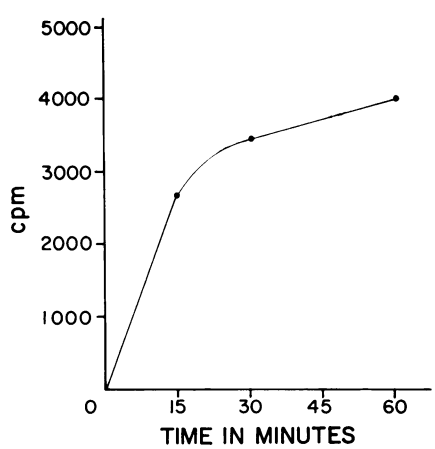

FIGURE 6 Assay of DNA-dependent RNA polymerase with 2 μg of denatured DNA for varying periods of time. Conditions were identical with those for Fig. 5 except that only 2 μg of denatured DNA was used, and the incubation period was varied from 15 to 60 min.

TABLE V

Effect of Ig Protein on the DNA Directed Synthesis of DNA and RNA

Group	Patient	DNA*	RNA* 10 min	RNA* 20 min
I				
	1	0.97‡	0.85	0.78
	2	0.93	0.86	0.99
	3	0.89	0.93	0.95
	4	0.89	0.92	0.92
	5	0.83	0.93	0.98
	6	0.85	0.91	0.84
II				
	7	0.84	0.89	0.88
	8	0.90	0.95	0.90
	9	0.93	0.84	0.88
	RB2§	0.89	0.89	0.94
III				
	10	0.86	0.82	0.89
	11	0.93	0.92	0.93
	12	0.90	0.86	0.94
	13	0.88	0.86	0.87
	14	0.93	0.79	0.81
	15	0.93	0.91	0.86
IV				
	16	0.90	0.82	0.86
	17	0.92	0.91	0.85
	18	0.81	0.86	0.92
	NR1‖	0.92	0.95	1.00

* Assay for DNA synthesis carried out at 37°C for 15 min. Assay for RNA synthesis done at 37°C.

‡ Numbers are ratios of the mμmoles of radioactive nucleotide incorporated in the presence of Ig protein divided by the amount incorporated in the absence of Ig protein. The amount of TMP incorporated in the absence of Ig was 8.9 mμmoles. 2.3 mμmoles of GMP was incorporated at 10 min and 2.6 mμmoles at 20 min in the absence of Ig protein.

§ Rabbit immunized with denatured DNA-MBSA.

‖ Unimmunized rabbit.

TABLE VI

Average Results of Ig Protein Effects on DNA and RNA Synthesis

Group	DNA*	RNA*	
		10 min	20 min.
I	0.89‡	0.90	0.91
II	0.89	0.89	0.90
III	0.90	0.86	0.88
IV	0.89	0.88	0.91

* DNA assay done at 37°C for 15 min. RNA assay done at both 10 and 20 min.

‡ Numbers are ratios determined as for Table V.

DNA were preincubated with the denatured DNA before the enzyme assay. There was a slightly greater amount of nonspecific inhibition with preincubation; however, there were only small but insignificant differences in the nucleotide incorporation in the presence and absence of antibodies to DNA (Table VII).

In order to establish that the antibodies to DNA were not inhibited from binding to DNA by the presence of deoxyribonucleotides acting as haptenes, complement fixation was carried out in the presence of both TTP alone and all four deoxyribonucleotides at the same final concentrations as were used in the DNA polymerase assays. The rabbit anti-DNA antibody in these C′ fixation tests, furthermore, was present in much smaller quantities than was the case in the polymerase assays. The amount of antibody available to fix C′ was that in 1 ml of a dilution of serum of 1/200, as compared to the relatively large quantity present in 1.0 mg of Ig fraction which was used in the polymerase assays. The data in Table VIII show that C′ fixation by DNA and anti-DNA antiserum was not inhibited by the presence of deoxyribonucleotides. The failure of antibodies to DNA to inhibit DNA polymerase activity, therefore, cannot be due to the blocking of the binding of the antibodies to the DNA template by haptene. Haptene inhibition assays with ribonucleotides were not carried out since they do not serve as haptene inhibitors of anti-DNA antibody (16).

