Clin Exp Immunol 1993; 94:134–139

Polynucleotide specificity of anti-reactive oxygen species (ROS) DNA antibodies

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(Accepted for publication 15 June 1993)

SUMMARY

Hydrogen peroxide in the presence of short wavelength UV light was able to induce alterations in native DNA fragments of 300 bp (ROS-DNA), thereby rendering it immunogenic in experimental animals. The specificity of induced antibodies was investigated by direct binding and competition ELISA. Inhibition studies revealed nearly 89% inhibition in the antibody binding by the immunogen and recognition of native B-, A- and allied conformations presented by various synthetic polynucleotides. Gel retardation assay reiterated the formation of immune complexes between induced antibodies and native and ROS-DNA fragments. It was observed that naturally occurring anti-DNA autoantibodies from systemic lupus erythematosus (SLE) sera recognize ROS-DNA. The comparison of the specificities of anti-DNA autoantibodies from 10 SLE patients showed a 20–50-fold preference for ROS-DNA over native DNA. These results demonstrate that anti-DNA antibodies can be induced by ROS-DNA, and that some of the autoimmune DNA binding antibodies found in SLE may result from response to reactive oxygen species.

Keywords anti-DNA antibodies hydroxyl radical antigenic specificity polynucleotides reactive oxygen species autoantibodies systemic lupus erythematosus

INTRODUCTION

Most attempts to induce anti-DNA antibodies by immunization with purified native DNA have been unsuccessful [1]. However, a number of synthetic analogues of DNA (e.g. Z-DNA, A-DNA, certain right-handed helical double-stranded polydeoxyribonucleotides) have been of considerable interest as immunogens [2,3]. This conformational variance from the B-form appears to be a prerequisite for the induction of antibody response, although the reason for this is not clear [4]. Antibodies that react with native DNA are found in individuals with autoimmune diseases, most characteristically in patients with systemic lupus erythematosus (SLE) or in animal models with similar autoimmune diseases [1,5–7].

The DNA-damaging and cytotoxic effects of hydrogen peroxide and other ROS are of considerable interest [8,9]. Hydrogen peroxide is a normal cell metabolite in aerobic organisms [10,11], and its production can be increased under several pathological conditions such as inflammation [12,13], or following cell exposure to agents like UV light, x-rays and many carcinogens or antineoplastic drugs [14–16]. Most of the deleterious effects of hydrogen peroxide are directly produced by hydroxyl radicals. Hydroxyl radicals are an extremely dangerous species and react with very high rate constants with most of the molecules present in a cell. The most important reactions are hydrogen abstraction and addition, and electron transfer [17,18].

Since naturally occurring autoantibodies have been found to recognize various conformations of nucleic acids, modified DNA may be considered as a stimulant for the production of antibodies showing cross-reactivity with B-conformation [1]. It has been reported that DNA after exposure to ROS presents a more discriminating antigen than native DNA for the binding of SLE autoantibodies [19,20]. Previously, we have shown that modification of double-stranded calf thymus DNA by ROS results in an increased binding of anti-DNA autoantibodies found in SLE sera [21,22]. In this study the hydroxyl radical modified DNA (300 bp) was found to be highly immunogenic in experimental animals. The antibodies induced by ROS-DNA also recognized native B-, A- and allied conformations of deoxyribonucleic acids.

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MATERIALS AND METHODS

Synthetic polynucleotides used in this study were obtained from Pharmacia, Uppsala (Sweden). Calf thymus DNA and nuclease S1 were purchased from Sigma Chemical Co. (St Louis, MO). DNA molecular weight markers were *Hae*III restriction nuclease digest of $\phi \times 174$ RF DNA (Pharmacia). The pVAT7 DNA was a kind gift of Professor S. M. Hadi (A.M.U. Aligarh, India). The sample typically contained 60–80% covalently closed supercoiled molecules, 20–40% open relaxed circular

molecules, and virtually no linear form. A short wavelength UV lamp (Spectroline; R-51 Black Light Eastern Inc., Westbury, NY) having maximum emission at 253·7 nm, was the source of irradiation. Single-stranded DNA was prepared by heat denaturation.

