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Antigen binding characteristics of antibodies induced against nitric oxide modified plasmid DNA

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Abstract

Nitric oxide (NO) generated by the reduction of sodium nitrite with sodium dithionite caused damage to plasmid Bluescript DNA leading to strand breaks and base modifications. The NO-plasmid DNA was highly immunogenic in rabbits. The antibody activity was inhibited to the extent of 86% with the immunogen as inhibitor, indicating the induction of immunogen specific antibodies. However, delineating the antigenic specificity of anti-NO-plasmid DNA antibodies by competition ELISA, multiple cross-reactivity was observed. The antibodies recognised B-, A- and allied conformations. The visual detection of immune complex formation with native and NO-plasmid DNA reiterated preferential binding with modified plasmid DNA. DNA modified by nitric oxide presents unique epitopes which may be one of the factors in antigen-driven autoimmune response in systemic lupus erythematosus.

Keywords: Nitric oxide-plasmid DNA; Nitric oxide; Systemic lupus erythematosus; Anti-DNA antibody; Autoantibody

1. Introduction

Nitric oxide (NO) is a well known environment pollutant [1,2] and a multifaceted bioregulatory agent, exhibiting an astonishing range of physiological functions, including suppression of pathogens, vasodilation and neurotransmission [3,4]. Macrophages also produce nitric oxide as a cytotoxic agent which may lead to DNA damage [5]. Nitric oxide, a highly reactive free radical [6,7], deaminates deoxynucleosides, deoxynucleotides and intact DNA at physiological pH [8] and causes strand break [9,10]. Besides these effects, nitric oxide also causes tissue injury by attacking the iron–sulphur centres in various key proteins [11], inhibiting ribonucleotide reductase [12] and several other DNA repair/replication enzymes [13,14].

Systemic lupus erythematosus (SLE) is an autoimmune disorder of unknown etiology. It is characterised by the presence of circulating anti-DNA antibodies which serve as a diagnostic marker for this disease [15]. The origin and nature of antigens that induce anti-double stranded DNA antibodies are not known [16]. However, it has been well established that native double stranded DNA (B-conformation) per se is not immunogenic [17–19]. Many studies suggest modified forms of DNA and polynucleotides to be immunogenic in animal models and the induced antibodies show SLE autoantibody-like characteristics [20–22]. Other studies suggest that anti-DNA antibodies may be generated by autoimmunisation with chromatin [23], immune response against modified self-determinants [24,25] or antibodies to self-proteins that cross-react with native DNA [26].

Nitric oxide is generated in vivo by many types of cells through the nitric oxide synthase (NOS) pathway [27,28]. It is an important mediator of inflammatory response. Activated macrophages produce nitric oxide which damages DNA resulting in mutations and strand breaks [7]. In the present study, we have investigated immunogenicity of nitric oxide modified plasmid DNA (NO-DNA). The induced antibodies showed diverse binding characteristics similar to that of SLE anti-DNA autoantibodies. The notable feature of this investigation is the high degree of recognition of nitric oxide modified conformers of various synthetic polynucleotides and nucleic acids polymers.

Abbreviations: SLE, systemic lupus erythematosus; NO-DNA, nitric oxide modified plasmid DNA; ROS, reactive oxygen species; NOS, nitric oxide synthase

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2. Materials and method

2.1. Materials

Sodium nitrite and sodium dithionite were purchased from Bio-Rad (USA). Calf thymus DNA, nuclease S1, *Eco*RI, methylated bovine serum albumin (BSA), anti-rabbit IgG alkaline phosphatase conjugate, Tween 20, Freund's complete and incomplete adjuvants, *p*-nitrophenyl phosphate, ethidium bromide, nitrogenous bases, cardiolipin, chondroitin sulphate, Ficoll-400 and bromophenol blue were obtained from Sigma (USA). Synthetic polynucleotides and agarose were procured from Pharmacia (Sweden). Polystyrene microtitre flat bottom ELISA plates (96 wells) and modules were products of Nunc (Denmark). All other chemicals were of analytical grade. Plasmid pBS was isolated from *Escherichia coli* $DH_5\ \alpha$ 'KS' using the alkali lysis method as described by Maniatis et al. [29]. Fragments of calf thymus DNA (200 bp) and DNA from human lymphocytes were isolated as described earlier [30].

