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Inhibition of Enzymatic DNA Synthesis by Certain Pathological Human Sera.* (28145)

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Sera from patients with systemic lupus erythematosus and certain other "collagen diseases" contain antibodies reacting with cell nuclei, nucleoprotein, and DNA‡ as demonstrated by complement fixation and precipitin reactions(1). At least two factors could be differentially adsorbed: one reacted with cell nuclei and was responsible for the "LE phenomenon"; another, present only in certain sera, reacted with purified DNA from different sources and species. Using a fluorescent antibody technic, Calabresi *et al.*(2) detected antinuclear globulins in the sera of all patients studied with SLE or with Felty's syndrome of rheumatoid arthritis. Williams and Schilling(3), however, did not find any marked effects of SLE and FS sera, compared to normal sera, on incorporation of adenine or thymidine into DNA of human blood leukocytes *in vitro*. Holman *et al.*(1), Lachman (4), and Rapp(5) found that the antinuclear factors present in SLE sera did not interfere with the growth of normal or tumor cells in tissue culture, and, from these and other data, concluded that the factors could not penetrate viable cells.

Inability of DNA-reactive antibody to penetrate to the nucleus of viable cells might explain the negligible effect of the antinuclear factors on incorporation or growth of whole cells *in vitro*. However, the possibility that anti-DNA factors present might affect the biosynthesis of DNA in cell free systems remained to be tested. The enzymatic incorporation of deoxyribonucleoside triphosphates into DNA in presence of a small amount of heat-denatured primer DNA, catalyzed by a purified DNA polymerase from calf thymus gland(6), provides a system to test this hypothesis.

In the present study, we measured the effect of pathological and normal sera on synthesis of DNA with purified calf thymus polymerase. Certain pathological sera do contain DNA-reactive material as demonstrated by inhibition of DNA synthesis. The method is sensitive, rapid, and provides a means of obtaining information on the combining sites of serum factors with DNA.

Materials and methods. Agar tube precipitin tests. Calf thymus DNA (Worthington) was dissolved in .01 tris buffer at pH 8.1, heated at 100°C for 2 min, and cooled immediately by immersion in an ice bath. The DNA was added to agar in .02 M sodium phosphate buffer, pH 7, held at 50°C. Final concentrations were 10 and 50 μg DNA/ml and 0.5% agar. Fifteen-mm columns of DNA- agar were pipetted into 5-mm ID tubes

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‡ The following abbreviations are used: DNA, deoxyribonucleic acid; SLE, systemic lupus erythematosus; FS, Felty's syndrome of rheumatoid arthritis; dCMP, the monophosphate of deoxycytidine; tris, tris-(hydroxymethyl)-aminomethane; dCTP, TTP, dATP, dGTP, and ATP, the triphosphates of deoxycytidine, thymidine, deoxyadenosine, deoxyguanosine, and adenosine; RA, rheumatoid arthritis.

TABLE I. Sera Used in Tests of DNA Synthesis.

Patient	Clinical diagnosis	LE prep	Sheep cell or latex	Nuclear fluor.*	DNA precipitin
D.B.	SLE	+	+	+	+
D.J.	"	+	—	+	+
I.L.	"	+	+	+	—
P.P.	"	+	—	+	—
X.R.	"	+	—	+	—
C.S.	"	+	—	+	—
K.S.	"	+	+	+	—
E.W.	"	+	—	+	+
E.B.	FS	—	+	+	+
I.B.	"	+	+	+	±
R S.	"	—	+	+	+
D.W.	"	—	+	+	—
C.E.	RA	—	+	+	—
L.P.	"	—	+	—	—
J.M.	Neutropenia†	—	—	—	—
M.R.	"	—	—	+	—
3 sera	Mult. myel.			—	—
7 sera	Normal			—	—

* Performed as described in(2).
† Neutropenia not obviously related to collagen disease.

and allowed to harden, and 15-mm columns of experimental sera (undiluted) were then pipetted above the agar. Results were read at 24 hours. Each serum was also tested after it had been heated for 30 min at 56°C. No difference in results with heated and unheated serum was found.

DNA polymerase assays. Sera were dialyzed for two 2-hour periods against two 100× volume portions of .05 M NaCl. This step was used because the salt concentrations of the undialyzed sera were inhibitory to the enzyme system, and precipitates were obtained during dialysis when the concentration of the dialyzing medium was less than .05 M.

