

Anti-DNA antibodies: a diagnostic and prognostic tool for systemic lupus erythematosus?

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Abstract

The clinical impact of anti-DNA antibodies lies on their diagnostic power for systemic lupus erythematosus (SLE), being a formal classification criterion. In spite of such a disease association, low-avidity anti-DNA antibodies might also be part of the natural autoantibody repertoire. Their switch to pathogenic high-avidity autoantibodies is the result of the autoimmune process leading to SLE.

Anti-DNA antibodies were shown to play a role in SLE pathogenesis and particularly in kidney damage. Accordingly, antibody titres might fluctuate in relation to disease activity, but their prognostic value for flares is still debated.

Several methods for anti-DNA detection were described and there is evidence that the assays identify different antibodies with different prognostic value. The results of a multicenter study on four different routine tests for anti-dsDNA antibody detection showed that: (i) Farr assay displays the best diagnostic specificity/sensitivity for SLE, followed by *Crithidia luciliae* method (CLIFT), (ii) the new generation of solid phase assay (EliA) shows an increased sensibility versus the classical enzyme linked immune assay (ELISA) but a decreased specificity. Antibody titre detected by EliA and Farr assay correlated with disease activity. These findings would suggest that more than one assay should be useful for SLE diagnosis and monitoring.

Keywords: DNA, autoantibodies, systemic lupus erythematosus, disease activity

Introduction

The presence of antinuclear antibodies (ANA) is widely accepted as a screening tool in the diagnostic workout for autoimmune diseases. Different antigenic targets might be responsible for the ANA positivity by indirect immunofluorescence, and most of them have been identified in the last decade. However, the first characterized nuclear antigen targeted by anti-nuclear antibodies was DNA back to 1957 [1].

Since then, autoantibodies to double stranded (ds)-DNA became an important serological marker for the diagnosis of SLE, and the American College of Rheumatology criteria for the disease included

the presence of anti-DNA antibodies as a formal classification tool [2].

DNA as antigen might occur as ds-DNA or as single-stranded (ss)-DNA, however, it is almost present *in vivo* in the form of nucleosomes. Since the epitopes situated on DNA partially reflect the repetitive charge of the molecule, synthetic polynucleotides can be often recognized by anti-DNA antibodies. A partial cross-reactivity with RNA has been also reported [3–6]. Although the autoantibodies appear to display a different reactivity towards DNA molecules from different species, however, a binding can be detected. So, DNA from different sources (calf thymus,

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eukaryotic cells, bacteria, bacteriophages) and even plasmidic DNA have been employed as antigenic target to detect anti-DNA autoantibodies [3–6].

Sequential and backbone DNA determinants can be recognized by anti-DNA antibodies. Backbone determinants on ss- and ds-DNA are short regions of DNA helix or short nucleotide sequences and the antibody binding appears to be mainly due to electrostatic interaction since it is sensitive to molarity and pH. Besides backbone recognition, the binding to defined nucleotide sequences has also been reported particularly for anti-ssDNA antibodies. Although antibodies specific for ss-DNA do exist, most of the anti-ssDNA activity can be due to cross-reacting low-avidity anti-dsDNA antibodies [3–6].

Origin of anti-dsDNA antibodies

Although anti-dsDNA are quite specific for SLE, however, some anti-DNA antibodies can be found in normal individuals. These antibodies belong to the normal immune repertoire and display the characteristic features of natural autoantibodies. In other words, they are mostly IgM encoded by germline DNA with few or no somatic mutations, display a polyreactivity and bind DNA with low-avidity. These antibodies are non-pathogenic and mainly react with ss-DNA. On the other hand, SLE pathogenic anti-DNA antibodies are thought to be high-avidity IgG reacting with double stranded molecules and are somatically mutated as expression of an antigen-driven selection process [7].

The specificity for ds-DNA would depend on certain V-region structures encoded by V-region genes and somatic mutants of these genes. Most mutations in the complementarity-determining regions (CDRs) of the V_H region are to arginine and also to lysine and asparagine. All these positively charged amino acids favor antibody binding to the negatively charged DNA molecules [7,8].

