

# Detection of Anti-DNA Antibody Using Synthetic Antigens

## CHARACTERIZATION AND CLINICAL SIGNIFICANCE OF BINDING OF POLY(DEOXYADENYLATE-DEOXYTHYMIDYLATE) BY SERUM

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**ABSTRACT** Virtually all preparations of DNA used to detect antibody to native DNA (nDNA) by binding assays have been found to be subtly contaminated by single stranded DNA. Because recent DNA binding data have directly challenged the unique role previously attributed to these antibodies in systemic lupus erythematosus (SLE), resolution of the consequent ambiguity is of theoretical and practical importance. It is proposed that a synthetic nDNA molecule (dAT) might circumvent this difficulty by being antigenically equivalent to nDNA while, on theoretical grounds, lacking significant contamination with single stranded DNA or other cellular antigens. These expectations were generally confirmed by biochemical and immunological analyses.

In clinical studies, sera from 124 patients with SLE and from controls were examined for their ability to bind dAT.

In contrast to results with KB binding, patients with non-SLE rheumatologic disorders were indistinguishable from normals by dAT binding.

dAT binding was elevated in 85% of sera from SLE patients with clinically-judged active nephritis but in only 9% of those with inactive renal disease. Active non-renal disease, including cerebritis, was not associated with increased dAT binding. Individual non-lupus sera which bound increased amounts of KB DNA, failed to bind dAT. It is suggested that such binding resulted from contaminating non-nDNA antigens. When elevated, dAT binding, like KB binding, varied with disease activity and might thus be useful as a parameter thereof. In several patients elevated dAT binding led to the finding, on biopsy, of clinically silent, active, diffuse proliferative nephritis.

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It is concluded that use of synthetic nDNA antigens such as dAT may offer theoretical and practical advantages over naturally-derived preparations in detecting anti-nDNA, both clinically and for investigational purposes. Also, caution is urged in interpreting DNA binding data derived from incompletely characterized systems, particularly with regard to the occurrence of anti-nDNA antibodies in serum.

### INTRODUCTION

Measurement of antibody to native DNA (nDNA)<sup>1</sup> has played a central role in our understanding of systemic lupus erythematosus (SLE) and in its diagnosis and clinical management. In recent years techniques of measuring the direct binding of radiolabeled antigen by antibody have become available. One of these in particular, the ammonium sulfate precipitation method (1, 2) has come into wide clinical use. Concomitantly, a major difficulty has emerged concerning the homogeneity of the radiolabeled DNA used as antigen in these assays. With storage, decreased specificity and increased binding by normal sera have been observed. In addition, variability of binding resulting from use of DNA from

<sup>1</sup> Abbreviations used in this paper are: A + T, deoxyadenosine plus deoxythymidine content of DNA as percent of total base content; anti-T, rabbit antithymidine antiserum; CT, DNA prepared from calf thymus; dAT, alternating copolymer of deoxyadenylate and deoxythymidylate; d(A,T), random copolymer of deoxyadenylate and deoxythymidylate; den-CT, heat denatured calf thymus DNA; dGC, alternating copolymer of deoxyguanidylate and deoxycytidylate; GN, glomerulonephritis; KB, DNA prepared from human KB cells; MAK, methylated albumin kieselguhr; 0.7MAK, KB eluting from MAK in 0.7 M NaCl; nDNA, native DNA; SLE, systemic lupus erythematosus; SN-CT, CT treated with ss-nuclease; ssDNA, single stranded (denatured) DNA; SN-KB, KB treated with ss-nuclease; ss-nuclease, single-strand-specific endonuclease from *N. crassa*.

different sources or even from different batches of the same source have also presented difficulty regardless of whether the radiolabeled moiety was incorporated during cell growth or after DNA extraction. Several recent reports have supported the possibility that at least some of this variability resulted from contamination with single-stranded or denatured DNA (ssDNA) (3, 4). This seems likely to have arisen during isolation and storage of the DNA and is probably related in part to the rigidity of the double helical molecule, long recognized as being vulnerable to breakage and partial denaturation as the result of exposure to shearing forces (5, 6).

Recently several reports have appeared describing apparent nDNA binding by sera from individuals who did not have SLE (7-10).

If, as suggested, this resulted from the presence of antibody to nDNA, important questions are raised regarding the unique role this antibody has been thought to play in the pathogenesis of SLE. Also, the clinical value of measuring DNA binding may be thereby diminished. Since these reports have all employed naturally derived preparations of DNA, interpretation depends on excluding the possibility that the observed binding was due to a non-nDNA contaminant such as ssDNA.

The purpose of this report is threefold: first, to confirm the apparently ubiquitous ssDNA contamination of routinely prepared mammalian nDNA preparation by using three independent methods; second, to propose and evaluate a stratagem for avoiding the consequent difficulty; and third to thereby re-examine the significance of antibodies to nDNA in sera from patients with SLE and related disorders.

It was reasoned that a self-complementary alternating copolymer of deoxyribonucleotides, because of the extreme rapidity of its renaturation reaction (11) (assumed to be of the "type I," or intramolecular, variety of Geiduschek [12]), ought to exhibit exceptional stability of the double stranded configuration at conditions under which the binding assay is performed. It would, in fact, on kinetic grounds, be expected to be incapable of maintaining a stable single stranded state, even if previously subjected to denaturing conditions.

Since the antigenic sites responsible for binding anti-nDNA are thought to be located entirely on the exposed deoxyribosephosphate backbone and to be independent of the individual bases (13), it might be expected that such a substance would possess all or most of the binding sites intrinsic to naturally-occurring nDNA. Hence, it ought to provide a defined, stable molecule free of contamination with either ssDNA or other cellular antigens.

It would also be expected to be easy to store and handle without risk of denaturation. Hence its use might

be expected to improve reliability and ease of detection of anti-nDNA. The data to be presented are considered to support these expectations for the alternating copolymer of deoxyadenylate and deoxythymidylate (dAT). An additional advantage resulting from the use of dAT in place of less homogeneous natural antigens may be closer correlation of its binding with activity of lupus nephritis.