2.2. Modification of DNA

Plasmid DNA was modified with nitric oxide [9] generated by the reduction of sodium nitrite with sodium dithionite. The modification was performed for 3 h at 37°C in a volume of 20 μl containing 20 mM Tris–HCl buffer, pH 7.5. The reaction sample contained plasmid DNA (0.4 mg), sodium nitrite (100 mM) and sodium dithionite (10 mM). After incubation, ethanol precipitation of NO modified and control samples of DNA was performed twice. Plasmid DNA incubated with 100 μM sodium nitrite or 10 mM sodium dithionite alone served as controls. The UV absorption characteristics of native and NO-DNA were recorded on a Shimadzu UV-240 spectrophotometer. All the other nucleic acid samples and DNA bases were similarly modified.

2.3. Agarose gel electrophoresis

Native and NO-DNA were mixed with a one-tenth volume of sample buffer (0.125% bromophenol blue, 30% Ficoll-400, 500 mM EDTA in 10×electrophoresis buffer). The samples were loaded in the wells and electrophoresed for 2 h at 30 mA. The gels were stained with ethidium bromide (0.5 μg/ml), viewed by illumination under UV light and photographed.

2.4. Nuclease S1 treatment

Nitric oxide induced modification was characterised by nuclease S1 treatment [31] followed by agarose gel electrophoresis [32]. The digesting capability of nuclease S1 under our experimental conditions was checked using heat denatured calf thymus DNA. It was almost completely digested, whereas no digestion was observed in the case of purified native DNA. One microgram each of native and NO-DNA was incubated with nuclease S1 (20 units/μg DNA) in acetate buffer, pH 5.0, at 37°C for 30 min. The reaction was stopped by adding a 1/10 volume of 0.2 M EDTA, pH 8.0 and the sample electrophoresed on 1% agarose gel for 2 h at 30 mA. The nucleic acid bands were visualised under UV light after staining with ethidium bromide.

2.5. Alkaline sucrose density gradient ultracentrifugation

Damage to DNA caused by nitric oxide radical was also analysed by alkaline sucrose density gradient ultracentrifugation [33]. *Eco*RI linearised native and NO-DNA (0.2 ml of 100 μg/ml) treated with 0.2 ml of 0.2 N NaOH were layered on top of a linear 4.6 ml 5–20% alkaline sucrose gradient containing 800 mM NaCl, 200 mM NaOH and 10 mM EDTA. The samples were centrifuged at 30 000 rpm for 1 h at 20°C in a SW 50.1 rotor (Beckman, USA). The bottoms of the tubes were pierced and 0.5 ml fractions were collected and absorbance was monitored at 260 nm.

2.6. Immunisation schedule

The immunisation of random bred, female, New Zealand white rabbits was performed as described previously [34]. Briefly, rabbits ($n=4$; two each for native and NO-plasmid DNA antigens) were immunised subcutaneously at multiple sites with 50 μg of antigen complexed with methylated BSA in the ratio of 1:1 (w/w) and emulsified with an equal volume of Freund's complete adjuvant. The animals were boosted intramuscularly in Freund's incomplete adjuvant at weekly intervals for 5 weeks with the same amount of antigen. Test bleeds were performed 7 days post boost which gave appropriate titre of the antibody. The animals were bled and the serum separated from the blood (preimmune and immune) was decomplemented by heating at 56°C for 30 min.

2.7. Purification of antibodies

Immunoglobulin was isolated from immune sera on a protein A-Sepharose 4B column (Pharmacia) [35]. The homogeneity of isolated IgG was verified by performing 7.5% SDS–polyacrylamide gel electrophoresis.

