Immunogenicity of superoxide radical modified-DNA: studies on induced antibodies and SLE anti-DNA autoantibodies

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Abstract

Superoxide anion radical (SAR) is formed in almost all aerobic cells and it is the most abundant species generated by several enzymatic and non-enzymatic pathways in mammalian tissues, leading to unfavorable alteration of biomolecules including DNA. The SAR-modified macromolecules have been implicated in several disease states including disorders of inflammation. The SAR-induced damage to DNA showed hyperchromicity, single strand breaks, decrease in melting temperature, and modification of bases. Superoxide modified-DNA in rabbits elicited high titer antibodies and showed diverse antigens binding characteristics. The induced antibodies recognized native DNA and other nucleic acid polymers. Anti-DNA IgG from SLE sera, purified on Protein-A-Sepharose matrix, exhibited increased recognition of superoxide anion radical modified-DNA than native DNA in competitive immunoassay. The visual formation of immune complex between induced antibodies and native DNA, and between SLE anti-DNA IgG and superoxide modified-DNA, is a clear indication of property sharing between SLE autoantibodies and experimentally induced antibodies against superoxide modified-DNA.

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Increasing number of research papers on oxidative stress in context of human diseases appears to have spelled out a near definite role of oxygen free radicals (OFR) in autoimmunity and inflammation. Production of pathogenic anti-DNA antibodies in SLE is being actively examined under OFR scanner. During the initial acute phase inflammatory response, neutrophils are recruited to the site of injury and activated to release proteolytic components and generate OFR (Griffiths and Lunec, 1996). Superoxide anion radical (O$_2^-$) is formed within all aerobic organisms by the autooxidation of a variety of reduced electron carriers and redox enzymes. Leakage of electrons from electron transport chain, fibroblasts, activated phagocytic cells, and macrophages are some of the in vivo source of superoxide anion (Waris et al., 2000).

Spontaneously produced pathogenic autoantibodies reactive with DNA have drawn wide scientific interest with respect to the mechanisms that lead to anti-DNA antibody formation in SLE. Systemic lupus erythematosus is a multi-system, inflammatory disorder characterized by the production of autoantibodies to nucleic acids, nucleosomal antigens, and intracellular proteins. Circulating antibodies to dsDNA appear to correlate most uniquely with disease (Aitcheson and Tan, 1982). The marked heterogeneity of SLE autoantibodies has been one of the impediments in understanding the disease (Alam and Ali, 1999; Ahmad et al., 2000; Dixit and Ali, 2001). Many theories have been proposed to explain the onset of SLE, but it seems like no single theory can account for its pathogenesis and literature records speaks of the complexities associated with respect to diagnosis and treatment of this disease.

Mammalina DNA per se is non-immunogenic (Isenberg et al., 1994) and all efforts to replicate the disease by immunization of normal animals with high doses of native DNA (dsDNA) have failed (Schwartz and Stollar, 1985). In contrast to native form of mammalian DNA, bacterial DNA is a strong immunogen (Pisetsky, 1996). The immunogenicity of bacterial DNA have been attributed to the presence of nucleotide hexamers containing unmethylated CpG motifs. Similarly, the role of unmethylated CpG dinucleotide sequences in the immunogenicity of plasmid DNA is well recognised (Kreig et al., 1995; Sato et al., 1996; Grifantini et al., 1998). Other modified forms of DNA and polynucleotides (Arif and Ali, 1996; Ahmad et al., 1997), modified self determinants (Mamula, 1995) and antibodies to self proteins (Zack et al., 1995) have been reported to be immunogenic and the antibodies thus generated are cross reactive with native DNA (nDNA). Mammalian DNA complexed with synthetic peptide Fus-1 elicited anti-dsDNA response in mice (Desai et al., 1993). This report led to the conclusion that the antigen drive in SLE involves either a substance other than DNA or DNA in a form (e.g. nucleosome) that is not readily mimicked by artificial complexes (Burlingame et al., 1994; Mohan et al., 1998). Accumulated evidence of last 10-12 years suggests that nucleosome is emerging as the most reactive substrate for SLE, especially anti-ds DNA negative SLE (Min et al., 2002). Nucleosome consists of the pairs of the 4 core histones (H2A, H2B, H3, and H4), which form an octamer around which 146 bp of DNA are wound twice with histone H1 at the outside (Grootscholten et al., 2003). Immunization of experimental animals with synthetic nucleic acid polymers like poly (dT), poly(dC), poly(dl), poly(G), left handed Z-DNA and double stranded DNA with the notable exception of native B-DNA can induce antibodies that react selectively with the immunogen (Stollar, 1986). Calf thymus DNA modified with OFR has been reported to induce antibodies against the modified polymer showing cross reactivity with nDNA (Ahmad et al., 1997; Alam et al., 1993). The binding diversity of lupus autoantibodies to a whole spectrum of modified nucleic acid conformers seems to be enormous. It appears that a fundamental defect in the immune system play an initiating role, environmental factors then contribute to the autoimmune response. In
present communication, we have studied the immunogenicity of superoxide anion radical modified-DNA (O$_2^-$ modified-DNA). The induced antibodies showed diverse antigen binding features similar to SLE anti-DNA autoantibodies.