The cellular origin of natural and autoimmune autoantibodies also appears different. The natural anti-DNA antibodies are in fact produced by B1 (CD5+) B cell subpopulation, while the pathogenic ones are secreted by B2 (CD5 neg) B lymphocytes [9].

B cell clones with V-region structures suitable for ds-DNA binding could be part of the native resting B repertoire. Such B cells are normally deleted or edited, but they may escape deletion or receptor editing being available for clonal expansion provided that an immunogenic DNA stimulus is present. More commonly, naïve B cells specific for ss-DNA may clonally expand if stimulated by immunogenic DNA and may gain specificity for ds-DNA as a consequence of somatic mutations under antigenic stimulation pressure [7].

One of the major conclusions of the studies on the mechanisms for anti-DNA production is that DNA must be coupled to a carrier protein to have full

potential as an immunogen. Histone- and nucleosome-specific T cells have been actually characterized in both murine and human SLE. However, these autoreactive T cells have been detected during active disease but not during remission, suggesting that autoimmune antigen-specific T cells might fluctuate between anergy and functionality. Their activation with the consequent generation of an immune response to DNA goes through the presentation of DNA–protein complexes to T-cells specific for the protein moiety and implies a subsequent shift of the immune response toward the DNA moiety of the complex. This can be accomplished through the stimulation of T cells specific for the non-self protein moiety with the secretion of interleukin 2 that in turn might trigger a non-antigen-selective cell division of anergic autoimmune histone-specific T cells, to which the DNA antigen was presented complexed with the non-self protein moiety. It has been suggested that autoimmune T cells may subsequently proliferate in an antigen-selective manner in response to the presented nuclear antigens [7].

It is now widely accepted that the normal immune system has the full potential to respond to an antigenic stimulus mediated by nucleosomes and DNA. The nature of the stimulus (transient or permanent) and its association with other genetically or acquired abnormalities might shift such a response from a transient to a pathological one [7,10]. Accordingly, different anti-DNA antibody populations can be produced. There is a general consensus on the observation that high-avidity anti-dsDNA antibodies might be pathogenic in comparison to low-avidity antibodies that frequently display a cross-reactivity with ssDNA molecules. However, exceptions of such a rule have been reported [3–7].

Detection assays

Several techniques to detect anti-DNA antibodies have been described; Table I reports the main methods that are currently used. While complement fixation and hemoagglutination assays are no more widely used, the most common are: *Crithidia luciliae* immunofluorescence test (CLIFT), Farr assay and the enzyme-linked immune assays (ELISA) [5,6].

Table I. Methods for anti-dsDNA antibody detection.

Method	Antibody avidity
Complement fixation	+
Haemoagglutination	+
Farr assay	++++
CLIFT*	+++
PEG [†]	++
ELISA	+

* CLIFT: *Crithidia luciliae* immunofluorescence test.

[†] PEG: polyethylenglycole assay.

More recently, additional methods have been described to detect anti-dsDNA antibodies such as the use of strips blotted with the DNA molecules (immunoblotting [IB]), the use of microarrays and of addressable laser bead immunoassays (ALBIA) [11–14]. Although promising results have been reported by using the above mentioned systems, there are no large studies that might draw definite conclusions up till now.

There is sound evidence that the different anti-dsDNA assays might detect different—although overlapping—subpopulations of autoantibodies. Low-avidity autoantibodies are actually detectable by ELISAs (and C' fixation, hemoagglutination and polyethylenglycol [PEG] assays), medium low-avidity autoantibodies by CLIFT, while high-avidity antibodies are identified by Farr assay only [5,6].

