Methodological update

Detection of antibodies to dsDNA: Current insights into its relevance

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ABSTRACT

Ever since its first discovery in 1957, anti-DNA has taken a special place amongst the other antinuclear antibodies. Even today, it stands out between these, because of its high specificity for one particular disease: systemic lupus erythematosus (SLE). Furthermore, anti-DNA has been shown to actually play a role in a key disease feature of SLE: lupus nephritis. Binding of anti-DNA to the glomerular basement membrane of the kidney has been shown to be mediated by nucleosomes. More recently, it has been shown that patients with SLE also have antibodies specific for nucleosomes in their circulation. It may well be that anti-nucleosome detection in the near future will prove to be of more relevance than anti-DNA detection. Nucleosomes also seem to play a key role in the induction of anti-DNA (and anti-nucleosome) production. Mechanisms involved in this process may include defects in apoptosis and/or clearance of apoptotic material. Studies of these mechanisms will help us to decipher the cause of autoantibody production, or indeed of autoimmune diseases such as SLE.

Antinuclear antibodies: A short history

Antibodies against antigens present in cell nuclei probably occur in the circulation of all human beings. Since such antibodies generally react with nuclei whatever their origin, they also react with antigens in the nuclei of the host. Therefore, these antibodies are termed autoantibodies. Levels of these so-called antinuclear antibodies (ANA), elevated significantly above the normal serum level, are found in all systemic rheumatic diseases, with sometimes high, sometimes rather loose associations between a particular ANA specificity and a particular rheumatic disease. Therefore, detection and identification of ANA has gained increasing acceptance by clinicians who use the information to help determine or confirm a diagnosis and in treatment follow up.

ANA were first demonstrated in 1957 by Holborow and by Friou, using the indirect immunofluorescence technique (IFT) as developed by Coons (1, 2). After more than 40 years, this method is still used as a screening technique, although the employed substrate has evolved from organ tissue to cultured cells. Since the molecular characterisation of (most) antigens, other techniques, such as ELISA and immunoblotting, have been developed that allow precise identification of a lot of ANA specificities. The more precise characterisation of the involved antigens has also taught us that some ANA actually react with antigens that do not predominantly occur in the nucleus, but more in the cytoplasm or on the membrane of the cell. Yet, the term “antinuclear antibodies” continues to be used also for these antibodies.

Most ANA are directed against nucleic acids or proteins associated with nucleic acids. The first description of ANA with specificity for DNA (anti-DNA) also dates back to 1957, when Ceppellini described serum components reactive with DNA in patients with systemic lupus erythematosus (SLE) (3). In the years that followed, a number of assays for the detection of anti-DNA were developed. Especially high avidity anti-DNA was found to be of considerable diagnostic value for SLE, in particular lupus nephritis. Nowadays, it is recognised that nucleosomes probably form the most prominent antigen in SLE (4, 5).

DNA as (auto)antigen

Particularly in the diagnosis and fol-

low-up of SLE, anti-DNA has always attracted a great deal of interest. That makes DNA an important (auto)antigen (6, 7). DNA as antigen may be either double-stranded (dsDNA) or single-stranded (ssDNA), but *in vivo* DNA will almost always occur in the form of nucleosomes. Nucleosomes form the basic structure of chromatin, and have an important function in the compaction of DNA in the nucleus of a cell. A nucleosome consists of dimers of the four core histones (H2A, H2B, H3 and H4), that together form a histone octamer around which 146 base pairs of DNA are twice wrapped. Two nucleosome subunits are connected via a stretch of linker DNA to which histone H1 is bound. ANA reactive with DNA, especially if measured using a sensitive and specific radioimmunoassay, are considered to be a hallmark of SLE. Because the epitopes situated on DNA are – at least in part – based on the repetitive negative charge of the molecule, synthetic polynucleotides are often also recognised by anti-DNA antibodies. Apart from DNA, ribonucleic acid (RNA) also constitutes an antigen in SLE. Antibody reactivity towards RNA may either be based on epitopes shared with DNA or form a separate entity (8, 9).

