Two-step magnetic beads based purification method for antibody reactive with single stranded DNA: a pilot study

Keywords: systemic lupus erythematous, autoimmune disease, anti-ssDNA antibodies, anti-DNA antibodies

Abbreviations: SLE, systemic lupus erythematosus; SS, single stranded; DS, double stranded; nervous systems

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Introduction

Systemic Lupus Erythematosus (SLE) is a chronic, potentially fatal autoimmune disease characterized by exacerbations and remissions with various clinical manifestations affecting multiple organ systems, including the skin, kidney, joints, cardiovascular, and nervous systems. The hallmark of SLE is the production of an array of IgG and IgM auto-antibodies (found in 70-90% of patients, especially those with nephritis) directed against one or more nuclear components, the most frequent of which are double stranded (ds) DNA and/or single stranded (ss) DNA.[1,2] The level of anti-DNA antibodies varies in different SLE patients' plasma, with high levels of anti-ssDNA and/or anti-dsDNA antibody being associated with the flare. Consequently, the level of anti-DNA antibodies in patients' sera is used to monitor disease activity and progression.[3-6] Although most clinicians ignore the occurrence of anti-ssDNA autoantibodies in lupus, the results from clinical and basic studies indicate that both anti-ssDNA and anti-dsDNA are involved in disease development, either due to the binding to some cellular proteins or due to their catalytic activities. They have even been eluted from the kidney biopsies of experimental murine models, as well as SLE patients.[7-9] While the anti-dsDNA autoantibodies occurring with high incidence in SLE are considered a "hallmark" of lupus disease, and therefore highly specific for the disease, the anti-ssDNA autoantibodies can be found in other autoimmune diseases, and therefore are not considered specific. Incidence of anti-ssDNA antibodies in different autoimmune diseases as a compilation from different sources is shown in Table 1. Yet, their abundant (up to 65%) presence in the sera of SLE patients constantly provokes scientists to determine the reason for their occurrence and persistence in the disease.[10,11]

Table 1 Incidence of Anti-ssDNA Antibodies in different autoimmune diseases

Disease Incidence %	
Systemic Lupus Erythematosus	65
Active	78
Inactive	43
Possible SLE	50
Rheumatoid Arthritis	35
Systemic Scleroderma	0
Localized Scleroderma	50
Normal Patients	0

Note: The frequency of anti-ss DNA antibodies in a disease are present compilation from the literature. The incidence varies depending upon the patient population

Due to the reasons described, many attempts have been made to efficiently purify and separate these two distinct types of anti-DNA antibodies in order to precisely address these multiple puzzling issues. However, none of the methods provide sufficient specificity for analyzing differences among distinct diseases and disease states. A particular problem in past approaches is the non-specific binding of a variety of antibodies to the DNA cellulose affinity matrix often employed in a broad spectrum of methods.[12-16]

The methods created and applied in the past were mainly performed with one goal in mind: to detect the anti-DNA structural and functional characteristics in order to understand their pathogenic activities (binding to DNA, DNA hydrolysis, and cytotoxicity).[17-22] It is important to prove that the antibody's catalytic activity is not due to a contamination, but that it is an intrinsic, constitutive property of the antibody itself since antibodies may form complexes with other proteins. In addition, the purification methods, described so far, have involved multiple steps under harsh and prolonged conditions, often leading to inactivation and denaturation of the antibodies. However, in those studies, there was no attempt to separate or distinguish between the antibodies against ssDNA and those against dsDNA, with the ultimate goal to determine and discriminate their particular/distinctive pathogenic roles and mechanisms of action in the course of, or their predictive role in the flare of SLE, or other autoimmune diseases.[3,4] The fundamental objection to the previous attempts at purifying anti-DNA antibodies directly from human plasma is that they yielded numerous bands on electrophoretic separation that were not consistent with the molecular weight of IgGs in non-reducing conditions.[17]

Hence, many scientists have shifted their attention to monoclonal anti-DNA antibody generation.[16,21,22-33] However, this does not reflect natural conditions, since anti-DNA antibodies in human diseases and SLE are polyclonal.[18,19]

Therefore, the development of a specific method for more efficient isolation and purification of each of the two categories of anti-DNA antibodies from human sera (anti-ss and anti-ds DNA) with the highest level of purity detected within highly sensitive system would be of crucial importance for use in studying their eventual individual pathogenic role, as well as their individual role in the ethio-pathogenesis of SLE and other autoimmune diseases.

Due to the reasons mentioned above, the objective of this study was to develop a novel, fast, specific, well-yielded, and relatively cheap method for isolation and purification of anti-DNA antibodies reactive with ssDNA, in order to investigate the possible differences among anti-ssDNA antibodies from normal individuals, SLE and B-CLL patients, in future. In this initial, pilot study, with the novel method[32] and its modification as a priority, we have tested only a minimal statistical number of controls and patients with B-CLL and SLE for anti-DNA antibody serum concentration, electrophoretic patterns, and their hydrolytic activity. The possibilities for their existence in these diseases are also discussed.

Material and methods

Principle of the method: The use of poly-thymidine oligomers for the specific removal of anti-DNA antibodies was based on X-ray crystallography data presented by Tanner et al.,[24] showing that mouse monoclonal anti-ssDNA antibody specifically binds to a motif of five thymidine nucleotides.[24] The order of base specificity for anti-DNA antibodies is dT>>dG>>dC; dA is not recognized by any of the antibodies.[7] Protein G affinity matrices are widely employed in the purification of the IgG isotype antibodies.

Material

Patients and sera

Whole blood was intravenously drawn, with written consent and under the guidance of protocols approved by the Florida Atlantic University's Institutional Review Board. The plasma of lupus (SLE), B-CLL patients and normal donors was obtained by centrifuging whole blood at 300 x g for 10minutes, to sediment the peripheral blood mononuclear fraction from the plasma. Supernatant fluid containing plasma was harvested and centrifuged at 600xg for 15minutes, in order to subsequently sediment and remove blood platelets. The plasma was stored frozen at -20°C until further use. Three individuals from each group (normal, B-CLL and SLE) were analyzed.