2.8. ELISA

Enzyme linked immunosorbent assay (ELISA) was performed on flat bottom 96-well, polystyrene immunoplates (maxisorp), as described previously [36]. Briefly, the plates were coated with 100 μl plasmid DNA (2.5 μg/ml) for 2 h at room temperature and overnight at 4°C. After washing three times with TBS-T (20 mM Tris, 2.68 mM Tris,

2.68 mM KCl, 150 mM NaCl, pH 7.4 containing 0.05% Tween 20), unoccupied sites were blocked with 1.5% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4–6 h at room temperature. The test serum serially diluted in TBS (100 μl/well) was adsorbed for 2 h at room temperature and overnight at 4°C. Bound antibodies were assayed with anti-rabbit IgG alkaline phosphatase conjugate using *p*-nitrophenyl phosphate as substrate. The absorbance of each well was monitored at 410 nm on an automatic microplate reader. Proper controls were included in each set of experiments.

2.9. Competition ELISA

Antibody specificity was ascertained by competitive binding assay [37]. Varying concentrations of inhibitors (0–20 μg/ml) were allowed to interact with a constant quantity of antibody (50 μg/ml IgG or 1/100 diluted serum) for 2 h at room temperature and overnight at 4°C. The mixture was added to antigen coated plates and the bound antibody was diluted as described in the direct binding ELISA. Inhibition was expressed as the amount of inhibitor required for 50% elimination in the antibody binding to the solid phase antigen.

2.10. Gel retardation assay

Electrophoresis was performed on 1% agarose gel in 40 mM Tris–acetate buffer (TAE), pH 8.0, for the visual detection of antigen–antibody binding and formation of immune complexes [38]. A constant amount of antigen (0.5 μg) was incubated with increasing concentrations (20–80 μg) of IgG for 2 h at room temperature and overnight at 4°C before loading onto the gels. On completion of electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV illumination.

3. Results

3.1. Characterisation of NO-plasmid DNA

Native plasmid DNA was modified by nitric oxide and the induced changes in DNA were analysed by UV absorption spectroscopy. The UV absorption spectra of NO-DNA revealed a marked hypochromicity (17.9%) at λ_{max} and exhibited a shift in the maxima and minima to the extent of 8 nm and 6 nm relative to native DNA, respectively (data not given).

To evaluate the effect of NO on the degradation of plasmid DNA, agarose gel electrophoresis was performed. Form I (supercoiled) and form II (relaxed form) of NO modified DNA showed appreciable loss of fluorescence intensity. Almost no change in linear form (III) could probably be due to compromised ethidium bromide fluorescence as a result of damage to all forms of plasmid DNA by NO radical (Fig. 1). After many nicks the supercoiled DNA becomes linear and further nicks lead to fragmentation of this linear form, rendering the molecule too short to appear as band.

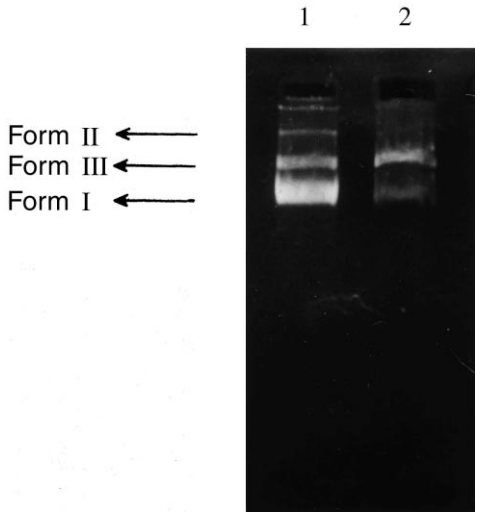

Fig. 1. Agarose gel electrophoresis of native plasmid DNA (lane 1) and NO-plasmid DNA (lane 2). Electrophoresis was carried out on 1% agarose gel for 2 h at 30 mA. Form I, supercoiled form; Form II, relaxed form; Form III, linear form.