TABLE VII

Effect of Preincubating Denatured DNA with Ig Protein before Assaying DNA Template Activity

Source of Ig protein	DNA*	RNA*
With antibodies to DNA		
Patient 7	0.84‡	0.84
Patient 8	0.72	0.77
Patient 9	0.83	0.82
RB2	0.89	0.89
Without antibodies to DNA		
Patient 13	0.91	0.74
NR1	0.85	1.00

* Assays for both DNA and RNA production carried out at 37°C for 15 min after a preincubation period of 30 min.

‡ Numbers are ratios determined as for Table V.

Competition between antibodies and enzymes for DNA

Because there could be demonstrated no specific inhibition of DNA template activity by antibodies to DNA, it was decided to investigate the possibility that the affinity of the polymerases for the DNA was greater than that of the antibodies. If the attachment site for the enzymes and the antigenic determinants for antibodies are in the same areas of the single-stranded DNA, a stronger association of enzyme with the DNA would reduce the fixation of C′ by preventing the interaction of

TABLE VIII

Complement Fixation by the Reaction of DNA and Rabbit Anti-DNA Antiserum in the Presence of Deoxynucleotides

Additions to standard reaction mixture			
DNA*	Antiserum ‡	TTP§	% C′ fixed
+	+	−	100
−	−	+	0
+	−	+	0
−	+	+	0
+	+	+	96
DNA*	Anti-serum‡	dXTP‖	% C′ fixed
+	+	−	100
−	−	+	3
+	−	+	0
−	+	+	0
+	+	+	90

* DNA concentration, 0.01 μg/reaction mixture.

‡ Rabbit anti-DNA–MBSA serum, 1.0 ml of a 1/200 dilution per reaction mixture.

§ TTP, 1.0 μmole/reaction mixture (final concentration 0.15×10^{-3} mole/liter).

‖ TTP, dCTP, dATP, and dGTP each at a concentration of 0.15×10^{-3} mole/liter.

the antibodies to DNA with the denatured DNA. This would be a phenomenon similar to haptene inhibition of antigen-antibody association. In this instance, however, the enzyme competitor would bind with antigen preventing its subsequent reaction with the antibody. A similar mechanism of action has been demonstrated to explain the inhibition by chloroquine of the reaction between denatured DNA and antibodies to DNA (29).

Several preliminary experiments were performed to establish the reliability of the system for studying this competition. Neither the solution of DNA polymerase nor that of DNA-dependent RNA polymerase was anti-complementary. The reaction of neither enzyme with DNA was accompanied by fixation of C′, and C′ was not fixed when either enzyme, in the absence of denatured DNA, was mixed with a solution containing antibodies to DNA. Denatured DNA preincubated alone at 37°C fixed the same amount of C′ with antibodies to DNA as did denatured DNA not preincubated.

Some DNase activity has been reported by others in both enzyme preparations used (21, 22). Degradation of DNA by DNase could also result in a decrease in the amount of C′ fixed. For this reason DNase determinations for both enzyme preparations were made. DNase activity, as measured by the release of acid soluble nucleotides, could not be detected in either enzyme solution in amounts up to 25 μg of enzyme solution protein. This was 5 μg more than was used in any of the competition studies, and several fold more than that used in most of the assays. The assay for polynucleotide endonuclease activity failed to demonstrate significant degradation of DNA by the DNA polymerase preparation. Fig. 7 *a* depicts a composite of sucrose gradient analyses of DNA before and after incubation with this solution. A more prolonged centrifugation moved both peaks to the bottom of the tube. The sedimentation characteristics of the DNA, therefore, were not changed even under alkaline conditions which are intended to denature DNA in order to detect single-strand breaks.