## METHODS

*DNA preparations.* [<sup>14</sup>C] human DNA (KB) extracted from KB cells grown in tissue culture was purchased in multiple batches from Electro Nucleonics, Bethesda, Md. An additional preparation of [<sup>14</sup>C] KB DNA as well as a preparation of calf thymus (CT) DNA, radiolabeled with <sup>125</sup>I, were the gifts of Dr. Robert Lightfoot. These preparations were stored at 4°C or as recommended by the supplier. They were used within 4 wk of receipt. [<sup>14</sup>C]dAT and the alternating copolymer of deoxyguanylate and deoxycytidylate (dGC) (sp act 2.5 μCi/μM) were obtained from General Biochemicals, Chagrin Falls, Ohio. Use of three different dAT preparations from this source resulted in identical binding results on multiple sera. Storage for up to 2 yr at -20°C produced no apparent adverse effect. Recently, material synthesized in a different laboratory and purchased from Grand Island Biological Co., Grand Island, N. Y. was examined. Although binding studies performed with this preparation were qualitatively similar to those utilizing the material from General Biochemicals, all sera, including normals, gave significantly higher binding results. Sonication of the material from Grand Island Biological Co. (with a Sonifier, model W185, Branson Sonic Power Co., Danbury, Conn. using the "special microtip" at a setting of "2" for 10±2 s at 4°C with a 200-μl sample containing 5 μg/ml dAT in the borate-saline buffer described below) resulted in binding values similar to those obtained using the material from General Biochemicals. However, experience with the newer preparation is still too limited to draw general conclusions. Neither deproteinization with phenol nor heat denaturation and subsequent renaturation affected the binding. Because of the effect of sonication, it was assumed that the significant difference between dAT preparations from these two sources is in dAT chain length. Preliminary results of chromatography of these preparations on Biogel A-150m (Bio-Rad Laboratories, Richmond, Calif.) support this interpretation. As a practical matter, it is recommended that, if necessary, an uncharacterized batch of dAT be first sonicated (graded DNase treatment might also suffice) just enough for normal sera to bind between 0 and 10%. Shearing alone, by passage through a 25 gauge needle, apparently did not lower the molecular weight of this material adequately. Also, excessive sonication was found capable of almost eliminating all binding, presumably by production of small fragments capable of inhibiting binding of longer chains.<sup>2</sup>

All data in the present report were obtained using dAT from General Biochemicals which did not require sonication.

The dGC preparation contained nonacid-precipitable radioactivity that was removed by passage through Sephadex G50 before use. Both dAT and dGC became cloudy on stor-

<sup>2</sup> Preliminary data suggest that sonicated dAT that is neither excluded from nor in the low molecular weight peak of an A-150m column has the approximate binding characteristics described.

age but could be clarified by addition of 0.5 M sodium chloride to the stock solutions. Before use, radiolabeled dAT preparations were held for 2 h at 47°C in 0.3 M sodium chloride containing 0.05 M borate buffer, pH 8.0 and then sheared by passage 10 times through a 25 gauge, ½ inch needle. CT DNA was purchased from Worthington Biochemical Corp., Freehold, N. J. Heat denatured CT DNA (den-CT) was prepared by heating a solution of CT DNA (at 1 mg/ml of 0.15 M sodium chloride containing 0.05 M sodium borate, pH 8.0 (borate-saline buffer) at 100°C for 10 min followed by rapid quenching in ice. The endonuclease from *Neurospora crassa* specific for ssDNA (ss-nuclease) (14) was purchased from Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.

SS-nuclease-treated KB DNA (SN-KB) or CT DNA (SN-CT) was prepared under the same conditions used for the ssDNA assay described below except that 5 U of enzyme was used/0.1 ml of reaction mixture and that incubation at 37°C was continued for 16 h after which 2 additional U/0.1 ml was added followed by 4 h of further incubation. No increase in acid-soluble radioactivity was found during the final 4 h. After incubation the reaction mixture was passed over Sephadex G50 in borate-saline buffer and the excluded peak of DNA collected. It was used without concentration. DNA fractions purified on methylated albumin kieselguhr (MAK) columns (see procedure below) and by ss-nuclease digestion were used within a week of preparation. Additional purification of SN-KB on MAK was found not to alter its binding and was not routinely performed. Freezing and exposure of these DNA preparations to unnecessary shear were avoided. They were stored at 4°C over chloroform.

*Sera and binding assays.* Sera were obtained in a routine manner, separated within 6 h of collection and stored at -20°C.

The ammonium sulfate binding assay was performed in duplicate as previously described (2), except that for all but the clinical studies the antigen concentration was 0.016 µg/100 µl of assay mixture. Under these conditions, normal sera bound less than 20% dAT. For the clinical studies 0.1 µg/100 µl was used. Duplicate assays not matching to within 10% were repeated. Data were analyzed by Student's *t* test (15) and are generally expressed as mean ± SEM.

Absorption of sera was performed by addition of the indicated nucleic acid preparation at 100 µg/ml, incubation at 37°C for 60 min and then at 4°C for at least 2 h. Counter-immunoelectrophoresis was performed as previously described (16). Rabbit antisera to human immunoglobulins and to human C'3 were purchased from Meloy Laboratories, Inc., Springfield, Va. Rabbit antiserum specific for thymidine (anti-T) was the gift of Dr. David Koffler. Its preparation and specificity have been previously described (17). When inhibitors were used, they were added to the reaction mixture before the radiolabeled antigen.

*Assays for ssDNA.* Anti-T was diluted fourfold in normal rabbit serum before use in binding assays. The latter were performed as in the usual ammonium sulfate precipitation technique described above.

MAK was prepared and used as previously described (18). DNA solutions were applied to MAK in columns constructed from Pasteur pipettes and were washed with 0.05 M sodium phosphate, pH 6.7, that was 0.1 M in sodium chloride. Sequential elution was then carried out with similar phosphate buffers containing 0.5 M, 0.7 M, and either 0.9 or 1.0 M sodium chloride. A final elution was performed with 1 M sodium phosphate, pH 11.6. Material eluted with buffer containing 0.7 M NaCl was considered "native" (0.7

MAK). That requiring higher molarity or increased pH was considered "denatured" (18). Results are reported as percent of total DNA recovered after having been bound to MAK. Total recoveries ranged from 65 to 95% for KB preparations and were greater than 85% for the alternating copolymers.

