ANTI-ROS DNA ANTIBODIES AS MOLECULAR PROBE TO DETECT OXIDATIVE DNA DAMAGE TO HUMAN GENOME

ABSTRACT

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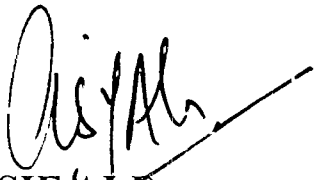
CERTIFICATE

I certify that the work presented in the following pages has been carried out by **Ms. Saba Khan** and is suitable for the award of **Ph.D.** degree in **Biochemistry**, of the Aligarh Muslim University, Aligarh.



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DEDICATED
TO
MY PARENTS

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ABSTRACT

Reactive oxygen species (ROS) are implicated in the inflammatory, autoimmune, connective tissue disease, systemic lupus erythematosus (SLE), particularly in respect of processes leading to the formation of pathogenic anti-DNA antibodies. Exposure to ROS increases the antigenicity of DNA. These reactive species are generally produced by ionizing radiation, redox-cycling drugs and many carcinogenic chemicals and can cause DNA damage, including single strand breaks and base modifications. Among these radicals, superoxide anion radical and singlet oxygen species are the most abundant. Superoxide is generated *in vivo* by several enzymatic and non-enzymatic pathways in mammalian tissues. Singlet oxygen is not a direct product of the enzymatic reactions, but rather a secondary reaction product and is a potent biological toxin. Both have been implicated in several disease states via alteration of biomolecules. Oxidative damage to cellular DNA can lead to mutations and may, therefore, play an important role in carcinogenesis and other biological processes.

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease involving both humoral and cellular aspects of the innate and acquired immune systems. Lupus is characterized by autoantibodies with a spectrum of specificities that participate in disease pathogenesis. In SLE, antibodies to DNA occur prominently and serve as markers of diagnostic and prognostic significance. The antibodies are predominantly IgG class and bind to conserved sites on the backbone of both single stranded (ss) as well as double-stranded (ds) DNA. Studies have emphasised that this disease is a complex genetic trait with contributions from major histocompatibility complex (MHC) genes and multiple non-MHC genes. The apoptosis genes *Fas* and *Fas* ligand (*Fas* L) are candidate contributory gene in human SLE, as mutations of these genes result in autoimmunity in several murine models of this disease.

In the present study, commercially available calf thymus DNA was purified and fragmented by using micrococcal nuclease and 200 bp fragments were selected for further studies. DNA was modified by singlet oxygen and superoxide anion radicals generated by riboflavin on exposure to UV light. The results indicated single strand breaks and base modifications. The induced modifications in DNA were analyzed by UV and fluorescence spectroscopic techniques. The modified DNA showed hyperchromicity

at 260 nm, as compared to native DNA, thereby, reflecting the change in DNA helix organisation. The Tm of $^1O_2$-$O_2^{\cdot-}$ -DNA was found to be 78°C, whereas, native DNA showed a Tm of 86°C. A net decrease of 8°C in the Tm value of $^1O_2$-$O_2^{\cdot-}$ -DNA indicates a partial destruction of its secondary structure. The single strand breaks, induced in DNA as a result of modification, were detected by nuclease S1 digestibility assay. The modified DNA fragments were employed in competitive assays to delineate the epitope recognition of induced antibodies against $^1O_2$-$O_2^{\cdot-}$ -DNA.

The antigenicity of $^1O_2$-$O_2^{\cdot-}$ -DNA was probed by inducing antibodies in rabbits. The repertoire of specificities of induced antibodies were evaluated by direct binding and competition ELISA. The induced antibodies exhibited polyspecificity, a property commonly associated with SLE anti-DNA autoantibodies. Anti-$^1O_2$-$O_2^{\cdot-}$ -DNA antibodies showed preferential binding to ROS-DNA.

Twenty four SLE sera were studied for their binding to native and $^1O_2$-$O_2^{\cdot-}$ -DNA. Specificity of antigen binding was assessed by inhibition ELISA. In direct binding assay, all SLE autoantibodies bound $^1O_2$-$O_2^{\cdot-}$ -DNA in preference to dsDNA. The preference was reiterated by inhibition ELISA. The binding specificity of native and $^1O_2$-$O_2^{\cdot-}$ -DNA with SLE anti-DNA autoantibodies was also confirmed by gel retardation assay.