The RNA polymerase preparation, on the other hand, did contain DNase activity. The sucrose gradient analyses shown in Fig. 7 *b* demonstrated that the sedimentation coefficient of the DNA after incubation with this solution is less than that of the control. In order to determine whether the DNA so degraded is still capable of functioning in the micro-C′ fixation assay, we extracted the DNA from the incubation mixture as described in Methods. This DNA functioned as well and at the same concentration as control DNA. Finally, in order to rule out the possibility that the DNA extraction procedure selected only intact DNA, the chloroform-extracted DNA was analyzed by alkaline sucrose gradient centrifugation. This DNA, which functions in the micro-C′ fixation test, had a sedimentation pattern identical with that of the postincubation DNA shown in Fig. 7 *b*.

Healy, Stollar, Simon, and Levine (30) have shown that C′ fixation by DNA–anti-DNA is inhibited by a molecular weight change from 2×10^7 to 3×10^6, a sixfold decrease. Stollar and Levine (31) have shown that shearing DNA to one quarter of its original size did not reduce its antigenic activity by more than 10%. The results

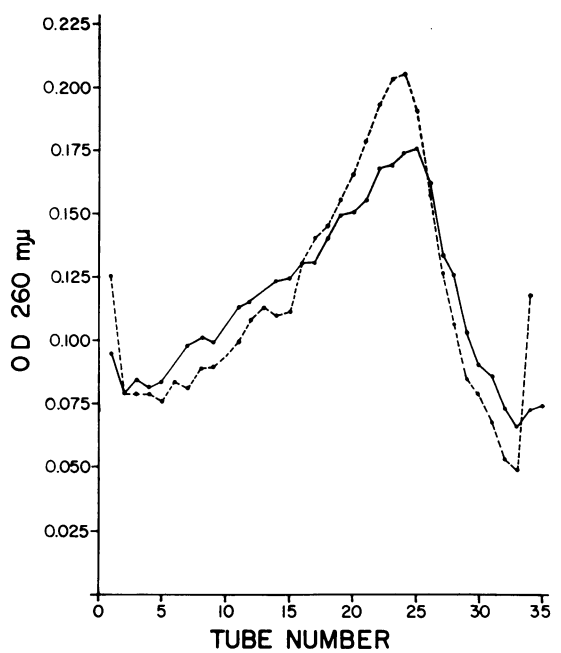

FIGURE 7 A Sucrose gradient analysis of DNA before and after incubation with DNA polymerase. The incubation mixture contained highly polymerized calf thymus DNA, 0.25 mg, sodium borate buffer, pH 8.8, 10 μmoles, $CaCl_2$, 0.5 μmoles, and 0.025 ml DNA polymerase solution in a final volume of 0.23 ml. Incubation was for 30 min at 37°C. Aliquots of the mixture before and after incubation were analyzed by alkaline sucrose gradient centrifugation as described under Methods. Centrifugation was for 8 hr at 50,000 rpm. ●——●, control; ●- - - -●, incubate.

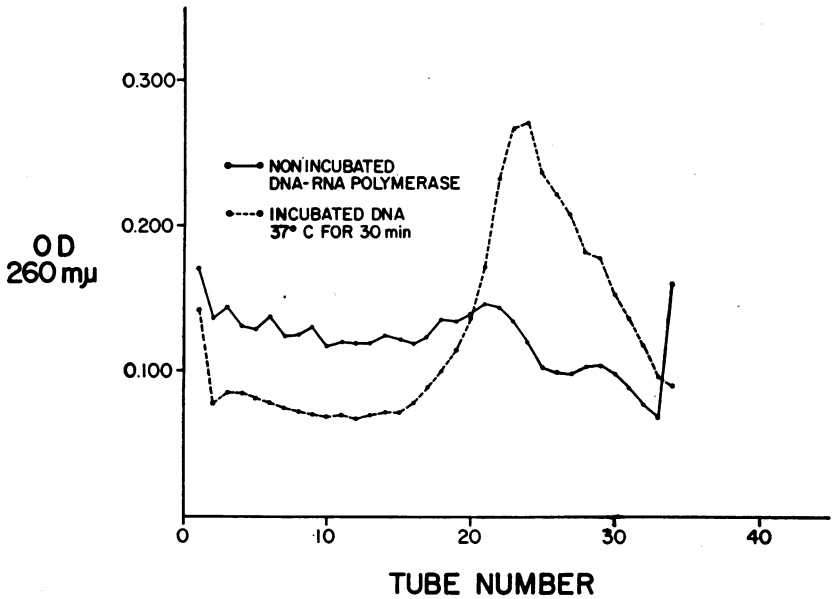

FIGURE 7 B Sucrose gradient analysis of DNA before and after incubation with DNA-dependent RNA polymerase. Conditions were as in Fig. 7 A except that 0.02 ml RNA polymerase solution was used, and centrifugation was for 16 hr at 40,000 rpm. ●——●, control; ●----●, incubate.