Reagents: essential

Biotinylated Oligo dT 20- mer (MWG Inc. BIOTECH, High Point, NC), dynalbeads M-280 (DYNAL INC, Lake Success, NY) and Dynal protein G beads (DYNAL INC., Lake Success, NY) were used to isolate anti-ssDNA antibodies. Oligo 18mer for hydrolytic analysis was obtained from Invitrogen™, Carlsbad, CA. SDS-PAGE (PolyAcrylamide Gel Electrophoresis) was performed using the Pharmacia PhastSystem™ and PhastGel™ Gradient 4-15 separation gels, PhastGel™ buffer strips and full-range rainbow molecular weight markers (GE Healthcare, Piscataway, NJ) A Microcon YM-50 centrifugal filter device (Millipore, Burlington, MA) was used for concentration of isolated and purified antibody. Anti-DNA antibody levels in normal individuals, SLE and CLL patients' sera were measured by using Oligo-(dT) 20mer (Eurofins MWG Operon, Huntsville, AL) coated Streptavidin microplates (Roche Diagnostics, Indianapolis, IN) for ELISA assay developed in our lab.

Methods

Magnetic beads

Preparation of magnetic beads coated with a thymidine oligomer (Oligo dT): Mathematical model from a stock solution containing a concentration of $1.7x10^8$/ml of streptavidin coated magnetic beads known by the trade name of dynalbeads M-280 (DYNAL INC, Lake Success, NY), 2.5 milligrams were removed and washed twice with Binding & Washing (B & W) buffer, containing

10mM Tris-HCl (pH 7.5), 1mM EDTA and 2.0 M NaCl, according to the manufacturer's directions for the use of the magnetic separating device (BD Biosciences, Palo Alto, CA). Following the second wash, the supernatant fluid was removed and the beads were re-suspended with 500 µl of B &W buffer. In order to bind the oligomer to the streptavidin coated beads, an equal volume of the following solution was added to the re-suspended beads: 19 µl of biotinylated Oligo dT 20- mer (MWG Inc. BIOTECH, High Point, NC) at a concentration of 132.1 pmol/µl (stock concentration) in 481µl of nanopure water. The mixed suspension of biotinylated Oligo (DT) and strepavid in coated dynal beads was incubated for 15minutes at room temperature. After incubation, the tube containing the suspension of Oligo (dT) coated beads was placed against the dynal magnet for 1minute and the supernatant fluid containing unbound oligo (dT) was discarded. The Oligo (DT) coupled M-280 magnetic beads were then washed twice with B & W buffer. After removal of the supernatant fluid, the Oligo(DT) bound beads were stored at +4°C in 1ml of storage buffer consisting of PBS and 0.01% sodium azide at a final bead concentration of $1.7x10^8$/ml. A schematic presentation of the preparation of magnetic beads is given in Figure 1 which is based on previously published principles of the original method.[32]
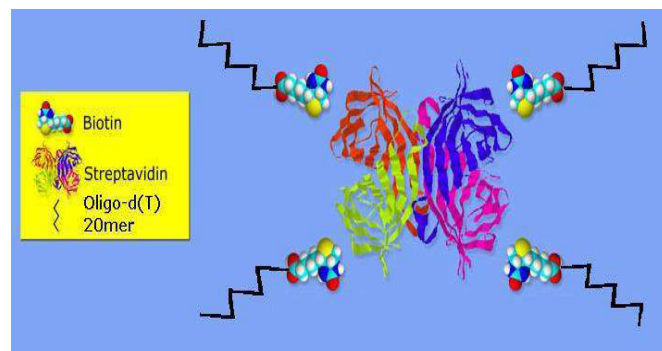

Figure 1 Magnetic SA Beads modeling and principles of function: Tetrameric SA molecule binds one biotin molecule per each monomer. Each biotin molecule binds one oligo-(DT), while one molecule of oligo-(DT) binds one anti-DNA autoantibody molecule.

Calculation of dynal oligo (DT) coupled M-280 magnetic beads and using dynal protein G beads for first and second steps of anti-DNA antibody purification: The theoretical number of oligo - T beads and protein G beads needed to maximize recovery of anti-DNA antibodies from normal individual, lupus and B-CLL patient's sera was determined. We know that 1IgG antibody weighs $2.58x10^{-19}$ g. Because 1 oligo T bead possesses approximately $1.8 x 10^6$ oligo dT on its surface, it can bind $1.8 x 10^6$ antibodies. So, 1 bead binds $1.8 x 10^6 x 2.58x 10^{-19}$ g=$4.65 x10^{-13}$g of antibodies. We assayed 66.55µg, 489.32µg and 301.16µg of anti-DNA antibodies in 1ml of normal individual, lupus and B-CLL patients' plasma, respectively. Thus, we need (a) 66.55µg/($4.65 x10^{-7}$µg)=$1.43x10^8$ beads, (b) 489.32µg/($4.65 x 10^{-7}$µg)=$1.05x10^9$ beads, (c) 301.16µg/($4.65 x 10^{-7}$ µg)=$6.48x10^8$ beads, to recover the total of anti-DNA antibodies from 1ml of normal individual, lupus and B-CLL patients' plasma respectively (Figure 2A). According to the Dynal protocol for protein G, the recovery rate of 100 µl of beads is around 40%. We sought to recover the maximum IgG from normal individual, lupus and B-CLL patients' sample by adding 250µl of dynal protein G beads to each sample. Theoretically, we need $1.43x10^8$, $1.05 x 10^9$, $6.48 x 10^8$ oligo-dT beads and 250µl of protein G beads respectively to pull out total anti-DNA antibodies from 1ml of normal individual, lupus and B-CLL patients' plasma (Figure 2A).