Materials and methods

Calf thymus DNA, nuclease S1, riboflavin, anti-human and anti-rabbit IgG alkaline phosphatase conjugates were purchased from Sigma Chemical Company, U.S.A. Nitrobluetetrazolium (NBT) was from Loba-Chemie, India. Polystyrene flat-bottom ELISA plates (96 wells) were obtained from NUNC, Denmark. All other chemicals used were of highest purity available in the country.

Modification of DNA

Calf thymus DNA, purified free of proteins, RNA, and single stranded regions (Ali et al., 1985), was modified with superoxide anion radicals. Briefly, solution (3.0 ml total volume) of DNA (100 µg/ml in 50 mM potassium phosphate buffer, pH 7.8), containing 0.1 mM EDTA, 0.06% Triton X-100, and 40 µM riboflavin was illuminated for 20 min at room temperature under 800 Lux of cool white fluorescent light. At the end of incubation, excess riboflavin and Triton were removed by extensive dialysis against potassium phosphate buffer, pH 7.8. DNA samples without riboflavin illuminated under identical conditions served as control. Superoxide anion production was confirmed by the reduction of NBT.

Ultracentrifugation

The damaging effect of O$_2^-$ on native DNA was analyzed by ultracentrifugation in alkaline sucrose density gradient (Alam et al., 1993). Equal amounts of native and O$_2^-$ modified-DNA were first treated with equal volume of 0.2 N NaOH and allowed to denature for 10 min at room temperature. The alkali-denatured samples (0.4 ml each) were layered separately on top of a 4.6 ml linear gradient of alkaline sucrose (5–20% sucrose in 0.1 NaOH and 10 mM EDTA). The tubes were centrifuged at 30,000 rpm at 25 °C for 1 hr in a swinging bucket rotor (Beckman, U.S.A.). At the end of centrifugation, tubes were carefully removed and pierced at the bottom. Fractions of 0.5 ml were collected, diluted and absorbance read at 260 nm.

Ion exchange chromatography

Native and modified DNA samples were mixed separately with perchloric acid (70%) in a stoppered tube and boiled for 1 hr to release the base. The sample, after neutralization, was placed on top of a DEAE Sephadex A–25 column (1.6 cm×50 cm) equilibrated with the same buffer at a flow rate of 40 ml/hr. Fractions (2.5 ml) were collected and monitored at 260 nm. Mixtures of equimolar concentrations of bases (A, G, C, T) were also passed to locate the elution pattern of unmodified bases. Native and modified bases were identified on the basis of their unique absorption profile (Waris et al., 2000; Alam et al., 1993; Hasan and Ali, 1990). The extent of base modification was calculated by measuring the peak area.
Immunization scheme

Preimmune blood was taken from rabbits. The animals were immunized intramuscularly with 50 μg each of native and O$_2^-$ modified-DNA, complexed with an equal amount (w/w) of methylated BSA (MBSA) and emulsified in Freund’s complete adjuvant. Subsequent injections were in incomplete adjuvant. Each animal received a total of 250 μg antigen during the course of immunization. The serum separated from preimmune and immunized blood was decomplemented by heating at 56 °C for 30 min and stored at –80 °C in aliquots with sodium azide as preservative.

Enzyme Linked Immunosorbent Assay (ELISA)

Inhibition ELISA was carried out on polystyrene plates with slight modification (Ali and Alam, 2002). The microtitre wells were coated with 100 μl of native DNA or superoxide modified-DNA (2.5 μg/ml) for 2 h at 37 °C and overnight at 4 °C. Each sample was coated in duplicate and half of the plate served as control devoid of only antigen coating. Unbound antigen was washed thrice with TBS-T (20 mM Tris, 150 mM NaCl pH 7.4, containing 0.05% Tween-20) and unoccupied sites were blocked with 1.5% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4–6 h at 4 °C and plates were washed once with TBS-T. In competitive binding assay, sera (100 μl of 1:100 dilution) were incubated with increasing concentration of inhibitors (native DNA or superoxide modified-DNA; 0–20 μg/ml) for 2 hr at room temperature and overnight at 4 °C. The immune complex thus formed was added to the antigen-coated wells and incubated for 2 h at 37 °C and overnight at 4 °C. In direct binding assay, the antibodies were directly added to antigen-coated wells. The plates were extensively washed with TBS-T and the bound antibodies were assayed by anti-human-IgG or anti-rabbit IgG alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The results were expressed as mean of difference of absorbance values in test and control wells.

