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Disease activity in systemic lupus erythematosus related to a range of antibodies binding DNA and synthetic polynucleotides

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SUMMARY Antibodies to dDNA, nDNA, Z-DNA, poly(dT), poly(I), poly(dG.dC), poly-(dA.dT), and total IgG and IgM were measured in five serial bleeds from 39 patients with systemic lupus erythematosus (SLE). The main findings were that those patients with renal disease form a distinct subset whose antibody levels correlate well with disease activity; anti-poly(dT) antibodies showed the best overall correlation with disease activity; and discriminant functional analysis demonstrated a major improvement in correlation of disease activity with combinations of antibodies to dDNA/nDNA/Z-DNA/poly(dT) (generally 50% or more were correctly classified) than with dDNA or nDNA alone (generally less than 25% correct). Serum IgG (but not IgM) correlated significantly ($p<0.01$) with six antibodies, suggesting that polyclonal activation plays a part in the development of these antibodies, though antibody cross reactivity is not excluded.

Key words: lupus nephritis.

It is clear that the sera of patients with systemic lupus erythematosus (SLE) contain a range of antibodies capable of binding many different target antigens.[1] Studies with hybridoma derived antibodies have shown cross reactions, indicating that the range of antibodies is probably more restricted than was originally thought.[2] For example antibodies binding single stranded (d) DNA or double stranded (n) DNA, or both, may also bind the phospholipid, cardiolipin,[3] Raji cells,[4] and vimentin.[5] Among those antibodies routinely measured some have been linked to particular disease manifestations. Anti-nDNA antibodies for example, are associated with renal disease according to some reports,[6–9] whereas anti-Ro(SS-A) antibodies have been found in many patients with photosensitive dermatitis[10] and congenital complete heart block.[11] In the main, however, the relation between individual autoantibodies and clinical manifestations remains poorly defined or weakly associated, or both. Raised levels of anti-DNA antibodies may be found even in patients with SLE whose disease is inactive.[12]

Antibodies to DNA have, however, proved the focus of considerable attention during the 30 years since they were identified.[13–16] We now describe a study of antibodies reactive with dDNA, nDNA, Z-DNA, and various synthetic polynucleotides which structurally resemble these forms of DNA in varying degrees. Our aim was to determine whether antibodies measured serially, and capable of distinguishing relatively subtle differences in epitopes, either singly or in combination, may reflect disease activity in general or disease in a particular organ or system. Does detection of antibodies with a particular nucleic acid or combination of nucleic acids offer a better guide to disease activity than those antinuclear antibodies currently sought on a routine basis in patients with SLE?

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Patients, materials, and methods

PATIENTS

Thirty nine patients with SLE were studied. Each

patient met four or more of the revised criteria of the American Rheumatism Association for the classification of the disease.[17] Five serial samples of blood from each patient were coded and studied 'blind'. Overall disease activity was graded according to a previously published index,[18] which has recently been favourably compared with an activity index generated by a computer program.[19] Patients in group 1 were judged to be inactive, while those in groups 2, 3, and 4 were thought to have mild, moderate, or severe activity respectively. Ten patients had predominant renal disease, nine had predominant disease of the central nervous system (CNS), eight had major serositis (heart/lung) without renal or CNS disease, and eight had relatively mild skin rash and arthralgia only. The clinical criteria for the organ or system involved have been published elsewhere.[20] Four patients formed a group with a positive lupus anticoagulant and combinations of recurrent thrombotic episodes, spontaneous abortions, thrombocytopenia or mild renal disease.