The reasons for such a difference lie on several points, however, the most important one is represented by the reaction conditions employed. Actually, the ammonium sulphate precipitation is carried out at high molarity that allows the detection of high-avidity but misses medium low-avidity antibodies [5,6]. In second antibody technique, as in CLIFT and ELISA, anti-human IgG antisera are frequently used, so permitting the detection of IgG anti-dsDNA only. The source and the presentation of the antigen do represent additional important aspects to explain the fact that different methods might detect different antibody subpopulations: DNA can be eukaryotic or pro-eukaryotic, ss-DNA contamination might be responsible for the detection of low-avidity antibodies cross-reacting between ss- and ds-DNA. Finally, DNA antigen presentation can be different, being in solution in the Farr assay, coated to the plastic surfaces in the ELISAs or presented in its naïve form in the nucleus of Crithidia. A peculiar situation has been described for the Farr assay. Radiolabelled DNA might actually be precipitated by histones or nucleosomes complexed to anti-nucleosome antibodies so displaying a positive result in the assay [15].

Which kind of antibodies and which assay?

Since anti-dsDNA antibodies have been claimed to be a serological marker for SLE, their search is most frequently required to support an initial diagnosis. Accordingly, it has been suggested that the assay to be used should display the highest specificity. Antibodies with high-avidity seem to be more specific; accordingly the Farr assay and the Crithidia test are preferred for this purpose [5,6]. On the other hand, a positive result obtained by assays not selective for high-avidity anti-DNA antibodies does not always support diagnosis of SLE, since low-avidity anti-DNA antibodies can also occur in diseases other than SLE. Therefore, the screening should be confirmed by an assay more selective for high-avidity antibodies.

The above statement has been supported by the predictive values for developing full-blown SLE in healthy subjects positive for the Farr or the PEG assay over the time. Up to 85% of the Farr positive subjects developed SLE after 5 years of follow-up versus 52% only in the group with a positivity for PEG but with a negativity for the Farr assay [16].

Longitudinal evaluation of anti-dsDNA antibodies showed titre fluctuations during disease flares and remissions. It has been actually reported that exacerbations are preceded by an antibody increase followed by a rapid drop [3–6]. Accordingly, a corticosteroid treatment started as soon as the antibody titre increase was found to result in the prevention of relapses in most cases without increasing the cumulative corticosteroid dosage [17]. However, a number of studies reported opposite results stating that serological tests—including anti-dsDNA antibodies—were unhelpful in detecting or preventing lupus flares [18–20].

So, while the relationship with renal damage is well established, the predicting power for disease flares is still a matter of debate. The heterogeneity of the antibodies detected by the anti-dsDNA assays used in these studies might be the reason for such a discrepancy. It would appear that high-avidity anti-DNA—as detected by Farr assay—might associate with disease flares closer than medium low-avidity antibodies [3–6]. On the other hand, since several additional pathogenic mechanisms are involved in lupus pathogenesis, it is likely that in some patients more than one mechanism could play a role at the same time. Accordingly, other biological markers exploring different pathogenic events—alone or in combination—might better reflect the disease activity than anti-DNA antibody titres alone. Accordingly, it has been reported that the detection of anti-C1q autoantibodies might offer a marker of renal involvement displaying a higher sensitivity than the anti-dsDNA titre fluctuation itself [21]. In addition, anti-nucleosome antibodies have been reported to be a useful diagnostic tool for SLE as well as a parameter for disease activity assessment [22,23].

Evaluation of different methods for the detection of anti-dsDNA antibodies

As stated above, different methods have been applied for the detection of anti-dsDNA in the clinical practice. Each of them was shown to be able to detect different subpopulations of autoantibodies only partially overlapping and displaying different diagnostic or prognostic value.

We measured the “likelihood ratio” (LR) of four different tests used in routine detection of anti-dsDNA antibodies in three different institutions and we also evaluated their sensitivity, specificity, correlation with disease activity and diagnostic utility. A total of 303 sera from healthy donors (HD), SLE patients, non-SLE

Table II. Number and diagnosis of the patients included in the study.

•136 SLE
•54 non-SLE-systemic autoimmune disorders
•6 MCTD*
•8 PAPS†
•2 Myositis
•7 Scleroderma
•Sjogren Syndrome
•23 UCTD‡
•2 RA [¶]
•DLE [§]
•32 Infectious diseases (29 HCV , 3 TBC [#])
•81 blood donors

* MCTD, mixed connective tissue disease.

† PAPS, primary antiphospholipid syndrome.

‡ UCTD, undifferentiated connective tissue disease.