The generation of single strand breaks in NO-DNA was demonstrated by nuclease S1 digestibility followed by agarose gel electrophoresis. On digestion, the data (Fig. 2) showed complete disappearance of the supercoiled form (form I) and considerably low intensity of form III of the NO-DNA. Nuclease S1 treatment of native plasmid

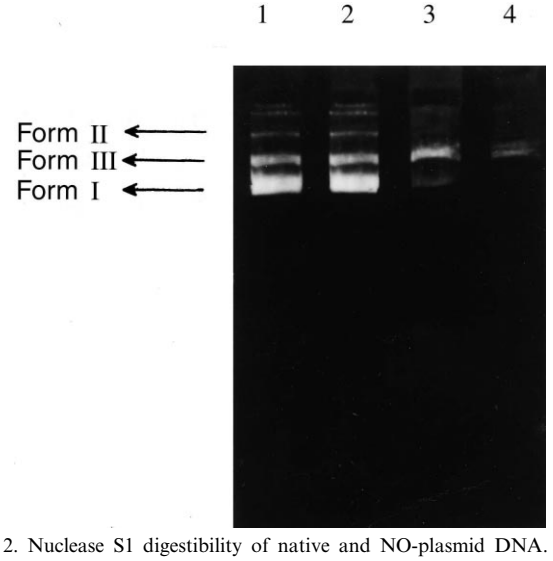

Fig. 2. Nuclease S1 digestibility of native and NO-plasmid DNA. Lane 1 contained native plasmid DNA, while lane 2 contained native plasmid DNA treated with nuclease S1. Lane 3 contained NO-plasmid DNA, while lane 4 contained NO-plasmid DNA treated with nuclease S1. Electrophoresis was carried out on 1% agarose gel for 2 h at 30 mA and nuclease S1 treatment was for 30 min. Form I, supercoiled form; Form II, relaxed form; Form III, linear form.

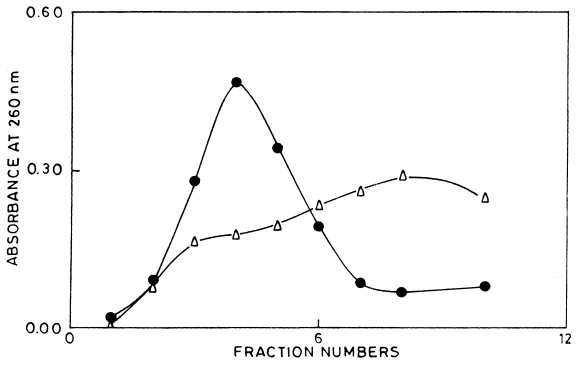

Fig. 3. Alkaline sucrose density gradient ultracentrifugation of native plasmid DNA (●) and NO-plasmid DNA (△).

DNA had no effect on the supercoiled and linear forms. Alkaline sucrose density ultracentrifugation of native plasmid DNA showed a single distinct species, whereas NO-plasmid DNA showed a diffused sedimentation profile (Fig. 3), indicating random distribution of single strands breaks which appear as fragments resulting in a diffused sedimentation pattern.

3.2. Antigenicity of NO-DNA

Immunisation of NO-DNA in rabbits induced high titre antibodies of $>1:12\,800$ (Fig. 4A). Protein A-Sepharose isolated anti-NO-DNA IgG showed similar binding with the immunogen (Fig. 4B). Preimmune IgG showed negligible binding.