METHODS

Flat bottomed enzyme linked immunosorbent assay (ELISA) plates (Immunlon I, Dynatech, Alexandria, VA) were pretreated by ultraviolet light irradiation overnight.[21] They were coated with 150 µl of the test polynucleotide (single stranded DNA, double stranded DNA, Z-DNA, poly(I), poly(dT), poly(dG.dC), or poly(dA.dT) at a concentration of 2·5 µg/ml in trometamol (TRIS) buffer saline containing 20 mM magnesium chloride (TBS/Mg^{++}), which was used throughout to maintain the stability of the Z-DNA. After two hours' incubation at room temperature the plates were washed three times in TBS/Mg^{++} and blocked with 150 µl of 1% bovine serum albumin (BSA; Sigma) in phosphate buffered saline containing 20 mM $MgCl_2$ (PBS-Mg^{++}). After a further two hour incubation at room temperature the plates were flicked dry. Test sera serially diluted in PBS-BSA (1%)/Mg^{++} were added to the wells and incubated for 90 minutes at room temperature. A positive control and two normal serum samples were diluted in the same way as the test sera on each plate. The same positive control serum—from a patient with severely active lupus—was used on every plate for each assay. Prior experiments had shown that this serum had high reactivity with each of the test antigens. The plates were washed with PBS-Tween (0·1%) containing 20 mM $MgCl_2$, then 100 µl of goat antihuman polyvalent immunoglobulin conjugated to alkaline phosphate (Sigma Chemical Co, St Louis, Missouri) diluted 1 in 1000 in TBS/Mg^{++} was added to each well. The plates were incubated for two hours at room temperature and then washed three times with PBS-Tween (0·1%)/Mg^{++}. The bound conjugate was detected by the addition of *p*-nitrophenyl phosphate. Absorbance at 405 nm was read on a Dynatech Model MR600 ELISA reader after 30 minutes. Standard curves were drawn for each sample and compared with the positive control curve on the same plate. The dilution giving an optical density (OD) value at the midpoint of each individual positive control curve was noted. (The midpoint OD value for the control varied from plate to plate but was invariably between 0·400 and 0·800.) The dilution of the test sera giving the same OD value was read off each test curve. The results were expressed as a percentage of the midpoint dilution of the positive control.

MEASUREMENT OF TOTAL IMMUNOGLOBULIN IGG AND IGM

Serum IgG and IgM levels of all the test sera were determined by an immunoturbidometric method using a Cobas Fara analyser (Roche). Serum samples were diluted 1:40 in phosphate buffer (0·1 M, pH 7·4) containing 10% polyethylene glycol. Rabbit antihuman IgG and IgM (Roche) were prepared at 1:15 in the same buffer. Test sera and antisera were mixed in the analyser. The reference human serum (Roche) was used to provide a reference calibration curve and to express the immunoglobulin concentrations in g/dl.

Results

Most of the results for each of the assays gave values of less than 100% of the positive control sera which was run on every plate. Among those sera from the patients judged to have moderate or severe disease, values of 200–300%, rarely higher, were sometimes recorded. The anti-poly(I) assay gave the highest number of values above 100% of the positive control. In contrast, the values for the healthy controls also tested on the same plates were usually less than 30% of the high positive control.

The Spearman correlation coefficient was used to compare the results for levels of individual antibodies and the total immunoglobulin (Table 1). In general there was a close correlation between the measurements of antibody with different polynucleotides. Serum IgG levels, sometimes raised to between two and three times normal, also correlated to a highly significant degree with six of the antibodies (anti-dDNA levels being the exception). In contrast, serum IgM levels, rarely much increased, correlated to a statistically significant degree only with total IgG.

Table 2 shows the relation between the individual

Table 1 *Comparison of individual antibody levels and total immunoglobulin levels using the Spearman correlation coefficient**

	dDNA	*nDNA*	*Z-DNA*	*Poly(I)*	*Poly(DT)*	*Poly (dG.dC)*	*Poly (dA.dT)*	*IgG*	*IgM*
dDNA	—								
nDNA	<0·0001	—							
Z-DNA	<0·0001	<0·0001	—						
Poly(I)	<0·0001	<0·0001	<0·0001						
Poly(DT)	<0·0001	<0·0001	0·006	<0·0001	—				
Poly(dG.dC)	<0·0001	<0·0001	<0·0001	<0·0001	0·14	—			
Poly(dA.dT)	<0·0001	<0·0001	<0·0001	<0·0001	<0·206	<0·0001	—		
IgG	0·071	0·005	0·001	0·019	0·009	0·001	0·007	—	
IgM	0·342	0·352	0·186	0·34	0·151	0·227	0·79	0·003	—

*Values are p values.