Anti-DNA antibody affinity purification with oligo (dT) M-280 magnetic beads and dynal protein G beads: sequence of events: The sequential affinity purification procedure used to purify anti-DNA antibodies from heparinized plasma was conducted at 4°C. After thawing, the fibrin clot was removed from the plasma by centrifuging at 300xg for 10minutes. The clarified plasma (serum) was incubated with Oligo (dT) M-280 magnetic beads (DYNAL INC., Lake Success, NY) (previously blocked with BSA by manufacturer) in Binding & Washing (B & W) buffer containing 20mM Tris (pH 7.5), 10mM NaCl for antibody isolation. After incubation, contaminants were removed by washing the bead-antibody complexes ten times in the Binding and Washing buffer using a magnetic separating device (BD Biosciences, Palo Alto, CA). The anti-DNA antibody was eluted from Oligo (dT) M-280 magnetic beads with 20 mM Tris and 1M NaCl, pH 7.5. The eluate was concentrated immediately to remove salt and exchange the buffer using a Microcon model YM-50 centrifugal filter device (Amicon/Millipore, Massachusetts) The eluate was concentrated to 5ul and an additional 100 µl of phosphate buffer (0.1M, pH=7) was added to wash the membrane and harvest the antibodies. At this stage, contaminants such as DNAse, other nucleases and DNA binding proteins discharged from apoptotic cells may have been co-eluted along with the anti-DNA antibody. Therefore, the Dynal protein G beads (DYNAL INC., Lake Success, NY) were used in a second step to specifically separate all four IgG isotypes of anti-DNA antibody from other contaminants. Purified antibodies were stored immediately in a storage buffer containing 20mM Tris-HCl, 50% glycerol and 0.01% thimerosal,(pH=5.0-7.0) at -20°C, to preserve binding activity for a further ELISA specific test-system.
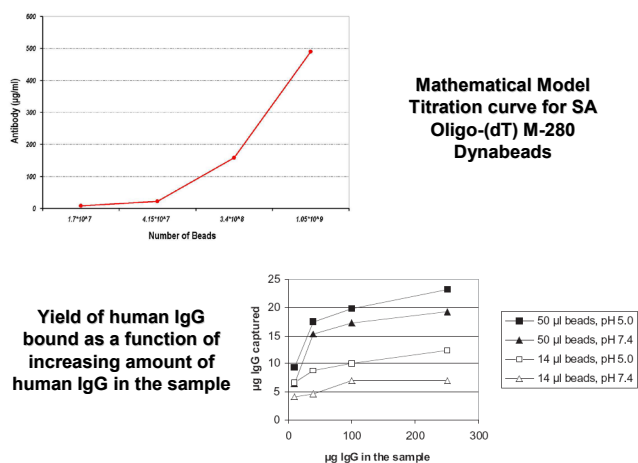

Mathematical Model Titration curve for SA Oligo-(dT) M-280 Dynabeads

Yield of human IgG bound as a function of increasing amount of human IgG in the sample

Figure 2 Experimental results of mathematical model expressed by means of SA Oligo-(dT) beads titration curve (A). The recovery efficiency can be greatly enhanced (from 20-40%) by adding the theoretical number of beads to the sample, and by lowering pH of protein G-beads elution buffer from 7 to 5 (B).

Testing of the antibody purity and efficiency of the isolation and purification method

The efficiency and resultant purity of this method was proven by SDS-PAGE, Western Blot, ELISA assay and recovery efficiency.

SDS-PAGE

SDS-PAGE was performed using the Pharmacia Phast System™ (Amersham Pharmacia Inc, Piscataway, NJ) with PhastGel™ Gradient 4-15 separation gels and PhastGel™ buffer strips. Full-range rainbow molecular weight markers were purchased from Amersham Biosciences (Amersham Biosciences, Corp, Piscataway, NJ). The

protein separation procedure was completed by following the PhastSystem™ Owner's Manual (Separation Technique File No.130). The detection range of the gradient 4-15 separation gel is 30 to > 300 kilodaltons. Any protein with a molecular weight below 30 KD may appear on the stained gel, but the migration distance may not be a linear proportion to the log of the molecular weight. According to the protocol, a molecular weight as low as 10 KD can be detected on the gel. This was observed using the rainbow markers, the molecular weight of which ranges from 10 to 250 KD. The silver staining technique in this system is derived from the method of Heukeshoven and Dernick.[25]

Western blot for confirmation of antibody subclasses

Proteins (antibodies) were separated by gel electrophoresis, using the PhastGel™ SDS-PAGE[4-15] gradient system located in our lab (GE Healthcare, Piscataway, NJ). Western blot was used to determine the identity of an electrophoretically separated protein and to measure relative amounts of the protein present in different samples, based upon densitometry. The proteins (antibodies) were transferred to a sheet of PVDF membranes (PhastSystem™ Development Technique File No.220). The proteins (antibodies) retained the same pattern of separation they had on the gel. The blot was incubated with a generic protein (such as milk protein) to bind to any remaining open binding sites on the membrane. A mouse anti-human antibody solution was incubated with the gel for 1 hour. After washing the membrane in a buffer to remove unbound mouse anti-human antibodies, a secondary goat anti-mouse antibody solution was added. The secondary antibody is conjugated with the enzyme (horseradish peroxidase). The location of the membrane bound antibody conjugate is revealed (visualized) by incubation with a colorless substrate.

Anti-DNA antibody quantification using ELISA

Purified anti-DNA antibodies were quantified using the Pierce Micro BCA™ Protein Assay kit (PIERCE Biotechnology, Rockford, IL) and used as a standard for the ELISA Assay. Anti-DNA antibody levels in normal individuals, SLE and B-CLL patients' sera were measured by using an Oligo (dT)-20mer coated Streptavidin microplate for ELISA assay. ELISA quantification was conducted as previously described.[26]

Recovery efficiency calculation

The level of anti-DNA antibody in patients' plasma was calculated from a standard curve determined by ELISA with affinity purified anti-DNA antibody quantified by the Pierce BCA microassay as described above. By comparing the amount of anti-DNA antibodies purified from 1 ml of patient's plasma to the total amount of anti-DNA antibodies assayed as present in 1 ml of patient's plasma, we obtained the recovery efficiency of Dynal Oligo (dT) coupled M-280 magnetic beads and Dynal protein G beads.