Methods are given in(6) for synthesis of deoxynucleoside triphosphates, preparation of deoxynucleoside monophosphates containing P^{32}, and phosphorylation of dCMP to the triphosphate level with a rat liver enzyme. The incubation medium consisted of 0.020 ml heat-denatured calf thymus DNA in .01 M tris buffer pH 8.1, 0.025 ml experimental serum or 0.05 M NaCl, 0.10 ml substrate mixture, and 0.005 ml DNA polymerase prepared from calf thymus(6). The DNA had been heated as described above. The substrate mixture contained the following compounds, in μmoles per 0.10 ml: 0.01 μmole $dCTP^{32}$, 0.10 μmole each of TTP and dATP, 0.20 μmole dGTP, 0.025 μmole each of ATP and 3-phosphoglycerate, 1.25 μmoles $MgCl_2$, 0.25 μmole tris buffer pH 8.1 (from phosphorylation of dCMP), and 5.00 μmoles potassium phosphate buffer pH 7.0. After mixture in an ice bath, samples were incubated for 30 min in small test tubes in a 35°C water bath. The tubes were then removed to an ice bath, and aliquots of 0.10 ml were pipetted onto filter paper discs and dropped into 5% trichloracetic acid. Details of the paper disc assay are given in(7). Radioactivities are expressed as cpm per 0.1 ml aliquot counted. During the course of the experiments reported below, the $dCTP^{32}$ standard decayed from 81,000 cpm/μmole to 63,000 cpm/μmole.

Where indicated in tables, preincubation of DNA with serum was carried out as follows. The DNA solution and serum were mixed at 0-4°, incubated in a 35°C water bath for 30 min, then removed to the ice bath. Substrate mixture and DNA polymerase were then added and DNA synthesis incubation was carried out as described above.

Results. The agar tube test for DNA precipitins was performed on 34 pathological and 11 normal sera; 3 SLE and 2 FS sera gave positive reactions. Twenty-six of these sera were tested for effect on DNA synthesis in the present experiments, and their reactivity in several immunological assays is described in Table I. The calf thymus DNA used in precipitin tests and DNA synthesis inhibition was thermally denatured. Heat denaturation

TABLE II. Effect of Human Sera on DNA Synthesis with Calf Thymus Polymerase and Calf Thymus DNA Primer.

cpm with serum/cpm with NaCl

Patient	Exp 1, 3 μg DNA		Exp 2, preincub		Exp 3, preincub
	Not preincub	Preincub*	3 μg DNA	1.5 μg DNA	18 μg DNA
SLE					
D.B.	.86	.35			.92
D.J.	95	.55			1.13
I.L.	1.05	.83	.83	.34	
P.P.	.99	.80		.58	1.06
X.R.	1.14	.65			
C.S.	1.07	.92 (.95)			
K.S.	.98	.79		.84	1.04
E.W.	1.02	.65	.50	.43	1.13
FS					
E.B.	.94	.19 (.32)			1.02
I.B.	.92	.77	.84	.36	
R.S.	.94	.60 (.48)		<.10	.97
D.W.	1.02	.61 (.48)		.33	1.06
RA					
C.E.	1.14	.75		.51	.99
L.P.	1.10	.73		.67	1.05
Normal					
M.E.	1.32	1.06			
F.M.	1.35	1.05			
L.S.	1.05 (1.07)	1.14			
J.S.	1.50	1.02			
F.V.	1.40	.99			
J.V.	1.27	1.10			
R.W.	1.25	.95			
Neutropenia					
J.M.		1.01			
M.R.		1.14			
3 mult myel		1.14, .99, .93			

* Serum was incubated with DNA for 30 min at 35°C before addition of reaction mixture and polymerase.

TABLE III. Effect of Human Sera on DNA Synthesis with Varying Amounts of Primer.*

cpm with serum/cpm with NaCl

Serum†	1.5 μg DNA	3.0 μg DNA	6.1 μg DNA	12.2 μg DNA
D.B. (SLE)	.18	.30	.26	.81
E.B. (FS)	.19	.37	.49	.92
R.S. (")	.13	.35	.35	.88
C.E. (RA)	.48	.53	.64	1.03
cpm NaCl control	522 ± 6	811 ± 27	1490 ± 63	2129 ± 78
L.S. (normal)	.98	1.14	1.13	1.19
J.S. (")	.89	1.02	—	1.08
J.V. (")	.83	1.10	—	1.19
O.E. (mult myel)	.76	1.14	1.20	—
E.H. (" ")	.84	.99	.98	—
A.S. (" ")	.76	.93	1.12	—

* The first 4 sera were tested together with the 4 levels of DNA in one incubation. Comparative data for the 6 normal and multiple myeloma sera were compiled from 2 incubations with 3 μg DNA and 1 each with the other levels.

† Serum was incubated with DNA for 30 minutes at 35°C before addition of reaction mixture and polymerase.

is an absolute requirement of the calf thymus DNA polymerase system(7). Non-fibrous deoxyribonucleoprotein and thermally denatured DNA have also been reported to be more reactive antigens than native DNA in complement fixation tests with SLE sera(8,9).