DNA is found in all prokaryotic and nucleated eukaryotic cells of all species. Whether DNA from various species differs in antigenicity has not been studied in great detail, but differences clearly exist. In general, anti-DNA antibodies seem to bind DNA of all species tested (though not to the same extent) (10). Comparable results have been reported for monoclonal anti-DNA (11). For use in anti-DNA assays, DNA can be purified from tissue (e.g. calf thymus), from (eukaryotic) cells, from bacteria or from bacteriophages. Plasmid DNA forms a suitable alternative; this DNA is easily iodinated after isolation for use in radioimmunoassays (12). Especially calf thymus DNA has been used often in anti-DNA assays. Care should be taken to avoid protein contamination of the employed preparation. A different approach is to make use of the hemoflagelate *Crithidia luciliae* for the measurement of anti-DNA; *Crithidia luciliae* contains a giant mitochondrion, the kinetoplast, composed of purely dsDNA not "contaminated" with proteins.

Immunobiology of anti-dsDNA: B cell epitopes

Sequential as well as backbone determinants of DNA can be the targets of anti-DNA recognition. Backbone determinants on either single-stranded or double-stranded DNA are short regions of DNA helix or short nucleotide sequences. The interaction between the B cell paratope and dsDNA epitope seems to be based on electrostatic interactions, since this binding is extremely sensitive to salt concentrations and pH (13). Yet, especially in the case of high avidity anti-DNA, secondary hydrogen bonding also plays a role (14). Most likely such dsDNA epitopes are constituted by the sugar-phosphate backbone of the DNA. Apart from backbone recognition there also is selective recognition of DNA sites, variably expressed on different DNAs (11). Such binding seems to be more pronounced in the case of single-stranded DNA and is presumably based on the recognition of defined nucleotide sequences (15). Although anti-DNA specific for ssDNA may exist as a separate entity, most of what is generally called anti-ssDNA reactivity actually is anti-dsDNA of low avidity. When dsDNA is denatured, the strands of DNA become more flexible. Upon cooling, internal duplex formation over short stretches of DNA occur. Reactivity of anti-dsDNA with ssDNA is mainly due to this kind of internal duplex formation (16). Epitopes formed by these internal duplexes are exposed quite differently than in dsDNA. The difference lies in the flexibility of the DNA backbone, which is of extreme importance in terms of allowing multipoint attachment (and thus high avidity binding) of antibodies to DNA. Therefore, the greater flexibility of ssDNA will lead to higher avidity binding.

The actual combining site of an anti-DNA autoantibody encompasses only about 6 nucleotides (17), but most anti-DNA antibodies require DNA fragments from 40 to several hundreds of base pairs in length for stable interaction. The size dependency, however, differs very much among antibodies (18). These findings suggest that both Fabs of an anti-DNA antibody need to be bound for a stable interaction via (monogamous) bivalent interactions with antigenic sites distributed along the DNA molecule.

Detection methods

It is not without significance that such a large array of assays has been developed for the measurement of anti-DNA. Of course, introduced assays have often reflected immunochemical innovations, but the need for ever more (and better ?) anti-DNA assays has probably been animated by discontent about existing assays. Continually methods were sought that correlated better with SLE, or with exacerbations or clinical manifestations of this disease. The earliest techniques used for anti-DNA detection were relatively insensitive precipitation methods such as complement fixation and hemagglutination. Currently, the mostly widely used assays are immunofluorescent techniques (e.g. IFT on *Crithidia luciliae*), radio immunoassays (RIAs, e.g. the Farr assay and PEG assay) and enzyme-linked immunosorbent assays (ELISAs) (12, 19-23). These methods can either be obtained in kit form or be employed as in-house assays. IFT on *Crithidia luciliae*, which specifically detects antibodies to dsDNA, is one of the preferred methods because it combines good sensitivity with high disease specificity.

In RIAs the choice of antigen is of great importance. The DNA employed has to be bigger than 10^5 but smaller than 10^7 kD. Furthermore, the DNA must be double-stranded and, to allow quantitation of antibody reactivity, monodisperse in size. This indicates that circular double-stranded bacteriophage DNA (such as from PM2) or plasmid DNA (such as pUC9) are preferred. In ELISA systems, DNA has to be coated to plastic. ssDNA can easily be coated directly, but dsDNA is often coated via intermediates such as poly-L-lysine, protamine or methylated BSA. Such pre-coats introduce problems

relating to the binding of immune complexes and/or immunoglobulins not directed against DNA to the plates (via the intermediate molecule) (24). An alternative is to make use of biotinylated DNA and coat this via streptavidin to the plates (25, 26).
More recent methods for the detection of anti-DNA make use of immunoblotting [such as InnoLIA from Innogenetics (27) or microarrays (28)].