Competition ELISA was performed using immunogen,

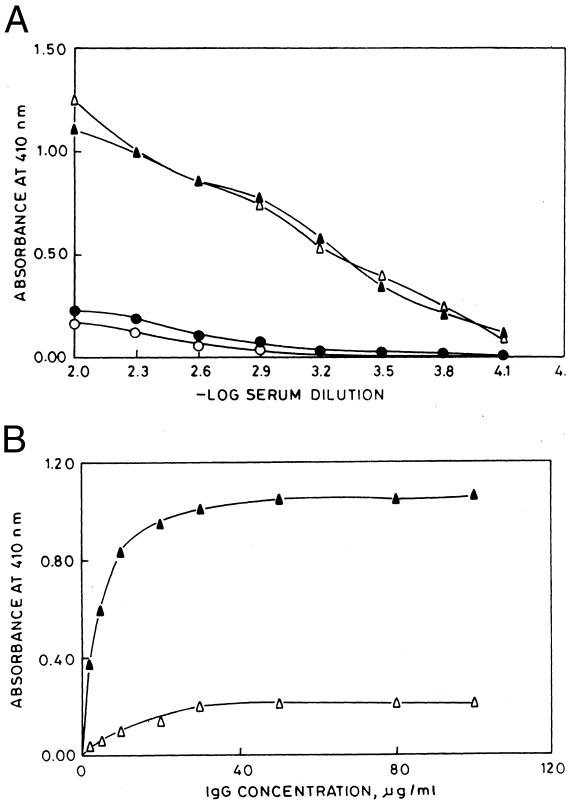

Fig. 4. (A) Direct binding ELISA of anti-NO-plasmid DNA antibody sera (△, ▲) and preimmune sera (○, ●). (B) Direct binding with affinity purified anti-NO-plasmid DNA IgG (▲) and preimmune IgG (△). The microtitre plates were coated with NO-plasmid DNA (2.5 μg/ml).

Table 1
Antigen binding specificity of anti-NO-plasmid DNA antibodies

Inhibitor	Maximum % inhibition at 20 μg/ml	Concentration for 50% inhibition (μg/ml)	Percent relative affinity
NO-plasmid DNA	86.2	1.6	100
Native plasmid DNA	58.7	16.4	9.75
Native 200 bp DNA	21.3	–[a]	–
NO-200 bp DNA	45.6	–	–
Native lymphocyte DNA	48.5	–	–
NO-lymphocyte DNA	54.2	17.6	9.09
Guanine	43.0	–	–
ROS-guanine	61.0	12.0	13.33
NO-guanine	69.0	8.7	18.37
Cytosine	21.0	–	–
ROS-cytosine	42.0	–	–
NO-cytosine	58.4	13.2	12.12
Adenine	14.0	–	–
ROS-adenine	30.0	–	–
NO-adenine	47.0	–	–
Cardiolipin	47.0	–	–
Chondroitin sulphate	33.0	–	–
Poly(G)	44.0	–	–
NO-poly(G)	65.0	15.0	10.6
Poly(dA-dG)·poly(dC-dT)	41.0	–	–
Poly(dA-dT)·poly(dA-dT)	33.0	–	–
Poly(rG)·poly(dC)	38.0	–	–

The microtitre plates were coated with NO-plasmid DNA (2.5 μg/ml).
[a]50% inhibition was not achieved.

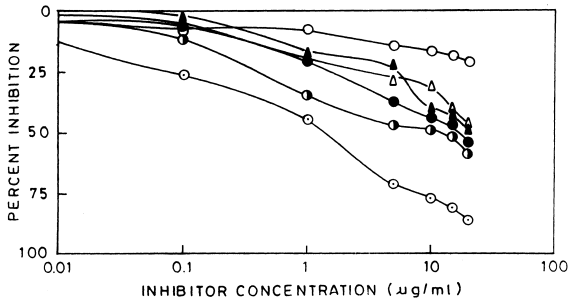

Fig. 5. Inhibition of anti-NO-plasmid DNA IgG binding to NO-plasmid DNA. The competitors were NO-plasmid DNA (⊙), native plasmid DNA (black and white circle), native lymphocyte DNA (▲), NO-lymphocyte DNA (●), native 200 bp DNA (○) and NO-200 bp DNA (△). The microtitre plates were coated with NO-plasmid DNA (2.5 μg/ml).