¶ RA, rheumatoid arthritis.

§ DLE, discoid lupus erythematosus.

|| HCV, hepatitis C virus.

TBC, tuberculosis.

systemic autoimmune disorders (AD) and infectious diseases (ID) have been blindly tested. The details of the patients and the subjects included in the study are reported in Table II. All the samples have been tested by: (i) Farr assay, based on ammonium sulphate precipitation (Anti-dsDNA kit, Amersham), (ii) immunofluorescence test on *Crithidia luciliae* (CLIFT—Immunoconcept), (iii) classical ELISA (VarElisa, Pharmacia Diagnostics) and (iv) a new automated fluorescence immunoassay (EliA, Pharmacia Diagnostics); both the last assays employed plasmidic DNA as antigen. To avoid selection bias, an equal number of SLE patients were recruited in three different institutions, where anti-dsDNA was routinely detected by three different assays (Farr assay, CLIFT, ELISA). All the CLIFT slides were performed and evaluated in the same institution to avoid different interpretation; anti-dsDNA antibodies detection by EliA was centrally carried out in one institution only. LR was calculated according to the quoted guidelines: positive LR (sensitivity/1-specificity) was considered very useful if > 5 , useful if $2 < 5$, not useful if < 2 , while negative LR (1-sensitivity/specificity) was considered very useful if < 0.2 , useful if $0.2 < 0.5$, not useful if > 0.5 [24,25].

All the assays displayed a statistically significant correlation among them (Table III).

Table IV reports the results relative to the sensitivity for SLE diagnosis, the specificity versus control groups (HD and AD or ID) as well as the values of positive and negative LR. The best specificity was displayed by CLIFT test, followed by VarElisa, Farr assay and EliA, while the highest sensitivity was displayed by Farr assay, followed by EliA, VarElisa and CLIFT. However, because of the high specificity and low sensitivity, a negative CLIFT test cannot rule out the diagnosis of SLE.

Table III. Correlation among the anti-dsDNA antibody assays (Spearman test).

	<i>r</i>	<i>P</i>
Farr vs. EliA	0.742	< 0.0001
Farr vs. Varelisa	0.782	< 0.0001
EliA vs. Varelisa	0.851	< 0.0001
Farr vs. CLIFT	0.39	< 0.0001
EliA vs. CLIFT	0.336	< 0.0001
Varelisa vs. CLIFT	0.361	< 0.0001

In this study, all the four anti-dsDNA assays, when positive, provided a “very useful” information for the diagnosis of SLE. Therefore we may assume that all the methods are able to identify antibodies closely involved in the disease pathogenesis. This appears to be particularly true for both Farr and EliA assays that displayed a quite good correlation with the disease activity evaluated according to the European Consensus Lupus Activity Measurement score (ECLAM; data not shown) [26].

Despite its old age, the Farr assay still appears the best combination of the positive and negative results providing the best diagnostic contribution. The high performance of this test is probably related to the fact that a positive result might be also due to the presence of immune complexes containing nucleosomes composed by all three immunoglobulin isotypes (IgG, IgA, and IgM), and by high-avidity antibodies [5,6,15].

Pathogenic mechanisms of anti-dsDNA antibodies

Anti-dsDNA antibodies are not only a helpful diagnostic tool for SLE, but have also been shown to play a central role in the pathogenesis of some clinical manifestations of the disease, in particular lupus nephritis.

Besides the clinical association between antibody titre fluctuation and lupus flares, there are additional experimental models in favor of a pathogenic role of anti-dsDNA antibodies.

Antibodies to DNA can be eluted from affected kidneys suggesting their direct involvement in the organ damage [27]. Anti-DNA binding to glomerular basement membrane (GBM) has been shown in rat kidneys perfused with histones, DNA and anti-DNA antibodies [28]. Although binding of anti-DNA antibodies to GBM was originally suggested to be due to the interaction with its component heparan sulphate, it has been showed that actually the binding was mediated by nucleosomes adhered to GBM [28]. In addition, mice injected with human or murine anti-dsDNA antibodies or with antibody secreting hybridomas have been shown to display many features of lupus nephritis [29–31]. Finally, non-autoimmune mice transgenic for the secreted form of the heavy and

Table IV. Sensitivity, specificity and positive and negative likelihood ratio for SLE diagnosis of the different anti-dsDNA assays investigated.