Enzymatic assays for ssDNA were carried out in a total volume of 0.1 ml containing 0.1 M Tris buffer, pH 8.0, 0.01 M MgCl<sub>2</sub>, 2 U ss-nuclease, and 1 µg DNA. In each case the result is expressed as percent nuclease-produced solubility in 10% cold perchloric acid (in the presence of "carrier" CT DNA, 500 µg/ml) after 2 h of incubation at 37°C. Timed portions were taken to assure completion of the reaction by this time. All samples were ≥95% precipitable before enzyme digestion. In two instances, ss-nuclease susceptibility was determined by retardation on Sephadex G50 chromatography. Addition of 0.003 M mercaptoethanol to the reaction mixture, increasing the enzyme concentration fivefold or prolonging the incubation fivefold did not significantly alter the results.

*Clinical studies.* All patients with SLE fulfilled the preliminary criteria of the American Rheumatism Association for diagnosis (19). Diagnoses on other patients were agreed upon by two experienced rheumatologists without knowledge of DNA binding results.

Active nephritis was said to be present when the patient exhibited one or more of the following: persistently abnormal urinary sediment after exclusion of other causes; clearly increasing proteinuria as measured on 24-h urine collections; and deteriorating renal function as reflected by progressively increasing serum creatinine concentrations. Renal biopsy criteria alone were used in only one patient who demonstrated diffuse proliferative glomerulonephritis. In other patients, biopsy findings supported clinical evidence of active nephritis by demonstrating more than minimal activity by light microscopy (20). In the absence of these findings, nephritis was said to be absent or inactive (in patients who had previously demonstrated active renal disease). It is recognized that these criteria are crude and will misclassify the occasional patient with clinically occult nephritis, particularly since renal biopsies were not usually performed except when there were clinical indications of renal involvement (with the exceptions noted below). All available biopsies were interpreted without knowledge of dAT binding results.

Neurologic involvement was said to be present if otherwise unexplained neurologic deficits appeared or if gross psychotic behavior was unequivocally improved after treatment. Patients with psychiatric disturbances alone that seemed explainable as a manifestation of corticosteroid toxicity were not included in this group.

## RESULTS

*Detection of single strandedness in natural DNA preparations.* Three techniques were used to examine radiolabeled antigen preparations intended for use in the DNA binding assay.

First, binding to rabbit anti-T was above 10% in all five preparations examined and above 25% in two of these. Normal rabbit serum bound <10% of these preparations. Since completely double-stranded DNA would be expected to lack exposed antigenic sites specific for the nucleoside bases this binding is taken as evidence of such exposure and therefore of single-

strandedness. As expected, heat denaturation increased the binding in all preparations. Second, susceptibility to the action of ss-nuclease varied from 9 to 37% in a group of nine different preparations. Undigested control DNA preparations were <5% acid soluble. The synthetic copolymer, dGC, assumed to lack ssDNA, was <5% acid soluble after ss-nuclease digestion. Again, these results are taken as evidence of single-strandedness in the natural nDNA preparations, subject to considerations of enzyme specificity as discussed below. Third, three preparations of KB were bound 26, 70, and 78%, respectively, by MAK under conditions where ss-DNA but not nDNA would be expected to bind (18). This was in contrast to the purified and synthetic preparations discussed below.

Thus, of 14 natural DNA preparations tested, all contained single-stranded regions detectable by one or more of these methods, confirming previous reports (3, 4).

*Attempted detection of single-strandedness in purified natural, and synthetic nDNA preparations.* To examine the antigenic structure of dAT it was intended to compare it with preparations of natural nDNA, either untreated as above, or treated by either ss-nuclease or MAK chromatography in an attempt to remove at least some of the contaminating ss-DNA. Accordingly these three preparations were examined for the presence of ss-DNA as before. Representative results of these assays are shown in Table I. In general, these results were confirmed on more than one preparation. Also shown for comparison are data on ss-DNA (den KB), and on dGC.

Treatment of dAT with ss-nuclease could not be used as a measure of single-strandedness because it was found that slow but complete hydrolysis of the polymer resulted with the enzyme preparation used. At least two explanations seem reasonable. The first is that the hydrolysis resulted from contamination of the enzyme by the recently described nuclease activity from *N. crassa* (21) specific for deoxyadenylate and deoxythymidylate copolymers (d[A,T]nuclease). Perhaps a more likely possibility is that the transiently available single stranded regions known to occur in dAT at 37°C ("breathing") (22) acted as substrate for the enzyme resulting in slow but ultimately complete hydrolysis in a manner analogous to that described with the cumulative temperature-dependent reaction of dAT with formaldehyde (23). Regardless of the explanation, demonstration that the enzyme lacked nuclease activity for synthetic double stranded DNA required use of dGC, which would be expected to have a secondary structure similar to that of dAT, but to be much more resistant to temperature-dependent "breathing." As noted, no hydrolysis of dGC resulted from treatment with ss-nuclease. Accordingly, the amount of hydrolysis found in

TABLE I  
Assays for ssDNA in Purified and Synthetic  
nDNA Preparations

DNA preparation	Anti-T binding*	Susceptibility to ss-nuclease	Behavior during MAK chromatography "native"† "denatured"‡	
	%	%	%	%
0.7 MAK	16	14	ND	ND
SN-KB	3	ND	91	9
den KB	74	81	10	90
dGC	ND¶	4	92	8
dAT	1 (3)**	100‡‡	98	2

\* Antigen concentration was 0.1 µg/100 µl.

† Fraction eluting at 0.7 M NaCl.

‡ Fraction eluting at >0.7 M NaCl or with increased pH.

¶ Shown for comparison.

¶¶ Not done.

\*\* Figure in parenthesis represents binding of dAT put through the usual heat denaturation procedure.

‡‡ See text for discussion.

the KB preparations is taken as a measure of their content of ss-DNA. An alternative interpretation would be to consider the extent of hydrolysis to be a reflection of the deoxyadenosine plus deoxythymidine (A + T) content (or distribution) in KB DNA. However, the d(A,T)-nuclease suspected of contaminating the ss-nuclease preparation has been reported to be incapable of producing acid-soluble fragments from a murine DNA preparation despite its demonstrated ability to produce double-stranded breaks in regions of high (A + T) content (21). Instead, it was found to produce fragments no smaller than a certain minimum (acid-insoluble) size, suggesting that the acid solubility produced from KB resulted from a different mechanism, presumably hydrolysis of ssDNA regions.