Comparison of anti-DNA assays

Through the years, we have extensively studied the aforementioned anti-DNA assays (ELISA, IFT on *Crithidia lucili - ae*, Farr assay and PEG assay). Based on many comparative studies, we concluded that ELISA is the most sensitive method, whereas the Farr assay is the most specific for SLE. Using the latter technique, only antibodies with relatively high avidity for DNA are detected. Mild forms of SLE, where patients only have anti-dsDNA of low avidity in their circulation, may easily be missed by this technique. On the other hand, low avidity anti-DNA occurs in diseases other than SLE as well. As is often the case, what you win in specificity you lose in sensitivity. Of the assays mentioned, the Crithidia test is merely a qualitative test (although it is of course possible to express anti-DNA measured with this technique in titres); the ELISA is a semi-quantitative assay, whereas both the PEG and Farr assays are true quantitative assays. Levels of anti-DNA should be expressed in IU/ml, based on the WHO standard Wo/80 (29).
From these studies, we conclude that the various anti-DNA assays all detect different (yet overlapping) parts of the spectrum of anti-dsDNA antibodies produced by patients. Careful studies have taught us that autoantibodies (including anti-dsDNA) can also be found in patients with autoimmune diseases other than SLE, or even in normal individuals (30,31). Therefore, if we want an assay to discriminate between SLE and non-SLE, we should choose an assay that detects only anti-dsDNA of high avidity. In practice, this means the use of a Farr assay. Although such assays are somewhat less sensitive, this is counterbalanced by the increase in disease specificity.
There are many reasons why different assays detect different populations of antibodies:
(a) the source of antigen differs: DNA may be of eukaryotic or prokaryotic origin, be double-stranded or single-stranded, be polydisperse in size or homogenous etc.;
(b) presentation of the antigen to the antibody differs: in RIAs it is generally in solution, in ELISAs it is coated to plastic; in the Crithidia test DNA is mostly presented intact in cells;
(c) reaction conditions are different: e.g. due to the ammonium sulphate precipitation step employed anti-dsDNA of low avidity is missed in the Farr assay; in second antibody techniques such as IFT and ELISA the choice of conjugated antibody is of importance; often, only IgG anti-DNA is measured with these techniques.
Comparisons between various anti-DNA assays have also been published by others (32-34). In general, significant correlations between anti-DNA values obtained with different assays were obtained. The Crithidia test was reported to be very specific, but lacking in sensitivity. Solid phase assays were found to be more sensitive than Farr and filter assays. This was ascribed to the detection of antibodies with lower affinity in solid phase assays. The low sensitivity of the Crithidia test observed by these authors could not be confirmed by us. We wonder whether this may have something to do with the quality of commercially available Crithidia slides (we make our own).

Clinical value of anti-dsDNA detection

Anti-dsDNA assays can be used for different purposes. If the major aim is to help the clinician in the diagnosis of SLE, it is imperative that the assay has a high specificity for this disease. In practice, this means that the Farr assay is preferable, followed by the Crithidia test. If screening for the presence of anti-dsDNA is the main objective, an assay that that is not selective for high avidity anti-dsDNA may be used. However, a positive result in such an assay does not always indicate that the patient has SLE: anti-dsDNA of lower avidity also occurs in diseases other than SLE (35). An evaluation of the diagnostic value of low avidity anti-dsDNA has shown that 52% of patients in whom anti-dsDNA was PEG-positive but Farr-negative were found to have SLE (36). When high avidity anti-dsDNA was present as well, 86% of the patients had SLE. Therefore, screening using a "broad spectrum" method should be followed by an assay that is selective for high avidity anti-dsDNA. Especially when only high avidity anti-DNA is detected, anti-DNA is really very specific for SLE, and thus of great relevance to the clinician. This was underlined years ago in a study by Swaak *et al*. (37); among a group of over 400 patients without SLE but with Farr assay-detectable anti-dsDNA in the circulation, 85% developed SLE within 5 years after the initial Farr-positive result.
Because of problems inherent to the use of an ELISA as a screening assay, we have chosen to use the Crithidia test for this purpose. Detection of anti-DNA with this technique is then followed by an estimation of the amount and the relative avidity of the anti-DNA present using the Farr assay and the PEG assay. In Figure 1, the protocol that is employed in our institute for anti-dsDNA screening is displayed.
If the detection of anti-dsDNA is carried out to study SLE patients longitudinally, it may be of value to be informed of the fluctuations in the anti-dsDNA level as well as of the relative avidity of the anti-dsDNA present. Farr assay and PEG assay results may readily be compared, as these assays only differ with respect to the way in which DNA/anti-dsDNA complexes are precipitated. A relative avidity index can be acquired by calculating the ratio between the results of both assays, expressed in terms of IU/ml. Using this approach we found that the anti-dsDNA avidity of patients with nephritis was significantly higher than that of patients with CNS involvement (38, 39). Recently, Williams *et al*. described that anti-DNA (and anti-nucleosome) affinity often mirror-image lupus neph-