Testing of the abzyme's functional (hydrolytic) activity: determination by UV spectrophotometry

To facilitate the antibody binding, modified Gololobov's oligo 18mer and terminated with 5 thymidines in concentration of 1µM/ml has been used as a substrate for anti-ssDNA binding and hydrolysis. Enzyme (abzyme) to substrate ratio was optimized to 1:2. The substrates were obtained from Eurofins MWG Operon (Huntsville, AL). The reaction mixture containing 22 mM Tris-HCl and different concentrations of $MgCl_2$ and $CaCl_2$ have been used to check for hydrolytic activity of purified anti-ssDNA antibody from healthy, B-CLL and SLE suffering subjects, as well as for DNA-se 1. The reaction was followed at 37°C in different time-intervals. After that, the optical density of released nucleotides was measured at 1.84 purity

levels in UV spectrophotometer (260nm). The results obtained from triplicates were plotted and compared.

Results

The concentration of purified anti-DNA antibodies is the lowest in normal individuals and the highest in SLE patients

The concentration of purified anti-DNA antibodies from one lupus patient is 319.79 µg/ml determined by using Micro BCA™ Protein assay reagent. The purity was confirmed by SDS-PAGE, at silver staining nanogram sensitivity level. The known concentration of anti-DNA antibodies was subsequently used as the standard for ELISA assay. In quantification ELISA assay, five-fold serial dilutions of each serum sample was calculated by a 4-parameter logistic log model integrated from a standard curve. The mean antibody concentrations, standard deviations and CVs are reported in Table 2. The within-

assay CV of the calculated concentration of antibody in each sample was 9.46%, 3.86% and 5.69% in normal individual, SLE and B-CLL respectively. The averages of five dilutions of anti-DNA antibody concentration of normal individual, lupus and B-CLL patients' plasma were 66.55µg/ml, 489.32µg/ml and 301.16µg/ml respectively. It is apparent that titer is the lowest in normal individuals (66.55µg/ml) and the highest in SLE patient (489.32µg/ml) (Tables 4 & 5).

Table 2 Anti-DNA antibody concentration, standard deviation and coefficient of variation thereof from the sera of normal (healthy) individual, SLE and CLL patients

Anti-DNA Antibody			
Samples	**Concentration(mg/ml)**	**Standard deviation**	**CV(%)**
Normal Individual	66.55	6.3	9.46
Lupus Patient	489.32	18.89	3.86
CLL Patient	301.16	17.15	5.69

Table 3 Anti-DNA antibody recovery rate

Samples	**Total Amount of Anti-DNA Antibodies in 1ml Plasma(µg)**	**Purified anti-DNA antibodies from 1ml plasma(µg)**	**Recovery rate(%)**
Normal Individual	66.55	11.80	17.73
B-CLL	489.32	96.91	19.81
SLE	301.16	85.15	28.27

Table 4 Theoretical numbers of Dynal beads and protein G beads for purification of the total amount of anti-DNA antibodies from 1ml of normal individual, B-CLL and SLE patients' plasma

Patient	**Total amount of anti-DNA antibodies in 1ml plasma (µg)**	**Oligo-(dT) bound beads**	**Protein G beads (µl)**
Normal Individual	66.55	1.43×10^8	250
B - CLL	301.16	6.48×10^8	250
SLE	489.32	1.05×10^9	250

Table 5 Reaction mixture for Anti-DNA antibodies and DNAse 1 reaction activities as measured in UV spectrophotometer

DNA hydrolysis experimental design			
Substrates only	**Control 1**	**Control 2**	**Anti-DNA antibody**
SS Oligo-(dT) 18-20 mer	DNAse 1 + Substrates	Reaction Mixture No DNAse 1 No Antibody No Substrates	Anti-DNA Antibody + Substrates

Anti-ssDNA recovery rate of the two-step affinity purification method, improved by modification

The two-step purification recovery rate based on the amount of purified anti-DNA antibody concentration/anti-DNA antibody concentration in a patient's plasma is approximately 20%, as shown in Table 3. The recovery efficiency can be greatly enhanced by modifying the initial method,[32] e.g. by adding the theoretical number of oligo-dT beads to the sample, and lowering pH of protein G-beads elution buffer from 7 to 5 (Table 3, Figure 2A) (Figure 2B). Figure 2B shows the dependence of the amount of antibody eluted under different protein G beads elution buffer pH. A lower pH (5) favors elution and contributes to the higher recovery rate of about 40%. Yet, a more thorough wash is needed when using higher numbers of beads in order to obtain highly purified antibodies (this has been proven by experimentation, the data of which are not shown).

SDS-PAGE purity testing: patients with SLE have an increased rate of apoptosis

Normal individual, SLE and B-CLL patients' sera and affinity purified anti-DNA antibody preparations from the first and second

steps were subjected to analysis by SDS-PAGE electrophoresis. Figure 3 shows the results of the first and second purification steps on SDS-PAGE Phast gel electrophoresis in non-reducing conditions. More bands are observed in the first step than second step, indicating that a further purification is necessary to remove contaminants from the first step. The result in Figure 3 may also suggest that patients with SLE have an increased rate of apoptosis. More bands and higher intensity are shown on the gel after first purification in SLE patients, although the same amount of sample was applied. These molecules may be the apoptotic wastes which were not removed by phagocytic cells. It may indirectly be proof of the impaired immune complex clearance, by phagocytes and other components of the clearing system, caused by SLE.