Both alternating copolymers as well as the nuclease-treated DNA preparations lacked significant ssDNA by all three methods (except for the sensitivity of dAT to ss-nuclease as noted). Also shown in Table I is the result of anti-T binding of a preparation of dAT that was subjected to the same heat denaturation procedure used on the natural DNA preparations to demonstrate the complete and rapid reversibility of heat denaturation of this substance (12).

The 0.7 MAK preparation appeared still to contain a small amount of ssDNA contamination as indicated by its ss-nuclease susceptibility and also perhaps by the slight elevation of its anti-T binding. This conclusion is supported by the comparative binding studies shown below.

*Counterimmunoelectrophoresis studies.* Serum from six patients with SLE which gave specific lines of precipitation with native CT DNA by counterimmunoelectrophoresis (as demonstrated by abolition of the lines

both by preabsorption of the sera with CT and by pre-treatment of the antigen with deoxyribonuclease) were examined by this technique for antibody to dAT.

In all cases distinct lines of precipitation were obtained which were completely abolished by preabsorption of the sera with CT. Similarly, absorption of the sera with dAT completely eliminated precipitation with CT. Absorption with RNA did not affect reactivity with either preparation.

*Immunoprecipitation of antibody-bound dAT.* To confirm that primary binding of dAT resulted from interaction with immunoglobulin the following experiment was performed. Three SLE and three non-SLE sera were allowed to react with dAT (at 0.016  $\mu\text{g}/100 \mu\text{l}$ ) in the usual manner (2). In place of saturated ammonium sulfate, 10  $\mu\text{l}$  of rabbit antihuman immunoglobulin or, as control, of antihuman C' 3 were added to each 100  $\mu\text{l}$  reaction mixture. The mixtures were then incubated at 37°C for 1 h and at 4°C overnight after which radioactivity in the supernates and precipitates was determined. Specific immunoglobulin binding of dAT ranged from 35 to 56% in the SLE sera and was <5% in the three non-SLE sera (all of which bound >50% KB).

*Comparative binding studies.* The binding results on selected sera from patients with SLE and non-SLE collagen diseases using dAT and either KB, 0.7 MAK or SN-KB were compared at both 0.016 and 0.1  $\mu\text{g}/100 \mu\text{l}$  (Fig. 1). Since the high and low antigen concentrations did not differ in their degree of correlation the data for both are shown together. Binding of dAT correlated best with SN-KB and least well with KB. The 0.7 MAK fraction differed only slightly if at all from the latter. The KB binding was uniformly high on those sera which gave high dAT binding results. All low KB binding sera bound negligible amounts of dAT. A similar pattern was seen even if the binding of 0.05  $\mu\text{g}$  of dAT was compared to that of 0.1  $\mu\text{g}$  of KB (data not shown). Although the percentage of dAT bound was uniformly increased in this way as expected, correlation with KB DNA binding was not improved.

A number of studies have demonstrated that most SLE sera with antibodies to nDNA also react with ssDNA (13, 24-26). Hence, one would expect that if dAT contains most or all of the antigens of nDNA, any serum containing antibody to it would also react with ssDNA, thus accounting for the high KB binding by all sera reacting significantly with dAT. Conversely all those sera lacking KB reactivity also lack dAT binding as would be expected. That the difference in binding between KB and dAT is largely due to ssDNA contamination of the former is further suggested by the close correlation between binding of dAT with KB after, but not before, treatment with ss-nuclease (i.e. with SN-KB). That the 0.7 MAK fraction may still not be free of ssDNA contamination, as suggested above, is further supported by the poorer correlation of its binding with dAT than was shown by SN-KB. It should be noted that even accepting the specificity of *N. crassa* nuclease for ssDNA, the possibility remains that not all antigenic sites sensitive to the action of this enzyme are in fact intrinsic to ssDNA since any antigen attached to such ssDNA regions or dependent on them for its active configuration might be eliminated by treatment with ss-nuclease. Hence the difference in antigenic behavior between SN-KB and KB need not necessarily reflect only the loss of ssDNA antigenic sites per se although this interpretation seems most likely. Similar considerations apply to the comparative binding studies above.

*Binding inhibition studies.* The ability of dAT to inhibit the binding by SLE sera of SN-KB and KB is shown in Table II. As before, KB was bound to a greater extent than was SN-KB. The striking difference in the ability of dAT to inhibit the binding of these two antigens is again consistent with the interpretation that dAT behaves immunologically like nDNA and that KB is bound to a large extent by virtue of non-nDNA antigens.

The binding of den-KB by anti-T was decreased from 74 to 46% in the presence of inhibiting SN-CT but was unchanged in the presence of dAT (75%) sug-

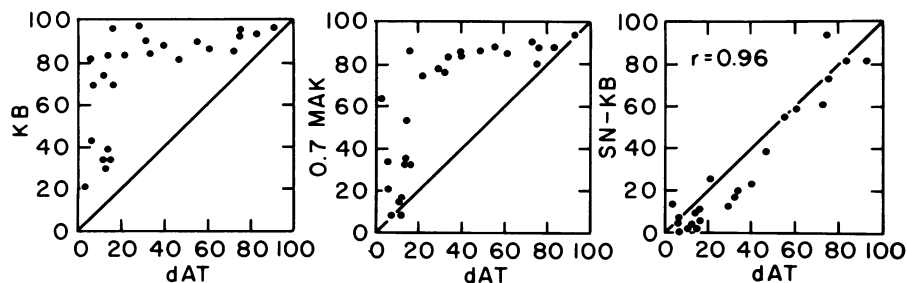


FIGURE 1 Comparison of dAT binding to that of KB, 0.7 MAK and SN-KB (as percent bound) using multiple sera. The correlation coefficient,  $r$ , is 0.96 for dAT with SN-KB binding.

TABLE II  
Binding, by SLE Sera, of KB and SN-KB and  
Inhibition by Unlabeled dAT

Radiolabeled antigen*	N‡	Binding	Inhibition by dAT§
		%	%
SN-KB	8	62.6±6.9	68.6±6.5
KB	10	91.1±1.7	9.7±3.6

\* Concentration 0.016 µg/100 µl.

‡ Number of sera tested (one serum per patient).

§ Concentration 0.1 µg/100 µl.

|| Significantly different from results with SN-KB ( $P < 0.001$ ).

gesting that SN-CT (and therefore, presumably SN-KB) remains subtly contaminated with ss-DNA although the latter does not appear to have been significant in the direct binding assays.