Serum samples

Sera from twelve SLE patients who satisfied the American Rheumatism Association criteria for the diagnosis of SLE [23] were investigated. The control group consisted of 25 healthy individuals. Serum samples were heated at 56°C for 30 min before use to inactivate complement.

DNA fragments

The commercial sample of DNA was purified free of proteins, RNA and single-stranded regions as described earlier [24]. Purified DNA was subjected to controlled digestion with micrococcal nuclease to obtain fragments varying from 70 bp to 800 bp. This was followed by the removal of single-stranded regions with nuclease S1 digestion [24].

Preparation of ROS-DNA fragments

Aqueous solutions of DNA fragments of 300 bp (0·15 mM) were irradiated under 254 nm light for 30 min at room temperature in the presence of hydrogen peroxide (15·1 mM). Excess of hydrogen peroxide was removed by extensive dialysis against PBS. DNA fragments exposed to hydrogen peroxide or UV light alone were used as corresponding controls.

Immunization schedule

Female rabbits were immunized intramuscularly with 50 μg of modified DNA (300 bp) complexed with an equal amount of methylated bovine serum albumin (MBSA) and emulsified in Freund's complete adjuvant (FCA). The rabbits received six similar injections, but with incomplete adjuvant at weekly intervals [25]. The serum separated from the immunized blood was decomplemented by heating at 56°C for 30 min.

Purification of antibodies

IgG was isolated from immune sera by DEAE Sephacel chromatography of 35% saturated ammonium sulphate-precipitated fraction. The isolated IgG was further purified through Sephadex G 200 column and eluted with 0·15 M phosphate buffer, pH 8·0 [26].

ELISA

ELISA on flat-bottomed 96-well polystyrene microtitre plate was performed as described earlier [27], with slight modifications. Polystyrene plate was preincubated with 100 μl poly-D-lysine (50 μg/ml in distilled water) for 30 min at room temperature. After washing three times with TBS (10 mM Tris, 150 mM NaCl, pH 7·4), wells were coated with 100 μl DNA (2·5 μg/ml in TBS) for 2 h at room temperature. One hundred microlitres of poly L-glutamate (50 μg/ml in TBS) were added to the wells for 2 h at room temperature. The plate was washed three times with TBS-T (20 mM Tris, 150 mM NaCl, 2·68 mM KCl, pH 7·4 containing 0·05% Tween-20) and unoccupied sites were blocked with 1·5% BSA in TBS for 6 h at room temperature. Antibodies (100 μl/well) were adsorbed for 2 h at room temperature and overnight at 4°C. Antibody specificity was ascertained by competitive binding assay [26]. 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. Antibodies in SLE sera were detected by ELISA as described above. Decomplemented sera were serially diluted in TBS-BSA and were added to antigen-coated plates for 2 h at room temperature and overnight at 4°C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate.

Table 1. Characteristics of native and modified DNA fragments under identical conditions

Parameter	Native DNA	Modified DNA
Absorbance ratio at 260/280 nm	1·8	2·0
Percent hyperchromicity at 95°C	27·7	18·9
Melting temperature (Tm)	55·0	47·0
Onset of duplex melting	50·0	42·0
Modification of adenine	—	21·7%
Modification of thymine	—	48·0%

The average size of DNA was 300 bp.

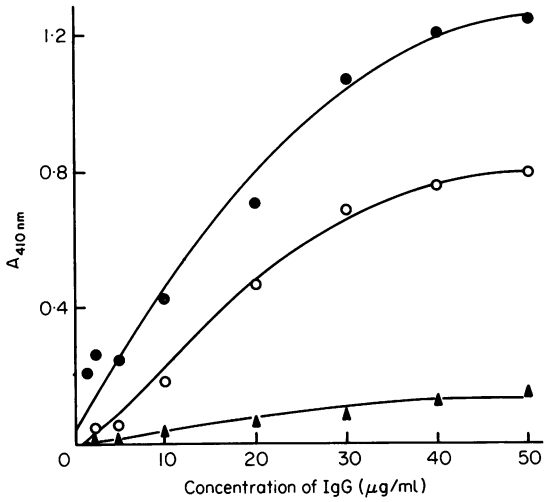

Fig. 1. Direct binding of anti-ROS-DNA antibodies with modified (●) and native DNA fragments of 300 bp (○), and of non-immune IgG with either antigen (▲).