Band shift assay

Band shift assay was performed to visualize antigen-antibody interaction in gel (Dixit and Ali, 2001). Constant amount of native and superoxide modified-DNA was incubated with varying quantities of immune IgG in PBS, pH 7.4 for 2 hr at 37 °C and overnight at 4 °C. The complex thus formed was electrophoresed on 1% agarose gel for 2 hr at 30 mA in TAE buffer (40 mM Tris-acetate, pH 8.0).

Results

Superoxide radical induced modification of DNA

Exposure of riboflavin to white fluorescent light generates highly reactive superoxide anion radical (SAR). Its formation was confirmed by the reduction of NBT as observed by increase in absorbance at 560 nm (data not shown). The optimum generation of superoxide radical was observed at 20 min of illumination (1.65 absorbance unit at 560 nm). In presence of 10 μg/ml of superoxide dismutase the formation of superoxide radical was significantly dropped as observed by decrease in absorbance from 1.65 (in absence of SOD) to 0.40 (in presence of SOD). Addition of heat inactivated SOD and native
catalase did not affect superoxide radical formation. DNA exposed to SAR showed hyperchromicity at 260 nm, decrease in melting temperature (Tm), and modification of thymine (51.8%), guanine (40.5%), and adenine (22.2%). Cytosine did not show modification by superoxide anion radical (Waris et al., 2000). Density gradient ultracentrifugation profile of native and SAR modified-DNA is given in Fig. 1. Pattern of profile shows the formation of low molecular weight species in modified-DNA as a consequence of strand breakage. Moreover, these breaks appears to be randomly present on sugar-phosphate backbone which under alkaline conditions sediment more slowly and presented a diffused pattern of sedimentation profile in comparison to native DNA that banded as a sharp peak.

Antigenicity of superoxide anion radical modified-DNA

Results of direct binding ELISA showed that superoxide anion radical modified-DNA is a potent immunogen and induced high titer (1:25600) antibodies (data not given). The IgG purified from immune serum showed high binding with immunogen and cross-reaction with unmodified DNA (Fig. 2). Preimmune IgG showed negligible binding under identical conditions. In competition ELISA, anti-\( \text{O}_2^- \)DNA IgG showed preference for immunogen (Fig. 3). Nonetheless, induced antibodies exhibited appreciable binding with native DNA. A maximum of 79% inhibition in antibody binding was observed.
using immunogen as competitor. Fifty percent inhibition was seen at immunogen concentration of 0.2 μg/ml only. Binding diversity of anti-O$_2^-$-DNA IgG with array of nucleic acid antigens is given in Table 1.

**Binding of SLE anti-DNA IgG with native DNA and superoxide radical modified-DNA**

In a pilot study blood samples of SLE patients were screened for anti-DNA autoantibodies using native DNA as antigen. SLE samples whose anti-DNA autoantibody titer was ≥12 800 were included in this study (data not given). Anti-DNA IgG was isolated from 12 SLE sera by affinity chromatography on Protein-A-Sepharose and were subjected to competitive-inhibition assay using native DNA and O$_2^-$ modified-DNA as competitors. The results are summarized in Table 2. Comparison of data suggests that epitopes of O$_2^-$ modified-DNA are better recognized by SLE IgG.

**Band shift assay**

Band shift assay was carried out in agarose gel to visualize the formation of immune complex between O$_2^-$-modified-DNA and immune IgG. As clearly evident form the data, with an increase in the amount of immune IgG there was progressive increase in the formation of high molecular weight

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
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<tbody>
<tr>
<td>O$_2^-$-DNA</td>
<td>79</td>
</tr>
<tr>
<td>Native DNA</td>
<td>51</td>
</tr>
<tr>
<td>RNA</td>
<td>44</td>
</tr>
<tr>
<td>O$_2^-$-RNA</td>
<td>61</td>
</tr>
<tr>
<td>Mitochondrial DNA (mtDNA)</td>
<td>26</td>
</tr>
<tr>
<td>O$_2^-$-mtDNA</td>
<td>47</td>
</tr>
<tr>
<td>Poly G</td>
<td>49</td>
</tr>
<tr>
<td>O$_2^-$-poly G</td>
<td>54</td>
</tr>
</tbody>
</table>

ELISA plate was coated with O$_2^-$ modified-DNA at 2.5 μg/ml.
immune complex having retarded mobility (Fig. 4). The recognition of native DNA and \(O_2^-\)-modified-DNA by SLE IgG was similarly demonstrated by decrease in electrophoretic mobility on immune complex formation. The data was consistent with the results shown in Table 2 and Fig. 5.