Table 2 *Relation between the individual antibody levels and disease activity in the total SLE population and subgroups*

Antibodies	*All patients with SLE (n=39)*	*SLE-renal (n=10)*	*SLE–CNS* (n=9)*	*SLE-arthralgia/ skin (n=8)*	*SLE-serositis (n=8)*	*SLE–DVT+* (n=4)*
dDNA						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	NS	p=0·006	NS	NS	NS	NS
1+2+3 *v* 4	NS	p=0·008	NS	—	NS	—
nDNA						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	NS	p=0·003	NS	NS	NS	NS
1+2+3 *v* 4	NS	p=0·003	NS	+	NS	—
Z-DNA						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	NS	p=0·049	NS	NS	NS	NS
1+2+3 *v* 4	NS	p=0·0008	NS	—	NS	—
Poly(I)						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	NS	p=0·049	NS	NS	NS	NS
1+2+3 *v* 4	NS	p=0·012	NS	—	NS	—
Poly(dT)						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	p=0·03	p=0·0004	NS	NS	NS	NS
1+2+3 *v* 4	p=0·004	p=0·0001	NS	—	NS	—
Poly(dG.dC)						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	NS	p=0·016	NS	NS	NS	NS
1+2+3 *v* 4	NS	p=0·009	NS	—	NS	—
Poly(dA.dT)						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	p=0·04	NS	NS	NS	NS	NS
1+2+3 *v* 4	NS	p=0·03	NS	—	p=0·03	—
IgG						
1 *v* 2	NS	NS	NS	p=0·002	NS	NS
1+2 *v* 3+4	NS	NS	NS	NS	NS	NS
1+2+3 *v* 4	NS	p=0·04	NS	—	NS	—
IgM						
1 *v* 2	NS	NS	NS	NS	NS	NS
1+2 *v* 3+4	NS	NS	NS	NS	NS	NS
1+2+3 *v* 4	NS	NS	NS	—	NS	—

*CNS=central nervous system; DVT+=deep vein thrombosis and related diseases.

antibody levels and disease activity in the group overall and in the designated subgroups. Three main findings were obvious. Renal involvement was clearly shown to distinguish a subset of patients with SLE. High levels of antibodies reactive with virtually all the antigens tested were found in those patients with the most active renal disease. Among the individual antibodies, those binding poly(dT) were the most useful in distinguishing disease activity overall. It was notable, however, that the antibody levels did not distinguish between inactive (group 1) and mildly active (group 2) disease. Furthermore, with the exception of the renal subgroup, there was little distinction between the patients with inactive and mild disease and the groups with more active disease, or between the group with severely active disease and the other groups combined. This last observation did not apply to the arthralgia/skin group or the deep vein thrombosis groups (DVT+), in which there were no severely active patients. Scrutiny of the antibody levels in individual patients showed clearly how titres of antibodies to virtually all the test antigens reflected disease activity very well. Thus in Fig. 1 four patients with renal disease are shown whose clinical activity varied significantly with time. In each case clear trends were seen in the levels of the antibodies to the test antigens, which correlated well with disease activity. There were exceptions to this, and the course of disease might have paralleled different sets of antibodies in different patients. Fig. 1a shows that the reactivity with poly(dG.dC) remained low throughout, while the other antibody titres were high to start with and then fell. Fig. 1b shows that while antibodies to six of the test antigens displayed marked fluctuation with disease activity, antibodies to poly(dA.dT) remained barely detectable. The antibody levels in the patient shown in Fig. 1c also showed a striking correlation, except for a rise in the anti-poly(I) titre when the patient had only mild disease. Fig. 1d shows that the high anti-poly(I) levels did not correlate well with disease activity, in contrast with antibodies binding dDNA, poly(dT), poly(dA.dT), and poly(dG.dC). In this