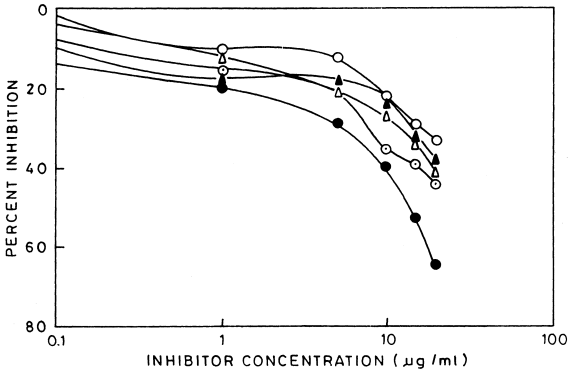

Fig. 7. Competition of anti-NO-plasmid DNA IgG binding to NO-plasmid DNA by poly(dA-dT)·poly(dA-dT) (○) poly(dA-dG)·poly(dC-dT) (△), poly(rG)·poly(dC) (▲), poly(G) (⊙), and NO-poly (G) (●).

nucleic acids, synthetic polynucleotides and bases as inhibitors to ascertain the antigenic binding specificity of induced antibodies. A maximum of 86% inhibition of antibody binding to solid phase bound antigen was observed with the immunogen as inhibitor (Fig. 5). The induced antibodies were highly specific for NO-DNA as only 1.6 μg/ml inhibited 50% antibody activity. The induced antibodies recognised native DNA and showed a maximum inhibition of 58.7% and 16.4 μg/ml was required for 50% inhibition of antibody binding. Total DNA from human lymphocytes and 200 bp fragments of calf thymus DNA showed maximum inhibition of 49% and 21% respectively, whereas their NO modified conformers were inhibitory to the extent of 54.2% and 45.6%, respectively and 17.6 μg/ml of NO-lymphocyte DNA was required for 50% inhibition (5; 1).

Guanine showed moderate inhibition of 43%. In contrast, reactive oxygen species (ROS) (hydroxyl) and NO modified conformers of guanine showed maximum inhibition of 61% and 69%, respectively; 11.9 μg/ml of ROS-guanine and 8.7 μg/ml of NO-guanine were required for 50% inhibition (Fig. 6). Cytosine, adenine and ROS-adenine showed negligible inhibition of antibody activity, whereas ROS-cytosine and NO-adenine exhibited a moderate inhibition of 42% and 47%, respectively. The NO modified form of cytosine showed a maximum inhibition of 58.4% and 13.2 μg/ml were required for 50% inhibition. Among the homopolymers, poly(dA-dG)·poly(dC-dT) showed a maximum inhibition of 41%, whereas poly(dA-dT)·poly(dA-dT) and poly(rG)·poly(dC) were moderately inhibitory (Fig. 7). The ribonucleotide polymer poly(G) showed a maximum inhibition of 44% while NO-poly(G) inhibited antibody binding to an extent of 65%. The concentration of NO-poly(G) required for 50% inhibition was 15 μg/ml.

The formation of immune complex by NO-plasmid DNA and IgG was visualised by band shift assay. As clearly evident, with an increase in the amount of IgG there was an increase in the formation of high molecular weight immune complex, having retarded mobility (Fig. 8A). The recognition of native plasmid DNA by anti-NO-plasmid DNA IgG was similarly demonstrated by the shift in electrophoretic mobility upon immune complex formation consistent with the data of Fig. 5 (Fig. 8B).