In conclusion, the results of these inhibition studies support the previously observed antigenic similarity between SN-KB and dAT on the one hand and their differences from KB and 0.7 MAK on the other. They are consistent with the interpretation that the latter pair of antigens, in contrast to the former, is highly contaminated with non-nDNA antigens, probably ss-DNA, at least in part.

*Comparative DNA binding by selected non-SLE sera.* Four sera from patients with diseases other than SLE

TABLE III  
Percent Binding by non-SLE Sera of Various DNA Antigens

Serum‡	Antigens*				
	KB	0.7 MAK	1 M MAK	SN-KB	dAT
Patient 4	81	10	82	4	6
Patient 5	69	4	22	7	7
Patient 6	74	19	46	3	12
Patient 8	69	3	62	5	16

\* Concentration 0.016 µg/100 µl.

‡ Diagnoses of patients 4, 5, 6, and 8 were, respectively, progressive systemic sclerosis, dermatomyositis, chronic systemic vasculitis, and progressive systemic sclerosis.

were selected for their ability to bind abnormal amounts of KB. As shown in Table III, this binding was virtually absent from the 0.7 MAK fraction. The 1 M fraction from MAK (presumably largely ssDNA) accounted for much of the original KB binding. These results are similar to those found previously using sera from patients with procainamide-induced SLE (4).

No significant binding was found with SN-KB or dAT, as would be expected if the KB binding were exclusively by virtue of non-nDNA antigenic sites.

*Binding by sera from SLE patients and controls.* In Fig. 2 are shown the results of binding assays on sera from three groups of patients: (a) those with SLE; (b) Patients with rheumatologic diseases other than

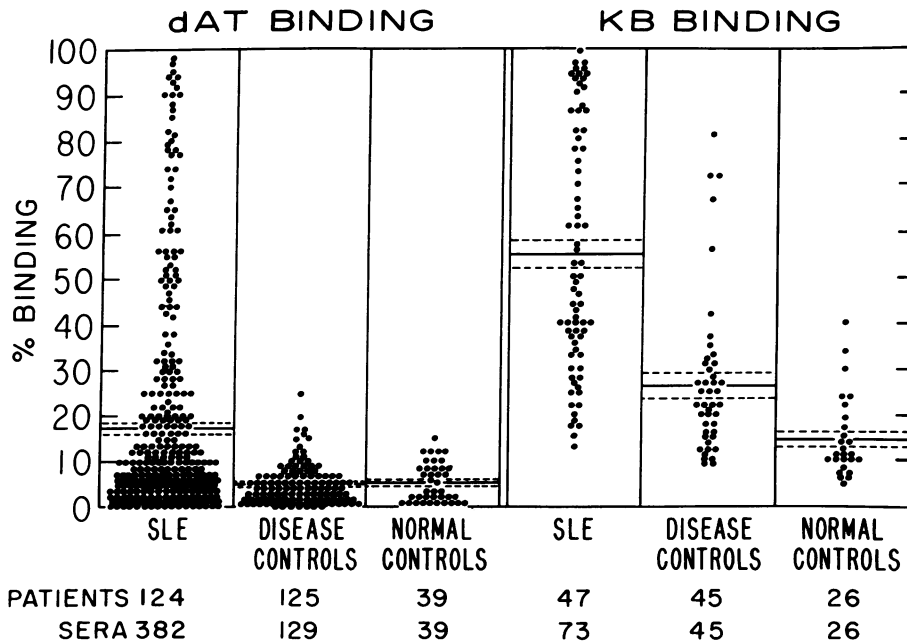


FIGURE 2 Binding of dAT and KB by sera from patients with SLE, with rheumatologic disorders other than SLE, and from normal individuals. In each group, the mean±SEM are indicated by solid and interrupted horizontal lines. In the lower portions of this and the following figure, some points are omitted because of space limitations.

TABLE IV  
Diagnoses of Patients in the Disease-Control Group Tested for dAT and KB Binding

Diagnosis	Number of sera tested by:	
	dAT binding	KB binding
Rheumatoid arthritis	50	15
Progressive systemic sclerosis	18	6
Vasculitis	17	3
Drug-induced SLE	11	1
Dermatomyositis	8	6
Unclassified arthritis	5	3
Raynaud's disease	3	3
Septic arthritis	3	1
Sjögren's syndrome	3	1
Reiter's syndrome	2	2
Mixed connective tissue syndrome	2	—
Sarcoid arthritis	1	1
Ankylosing spondylitis	—	1
Behcet's syndrome	1	1
Erythema nodosum	1	—
Chondrocalcinosis	1	—
Serum sickness	1	—
Myasthenia gravis and arthritis	1	1
Temporal arteritis	1	—
Total	129	45

SLE; and (c) normal controls. Diagnoses in the non-SLE disease control group are shown in Table IV. For comparison, similar data using commercial KB DNA are shown. In the case of dAT, no significant difference was found between the two control groups consisting of non-SLE rheumatologic disease sera and normals ( $P > 0.5$ ) in contrast to corresponding results for KB where the former control group bound significantly more DNA ( $P < 0.001$ ) than did the latter. The five disease control sera which gave the highest KB binding all bound less than 10% of dAT. The data for both dAT and KB include several different preparations of each. Although the dAT preparations gave similar results, KB varied somewhat from batch to batch with resulting differences in binding by normal sera. However, the five high KB binding sera in the disease control group noted above gave similar abnormal bindings with several batches of KB. This was also true using a selected batch of KB DNA whose mean binding was less than 10% by normal control sera. In this regard, it should be noted that all KB DNA batches used (including the one giving  $< 10\%$  normal binding) were shown to contain detectable ssDNA by at least two of the three methods previously described. If the amount of dAT per assay was reduced to 0.05  $\mu\text{g}$ , the percentage bound by all sera was increased but the clear difference

between binding by control and by SLE sera was maintained ( $P < 0.001$ ). To compensate for possible bias introduced by the use of multiple sera from individual patients in the SLE group, statistics were also calculated using only one specimen per patient and selecting the serum giving the lowest binding when more than one were available. The above conclusions remained unchanged with  $P < 0.001$ .