Effects of the sera on DNA synthesis are reported in Tables II-IV. The first and last

TABLE IV. Effect of Preincubating Enzyme with 1.5 μg DNA Primer Before Addition of Serum. cpm with serum/cpm with NaCl

Serum	DNA and enzyme preincubated together	DNA and enzyme preincubated separately
D.J. (SLE)	.67	.23
E.B. (FS)	.61	.10
I.B. (")	.81	.44
R.S. (")	.92	.27
cpm NaCl control	531 ± 36	245 ± 0

tubes of each series of incubations were controls containing .05 M NaCl (against which the sera had been dialyzed) in place of serum. Results are expressed as quotient of radioactivity in DNA with serum divided by radioactivity with NaCl controls. The averages of 29 of the 34 duplicate controls run during these experiments were within 4% of the duplicate mean; the remaining 5 were within 7%. To include a larger number of different pathological and normal sera in the same incubation, duplicate serum samples were not routinely tested. It was felt that a comparison of values for the same serum in different incubations would be more valuable. However, duplicates were included in several experiments involving preincubation of serum with DNA. Averages of duplicates with 3 normal sera and 5 inhibitory pathological sera were within 6% of the duplicate mean.

Two preparations of calf thymus polymerase, referred to as P1 and P2, were used in these experiments. With both preparations in the reaction system employed, 6 μg or less of primer DNA was rate-limiting and 12 μg or more was non-limiting in a 30-min incubation period. Table II summarizes data obtained with P1 and 3 levels of DNA. Experiment 1 consisted of 3 incubations, each including both normal and pathological sera. Corresponding preincubated and non-preincubated samples were tested in the same incubation. Results in parenthesis are from a second incubation of the serum under the given condition. Radioactivity of NaCl controls ranged from 1608 ± 46 to 2237 ± 22 cpm.

Of the 14 sera from collagen diseases, all but one were less than 0.9 of the NaCl control value when the samples were preincubated; 7 were .65 or less of control value. Without preincubation, 13 did not inhibit (.92-1.14) and D.B. was markedly less inhibitory. Seven normal sera and 5 from multiple myeloma and neutropenias not associated with collagen disease did not inhibit when preincubated (.93-1.14 × the control value). All but one of the normal sera were stimulatory when not preincubated (1.25-1.50).

Experiment 2 consisted of one incubation with 1.5 μg DNA and 9 collagen disease sera which had shown varying degrees of inhibition with 3 μg DNA. Three of these sera were also tested with 3 μg DNA; values corresponded reasonably well with those for the same sera in Exp. 1. Two sera (I.L. and I.B.) which were not very active with 3 μg DNA were more than twice as inhibitory with 1.5 μg, whereas another serum (K.S.) showed little inhibition with either level of DNA. Experiment 3 consisted of one incubation of 10 sera which had inhibited when 3 μg DNA was used. With a surplus of primer, 18 μg, none of these sera was inhibitory (.92-1.13 × the control value).

Since preincubation with a limiting amount of DNA was required to demonstrate marked inhibition by the collagen disease sera, it was apparent that inhibition of enzyme *per se* was not a major effect. Rather, the sera must be affecting the combining of enzyme with DNA primer. This would occur if a serum factor(s) combined with DNA on at least part of the active site for the polymerase (or in such a way that part of the active site for the enzyme was covered). Accordingly, 4 inhibitory sera were tested with 3 limiting levels and 1 non-limiting level of primer, and enzyme P_2. Results are shown in the upper half of Table III. The combination of serum factor(s) with DNA may be partly dissociable or may not occur completely within the 30-min preincubation of serum with DNA, since with the SLE and FS sera approximately the same amount of inhibition was found at 6 μg as at 3 μg. Additional RA sera would have to be tested to determine if the lesser inhibition, which did not differ greatly at the 3 limiting levels of DNA, was attributable to the same type of factor(s) present in lower concentrations.

For comparison, results with 3 multiple

myeloma sera (containing excess globulin) and 3 normal sera are compiled in the lower half of Table III. Data for 3 μg DNA (and P1) are taken from Table II. Data for 6 and 12 μg are from one incubation with each of these levels of DNA and enzyme P1. The normal and multiple myeloma sera were tested with 1.5 μg DNA and P2 in one incubation run consecutively with the experiment in the upper half of Table III. With this very small amount of DNA there was some inhibition by normal and multiple myeloma sera; such results point out the importance of the primer level in testing effects of sera on DNA polymerase systems.