Polynucleotide inhibition ELISA

Anti-ROS-DNA antibodies, diluted to 30 μg IgG/ml, were mixed with increasing amounts of various polynucleotides. Following incubation for 2 h at room temperature and overnight at 4°C, duplicate 100-μl aliquots of immune complex were added to ROS-DNA coated plates and the direct binding ELISA was performed. Inhibition was expressed as the amount of polynucleotide resulting in a 50% decrease in antibody binding to the solid-phase antigen.

Gel retardation assay

For the visual detection of antigen–antibody binding and formation of immune complexes, electrophoresis was per-

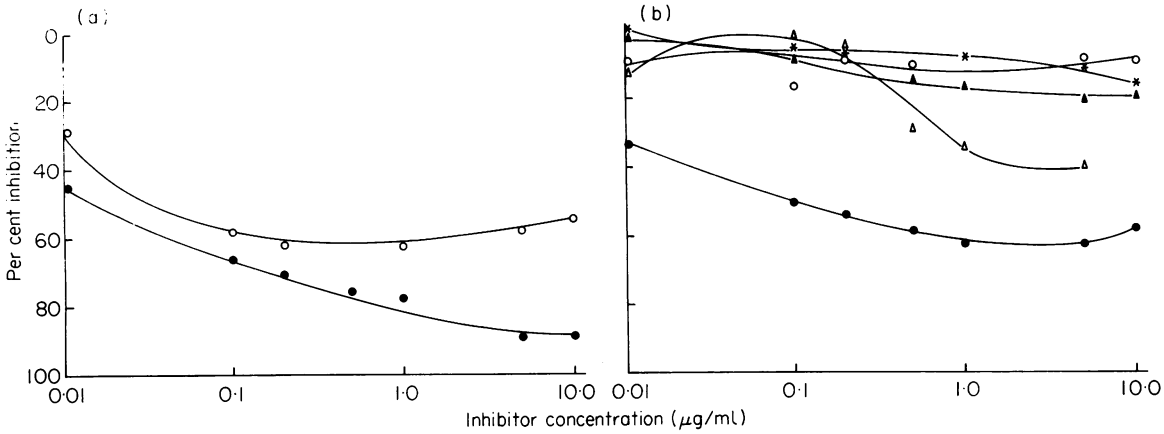

Fig. 2. (a) Inhibition of anti-ROS-DNA antibody binding to modified (●) and native DNA fragments (○). The microtitre plate was coated with modified DNA (2·5 μg/ml) and IgG was used at a concentration of 10 μg/ml. (b) Competition immunoassay of anti-ROS-DNA antibodies with nucleic acid polymers. The competitors were: heat-denatured calf thymus DNA (●); poly(rG)·poly(dC) (△); poly(dT) (▲); poly(dG) (*) and poly(dA) (○).

formed with 1% agarose gel [28]. The gels were run with 40 mM Tris-acetate buffer, pH 8·0. Samples were prepared by incubating constant amounts of antigen and antibody in PBS for 2 h at room temperature and overnight at 4°C before loading them onto the gel. In the case of native DNA, 5 μg of antigen was incubated with increasing amounts (25–100 μg) of immune IgG. On completion of electrophoresis, gels were stained with ethidium bromide (0·5 μg/ml) and photographed under UV illumination. Interaction between immunized antibodies and supercoiled plasmid pVAT7 DNA was also facilitated by gel retardation assay. A constant amount of plasmid DNA (1·0 μg) was incubated with increasing amounts (5–25 μg) of immune IgG for 2 h at room temperature and overnight at 4°C. The electrophoretic separation of free antigen from its protein-bound counterpart was achieved in 1·0% agarose gels, buffered with TAE, pH 8·0.