reported here, therefore, support the probability that any effect on C′ fixation by the DNA incubated with DNA polymerase and RNA polymerase was not due to degradation of the DNA substrate by endonuclease activity.

A 1:400 dilution of serum from Rabbit RB2 containing anti-DNA antibodies and 0.01 μg of denatured calf thymus DNA which would cause 75% C′ fixation were used for all the competition studies. DNA polymerase inhibited the fixation of C′ by denatured DNA with antibodies to DNA

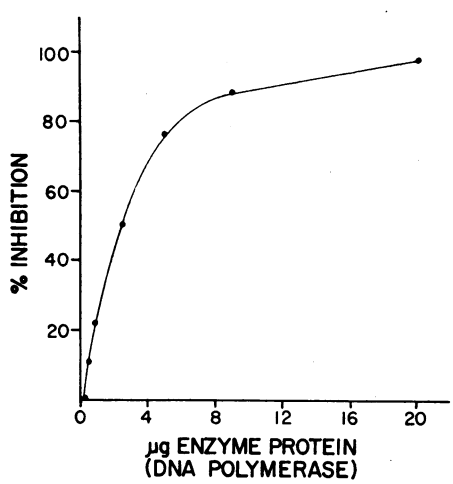

FIGURE 8 Inhibition of C′ fixation of denatured DNA with antibodies to DNA by DNA polymerase. 0–20.0 μg of DNA polymerase protein was preincubated with 0.01 μg of denatured DNA at 37°C for 10 min. The second and third incubation periods were conducted as described in Fig. 10. Specific activity of the DNA polymerase used was 2180 U/mg.

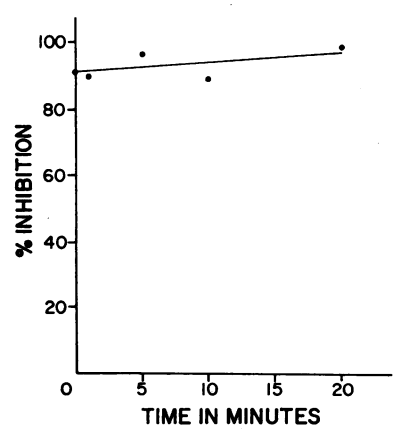

FIGURE 9 Length of preincubation of denatured DNA and DNA polymerase and inhibition of the reaction between antibodies to DNA and denatured DNA. Conditions were the same as for Fig. 8 except that the preincubation of 10.0 μg of DNA polymerase protein and 0.01 μg of denatured DNA at 37°C was varied from 0 to 20 min.

(Fig. 8). DNA polymerase solution containing 0.5 μg of protein produced 10% inhibition and that with 1.5 μg of protein produced 30%. The degree of inhibition was increased by using larger amounts of DNA polymerase, with 100% inhibition being approached with 20.0 μg. The inhibition by DNA polymerase did not depend upon preincubation of enzyme and DNA, and there was no significant change in inhibitory effect when the period of preincubation at 37°C was extended to 20 min (Fig. 9).

DNA-dependent RNA polymerase was shown to be a potent inhibitor of the C′ fixation by the binding of DNA antibodies to denatured DNA (Fig. 10); 30% inhibition was detected with 50 mμg of DNA-dependent RNA polymerase protein. Increasing amounts of inhibition were observed with larger amounts of enzyme until a maximum inhibition of about 60% was attained when 500 mμg of RNA polymerase solution protein was present. Inhibition occurred when DNA-dependent RNA polymerase solution was mixed with denatured DNA at 0°C; however, preincubation of enzyme and DNA at 37°C was accompanied by an increased degree of inhibition (Fig. 11). A maximum inhibitory effect was reached after 5 min of preincubation at 37°C, but inhibition of C′ fixations was never 100%, even after 20 min of preincubation.