SDS-PAGE electrophoresis: anti-DNA antibodies purified from SLE express different pattern in comparison to those in normal individuals and CLL patients

IgG Silver stained SDS-PAGE electrophoresis for purified anti-DNA antibodies of two normal individuals, SLE and B-CLL patient'

are shown in Figure 4. Anti-DNA antibodies from both normal individuals and B-CLL patients have two bands with similar molecular weights ranging from 150 to 180kD. The two bands seen on the gels loaded with affinity-purified anti-ssDNA from normal individuals are parallel to the bands seen in the B-CLL patients as shown in Figure 4. This value range corresponds to the molecular weight of human IgGs on the gel under non-reducing conditions. However, anti-DNA antibodies purified from SLE have higher molecular weights than standard human IgG (ranging from 150,000-180,000 KD) and the numbers of bands within the spectrum is higher than those of normal individuals and B-CLL patients. The differences with respect to MW and banding patterns are also seen between individual lupus patients. Therefore, our results strongly suggest that anti-DNA antibodies from SLE patients belong to more than two subclasses.
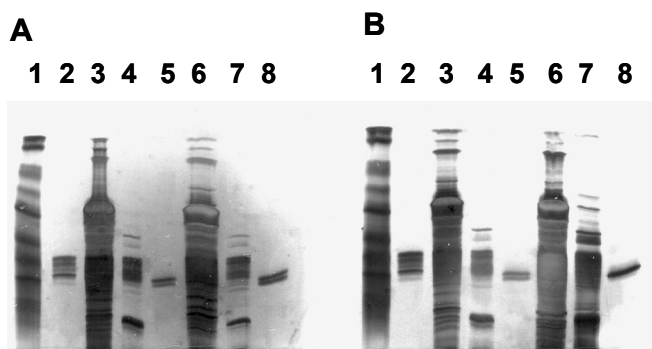
Figure 3 Anti-DNA antibody detection from sera: first and second step of purification. Eluates after each step analyzed on SDS-PAGE gel (non-reducing conditions). A. Lanes 1 to 5: marker, human IgG, normal individual's serum, eluates after first and second step purification. Lane 6 to 8: B-CLL patient's serum, eluates after first and second step purification. B. Lane 1 to 5: marker, human IgG, normal individual's serum, eluates after first and second step purification. Lane 6 to 8: SLE patient's serum, eluates after first and second step of purification.
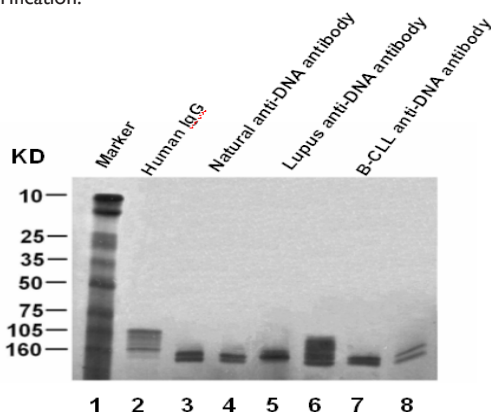
Figure 4 Purified anti-DNA antibodies from normal individual, SLE and CLL patients are detected on Silver-Stain SDS-PAGE gel in non-reducing conditions. Lane 1: Marker Lane2: Human IgG. Lane 3, 4: Purified anti-DNA antibody from healthy individuals. Lane 5, 6: Purified anti-DNA antibody from lupus patients. Lane 7, 8: Purified anti-DNA antibody from B-CLL patients. Banding patterns for SLE anti-ssDNA purified autoantibody are different (1-4 bands).

Confirmation of anti-DNA antibody IgG with Western blot

A Western Blot analysis of electrophoretically separated, affinity purified, anti-ssDNA is shown in Figure 5. The reaction, visualized at the molecular weight for IgG, provides conclusive evidence that the protein we purified is human IgG. The number of bands detected in the molecular weight range of IgGs was similar to that found by staining the original electrophoretic gel (Figure 5A).
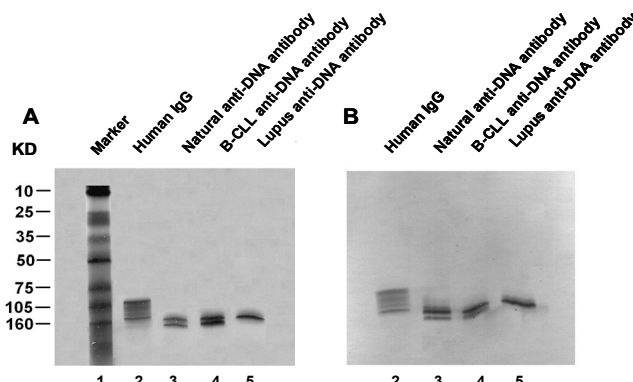
Figure 5 Western Blot confirmations of purified anti-DNA antibodies from normal individual, B-CLL and SLE. A. Marker (lane1), Human IgG (lane 2), Purified anti-DNA antibody from normal individual (lane3), Purified anti-DNA antibody from CLL (lane 4), Purified anti-DNA antibody from SLE (lane 5) on Silver-Stain SDS-PAGE gel in non-reducing condition. B. Corresponding Western blot result, shown on the right. Lanes 2 to 5 correspond to SDS-PAGE gel counterpart.

Hydrolytic activity of purified anti-DNA antibody is conserved during purification procedure