RESULTS

Characterization of ROS-DNA

Calf thymus DNA fragments of 300 bp on exposure to UV light in presence of hydrogen peroxide showed increased absorption at 260 nm, decrease in melting temperature, and modification of adenine and thymine bases. The physicochemical characteristics of native and modified fragments are presented in Table 1.

Antigenicity of ROS-DNA fragments

The modified polymer was highly immunogenic in rabbits. The antisera showed titres greater than 3200 by direct binding ELISA. DEAE Sephacel purified immune IgG showed effective binding with nDNA besides immunogen. Preimmune IgG as control showed negligible binding to these antigens (Fig. 1).

To ascertain the antibody specificity, competition ELISA was performed by employing modified polymer as inhibitor. A maximum of 89% inhibition was recorded at a concentration of 5 μg/ml (Fig. 2a). Only 0·015 μg of antibody was required for 50% inhibition in binding of immunogen. With native DNA a maximum of 62% inhibition was recorded at a concentration of 1·0 μg/ml. While DNA proved to be an effective inhibitor, total RNA from buffalo thymus, calf thymus DNA brominated in 4·0 M NaCl and single-stranded polymers (poly dG, poly dT and poly dA) showed poor inhibition of antibody binding. However, heat-denatured DNA showed appreciable inhibition in antibody activity (Fig. 2b). Considerable inhibition was obtained with poly (rG)·poly(dC), a polymer known to attain A-/analogous conformation in solution. The binding characteristics of anti-ROS-DNA antibodies are given in Table 2.

Table 2. Inhibition of the binding of anti-ROS-DNA antibodies by polynucleotides

Competitor	Concentration for 50% inhibition (μg/ml)	Maximum % inhibition at 10 μg/ml	Relative affinity, %
ROS-DNA	0·015	89	100
nDNA	0·04	62	37·5
Poly(dA·dT)·poly(dA·dT)	0·50	58	16
Poly(dA·dU)·poly(dA·dU)	5·0	58	1·6
Poly(dA·dG)·poly(dC·dT)	5·0	56	1·6
dDNA	0·1	60	15
Poly(dG)·poly(dC)	5·0	50	1·6
Poly(dI·dC)·poly(dI·dC)	5·0	25	1·6
Poly(rG)·poly(dC)	Nil	40	—
Br-DNA	Nil	9	—
RNA	Nil	9	—
Poly(dG)	Nil	15	—
Poly(dT)	Nil	20	—
Poly(dA)	Nil	9	—

Inhibition studies with polynucleotides

Besides native DNA, certain right-handed helical double-stranded polydeoxyribonucleotides which differ slightly from the average B-DNA helix were also tested for their ability to inhibit anti-ROS-DNA antibody binding. A varying degree of inhibition in antibody binding was obtained with these double-stranded polynucleotides (Fig. 3, Table 2).

Binding of anti-ROS-DNA antibodies to modified and native DNA

The antibody binding to modified and native DNA was studied by agarose gel electrophoresis. Retardation in the mobility of

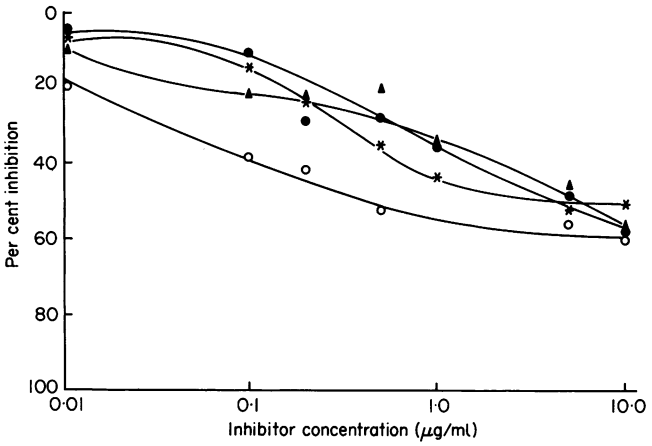

Fig. 3. Inhibition of anti-ROS-DNA antibody binding by polynucleotides. The antibodies were incubated with various polynucleotides and then tested for residual binding to modified DNA. The polynucleotides were: poly(dA-dG)·poly(dC-dT) (●); poly(dG)·poly(dC) (*); poly(dA-dU)·poly(dA-dU) (▲); and poly (dA-dT)·poly(dA-dT) (○).