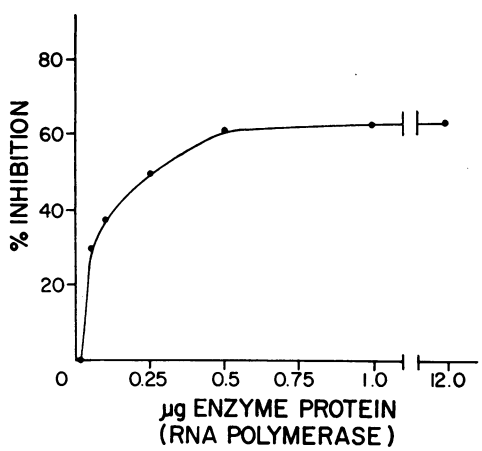

FIGURE 10 Inhibition of the reaction between denatured DNA and antibodies to DNA by DNA-dependent RNA polymerase. 0–12.0 μg of DNA-dependent RNA polymerase protein was preincubated with 0.01 μg of denatured DNA at 37°C for 10 min before being mixed with a 1:400 dilution of anti-DNA serum and C′. This mixture was incubated at 37°C for 1 hr. After the addition of sensitized sheep erythrocytes, a third incubation period at 37°C was allowed, and the extent of hemolysis was determined. Specific activity of the enzyme preparation used was 920 U/mg.

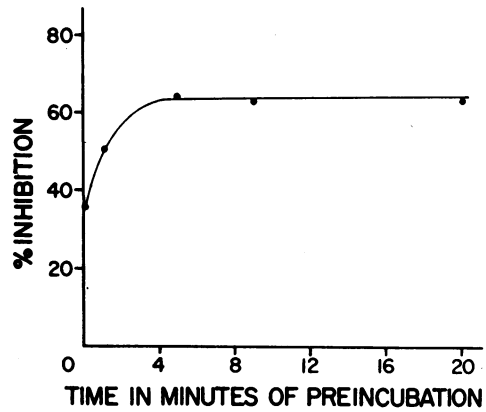

FIGURE 11 Relationship of duration of preincubation of denatured DNA and DNA-dependent RNA polymerase and inhibition of the reaction between anti-DNA antibody and denatured DNA. Conditions were similar to Fig. 7, except for the preincubation stage during which 1.0 μg of DNA-dependent RNA polymerase protein was incubated with 0.01 μg of denatured DNA at 37°C for 0–20 min.

DISCUSSION

These experiments were carried out in order to determine whether antibodies to DNA have any effect on the self-replicative and transcriptive template functions of DNA. In order to demonstrate conclusively that antibodies to DNA have specific effects on activities directed by DNA, several requirements were met. The presence of anti-DNA antibodies, both naturally occurring and experimentally produced, was demonstrated by micro-C′ fixation with denatured DNA serving as the antigen. The hypothesis that a specific marginal-type of immunofluorescent nuclear staining pattern is characteristic of antibodies to DNA was disproven both by the lack of correlation between the C′ fixation tests and their staining pattern, and by the failure to remove the marginal stain by absorption with denatured DNA. Rather than removing the marginal-type staining pattern, the absorption with DNA was followed by an alteration in the staining pattern to a homogeneous type which is usually related to an antibody to deoxynucleoprotein (10). Our results are similar to those of others (15, 32, 33) who have found many exceptions to the reported correlation between marginal nuclear staining patterns and antibodies to DNA

(34). The reason is unknown for the change of nuclear staining pattern from marginal to homogeneous after absorption of the sera with denatured DNA. Sera with DNA antibodies will usually contain antibodies to other nuclear constituents also (9), so it is possible that a blocking factor has been removed (35). Since the titer of an unrelated antibody, anti-BSA, was not reduced after absorption with denatured DNA, the removal by the DNA of a specific antinuclear factor is suggested.