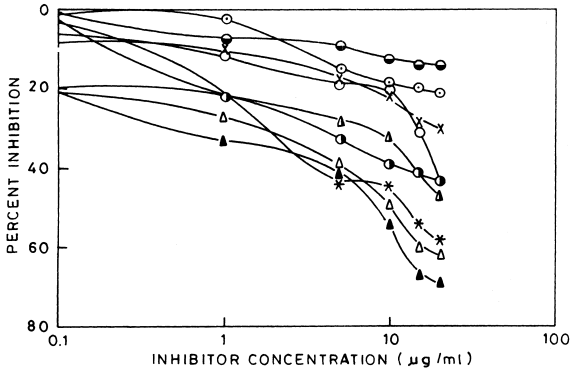

Fig. 6. Inhibition of anti-NO-plasmid DNA IgG biding to NO-plasmid DNA by guanine (vertical black and white circle), ROS-guanine (△), NO-guanine (▲), cytosine (⊙), ROS-cytosine (○), NO-cytosine (*), adenine (horizontal black and white circle), ROS-adenine (×), and NO-adenine (black and white triangle).

4. Discussion

Nitric oxide, a multifaceted bioregulatory agent, shows an unusual divergence of action. It is demonstrated to play a major role as neurotransmitter and a regulator of blood pressure. The role of nitric oxide in numerous disease states has generated considerable discussion over the past several years. Nitric oxide mediated cell injury may arise by a variety of mechanisms, including disruption of mitochondrial respiration, enzymes inhibition, lipid peroxidation and genetic mutation. Toxicity is largely mediated via intermediates such as N_2O_3 and peroxynitrite, arising from the reaction of nitric oxide with either molecular oxygen or reactive oxygen species. In general, such reactions become significant only when high concentrations of nitric oxide are generated by the induction of NOS [39–41]. Macrophages also use nitric oxide as a cytotoxic agent which may lead to DNA damage. The effects of nitric

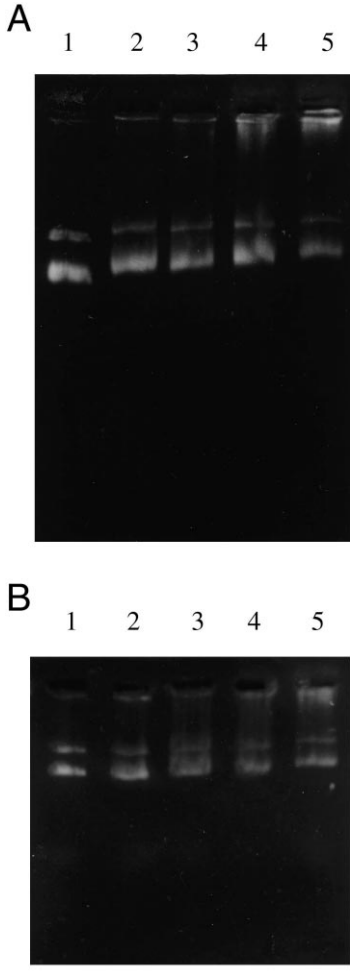

Fig. 8. Band shift assay of anti-NO-plasmid DNA IgG binding to NO-plasmid DNA and native plasmid DNA. (A) NO-plasmid DNA (0.5 μg) was incubated with 20, 40, 60 and 80 μg IgG (lanes 2–5), while lane 1 contained NO-plasmid DNA. (B) Native plasmid DNA (0.5 μg) was incubated with 20, 30, 40, 60 and 80 μg immune IgG (lanes 2–6) for 2 h at 37°C and overnight at 4°C. Lane 1 contained native plasmid alone. Electrophoresis was performed on 1% agarose for 2 h at 30 mA.

oxide at the DNA level are complex and involve formation of *N*-nitrosamines, deamination of purines and pyrimidines or damage induced by peroxynitrite. Complex oxidation chemistry can also occur, causing DNA base and sugar oxidative modifications [13,14,42].

Purines are more readily deaminated than pyrimidines. However, some unexpected bases like 8-hydroxyadenine and 8-hydroxyguanine have been reported on exposure of nucleic acid bases by nitric oxide [43]. The GC→AT transitions predominate in the molecular mechanism of mutagenesis in cells exposed to nitric oxide. Nitric oxide damages DNA by diazotisation of primary arylamines in an oxidising environment [44,45]. Xanthine and hypoxanthine from deamination of guanine and adenine respectively lead to mispairing [46,47] and the instability of hypoxanthine and xanthine in DNA leads to depurination and subsequent strand breakage [48]. Both of these phenomena have been observed in calf thymus DNA and in intact cells and potentially contribute to both cytotoxicity and mutagenicity [49]. A third possibility is the formation of intrastrand cross-links via attack of any diazonium ion of one purine on the free amino group of a second purine in the matching strand [50].