The single patient in the disease control group with clearly elevated dAT binding had clinically typical rheumatoid arthritis accompanied by Raynaud's phenomenon and Sjögren's syndrome. She lacked definite stigmata of SLE. Five other patients with Sjögren's syndrome (including two with rheumatoid arthritis under which they are listed in Table IV) demonstrated normal dAT binding and a later serum from the same patient bound less than 10% dAT. Although it is possible that this patient will yet develop manifestations of SLE, she is considered an exception to the otherwise complete specificity of elevated dAT binding for SLE. This specificity was also demonstrated by uniformly normal dAT binding by eleven patients with drug-induced lupus. Ten had received procainamide and one hydralazine. Normal binding in these patients has also been reported using MAK-purified KB DNA (4).

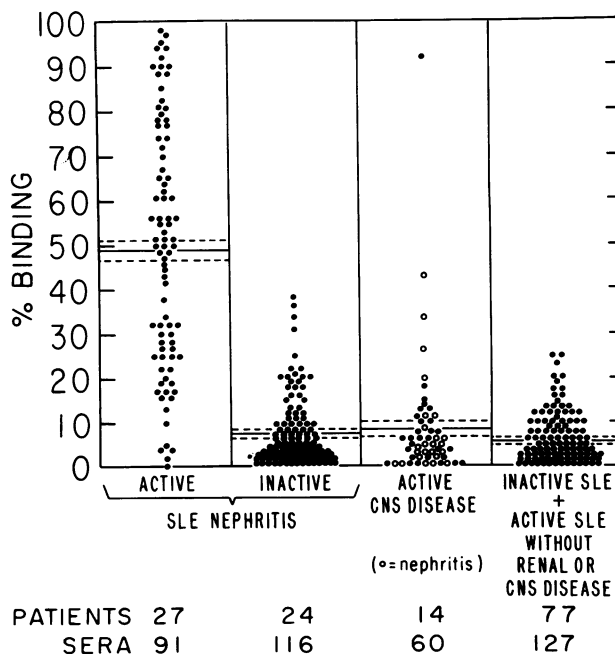


FIGURE 3 Binding of dAT by sera from SLE patients grouped according to activity of disease and type of involvement (renal or central nervous system [CNS]). In the group with active CNS disease, co-existent nephritis is indicated by the open circles (O). As in Fig. 1, mean  $\pm$  SEM is shown in each case.

*Correlation with activity of disease.* The degree of dAT binding appeared to correlate strongly with the presence of active nephritis (Fig. 3) as defined above. 85% of sera from patients with nephritis judged to be active bound more than 20% dAT while this was true for 9% of sera from patients with inactive nephritis. Only 2% of sera from patients with no clinically apparent renal involvement bound more than 20% of dAT. However, as discussed below, this figure is biased by the fact that two patients who would otherwise be included in this group underwent renal biopsy on the basis of elevated dAT binding and were thereby classified as having active nephritis. Correction as above for possible bias due to inclusion of multiple sera from single patients did not significantly alter these findings. This was true regardless of whether the specimen giving the highest or lowest binding for each patient was used for comparison of active and inactive renal disease groups.

Active neurological disease was associated with increased binding only in the presence of co-existent active nephritis with a single exception. This patient had unequivocal neurological disease with psychosis and extrapyramidal abnormalities that became clinically apparent several months after the high dAT binding was found and appeared to improve upon treatment with glucocorticoids and cytostatic agents. However, during the period of active neurological disease, dAT binding was normal. Because this activity occurred sufficiently close to the time of the high dAT binding to make her inclusion in this group uncertain, she is so included. At the time of the high dAT binding, she lacked all evidence of renal disease. Biopsy was not performed. 2 yr later, she presented with overt nephrosis and psychosis, at another institution. Neither serum nor pathological material were available at that time. Although it seems possible in retrospect that clinically inapparent nephritis may have been present at least as a transitory event at the time of elevated dAT binding, this patient is considered an exception.

The group including patients with clinically inactive SLE as well as patients with active SLE who lacked renal or neurological involvement was barely distinguishable by dAT binding from the group with clinically inactive nephritis ( $0.25 < P < 0.05$ ). The data do not allow conclusions as to whether the elevated dAT binding exhibited by individual sera in both these groups reflected the prevalence of occult active nephritis although this is one possible interpretation.

*Serial assays on individual patients.* Increased binding of dAT has preceded or accompanied clinical exacerbations of SLE nephritis in several patients followed over the past 3 yr. As a harbinger of clinical activity it seems to be neither more nor less sensitive, nor to give earlier changes, than the appearance of

elevated KB binding, although experience with serial studies is still too limited to be certain of these conclusions. Fig. 4 illustrates a clinical exacerbation of nephritis accompanied or slightly preceded by increasing dAT (and KB) binding. This patient also illustrates, incidentally, conversion of the mesangial to membranous pattern of glomerulonephritis (GN), both periods of activity being associated with elevated dAT binding.

The patient whose clinical course is shown in Fig. 5 underwent biopsy solely on the basis of elevated dAT binding, having had completely normal urinalyses and parameters of renal function. The biopsy demonstrated diffuse proliferative nephritis. Severe symptomatic pericarditis required treatment with prednisone and was accompanied both by suppression of clinical extra-renal disease and a fall in dAT (and KB) binding. One additional patient underwent renal biopsy primarily because of the elevated dAT binding, having had only minimal proteinuria over the previous year. A diffuse proliferative lesion was again found. Over the 3 mo after biopsy, progressive and irreversible renal failure appeared for the first time.

## DISCUSSION

From a large body of data, it has been concluded that sera from patients with SLE are, in general, able to recognize at least three groups of antigens on DNA (13, 25, 26). The first is found only on ssDNA and generally involves one or more specific bases. Antibody recognizing such antigens may be prepared by immunization of experimental animals. Since the individual bases are located within the double helix of nDNA they are sterically protected from interaction with antibody thus accounting for their detection on ssDNA exclusively. The second group of antigens is found on the deoxyribosephosphate backbone which is exposed both in nDNA and in ssDNA. Hence the two forms of DNA cross-react with antibody to this group of antigens. The third group is found exclusively on nDNA and probably requires the double helical configuration of the molecule, presumably the common B-form described by x-ray crystallography and known to be present in dAT (27).