Figure 6 shows that anti-ssDNA antibodies from normal individual and B-CLL did not cleave Gololobov's modified ss-DNA-mer, while there was a gradual cleavage with time (range 0-3h) in the case of lupus patient's antibody. On the other hand, DNAse1 cleavage was different. Optimal reaction mixture at the same substrate: enzyme ratio cleaved the same substrate at a much faster rate (for half an hour), indicating that it is a quite different entity (Figure 7). The reaction mixture for both (Abs and DNAse1) enzymatic reactions is given in Table 5. The Gololobov's modified oligo probe, in appropriate reaction mixture did not show any changes in OD, meaning that there was no spontaneous hydrolysis.
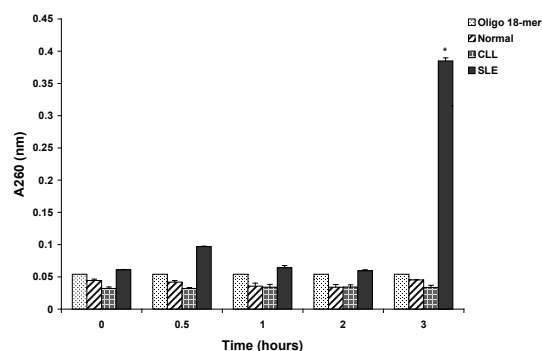
Figure 6 Determination of hydrolytic activity of purified anti-ssDNA antibodies in healthy individual, versus SLE and B-CLL patients, by UV spectrophotometry. Anti-ssDNA antibodies from sera of normal and B-CLL patients do not show any hydrolytic activity compared to Oligo 18-mer, alone. However, anti-ssDNA antibodies purified from SLE sera show the cleavage ability during the 3hour of incubation time-interval. (*P<0.01: normal individuals versus SLE patients).

As extension of our previous work[32] our results strongly suggest that anti-ssDNA antibodies isolated and purified from sera of SLE patient are DNA hydrolytic abzymes in their nature and therefore, most probably, involved in the pathogenicity of lupus disease.

Table 6 Optimization of concentrations of substrates, DNAse and Antibodies, Reaction Mixture, and time and temperature of completed reaction

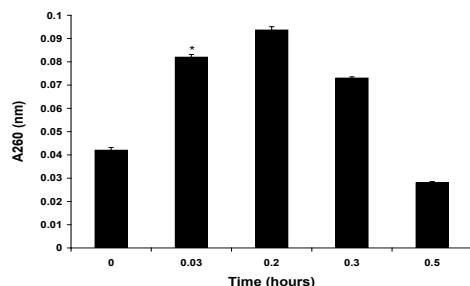| Reaction mixture: Control 1	Anti-DNA antibody
22mM Tris-HCl	22mM Tris-HCl
50mM NaCl	5mM NaCl
10mM MgCl$_2$	0.5mM MgCl$_2$

Figure 7 Determination of hydrolytic activity of commercial DNAse1. In comparison to Ab hydrolytic reaction, with difference in speed and reaction mixture components, this result proves the identity of the enzyme and its difference in relation to abzyme (Ab). Hydrolytic activity of DNA-se1 has different kinetics compared to kinetics of anti-ssDNA antibodies from SLE patients (Figure 6). It starts at 0.03hour, peaks at 0.2hours and subsides at 0.5hours which is much faster than kinetics of anti-ss DNA antibody isolated from sera of SLE patients (*P<0.01 for 0hour versus 0.03 hour).

Discussion

Anti-DNA antibodies are germline gene-encoded autoantibodies widely present in normal individuals, as well as those with autoimmune and lympho proliferative diseases.[18–28] The physiological role of naturally occurring auto-antibodies in healthy individuals is still unknown. The natural autoantibodies are of IgM and IgG isotypes with a low affinity for ssDNA, while most pathogenic anti-DNA antibodies are of the IgG isotype (IgG$_1$ and IgG$_3$), exhibiting high affinity for dsDNA and/or ssDNA.[18–20] A striking feature of pathogenic anti-DNA antibodies is nephrotoxicity; their ability to cause glomerulonephritis in SLE due to their cross-reactivity with alpha actinin in glomerular podocytes and mesangial cells.[8,29] Furthermore, the pathogenicity of the anti-DNA autoantibodies may also arise as the consequence of formation of complexes with DNA deposited in the renal glomeruli cells, and the subsequent hydrolytic and cytotoxic activities of the anti-DNA antibodies.[18,19] Pathogenic anti-dsDNA antibodies primarily recognize determinants on the deoxyribose-phosphate backbone whereas anti-ssDNA antibodies recognize thymidine bases in DNA[24] Catalytic anti-DNA antibodies, called abzymes according to Linus Pauling (1940), are detected in the plasma of SLE and B-CLL patients with concomitant autoimmune B-cell-mediated syndromes. Thus, they are implicated in the causation of symptoms in autoimmune disorders. However, their role and reason for developing in B-CLL and SLE are still unknown; neither is it known what actually lupus B-cell is.

Anti-DNA antibodies are the hallmark of autoimmune diseases. It has been widely used to diagnose and predict the development of SLE. Although anti-DNA antibodies are specific to SLE, they also exist in normal individuals as well as in lymphoproliferative diseases such as B-CLL. The difference among anti-DNA antibodies in normal individuals, SLE and B-CLL is uncertain due to the lack of purified, intact, anti-DNA antibodies from patients' sera.

We developed a two-step purification method to isolate intact anti-DNA antibodies from normal individual, B-CLL and SLE