If the major cause of the decreased polymerization is a competition between enzyme and serum factors for active sites on DNA primer, an initial preincubation of enzyme with DNA should decrease the inhibitory effect of serum. The experiment of Table IV was carried out as follows. In the first group, enzyme P2 was incubated with 1.5 μg DNA at 35°C for 15 min before serum was added to the mixture and the incubation continued for 30 min. Radioactive substrate was then added to start the reaction. In the second group, DNA and enzyme were incubated separately for 15 min, serum was then added to the DNA, and incubation of this mixture and of the enzyme (held separately) was continued for 30 min. Substrate and enzyme were then added to start the reaction. Inhibition was 2-6× as great in the group where the enzyme was not preincubated with DNA (Table IV). These data can also be interpreted to show that the serum factor is directed against DNA rather than against a DNA enzyme protein complex formed during the incubation. The leading evidence against this possibility, however, is the requirement for preincubtaion of serum with DNA to demonstrate marked inhibition.

Discussion. Stollar *et al.*(10) have used inhibition of complement fixation by oligonucleotides to investigate the antigenic determinant of denatured DNA reacting with antibodies in 2 SLE sera. They concluded that the determinant was a pentathymidylic acid with one serum and a tetrathymidylic acid with the other. Such thymidylate units might reasonably be expected to form part of the active site for DNA polymerase.

The 5 sera which showed precipitins against DNA in the agar tube test were all markedly inhibitory in the polymerase assay. Only 2 of the other sera (X.R. and D.W.) were in the same inhibitory range; an explanation is lacking for the negative precipitin results with these 2 sera. However, a more sensitive immunological test might have detected antibodies in additional sera. Also, levels of other factors in these sera could be important in determining the results of the two tests.

Normal and pathological sera may be expected to contain various factors, both inhibitory and stimulatory, which would affect the polymerase reaction. Care must obviously be exercised in interpreting the effects of sera on such enzyme systems, since the relative importance of these factors might change with varying conditions of the assay. For example, the stimulation by normal serum did not occur to any great extent after the serum was preincubated with DNA. We have not attempted to define these factors. Levels of serum enzymes, particularly deoxyribonucleases, would be important. However, under the conditions of our assay, certain sera from collagen diseases have caused reproducible inhibitions not obtained with other pathological sera containing elevated globulin levels or with normal sera.

It is interesting that the 4 FS sera were as inhibitory in the polymerase system as the most active SLE sera tested. These FS sera, all from patients with severe leukopenias, had shown no marked effect over that of normal sera on DNA synthesis by white blood cells *in vitro*(3). The activity of these sera in the soluble enzyme system does not prove the inability of the serum factors to penetrate to the nuclei of viable cells. However, it does demonstrate that once these serum factors gain access to DNA, an inhibition of DNA synthesis can result.

Summary. The effect of normal and pathological sera on synthesis of DNA with purified calf thymus polymerase was tested. Twelve of 14 sera from collagen diseases were inhibitory. A limiting amount of DNA primer and

preincubation of serum with this primer before addition of enzyme were necessary to demonstrate marked inhibition. Seven normal sera and 5 from multiple myeloma and neutropenias not associated with collagen disease did not inhibit after preincubation of serum and primer. Without preincubation, the normal sera were stimulatory. Incubation of enzyme with primer before addition of inhibitory serum markedly reduced the inhibition. Results indicate that the combination of serum factor(s) with DNA covers up at least part of the active site for the polymerase.

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Disposition of S^{35}-Prochlorperazine in the Jaundiced Rat.* (28146)

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We have recently reported on the excretion of S^{35}-prochlorperazine in experimentally stressed rats(1) and the disposition of this agent in normal as well as chronically-treated rats(2). These studies revealed that information derived from certain investigations in normal animals may be misleading when applied to agents which will be used in abnormal physiological states. They also demonstrated the major importance of the fecal route of excretion of prochlorperazine and the contribution of the biliary route to elimination in the feces. For this reason we felt it of interest to investigate the distribution and excretion of this agent in rats subjected to ligation of the bile duct.

Methods. Male albino Holtzman rats having an initial weight of 250 to 350 g were employed. All animals were allowed food and water *ad libitum* up to the time of drug administration. Rats employed in excretion studies were allowed free access to food and water during the entire period. Each rat received 25 mg/kg of prochlorperazine base (as the dimaleate) as a 1.2% suspension in 4% acacia intraperitoneally. Within the separate studies, all animals were dosed from the same freshly prepared drug suspension. Each rat received 10 μc of S^{35} per 275 g of body weight.

Rats to be made jaundiced were anesthetized with ether and the bile duct was ligated with a single suture placed at about one-third of the length of the duct from the liver. Rats prepared in this manner were used 12 hours later. Preliminary studies revealed that rats operated on as above lived longer than 96 hours, the maximum duration employed in this investigation.

Distribution and excretion studies were conducted as previously described(2).

Results. Fig. 1 and 2 represent S^{35} levels in cerebrum, liver, kidney, fat, muscle, and blood of jaundiced and control rats, respectively, following administration of S^{35}-prochlorperazine. With the exception of fat, S^{35} levels one hour after drug administration were higher in all tissues in jaundiced than in control rats. Despite the higher initial levels,

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