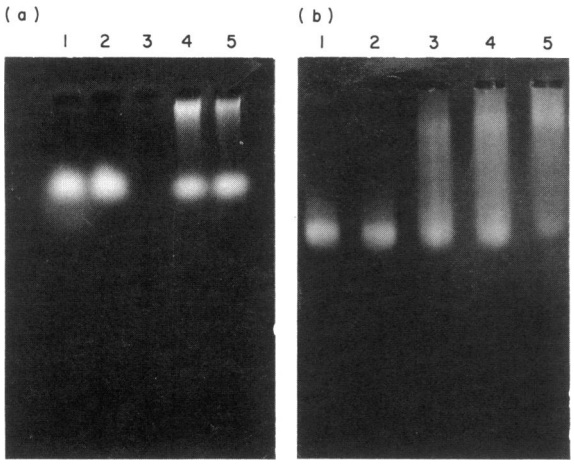

Fig. 4. (a) Binding of anti-ROS-DNA antibodies to modified and native DNA fragments as analysed by gel retardation assay. Modified and native DNA fragments (5·0 μg) were incubated with buffer (lanes 1 and 2) and with 30 μg of antibodies (lanes 4 and 5). (b) Binding of anti-ROS-DNA antibodies to native DNA fragments of 300 bp. Native DNA fragments (5·0 μg) were incubated with buffer (lane 1) and increasing concentrations of anti-ROS-DNA antibodies (25 μg, lane 2; 50 μg, lane 3; 75 μg, lane 4; and 100 μg, lane 5) for 2 h at room temperature and overnight at 4°C.

antigen was observed as a consequence of immune complex formation (Fig. 4a). When mixtures of antibody and native DNA fragments (average size 300 bp) were subjected to electrophoresis, the antigen–antibody complexes showed a varying degree of retarded mobility (Fig. 4b). With increasing concentration of antibody, the amount of immune complexes, as judged by their fluorescence intensity, was increased, whereas the amount of unbound DNA fragments indicated a proportional decrease in their concentration (lanes 2–5). Preimmune rabbit IgG did not show immune complex formation (data not shown).

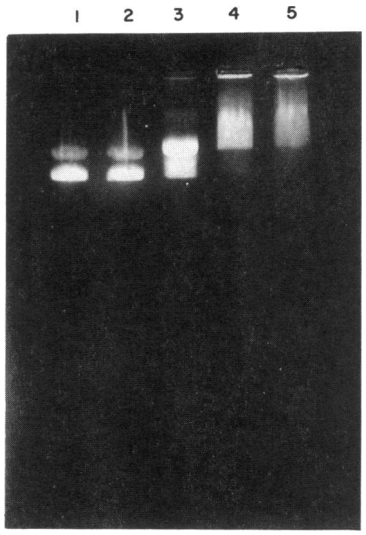

Fig. 5. Gel retardation assay of the binding of immune IgG to supercoiled plasmid pVAT7 DNA. The plasmid was incubated with buffer (lane 1), normal rabbit IgG (lane 2) and with 5 μg (lane 3), 20 μg (lane 4) and 25 μg (lane 5) of immune IgG. The electrophoresis was carried out with 1·0% agarose gels.