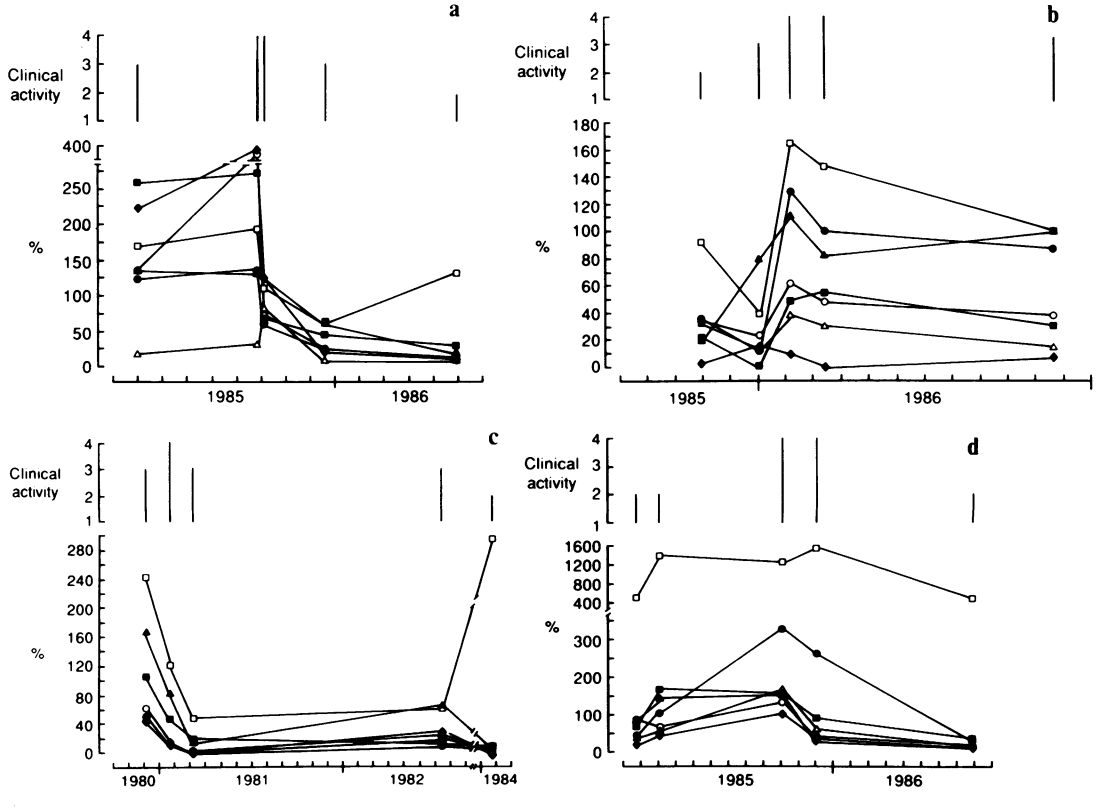

Fig. 1 *A composite showing four patients with renal disease whose autoantibody levels fluctuate with disease activity. The symbols are:* ▲*=poly(dT);* □*=poly(I);* ■*=Z-DNA;* ◆*=poly(dA.dT);* ●*=dDNA;* ○*=nDNA;* △*=poly(dG.dC).*

patient, however, there was little binding to Z-DNA or nDNA.

Correlation between antibody levels and disease activity occurred in certain patients with no renal disease, even though this was not seen for the subgroups as a whole. Thus Fig. 2a shows a patient with serositis whose antibody levels generally correlated with each other and with disease activity, though the level of anti-poly(I) antibodies remained high throughout. In contrast, and as a form of negative control, in Fig. 2b a patient with persistent but mild renal disease followed up for seven years is shown. During this period the patient did not have a single major disease exacerbation, and it is notable that her antibody titres remained virtually constant throughout, only the antibodies to poly(dT) and poly(I) ever exceeding 30% of the positive control.