Antibodies directed to the second and third groups of antigens, that is, those found on nDNA, have not convincingly been shown to occur in any situation, either experimental or natural, other than SLE in humans or animals (13). Hence a central requirement for all proposed pathogenetic mechanisms for the disease has been to account for this unique phenomenon. In addition, it has formed the basis of a diagnostic test which has also proved a useful guide to therapy since the antibody as measured by the Farr assay appears to vary in amount with disease activity, particularly in patients with nephritis (2, 28). Recently these concepts have been challenged by several reports suggesting the oc-



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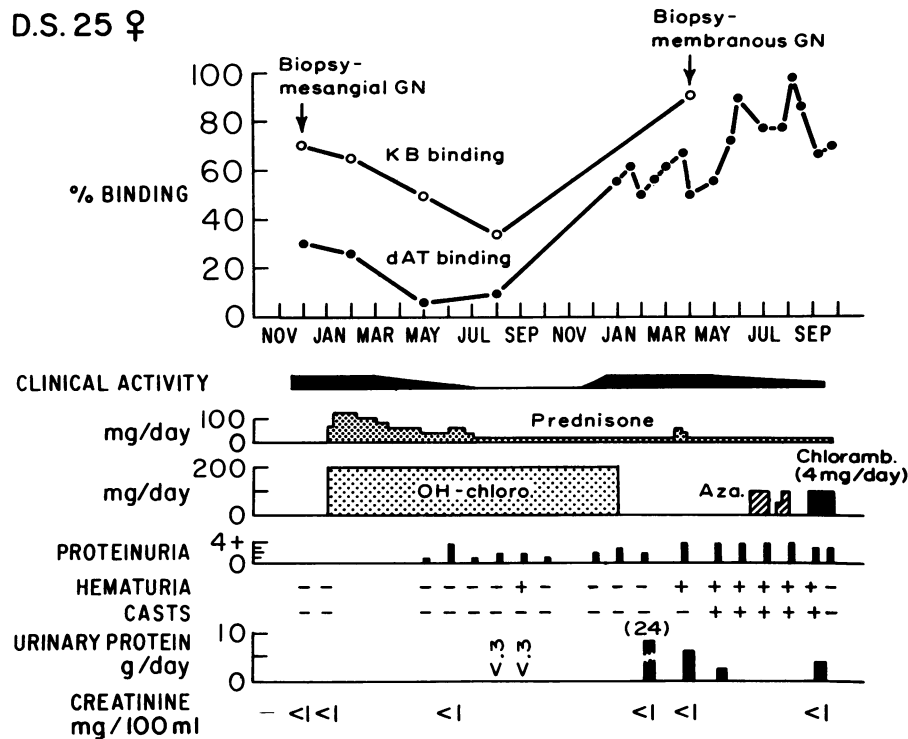


FIGURE 4 Course of a patient with SLE illustrating the correlation between dAT (and KB) binding and activity of nephritis. Clinical activity reflects the presence of fever, skin, and joint involvement. Treatment included prednisone, hydroxychloroquine (OH-chloro), azathioprine (Aza), and chlorambucil (Chloramb).

currence of antibody to nDNA in diseases other than SLE (7-9) as well as in normals (10). The assays used have invariably employed naturally derived nDNA as antigen. However, other reports (3, 4) suggesting contamination of most, if not all, naturally derived nDNA preparations with ssDNA as confirmed above, necessitate reevaluation of such data.

The difficulties of preparing and maintaining an antigenically pure nDNA preparation make it desirable to find a model compound which obviates them. Self-complementary alternating copolymers such as dAT are candidates for this role. Under the conditions used here, it appears that dAT is free of detectable ssDNA and nucleoprotein contaminants and is stable on storage. Also it is available commercially at a cost much lower than that of commercially-obtained <sup>14</sup>C KB DNA. In addition, its binding correlates closely with that of SN-KB, a natural substance that would be expected to contain all the antigenic sites intrinsic to the nDNA structure. The inability of dAT to completely inhibit binding of SN-KB could be due either to antigenic sites intrinsic to nDNA but lacking in dAT or, more likely, to residual contaminants in SN-KB, one of which, ssDNA, was shown indirectly to be still detectable, albeit by

only a single assay method. These alternatives cannot now be distinguished and are of theoretical rather than practical importance given the high degree of correlation between the binding of the two antigens.

Purification of natural nDNA on MAK has been proposed as another method of improving the specificity of binding assays (29).

The above data confirm the value of this procedure for helping to distinguish between the SLE patients and the disease controls. However, at least by methods used here, it appears likely that such MAK-purified nDNA still contains contaminating non-nDNA antigens. In addition, the difficulties of storage would be expected to apply to this material just as they would to any complex nDNA.

Perhaps a more nearly ideal natural nDNA antigen would be SN-KB as prepared above since it appears to be almost free of ssDNA. However, the difficulty and expense of its preparation and the problems of storage as well as the possible occurrence of chemically undetectable but immunologically significant contamination with non-nDNA antigens are limitations to its usefulness.

The practical value of using dAT in place of KB

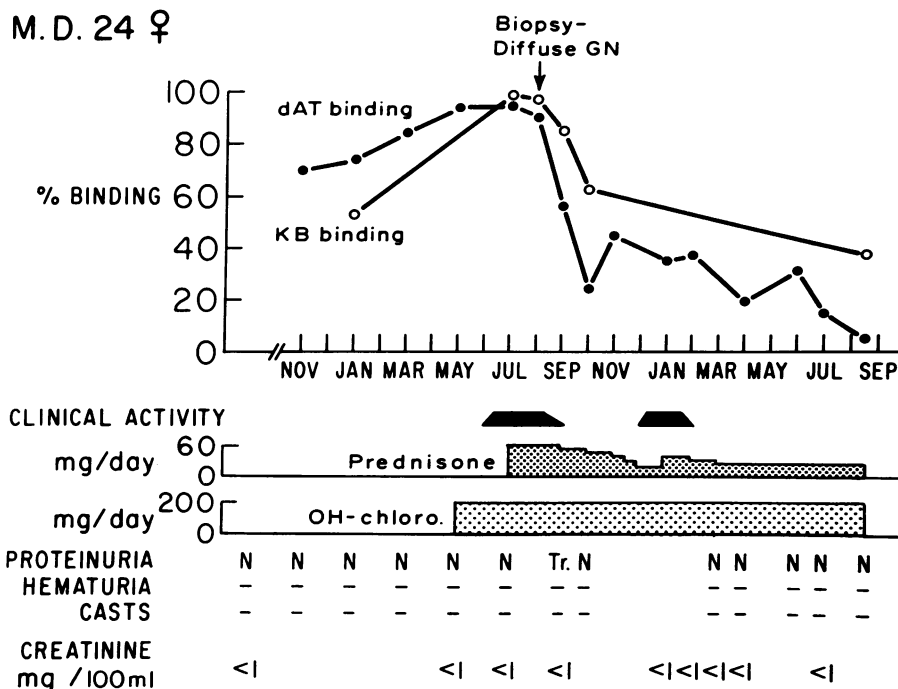


FIGURE 5 Course of a patient who lacked clinical evidence of active nephritis before renal biopsy. Clinical activity included fever, rash, arthritis, and serositis.