	Farr assay	CLIFT	VarElisa	EliA
Sensitivity	111/130 (85.4%)	62/132 (46.9%)	85/136 (62.5%)	99/136 (72.8%)
Specificity vs. HD*	1/81 (98.8%)	0/81 (100%)	0/79 (100%)	4/81 (95%)
Specificity vs. AD†	4/86 (95.3%)	2/85 (97.6%)	3/84 (96.4%)	5/85 (94%)
Positive LR‡	28.4	39.08	34.72	13.29
Negative LR	0.15	0.54	0.38	0.29

* HD, healthy donors.

† AD, non-SLE autoimmune disorders.

‡ LR, likelihood ratio.

light chain of an anti-DNA antibody display signs of nephritis [32].

In general IgG antibodies are of greater relevance to the disease than IgM antibodies [3–6].

Although the assumption of a pathogenic role for anti-dsDNA antibodies is now widely accepted, still matter of research is the precise mechanism (or mechanisms) by which these autoantibodies can induce renal damage.

Three main mechanisms have been originally proposed: (i) anti-dsDNA antibodies bind circulating nucleosomal DNA and induce a classical immune complex-related damage; (ii) anti-dsDNA cross-react with non-DNA kidney-specific antigens inducing an antibody mediated injury; and (iii) anti-dsDNA bind to planted antigens, either DNA or nucleosomes previously bound to renal tissue, leading to the *in situ* immune complexes formation [33].

More recently it has been suggested that some anti-DNA antibodies can bind to the cell surface, penetrate the cells, and localize in the cytoplasm, or deposit in the nucleus. Different cell types can be penetrated by anti-DNA antibodies *in vitro*, while following *in vivo* injection, staining of nuclei in liver, spleen and skin was found. Interestingly, this finding is reminiscent of the presence of intranuclear immunoglobulin deposit in tissues of some lupus patients described several years ago [34,35]. Penetration into living cells was shown to be a metabolically active phenomenon mediated by the F(ab)₂ fragment of the antibodies. It has been reported that penetrating antibodies may play a pathogenic role by enhancing cell growth and proliferation and by inducing cell death or apoptosis [34].

Finally, *in vitro* studies demonstrated that anti-dsDNA antibodies can bind to membrane structures of different cell types and induce cell perturbation leading to the expression of a pro-inflammatory and a pro-coagulant phenotype. Such an effect was related to antibody penetration in one study, but in others penetration was not investigated [36–44]. In some cases, the antibodies have been found to recognize DNA/histone complexes “planted” on the cell membranes because of electric charge interaction, while in other experimental models the antibodies

apparently reacted with constitutive not yet identified molecules [36–44]. These effects have been suggested to contribute to tissue inflammation and damage, particularly in the kidney [34].

Although there is a sound clinical and experimental evidence for a pathogenic (nephritogenic) role of anti-dsDNA antibodies, a relatively low proportion of patients with the autoantibodies undergo proliferative nephritis. Such a clash has been explained by the heterogeneity of the anti-dsDNA populations, only some of them being directly involved [33].

Conclusions

Dogmatically anti-DNA antibodies have been associated to SLE, however, the recent demonstration of the potential to produce anti-dsDNA antibodies also by a normal immune system raised some doubts on such a dogma. At the same time it appeared that only some populations of the anti-dsDNA antibodies do display a clear nephritogenic activity. There is evidence that both the diagnostic and prognostic power of anti-dsDNA antibodies are dependent on certain autoantibody populations rather than on the whole population in general. The same is also likely for the nephritogenic activity. Antibodies displaying high-avidity as well as cross-reactivity with kidney structures have been suggested to represent the clue autoantibodies, but a definite demonstration of such a theory is still a matter of research. Insight into these issues might give information on the characteristics of the diagnostic assays useful for diagnosis and prognosis of the lupus disease.

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