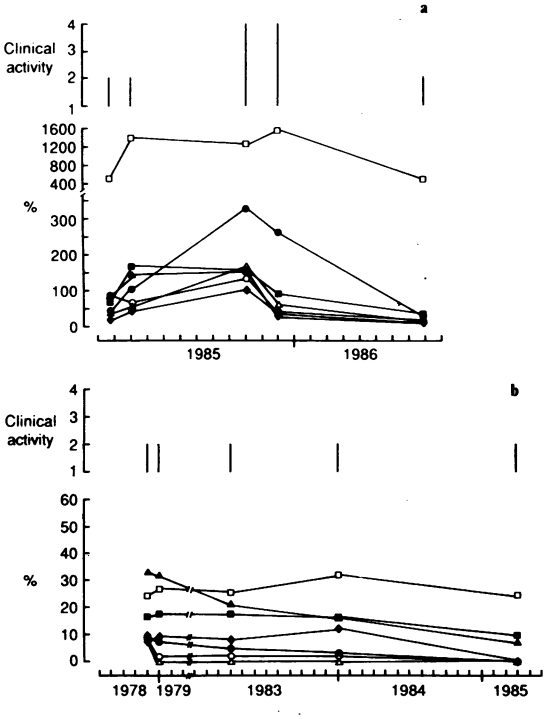

Fig. 2 *A composite showing (a) a patient with serositis whose autoantibodies fluctuate with disease and (b) a patient with chronic renal disease. The symbols are the same as in Fig. 1.*

Table 3 shows the results of the discriminant function analysis. The mathematical objective of this technique is to weight and linearly combine discriminating variables (in this case the antibody and total immunoglobulin results) so that the subgroups become statistically distinct. As shown, when analysed in this way, the numbers of cases correctly classified increased when two or more variables were examined together compared with the current method of using either antibodies to dDNA or nDNA. With the exception of the group with DVT+, when analysed by all seven variables, none of the discriminant functional analysis scores was higher than 80%.

Discussion

This study describes a detailed correlation between antibodies to seven different polynucleotides and disease manifestations in SLE. Our major findings were the clear distinction of the renal subgroup, especially those with moderate or severe disease; the close relation between antibodies binding all or most of the seven test antigens in some of the patients; but the lack of overall 'clinical distinguishing ability' of the antibodies for patients in the CNS, arthritis/skin, serositis, and DVT+ groups. In a previous study discriminant function

Table 3 *Numbers of cases currently classified by discriminant functional analysis (in %)*

Patient groups	*Antibodies to:*						
	dDNA	*nDNA*	*d/nDNA*	*d/n/Z-DNA*	*d/n/Z-DNA+ +poly(DT)*	*d/n/Z-DNA+ poly(dT)+ poly(dA.dT)*	*All antibodies +IgG+IgM*
All patients with SLE	19	22	20	42	43	43	56
Renal	24	50	62	64	56	56	59
CNS*	20	24	24	28	31	33	53
Serositis	28	46	36	61	61	61	76
Skin/joints	22	17	37	40	52	57	77
DVT+*	15	75	65	70	70	70	100

*CNS=central nervous system; DVT+=deep vein thrombosis and related disease.

analysis using lymphocyte count, erythrocyte sedimentation rate, serum C3, and DNA binding (by the Amersham kit) could only correctly classify 44% of lupus patients.[20] In this study, despite increasing the number of disease activity groups from three to four, over 50% of the subgroups were often correctly classified. To achieve clinical utility, however, this figure would have to be improved.

Although previous studies have described increased polynucleotide binding with clinically active disease in some cases, no consensus about the value of detecting these antibodies seems to have emerged.[22–33] An exception to this is the link between renal disease and antibodies to nDNA. Our data do not support a recommendation that seven different antigens be tested routinely.

More recently there has been much interest in the detection of antibodies binding the left-handed Z-DNA.[34] Sibley *et al* in a study of antibodies to Z-DNA (and five synthetic duplex DNA antigens) found that these antibodies were detected more frequently in the sera of patients with rheumatoid arthritis than SLE, and among the SLE groups they could not correlate the levels with disease type or activity.[35] They did not, however, examine serial bleeds from their patients.