patients, based on the fact that anti-ssDNA antibody has a strong binding preference to thymine bases, confirmed by analysis of the crystal structure of a complex of Oligo (dT) and mouse monoclonal anti-ssDNA antibody.[24] By using this information, we were able to design and create biotinylated oligo (dT) 20mer bound to streptavidin coated magnetic dynabeads from DYNAL, to isolate anti-ssDNA antibodies specific for this single strand of thymine nucleotides. In order to purify the intact anti-DNA antibodies from patients' sera, a two-step purification method was developed in our lab using Dynal Oligo (dT) coupled, M-280 streptavidin coated, magnetic beads, followed by the use of Dynal protein G beads.[32] The order of two-step purification method cannot be reversed, as confirmed in our lab. No anti-DNA antibody was extracted if Dynal protein G beads were applied as the first step. The characteristics of Dynabeads make them suitable for molecular purification. Dynabeads M-280 Streptavidin coupled with Biotinylated Oligo-(dT) have an excellent stability and a high lot-to-lot reproducibility due to a low nonspecific binding characteristic of streptavidin and high binding affinity of the streptavidin/biotin interaction (Kd=10-15) allowing for efficient isolation of the target molecules. The beads were blocked during the manufacturing procedure (personal report from the manufacturer) with 3% BSA to eliminate non-specific binding for S-A, and stored in a buffer containing 0.1% BSA. Protein G Dynabeads are designed for IgG isotype purification. Protein G has a strong binding affinity to four different subclasses of IgG and there is no leakage of affinity-bound substrate from protein G beads during purification (checked by electrophoresis of eluate). Moreover, Dynabeads freely suspended in solution can be washed many times to eliminate nonspecific binding. Our anti-DNA antibodies are very pure, as shown in Figure 5. The Western Blot result provides conclusive evidence that the protein we purified is human IgG. Another advantage of this method is that we can calculate the optimal number of the beads required for purifying a certain amount of antibodies in the plasma, determined by antibody quantification (Figure 2A) (Table 4). The oligo-(dT) coupled to the strepavidin dynalbeads is a 20mer poly T which has the features of DNA, but is too short to bridge two antibody molecules.[7] Because of this characteristic of the oligo-(dT) 20mer, we are able to estimate the maximal amount of the beads used to recover all anti-DNA antibodies from 1ml of patient's plasma. Our results showed that theoretically we need 1.43×10^8, 1.05×10^9, 6.48×10^8 oligo-(dT) beads and 250μl of protein G beads to pull out corresponding values of total anti-DNA antibodies from 1ml of plasma. As it is shown in Table 3 & 4, corresponding values of purified antibodies' concentrations for already mentioned amount of beads are: 67μg/ml, for normal individual: 489μg/ml for SLE and CLL 301μg/ml for B-CLL individuals (Table 4). According to our knowledge, this is the first time that the concentration of these autoantibodies is expressed in absolute numbers and not in international units. The two-step purification recovery rate, based on the amount of purified anti-DNA antibody concentration/anti-DNA antibody concentration in a patient's plasma, is approximately 20% (Table 2). The possible reason for the slight differences between recovery rates from normal individual, SLE and B-CLL patients is that binding affinity of each antibody from the selected samples is slightly different. This can be investigated further with binding affinity studies, but this was not the purpose of our work. The recovery efficiency can be increased two-fold by lowering pH (from 7 to 5) of the protein G beads elution buffer, bringing the yield of eluted antibody to about 40% (Figure 2B). This makes the method very precise and economical for application. It overcomes the previous problems present in the purification steps: laborious and time-consuming procedures, and antibodies that are prone to denature under harsh conditions.[1,14,15,21]

Since we have used a moderate procedure to purify anti-DNA antibody, the binding activity of the auto-antibody is retained after purification. The successful anti-DNA antibody purification enables us

to compare the band patterns and hydrolytic activity of autoantibodies among normal individual, SLE and B-CLL patients. As seen in Figure 3, we analyzed the sera, and first and second purification elute from three different patients. By comparing Lanes 4 and 7 from gels A and B, we can see that the SLE patient has more bands in the first purification than those in the other two. The molecules from the bands of first purification might be RNA, DNA and DNA binding proteins and other proteins discharged from apoptotic cells isolated along with anti-DNA antibodies from sera during the purification procedure. The result in Figure 3 may also suggest that patients with SLE have an increased rate of apoptosis. The defect in clearance of phagocytic cells leads to the increased level of apoptotic wastes in the body of SLE patients. These apoptotic wastes sequentially trigger the autoantibody-producing cells to generate more anti-DNA antibodies (Table 2). Most recently, it has been proposed that defects in clearance of the apoptotic material may be critical in perpetuating the disease.[30–33] If this material is not cleared at a normal rate, its persistence may lead to the production of anti-nuclear autoantibodies, the category to which anti-DNA antibodies belong. If this situation is coupled with an unusual degree of somatic mutations occurring during autoantibody production, the harmful high affinity antibodies, such as dsDNA antibodies, may be generated.[30]

Unlike SLE, B-CLL patients do not show an abnormal amount of apoptotic molecules in plasma (Figure 3). The band pattern from the first and second purification is the same in normal individual and B-CLL patient. However, anti-DNA antibody concentration in B-CLL patient is much higher than that in normal individual (Table 2). One model may be used to explain this situation.[34] In normal individuals, natural autoantibody IgG is kept in control by IgM. Because B-CLL cells in most patients express IgM autoantibodies, the enormous amount of abnormal IgM dysregulates the immune system, leading to a change of antibody repertoires in B-CLL patients. The abnormal IgM produced by B-CLL cells fails to control the autoantibody IgG isotype from attacking its self-antigen. Intravenous infusions of immunoglobulins collected from the blood of healthy volunteers which contain normal IgM can restore the immune system. In this model, autoantibodies are considered to be produced by normal B cells, which correspond to our result: anti-DNA antibodies from normal individual and B-CLL patient have the same pattern. However, another model shows that anti-DNA antibodies from B-CLL patients have the cytotoxic ability,[35] the hallmark of pathogenic anti-DNA antibodies. The results showed that treatment of tumor cells with anti-DNA antibodies induced internucleosomal DNA fragmentation with evidence of laddering, suggesting a toxic (apoptotic) event. This is not supported by our result; the anti-DNA antibodies from particular B-CLL patient do not have hydrolytic activity (Figure 6). It is suggested that underlying mechanisms of DNA hydrolysis and cytotoxicity are different,[17] which, beside small sample size, can explain the result obtained in our lab.