Nitric oxide radical caused substantial damage to plasmid DNA as evident from hypochromicity as well as a shift in λ_{max} and λ_{min} of the spectral curve of NO-plasmid DNA in comparison to the native plasmid DNA. This could be attributed to the generation of strand breaks and modification of the nitrogenous bases which result in the destruction of the chromophoric groups. No change was observed in plasmid DNA on incubation with either sodium nitrite or sodium dithionite alone.

Appreciable evidence for the generation of strand breaks (single and double) in the plasmid DNA as a consequence of nitric oxide modification was resolved by nuclease S1 digestibility and alkaline sucrose density gradient centrifugation. The data are compatible with earlier reports indicating nitric oxide radicals result in strand breaks [9,51].

The NO-plasmid DNA was a potent immunising stimulus inducing high titre antibodies in rabbits. The antibodies were specific for the immunogen but showed cross-reactivity with native plasmid DNA. Modification of DNA by NO might have generated potential epitopes against which antibodies are raised. The cross-reactivity with plasmid DNA may be due to the recognition of the antigenic determinant common to both NO-plasmid DNA and native plasmid DNA and could possibly be the sugar phosphate backbone [52,53]. Gel retardation data reiterated the preferential recognition of NO modified plasmid over native plasmid.

Total DNA from human lymphocytes and 200 bp fragments of calf thymus DNA on NO modification were more inhibitory than their unmodified counterparts. The immune IgG was relatively more specific to NO modified guanine, cytosine and adenine [9,43,49]. Nitric oxide attacks guanine bases as the most preferential cleaving sites and the frequency of the nitric oxide induced cutting decreases in the order G > C > A > T [9].

In addition, the induced antibodies showed recognition of various synthetic polynucleotides, recognising, B-, A- and allied conformations, particularly those in B-conformation. The ability of NO-DNA to induce antibodies cross-reactive to double stranded polydeoxyribonucleotides indicates that some features of B-conformation are present on the antigen. Besides binding to various nucleic acids and synthetic polynucleotides, the anti-NO-plasmid DNA IgG also showed cross-reactivity towards chondroitin sulphate and cardiolipin. It has been suggested that the phosphate-sugar-phosphate moiety of cardiolipin mimics the backbone of DNA, thus explaining the cross-reactivity of immune IgG [5]. Analysis of the data indicates that anti-NO-plasmid DNA IgG is immunogen specific and

the varying degree of cross-reactivity is due to sharing of common antigenic determinants.

SLE is an autoimmune disease characterised by the presence of circulating anti-DNA autoantibodies. Autoantibody production in lupus has been attributed to either selective stimulation of autoreactive B cells by self-antigens or antigens cross-reactive with self [54]. Numerous modified forms of DNA have been found to be immunogenic and are recognised by SLE anti-DNA antibodies [20–22]. According to Hardin [54] and Diamond et al. [55] the persistence of anti-DNA antibodies in SLE patients, despite systems to suppress self-recognition, suggests that the response is driven by an antigen resembling native DNA, together with somatic mutations in the variable regions within B cells. Our preliminary studies using protein A-Sepharose 4B purified anti-native DNA IgG from SLE sera showed higher inhibition by NO-plasmid DNA in comparison to native calf thymus and plasmid DNA when the immobilised antigen was native calf thymus DNA. The present study clearly shows the perturbations in plasmid DNA by nitric oxide radical rendering it immunogenic and the neo-epitopes might play a role in the induction of circulating anti-DNA antibodies in SLE.

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