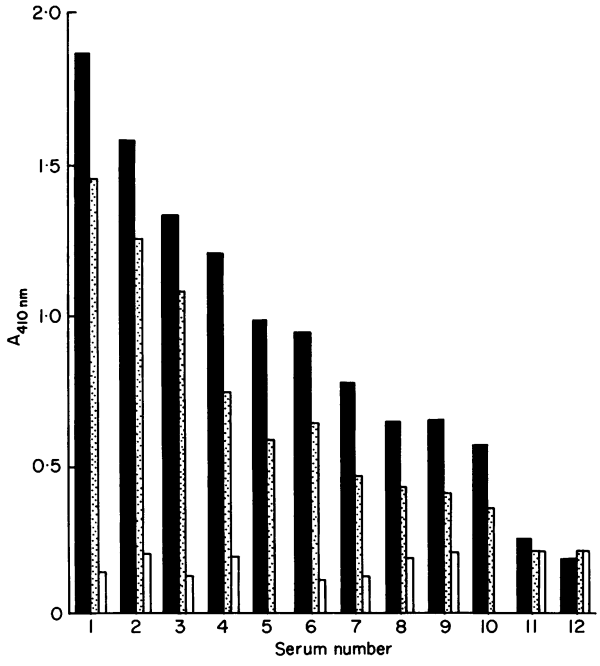

Fig. 6. Direct binding of systemic lupus erythematosus (SLE) serum antibodies to ROS-modified (■) and native (▩) DNA of 300 bp. □, Normal human sera with either of the antigens.

To confirm the binding of immune IgG to double-stranded DNA, supercoiled plasmid DNA (pVAT7) was allowed to interact with immune IgG under equilibrium conditions and subjected to electrophoresis in 1% agarose gels. The formation of immune complex(s) was observed (Fig. 5, lanes 3–5) and their concentration (in terms of fluorescence intensity) was propor-

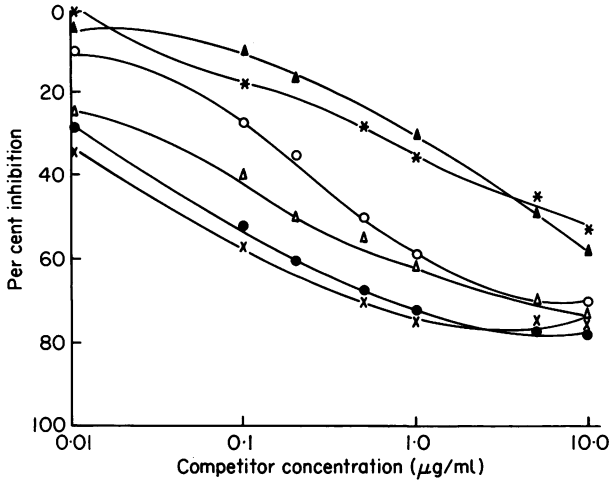

Fig. 7. Inhibition of anti-DNA antibody binding by three systemic lupus erythematosus sera to native (▲), (○), (*) and ROS-DNA (△), (●), (X). The plates were coated with native DNA (2·5 μg/ml).

Table 3. Inhibition of binding of systemic lupus erythematosus (SLE) autoantibodies by native and ROS-DNA

	Inhibitors	
Patients	Native DNA	ROS-DNA
1	4·0* (60)†	0·05* (75)†
2	5·0 (58)	0·10 (76)
3	7·0 (55)	0·10 (72)
4	4·0 (58)	0·20 (78)
5	7·5 (52)	0·50 (70)
6	7·5 (55)	0·20 (75)
7	8·0 (56)	0·50 (70)
8	10·0 (50)	0·20 (72)
9	5·0 (60)	0·10 (74)
10	0·5 (70)	0·50 (68)

The size of nucleic acid was 300 bp.

* Amount of competitor (μg/ml) required to inhibit 50% of DNA-anti-DNA antibody binding.

† Percentage inhibition at the maximum concentration of competitor used.

tional to the antibody concentration. At an antibody concentration of 25 μg, no free antigen bands were noticed (lane 5). Normal rabbit IgG did not show immune complex formation (lane 2). These results clearly demonstrate the recognition of double-stranded DNA by anti-ROS-DNA antibodies.

Preferential binding of human anti-DNA autoantibodies by ROS-DNA

Sera from 12 patients with SLE having high titre anti-DNA antibodies with preference for native DNA over single-stranded DNA were tested for reactivity with ROS-DNA. Such antibodies were found in 10/12 SLE patients with various titres. The results of direct binding of these autoantibodies showed that all these antibodies had stronger binding to ROS-DNA, while significantly lower binding was observed with native DNA fragments (Fig. 6). In contrast, no anti-ROS-DNA antibodies could be demonstrated in the sera of 25 normal human subjects (defining specific DNA binding as absorbance values of > 2 s.d. above the mean of 0·2 for the 25 normal human serum controls).