ritis: residual anti-DNA antibody affinity for DNA is often low during phases of active SLE nephritis. When nephritis improves or precipitates chronic renal failure, serum anti-DNA antibody affinity increases again (40). They concluded that the measurement of anti-DNA antibody affinity may provide a useful indicator of renal disease activity.

Anti-dsDNA as prognostic tool

Longitudinal studies of anti-DNA have primarily been performed with the Farr assay (41,42) and the PEG assay (43, 44). With respect to the Farr assay, Swaak *et al*. found that major disease symptoms in SLE patients only occurred during a rapid decrease of the anti-dsDNA level (45). All exacerbations were preceded by a continuous increase of anti-dsDNA levels and followed by a sharp drop of these levels (46). These observations have been confirmed by Ter Borg *et al*. (47). Following the suggestion that an SLE patient whose anti-dsDNA level is rapidly increasing is bound to develop an exacerbation, Bootsma *et al*. treated SLE patients with prednisone as soon as a significant rise in anti-dsDNA occurred; they reported that this prevented relapses in most cases without increasing the cumulative dose of prednisone given (48).

Longitudinal studies by Nossent *et al*. (44) showed that the anti-dsDNA avidity of SLE patients remains more or less constant over time. This held for patients who at the beginning of the study had only low avidity anti-dsDNA in their serum, as well as for patients with higher avidity anti-dsDNA. Exceptions were mostly found among patients who developed nephritis during the course of the disease. Clinically, patients with only low avidity anti-dsDNA in their circulation had a rather mild disease course with the absence of renal involvement. The PEG assay was found to have a rather limited predictive value but a high specificity (90%) for clinical exacerbations.

The role of anti-dsDNA in the pathogenesis of SLE

The concept of anti-DNA playing a direct role in the pathogenesis of SLE is based on a large body of circumstantial evidence. The various pieces of evidence pointing in the direction of an active role in the pathogenesis are:

1. Anti-DNA fluctuates in time, in close association with exacerbations and remissions of the disease;
2. Patients who do not have SLE at the time that anti-DNA is first detected in their circulation generally develop SLE within the next 5 years;
3. Antibodies to DNA can be eluted from affected kidneys (49-51);
4. Perfusion of rat kidneys with histones, DNA and antibodies to DNA leads to binding of anti-DNA to the glomerular basement membrane (GBM) (52).

Taken together, these pieces of evidence indicate that anti-DNA is directly implicated in the induction and propagation of inflammatory reactions in affected tissues. The mechanism through which anti-DNA binds to tissues has been a matter of debate throughout the years. Traditionally, SLE was considered to be an immune complex disease (53). In this concept, binding of anti-dsDNA to DNA leads to immune complexes that deposit in tissue. Binding of DNA by antibodies may either occur in the circulation or *in situ* (54). At the site of deposition, subsequent complement activation then leads to inflammation and the characteristic disease features of SLE.