DNA in the ammonium sulfate binding assay for clinical application appears confirmed by the clinical data given above. It seems likely that dAT would offer similar advantages over the use of other natural nDNA preparations although this was not demonstrated here.

An important practical disadvantage to the use of dAT should be noted. Because of its apparent freedom from contamination with non-nDNA antigens, it would not be expected to be bound by SLE sera containing antibody only to those antigens. In SLE, particularly in the absence of active nephritis, antibody to nDNA appears to occur less frequently than does antibody to ssDNA. When present, reactivity with the former is almost always accompanied by antibody to the latter although the converse is not so (13). Hence fewer SLE sera would be expected to bind dAT than KB. This can be viewed as a loss of specificity in exchange for increased sensitivity when using KB DNA. However, this may be misleading since use of the two antigens appears to allow detection of different (although overlapping) populations of antibody which may have different clinical and pathological implications. Nonetheless, the dAT binding assay would not be as useful as that using less homogeneous preparations of DNA for following the activity of disease in SLE patients who lack anti-dAT (and therefore, presumably, anti-nDNA) but might still demonstrate elevated binding of other

nuclear antigens present in these DNA preparations. However, it remains to be established whether, in the absence of increased dAT binding, elevated KB binding provides information regarding SLE disease activity that is more useful clinically than do the more easily measured general parameters of inflammation such as the erythrocyte sedimentation rate.

A major practical advantage of using a better defined nDNA antigen in the binding assay may be a closer correlation with active nephritis than has been reported using KB DNA (2). Since clinical methods of assessing activity of nephritis are relatively crude, it seems surprising that the correlation with dAT binding is as close as was found. Because of the importance of this apparent correlation, a comparison with renal biopsy results seems indicated.

As noted above, previous reports interpreted as demonstrating anti-nDNA in non-SLE sera have all employed naturally-derived DNA preparations. In view of the evidence of ssDNA contamination of such preparations, these data cannot yet be said to challenge the previously held view that antibody to nDNA occurs exclusively in human or animal SLE (13). The apparent specificity of dAT binding for SLE sera found in the present study tends to support this view although additional data are needed to strengthen this conclusion.

The convincing demonstration of antibody to nDNA

has been impeded by several sources of ambiguity. These relate not only to questions surrounding homogeneity of the antigen, as discussed above, but also to recently described non-immunoglobulin, DNA-binding proteins in animal, (30) and human (31) serum as well as human cerebrospinal fluid (32).

Therefore, because of its theoretical importance, particularly stringent criteria would seem desirable for concluding that anti-nDNA was present in patients who do not have SLE. Hence it is proposed that before such conclusions are drawn, the antigen used be shown to be free of contaminating non-nDNA antigens by several sensitive methods, preferably under conditions similar to those at which binding assays are performed. It must be emphasized that the commonly used criteria such as "percent hyperchromicity" after denaturation (to demonstrate the degree of double strandedness) or the ratio of OD at 260 nm to that at 280 nm (to demonstrate freedom from protein contamination) are far too crude to have immunologic significance. Perhaps a useful additional method of detecting non-nDNA contaminants would be to demonstrate that the putative nDNA antigen is not bound by a panel of sera that are known to bind to preparations of relatively pure natural nDNA but not to dAT and that therefore are assumed to contain antibody to non-nDNA contaminants, some of which may be present in these natural preparations. In addition, it would seem appropriate to require evidence that the nDNA antigen was bound to immunoglobulin and that this binding occurred by the direct interaction between the Fab portion of the immunoglobulin molecule and the antigen without mediation by intervening molecules. Ideally, isolated purified anti-nDNA immunoglobulin should be obtained for this demonstration.

It is recognized that these requirements may be so strict as to be difficult, if not impossible to meet currently. Indeed, they have not as yet been fulfilled even for the demonstration of anti-nDNA in SLE sera (although the large and consistent body of work demonstrating their presence by independent means would seem to leave little doubt as to their occurrence in this disease). Nonetheless, these criteria may be helpful in evaluating data that bear on this important question.

Finally, it must be noted that, regardless of any practical clinical value they might possess, both dAT and SN-KB have major limitations as model nDNA antigens which should be recognized. The possible persistence of ssDNA and other potential contaminants in SN-KB has already been discussed. dAT might present other difficulties which have not yet been evaluated. For example, contamination of a preparation with the DNA polymerase used to synthesize it might lead to artifactual ammonium sulfate precipitation and appar-

ently high binding. More serious, perhaps, is the fact that the molecule transiently possesses short single stranded segments as a result of its so-called "breathing" (23). Such regions might hypothetically bind to anti-ssDNA which could then stabilize them. Since the occurrence of these segments is temperature-dependent, binding assays performed at room temperature and above (as opposed to the ammonium sulfate assay with its prolonged 4°C incubation) might be particularly vulnerable to ambiguous interpretation on this account. For this reason dGC might be a more nearly ideal model nDNA compound particularly for use at these higher temperatures because of its greater thermal stability. However, lack of availability in radiolabeled form currently limits its value. In addition, information about its secondary structure is less complete than for dAT.

An important variable whose effect on binding has not yet been thoroughly explored is the average DNA chain length. In this regard, it seems likely that the presence of very short dAT chains would decrease the percentage bound by a given antiserum whereas the reverse might result if there were relatively few long chains or complex networks of dAT.

Limited experience with a preparation of dAT that had apparently been prepared differently from that used in the present study supports this concept and suggests that control of this variable may be important for obtaining reproducible results with dAT binding.

It is concluded that use of more closely defined nucleic acid antigens such as dAT in investigating antibodies in SLE and related disorders appears to offer both practical and theoretical advantages over the use of more heterogeneous materials and that further investigation seems justified.

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