The antibodies which could possibly interfere with DNA activity must be permitted access to the DNA. Antinuclear antibodies are incapable of entering an intact mammalian cell (36–38) so that tissue culture systems are not suitable for examining the effects of antibodies to DNA on DNA-directed activities. The cell-free systems used for assaying DNA template activity provided conditions in which DNA was exposed to its antibodies. Because nearly all antibodies to DNA react much better with denatured than with double-stranded DNA (31), the use of single-stranded DNA in the assay presented a maximum opportunity for the binding to DNA by DNA antibodies.

In the in vitro assay of DNA template activity, the concentration of DNA present was limiting. Under these conditions, any decrease in DNA accessible to the polymerases would have been recorded as a decrease in DNA template activity as manifested by a decrease in amount of the final product. The amount of denatured DNA used in assaying DNA template activity with both DNA polymerase and DNA-dependent RNA polymerase, was shown to be limiting on the basis of an assay using varying denatured DNA concentrations.

Decreases in DNA template activity assayed in the presence of serum or serum products can be the result of actions other than the binding of template by antibody, and such mechanisms must be recognized. Nonspecific effects due to the presence of large amounts of protein or other material which might bind nucleotides or chelate essential ions should be reduced to a minimum. In the assay of DNA polymerase the nonspecific inhibition was reduced by increasing magnesium and nucleotide concentrations. These conditions were shown not to block C′ fixation of the DNA–anti-DNA reaction by haptene inhibition. In the case of DNA-dependent RNA polymerase the nonspecific inhibition was overcome by using single-stranded rather than double-stranded DNA. Other, more specific, inhibitory effects can be produced by nuclease activity, and, without nuclease determinations, any inhibitory effects on DNA template activity by antibodies binding to DNA cannot be separated from the destruction of DNA template and (or) product by nuclease attack. The assays for DNase and RNase revealed no significant contamination of the enzyme or immunoglobulin preparations with these nucleases. This finding, associated with the lack of effect of immunoglobulin on DNA template activity, supported the conclusion that the inhibition of complement fixation by the polymerases was due to blocking of the antigen-antibody reaction rather than to degradation of the substrate.

The data presented clearly showed that the binding of DNA antibodies with denatured DNA did not reduce the over-all template activity of denatured DNA for DNA and RNA production. Even with preincubation of DNA with anti-DNA antibody, there was no significant decrease in DNA template activity. Since no effect was found with whole Ig fraction which contained both 7S and 19S molecules, no attempt was made to separate the two fractions to see if either had an effect alone. There have been reports that antibodies which react with DNA will interfere with DNA activity. Williams and Bollum (39) observed a decrease in the template activity of denatured DNA with calf thymus DNA polymerase when the denatured DNA was preincubated with dialyzed whole sera, some of which contained antibodies to DNA. This effect of anti-DNA antibody occurred in the presence of relatively high concentration of deoxyribonucleotides. This decrease in template activity was attributed to the binding of the serum factors to the enzyme active sites of DNA. DNase activity, however, was not determined, so that nuclease activity may have accounted for some of the reduction. The difference in the ability of DNA polymerase from different sources to act on an "altered" DNA template might also be of importance in explaining the disagreement between the findings of Williams and Bollum and ours. The dose of irradiation which greatly reduces DNA template activity with *E coli* DNA polymerase (40), has no effect on the tem-

plate activity with DNA polymerase from calf thymus (41).

The effect of anti-DNA antibodies on the in vivo biological activity of DNA has been investigated. Rosenkranz, Erlanger, Tanenbaum, and Beiser (42) demonstrated an arrest in the development of fertilized sea urchin eggs exposed to antinucleoside antibodies which cross react with denatured DNA. The sea urchin eggs, in contrast to mammalian cells, are permeable to the antibody. Antibodies to calf thymus DNA can impair the transforming capacity of DNA from *Diplococcus pneumoniae* and *Bacillus subtilis* (43). Biological tests such as these may detect qualitative changes in the DNA that are not measured by a biochemical assay. A qualitative alteration in the types of RNA and DNA produced such as might be caused by interference with the fidelity of transcription or with the readout of specific cistrons, need not necessarily be accompanied by a quantitative change in over-all in vitro template activity.