For some years, another hypothesis has been attracting increasing interest. It is based on the observed crossreactions of (monoclonal) anti-DNA with polyneg-

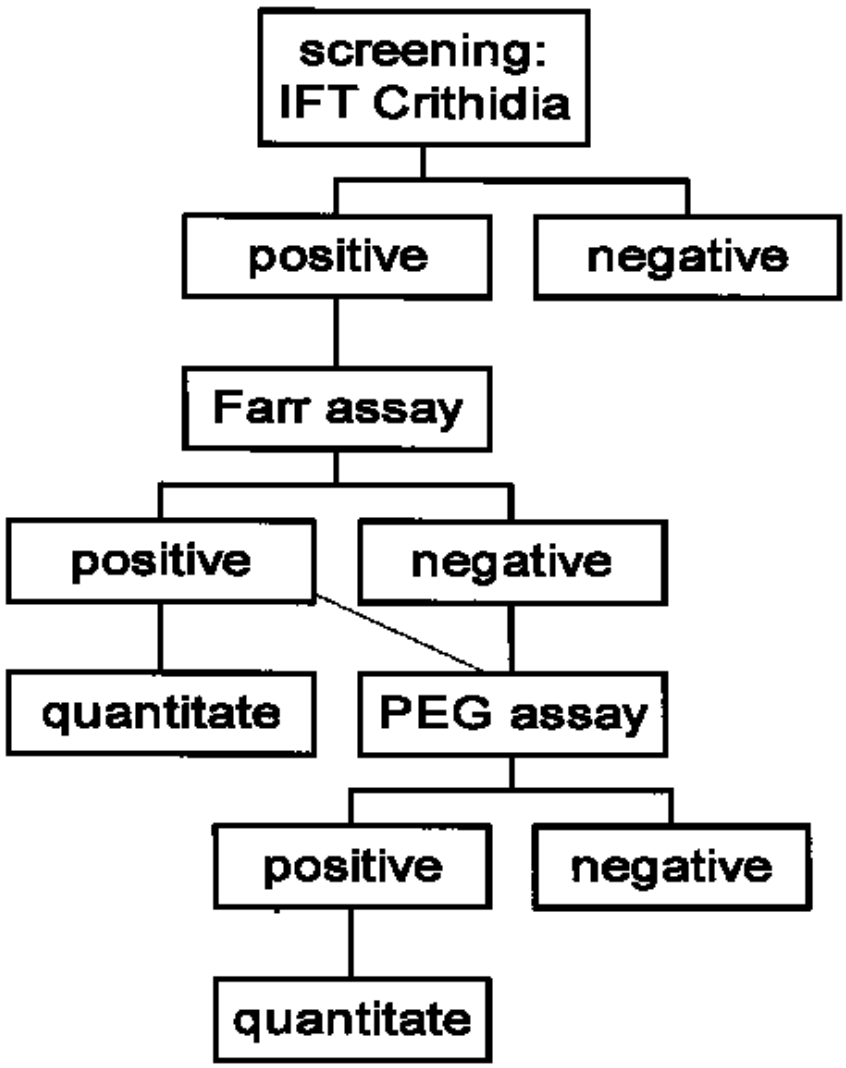

Fig. 1. Routine detection of anti-DNA.

ative structures, including heparan sulphate (55). Since heparan sulphate constitutes the major glycosaminoglycan of the GBM, direct binding of anti-DNA seemed to be a plausible mechanism for the induction of nephritis. More recently, however, it was shown that the binding of anti-DNA to heparan sulphate is mediated by nucleosomes (56-58). In support of this, Schmiedeke *et al.* (59) have shown that histones have a high intrinsic affinity for the GBM. The suggested target for this binding was (again) heparan sulphate. Bound histones retain the capacity to bind specific antibodies and DNA.

These findings again suggest an immune complex mediated pathway leading to the induction of disease features; only now the binding of autoantibodies to the GBM is mediated via nucleosomes (60, 61).

Origin of antibodies to DNA

The induction of autoantibody production towards DNA was originally thought to be based on the polyclonal activation of B cells. In such a scheme T cells with specificity for DNA are not to be found. However, several lines of evidence have challenged this hypothesis.

Biochemical studies of anti-DNA antibodies have illustrated the presence of somatic mutations in these antibodies (62,63). These data are in favour of antigenic stimulation of the anti-DNA response in a T-cell dependent way. Datta *et al.* were amongst the first to clone autoimmune T-helper (Th) cells from patients as well as from mice with lupus nephritis (64). Less than 15% of these "autoreactive" T cell clones had the functional ability to selectively induce the production of pathogenic anti-DNA autoantibodies (65). Later on, they showed that histone-derived peptides from nucleosomes could stimulate pathogenic T cell clones (66). Although the half-life of DNA in the blood has been reported to be only about 4 minutes in haemodialysis patients (67), the persistence of DNA (in the form of nucleosome-like structures) could immunise patients in a T-cell dependent way.