**Discussion**

The aim of this study was to compare the antigen binding characteristics of SLE anti-DNA autoantibodies with experimentally induced antibodies against superoxide radical modified-DNA. Production of superoxide anion radical in mammalian tissues by enzymatic and non-enzymatic pathways is well documented (Min et al., 2002). These radicals have been implicated in several disease states and often produce undesirable changes in DNA and proteins.

![Fig. 4. Band shift assay of immune IgG with \(O_2^-\)-DNA. One \(\mu\)g of modified DNA (lane 1) was incubated with 30, 40, 50, and 60 \(\mu\)g of immune IgG (lanes 2–5) for 2 hr at 37 °C and overnight at 4 °C. The samples were subjected to electrophoresis in 1% agarose gel for 2 hr and visualized under UV light in presence of ethidium bromide.](image)
In present communication, DNA was exposed to superoxide anion radical generated from riboflavin. The superoxide modified-DNA showed hyperchromicity, decrease in melting temperature, and single strand breaks. All these changes are attributed to partial loss of secondary structure as a result of superoxide modification. That the superoxide radicals have produced random nicks along the sugar-phosphate backbone of DNA was ascertained by ultracentrifugation of modified sample in alkaline sucrose density gradient. The appearance of broader peak of diffused nature in case of superoxide modified-DNA is suggestive of random breaks on backbone generating DNA fragments of varying size. Barring cytosine, other bases were modified to varying degree and the maximum modification occurred in thymine.

The origin of autoantibodies remains an enigma and the production of anti-DNA antibodies is even more complicated. Even though nucleic acid antigens are by themselves poorly immunogenic, their antigenicity can be enhanced by modification with ROS (Waris et al., 2000; Alam et al., 1993), mixing with Fus-1 (Desai et al., 1993) etc. An overwhelming number of studies support the role of free radicals in the initiation and progression of autoimmunity in general, and SLE in particular (Ahsan et al., 2003). It is therefore postulated that in chronic inflammatory diseases, ROS generated by phagocytic cells may cause damage to DNA generating neoepitopes leading to the production of antibodies cross reacting with nDNA. The undisputed presence of 8-hydroxyguanosine in the DNA of immune complex derived from SLE patients (Lunec et al., 1994) reiterates the already suggested role of ROS in inflammatory diseases like SLE.

SLE is a multisystem autoimmune disease in which anti-double stranded DNA antibody is a classic autoantibody that characterizes SLE. However, native DNA (B-form) is a poor immunogen and rarely induces antibodies in experimental animals. It has been reported that DNA exposed to ROS presents a more discriminating antigen for SLE anti-DNA autoantibodies (Blount et al., 1989; Cooke et al., 1997). The preferential binding of affinity purified SLE anti-DNA IgG with superoxide modified-DNA as compared to native DNA further supports the already suspected role of ROS in inflammatory disease.

Modification of DNA by superoxide anion radical made it highly immunogenic. A previous study reported that native DNA treated with xanthine-xanthine oxidase system produced superoxide anion radicals and generated antibodies against superoxide radical modified-DNA (Sah et al., 1995). In this study \( \text{O}_2^- \)-DNA induced high titer anti-immunogen antibodies. Binding of induced antibodies with an array of nucleic acid antigens demonstrates their heterogeneous antigen binding characteristics. It may be
mentioned here that SLE anti-DNA autoantibodies as well exhibit polyspecificity with respect to antigen binding (Alam and Ali, 1999; Ahmad et al., 2000; Dixit and Ali, 2001). Antibody affinity for the immunogen was clearly evident in band shift assay results.

Results of the present study demonstrate that treatment of native DNA with superoxide anion radical conferred immunogenicity. The body’s immunosurveillance may prove ineffective if the generation of superoxide anion radical is enhanced tremendously, as seen in chronic inflammation and in injured tissues. There can be a situation in which the native DNA released from natural apoptosis of the cells may fall into the well of superoxide anion radical generating system. Once inside, the damage and modification would be inevitable and the immunoregulatory network would be activated to deal with this alien modified-DNA. The subsequent production of autoantibodies would be a natural step.

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References