Binding specificity of SLE autoantibodies

The specificity of each SLE serum for native and ROS-DNA fragments was evaluated in inhibition ELISA. In this assay, the antibody was first incubated with the putative inhibitor (native or ROS-DNA) and then assayed for residual binding to native DNA. Antibodies from 10 SLE sera out of 12 studied showed more than 70% inhibition in binding to DNA by ROS-DNA fragments. Figure 7 shows the inhibition curves for three representative SLE sera. By comparison, the inhibition by nDNA fragments was less than 60% (Table 3). Only in one serum sample was the competitive efficiency of nDNA more than ROS-DNA (70%).

DISCUSSION

DNA damage caused by reactive oxygen species is known to be mutagenic, and probably represents a major natural hazard for the genomic stability of living cells [12,29,30]. Damage to DNA occurs at a site where a reduced metal bound to DNA reacts with hydrogen peroxide. Hydroxyl radical generated at the metal binding site may attack DNA at either the sugar or the base, ultimately leading to DNA strand breaks [31,32].

In this study we have investigated the antigenicity of free radical modified DNA fragments of 300 bp in length. The alterations in native calf thymus DNA fragments following exposure to 254 nm light in the presence of hydrogen peroxide were demonstrated by an increase in UV absorption, decrease in melting temperature (Tm) and formation of modified DNA bases. Among the bases, adenine and thymine were modified to the extent of 21·7% and 48% respectively. No modification of cytosine and guanine was observed. This is consistent with earlier observation that hydroxyl radical modifies predominantly thymine residues [17].

The modified polymer was highly immunogenic, inducing high titre antibodies in rabbits. Analysis of binding characteristics of induced antibodies indicated the recognition of modified part of the polymer and the sugar phosphate backbone. Native B-conformation, A- and allied conformations were also recognized. The high degree of inhibition in antibody binding by double-stranded polydeoxyribonucleotides (in B-conformation), demonstrates the polyspecificity of the induced antibodies. The single-stranded polymers (poly dG, poly dT and poly dA) used in this study do not show significant binding with the antibodies, and would therefore be expected to contain very little, if any, secondary structure. Gel retardation assay with native (calf thymus and supercoiled pVAT7 DNA) and modified DNA (300 bp) reiterated the binding of both native and modified DNA by the immune IgG.

It could be assumed that the structural alterations in double-stranded DNA, on interaction with chemical and physical agents, render it highly immunogenic, resulting in the formation of high titre antibodies. The ability of ROS-DNA to induce antibody that cross-reacted with double-stranded polydeoxyribonucleotides indicates that some features of B-conformation are present in this antigen. The broad antigen binding specificity

of induced antibodies in a way resembles the binding characteristics of SLE anti-DNA autoantibodies. This binding reactivity could be due to the presence of common epitopes on the sugar phosphate backbone of native and modified nucleic acids.

Our study of anti-DNA antibodies in the sera of SLE indicates that each individual serum has a significantly greater capacity to bind to ROS-DNA than to untreated native DNA samples. Competition experiments revealed that in all cases ROS-DNA competed better than double-stranded native fragments. There was a wide range in the native DNA concentration required for 50% inhibition in the ELISA, varying from 0·5 to 10·0 μg/ml. In the case of ROS-DNA, approximately 0·2 μg/ml of antigen was required for a similar level of inhibition. Earlier studies have shown that DNA denatured by ROS served as a better antigen for antibodies found in SLE sera [19,20].

The mechanism of anti-DNA antibody production in SLE has not yet been identified. If antigen selection is an important aspect of differentiation, the nature of the stimulating agent also remains to be determined. It could be DNA or a cross-reacting antigen. It is therefore possible that in SLE, the consequent production of antibodies may be the result of ROS attack on DNA, causing changes in structure at the macromolecular level.

ACKNOWLEDGMENTS

This work was supported in part by a grant (4/5/88-G) to R. A. from the Department of Atomic Energy, Government of India.

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