A possible clue to the question of how nuclear antigens are exposed to the immune system comes from work by Casciola-Rosen *et al*. (68), who reported the presence of nucleosomes and other nuclear antigens in the surface blebs of cells dying from apoptosis. Several studies have provided data in support of defective apoptosis playing a role in the induction of autoantibody production. Indications of increased apoptosis in SLE have been found in the increased levels of soluble Fas; these levels are the highest in patients bound to develop an exacerbation (69). Indeed, increased levels of nucleosomes have been identified in the circulation (70,71). Our own results (to be published) even show nucleosome levels to be inversely related to anti-DNA levels. Although prolonged increased levels of nucleosomes in the circulation may result from defects in apoptosis, they can also result from the improper elimination of apoptotic material. Inherited deficiencies in early factors of the complement system often present with SLE. Early complement factors are important for the clearance of immune complexes, but also play a role in the clearance of apoptotic cells (72). Indeed, mice made deficient for C1q by gene targeting develop ANA and glomerulonephritis (73).

Recently, it was shown that mice with targeted deletion of the gene coding for serum amyloid P component (SAP) spontaneously develop ANA and severe glomerulonephritis (74). SAP, a highly conserved plasma protein named for its universal presence in amyloid, binds specifically to chromatin under physiological conditions by the displacement of histone H1. This leads to solubilization of chromatin, which is otherwise insoluble in plasma. Furthermore, SAP binds *in vivo* both to apoptotic cells, to the surface blebs which bear chromatin fragments, and to the nuclear debris released by necrosis. SAP may therefore participate in the clearance of chromatin exposed by cell death. It has indeed been shown that levels of DNA/SAP complexes in normal plasma are higher than in the plasma of SLE patients (75). These findings indicate that SAP, mediating the clearance of nuclear material, prevents the formation of pathogenic autoantibodies against chromatin and DNA.

Anti-DNA: Which way from here?

Forty-four years after they were first discovered, antibodies to DNA still have an important place in the diagnosis and follow-up of SLE patients. This is mainly due to the specificity of anti-DNA for SLE and the good correlation of levels of anti-DNA with disease features. But will we still be measuring anti-DNA in 10 or 20 years? There are many reasons to doubt this. The first is that it is not really certain that what we call "anti-DNA" really is anti-DNA. For instance, Hylkema in our lab discovered that much of the reactivity in a Farr assay actually is not attributable to anti-DNA but to complexes of autoantibodies and nucleosomes (76). Alternatively, recent reports have demonstrated that it may be more relevant to measure anti-nucleosome antibodies rather than anti-DNA (77, 78): these may be more specific for SLE and more directly involved in the disease features.

Another approach to the measurement of anti-DNA has been taken by Sun *et al.*, who looked for structures reactive with anti-DNA antibodies in peptide libraries (79). These authors have come up with a peptide of 10 amino acids mimicking the antigenic determinant of anti-DNA. The chemically synthesized peptide (RLTSSLRYNP) was recognized by 88% (37 out of 42) of anti-dsDNA antibody-positive SLE sera. Especially if the use of such mimotopes would allow for easy discrimination between anti-DNA and anti-nucleosome reactivity, they may (in due time) gain in relevance for the routine detection of these autoantibodies. Other assays that one day soon may replace the traditional fluorescence and radioimmunoassay techniques are based on microarray technology or multi-analysers (e.g. Pharmacia). Introduction of these new technologies will undoubtly introduce new questions: concepts such as the "SLE-specificity" of anti-DNA, or even the use of the Wo/80 standard for quantitation of anti-DNA have been defined using tradi-

tional assays. It is by no means obvious that such concepts also hold for anti-DNA detected with these new assays. It will be of the utmost importance to validate such assays ourselves, in our own labs, using our own panels of sera of patients with different autoimmune diseases.

Of even more relevance, of course, will be the deciphering of what causes anti-DNA production in patients. This will bring us insight into the cause of autoimmune disease and may help us to conquer it in the coming 44 years.

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