There are several possible explanations for the failure of anti-DNA antibodies to decrease DNA template activity. First, the DNA antibodies and enzymes which utilize a denatured DNA template may attach to different areas of the DNA. In the case of DNA-dependent RNA polymerase, it is known that there are certain initiator sites on the DNA to which the enzyme attaches (44), and these initiator sites could be different from the antigenic-determinant sites. In such a situation, there would be little interference with enzyme attachment by the binding of the antibody, and product synthesis would be on a jump-skip basis. This seems unlikely, however, because the antigenic determinants for the naturally occurring antibodies to DNA have been different in all cases studied (45, 46) and are certain to be repeated throughout the DNA molecule. The antigenic determinants for the DNA antibodies experimentally produced by denatured DNA-MBSA immunization have not been completely identified but, as evidenced by haptene inhibition studies (16), are at least the size of oligonucleotides.

There is always a possibility that anti-DNA antisera which are specific for a polynucleotide sequence which is responsible for initiation (47) might have an inhibitory effect on transcription which is not seen with other antisera, and may even compete more successfully with the polymerase. Although the specificities of the antibody used were not determined, inasmuch as whole DNA was used as the antigen, it is probably justified in assuming that it had multiple specificities, some of which may have been against initiation sequences. In any event, the point to be made was that an antibody which was shown to be able to bind to DNA was prevented from so doing by an enzyme which also is known to bind to DNA. Since the antibodies to DNA in SLE sera are heterogeneous, a systematic survey to correlate antigenic specificity, affinity to substrate, and effect on template activity of a series of such sera may be worthwhile.

A second possibility is that the antibodies to DNA and enzymes which utilize a DNA template do attach to identical or similar areas, but with different binding affinities. Thus, the enzymes, which have a greater affinity, are able to block or displace the DNA antibodies from the single-stranded DNA. The competition experiments in this study were designed to test these possibilities. They showed that denatured DNA linked with DNA polymerase or DNA-dependent RNA polymerase reacts less effectively with antibodies to DNA, as evidenced by decreased C′ fixation. This inhibition suggests that the attachment sites for the enzymes and the antigenic determinants for the antibodies are in the same or adjacent areas of the denatured DNA. If the enzymes had attached to sites at a distance from the antigenic determinants for the antibodies, no inhibition of the reaction between DNA and its antibodies would have been observed. The similarity of the sites for enzyme attachment and antibody binding was reported by Tanenbaum, Burke, and Beiser (48) who observed an homology between the combining regions of anti-haptene antibodies and enzymes induced by specific β-D-glucosides. These findings, moreover, support the hypothesis that the reason anti-DNA antibodies do not decrease the over-all DNA template activity is that the enzymes at the concentrations used in the assays bind with greater affinity than the antibodies to the denatured DNA template and can displace them from the molecule.

This hypothesis is supported by the failure to demonstrate DNase activity in the DNA polymerase solution, ruling out degradation of the DNA as the cause of C′ fixation inhibition. Although the

DNA-dependent RNA polymerase has some DNase activity at relatively high concentrations, the degree of degradation of the DNA was shown not to diminish its activity in the micro-C′ fixation test. This is supported by the observation that maximum but incomplete inhibition of C′ fixation by RNA polymerase is achieved after 5 min of preincubation, whereas, DNase degradation of the DNA should cause increasing inhibition to 100%, if this were the actual mechanism.

DNA-dependent RNA polymerase has great affinity for denatured DNA (28), and the interaction between the enzyme and template occurs in the absence of metal ions and ribonucleotides necessary for RNA production (49). RNA polymerase attaches with less affinity to native DNA (28), but even in this case, the complex formed is sufficiently indissociable to prevent the DNA from being acted upon by DNA polymerase or exonucleases (50). In contrast, DNA polymerase probably forms a more readily dissociable complex (50).

These studies have revealed that anti-DNA antibodies cause no decrease in the in vitro template activity of denatured DNA and, in view of the competition experiments with the nucleic acid polymerases, they should not be expected to have so functioned. Such findings suggest that antibodies to DNA, with respect to interference with biochemical genetic activities, may not be of significance in the subcellular pathophysiology of SLE.

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