These earlier studies must all be considered in the context of the type of assays used to measure the antibodies and the clinical condition of the patients studied. Furthermore, particular properties of antibodies, such as their ability to fix complement, may be related to particular disease manifestations. Two recent studies highlight these points.[36 37] In the first Smeenk compared four different types of assay to measure anti-DNA antibodies: the polyethylene glycol assay, an ELISA, a Farr assay, and the *Crithidia* test.[36] Of 289 sera assessed, 158 were negative by all four techniques and only 22 positive by all four. While discussing his results, Smeenk reiterates that only the ELISA and *Crithidia* test incorporate an inherent check on the immunoglobulin nature of the anti-DNA antibody. In contrast, in the radioimmunoassays (RIA) non-specific DNA binding cannot be discriminated from immunoglobulin binding. Provided that purely double stranded DNA is used, however, Smeenk reported that the Farr assay gave the greatest specificity for SLE.

The second report describes an assessment by an ELISA, three RIA (including two commercial kits), and the *Crithidia* test of 60 SLE sera and 70 disease controls.[37] In this study the *Crithidia* test was the most specific (none of the controls was positive) but the least sensitive (13% only of the patients with SLE were positive). In contrast, the results for the IgG and IgM anti-nDNA ELISA and the three RIA were broadly similar (28–57% of the patients with SLE were positive). The patients with SLE categorised as severely ill had raised antibodies by all of these methods, but even among these inactive patients some positive results were reported with each technique. The implication of this report, that those patients judged severely ill were a distinct group, is to some extent confirmed in the 'renal' patients we have studied.

SLE is a heterogeneous condition both clinically and serologically. To determine whether individual antibodies could be matched to particular clinical features we selected for study patients who had relatively homogeneous disease. As is obvious from the scoring system used, however, the classification of the patients with SLE did require analysis of additional systemic features in determining disease severity. In the main, however, these were non-specific (e.g., lymphadenopathy, fever, Raynaud's phenomenon, corticosteroid requirement) and should not have interfered with the activity assessment of disease in the most affected organ/system in any individual patient. The published work abounds with lupus clinical activity scores but very often few details of precisely how a patient was categorised and which particular manifestations (renal, joints, skin, etc.) were present at the time of the bleed are provided. The UCH/Middlesex criteria for disease activity used in this study have recently[19] been compared with a computer based index formulated by the British Isles Lupus Assessment Group (BILAG—a group of interested physicians in the Bloomsbury rheumatology group, London and in the rheumatology departments of Bath, Birmingham, and Glasgow). An 85% overlap of identification of active patients was noted between the two systems scored independently by the physicians from the four different centres. The overall inability of the assays we have studied to distinguish clinically inactive disease from mild activity is probably because of problems of sensitivity with the scoring system used.

A longstanding problem in analysing SLE has been the difficulty in knowing whether antibodies to all of the many antigens tested are cross reactive or whether they represent individual clonal expansions. The evidence from studies performed with hybridoma derived antibodies (reviewed in refs 2 and 38) strongly supports the view that autoantibodies may be strikingly cross reactive. The close relation between the rise and fall of many of the antibody titres we have studied (see Figs 1 and 2) could also be interpreted as supporting this view. Clearly, however, not all antibodies are cross reactive and some may show very particular specificities.[34] In contrast, the statistically significant

correlation between total IgG and six of the autoantibody levels measured implies that polyclonal expansion may also be part of the explanation for our results. It may be possible to reconcile these opposing views in SLE, by considering the 'anti-DNA' antibody family as representing overlapping populations of closely related molecules.

Given the variation in antibody detection techniques, the ability of DNA to present several kinds of antigenic sites, and the clinically diverse nature of SLE, it is unlikely that agreement can be reached as to the best method of measuring 'anti-DNA antibodies'. Such a method would have to be simple to perform, have good reproducibility, show disease specificity, and reflect disease activity. A widely accepted anti-DNA antibody standard would have to be adopted and a generally agreed method of judging lupus activity established. Our observations with ELISAs to seven varieties of DNA/polynucleotide support the view that overall renal involvement represents a distinct subset in SLE. In contrast, the other clinical subsets of patients with SLE were not distinguished, and thus the detection of antibodies to the related, though distinct, antigens of the synthetic polynucleotides tested do not seem to add anything to the more routine detection of antibodies to nDNA or even dDNA.

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