In addition to an abnormal amount of apoptotic molecules in the blood, SLE patients also have a different pattern of anti-DNA antibodies compared to normal individuals and B-CLL patients. It is considered that they originate from both self-reactive and non-self-reactive B-cells.[36] In Figure 4, lanes 3 and 4, 5 and 6, 7 and 8 represent purified anti-DNA antibodies from normal individuals, SLE patients and B-CLL patients, respectively. It is noteworthy that anti-DNA antibodies from lupus patients exhibited a different number of bands between individual patients, in contrast to control and B-CLL patients, which exhibited strictly 2 bands. The number of bands in SLE patients were between 1 band and 4 bands, with MW between 150-180kD (four banded serum sample, personal data, is not shown). These differences of anti-DNA antibodies, in the number and MW of electrophoretic patterns among normal, B-CLL and SLE patients,

might reflect different mechanisms involved in modulation of autoantibody production, the reasons for which are as yet, unknown. They may be a product of mutation,[37–42] chain elongation[43] or glycosylation.[43–51] The immunoglobulin genes encoding the anti-DNA antibody from lupus patients might undergo more somatic mutations and encode more basic amino acid residues than genes encoding the natural anti-DNA antibody found in healthy subjects, and patients with B-CLL. A computer model of the antibody combining site of IgG anti-DNA antibodies from NZB/NZW mice, is shown to be dominated by arginine and tyrosine side chains.[52] In general, somatic mutations are not germ-line inherited. They are point mutations occurring in the activated B-cells driven by the autoantigen. Autoantibody VH and VL regions harbor numerous somatic mutations characteristic of an antigen-driven immune response.[53] In the presence of a large amount of apoptotic waste, autoantibody producing B- cells undergoes somatic mutation and generates the higher affinity- binding anti-DNA antibodies.

It is known that IgG antibodies are potent inducers of proinflammatory responses.[44–52] During autoimmune diseases, such as SLE and arthritis, IgG autoantibodies are responsible for the chronic inflammation and destruction of healthy tissues by cross-linking Fc receptors on innate immune effector cells. A critical factor for the overall structure and function of the molecule is the sugar moiety attached to the asparagine-297 residue in the constant domain of the antibody.[48] Removal of this sugar domain through enzymatic cleavage leads to the loss of proinflammatory activity, suggesting that in vivo modulation of antibody glycosylation might be a strategy to interfere with autoimmune processes.[50] Does glycosylation have something to do with hydrolytic activity, and is it a factor in the banding pattern variations observed in SLE patients' antibodies? This has yet to be determined.

The abzyme's hydrolytic activity determination (used as a fast test) by UV spectrophotometry strongly suggests that SLE patient's isolated anti-ssDNA antibody binds to, and degrades Gololobiv's modified probe, by enzymatic cleavage after 3h in medium with Mg^{2+} and Ca^{2+} in contrast to commercially available DNAse1, with completely different kinetics and components of reaction mixture (Figures 6 & 7). This also suggests that anti-ssDNA antibody from that particular SLE patient is hydrolytic, which means also pathogenic if hydrolysis is a mechanism of pathogenicity. In the living cell, the underlying mechanisms for hydrolysis are the most probably: penetration into the living cell and translocation into the nucleus, recognition of DNA in nucleus and mechanism of binding to DNA. If so, the anti-ssDNA antibody would play the role in maintenance and perpetuation of vicious cycle in the diseases and could serve as the predictor of the flare, as some already suggested.[31–33]

It can also be considered as part of the antimicrobial response of organism to the ssDNA of infectious agent, which might trigger SLE or other autoimmune diseases.[19,20] Pathogenic (hydrolytic and/ or cytotoxic) anti-DNA antibodies, in diverse autoimmune and lymphoproliferative diseases, may derive from somatic mutations in the genes coding for hypervariable regions in the antibody structure.[53] Recent data suggest that their production can also be triggered by different infectious agents, especially viruses.[64–67] However, their exact role in normalcy and pathology is still to be determined.

Conclusion

To our knowledge, our lab was the first to develop a simple,

relatively fast (4.5h), inexpensive and highly specific method for isolating and purifying human anti-ssDNA (IgG) antibodies from human sera. Besides specific separation of anti-ssDNA autoantibodies, based on specific binding,[24,32] the method has several advantages over existing ones. It can easily be modified in order to study other antibody isotypes. Furthermore, the principle of this method can be used to purify any antibody specific for a particular antigen. The new method will also allow for a comparison between anti-DNA IgG and anti-DNA IgM hydrolytic activities. The system is flexible and can be modified to increase the yield of purified product by increasing the number of beads and the volume of the sample to allow for studying larger antibody concentrations.

To our knowledge, this is the first time that electrophoretic analysis of the intact molecules of the anti-ssDNA antibody purified from normal individuals, B-CLL and SLE patients' plasma has been reported. According to SDS-PAGE data, it seems that IgGs from SLE patients have different MW compared to normal IgGs (over 150 000). The banding pattern of IgGs from normal individuals and B-CLL patients is the same in this study, but a larger sample size is necessary to generalize conclusions.

In general, the high purity of the method[54–56] allows for structural, binding, and pathogenicity studies and for improved applications in immunotherapy, due to its high purity. The amount and concentration of antibody obtained, and its purity, is sufficient for the studies of functional properties of antibodies (e.g. binding, and hydrolysis).In comparison to our first design[32] the yield improved by modifying the number of the beads and the pH of the second elution buffer.

Hydrolysis of DNA by human lupus anti-ssDNA antibody (determined by UV spectrophotometry) seems to be an intrinsic, constitutive activity of the antibody molecule, since the kinetics and conditions of the reaction are quite distinctive with regards to commercially available DNAse1.

The study of antibody structure, coupled with molecular synthetic techniques, could lead to the development of biologically active substances that would prevent anti-DNA antibody penetration into cells and translocation into the nucleus followed by its harmful hydrolytic and/or cytotoxic effects.[57–63]

Summary

In summary, we are hopeful that our technique will provide investigators a means to further explore the etiology and development of autoimmune diseases using higher numbers of patients in clinical studies.[32] Our method, and the product it provides, may assist in the design of methods or compounds that may someday cure autoimmune and lymphoproliferative diseases. The application of the method of anti-ssDNA antibody isolation and purification proposed by our design would be of fundamental importance for clarifying that antibodies' role in lupus diagnosis, flare prediction, monitoring of the disease, targeted and preventive therapy, and will give a deeper insight into molecular mechanisms of its pathogenesis.[32,64–66]

Acknowledgments

Conflict of interest

Author declares that there is no conflict of interest.

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