Similarly, cancer sera were screened for the presence of antibodies reactive with native and $^1O_2$-$O_2^{\cdot-}$ -DNA. The study consisted of 34 sera from patients with various types of malignancies. Direct binding ELISA showed greater recognition for $^1O_2$-$O_2^{\cdot-}$ -DNA as compared to native form. Four sera from breast cancer showed higher recognition of $^1O_2$-$O_2^{\cdot-}$ -DNA than native DNA. Five sera from cancer of urinary bladder showed higher reactivity with $^1O_2$-$O_2^{\cdot-}$ -DNA. Four sera from cancer of gall bladder also showed higher recognition with $^1O_2$-$O_2^{\cdot-}$ -DNA. Three sera from prostrate cancer showed moderate recognition with $^1O_2$-$O_2^{\cdot-}$ -DNA, while only one sera showed almost equal inhibition with both native and $^1O_2$-$O_2^{\cdot-}$ -DNA. All the sera of lung cancer, having a history of smoking showed higher recognition of $^1O_2$-$O_2^{\cdot-}$ -DNA. Among four sera from oral cancer, two showed higher recognition of $^1O_2$-$O_2^{\cdot-}$ -DNA, one showed moderate recognition, while one sera showed higher recognition of nDNA as compared to $^1O_2$-$O_2^{\cdot-}$ -DNA. Four sera from cancer of head and neck, two from vulva, one serum each from CML and

Hodgkin's lymphoma showed higher recognition for $^1O_2$-$O_2^{\cdot -}$ -DNA as compared to nDNA. The results indicate that ROS appears to enhance the antigenicity of native DNA, suggesting role of ROS damaged DNA in the production of autoantibodies in cancer patients.

The role of ROS in the development of cancer was further supported by detection of oxidative lesions in DNA isolated from lymphocytes of various cancer patients, using anti-$^1O_2$-$O_2^{\cdot -}$ -DNA lgG as probe. DNA from two patients with lung cancer recognized anti-$^1O_2$-$O_2^{\cdot -}$ -DNA IgG appreciably and inhibited its activity to 64% and 68%. DNA from patients with oral and prostrate carcinoma too inhibited antibody activity appreciably.

In conclusion, singlet oxygen and superoxide anion radical causes damage to DNA and renders it highly immunogenic. Antibodies against $^1O_2$-$O_2^{\cdot -}$-DNA are polyspecific in nature resembling the antigen binding characteristics of SLE anti-DNA autoantibodies. It is postulated that $^1O_2$-$O_2^{\cdot -}$-DNA may play a major role in the production of SLE anti-DNA autoantibodies by the modification of DNA (or nucleosomes) thus forming neoantigen(s) resulting in production of autoantibodies. Further the anti-$^1O_2$-$O_2^{\cdot -}$ -DNA IgG raised in experimental animal was able to detect oxidative lesions in DNA of cancer patients.

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ABBREVIATIONS

A_{260}	:	Absorbance at 260 nm
A_{280}	:	Absorbance at 280 nm
BSA	:	Bovine serum albumin
bp	:	Base pair
CT DNA	:	Calf thymus DNA
DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylene diamine tetraacetic acid
ELISA	:	Enzyme linked immunosorbent assay
EtBr	:	Ethidium bromide
H_2O_2	:	Hydrogen peroxide
$^{\bullet}OH$	:	Hydroxyl radical
IgG	:	Immunoglobulin G
MBSA	:	Methylated bovine serum albumin
nDNA	:	Native DNA
PBS	:	Phosphate buffered saline
$O_2^{\bullet -}$	:	Superoxide anion radical
$^1O_2$	:	Singlet oxygen
RNA	:	Ribonucleic acid
SLE	:	Systemic lupus erythematosus
Tris	:	Tris (hydroxymethyl) amino methane
TEMED	:	N,N,N,N-tetraethylmethylene diamine

UV	:	Ultraviolet
SDS-PAGE	:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
dsDNA	:	Double stranded DNA
ssDNA	:	Single stranded DNA
ROS	:	Reactive oxygen species
Tm	:	Melting temperature
µl	:	Microlitre
µg	:	Microgram
λ_{max}	:	Maximum wavelength
SOD	:	Superoxide dismutase
NBT	:	Nitroblue tetrazolium
pRNO	:	p-nitrosodimethylaniline

In 1953, James Watson and Francis Crick first enunciated the double helical structure of DNA with the axis of the helix running perpendicular to the nitrogenous bases. This model of DNA, known as B-DNA, is the most widely accepted structure of DNA in solution. However, DNA can adopt many more different conformations or structures (Rich, 1984). The major physical evidence for the structure of DNA comes from X-ray diffraction studies of fibres (Langridge *et al.,* 1960). The differences in molecular forms are of crucial importance in understanding the manner in which proteins interact and recognize nucleotide sequences in B-DNA. Wang *et al.* (1979) described a variant of B-DNA double helix in a DNA hexamer with a sequence of (CGCGCG). The two strands of the duplex were antiparallel and connected by Watson-Crick base pairs, but the helix was left-handed. The backbone had a zig-zag shape and was termed as Z-DNA. The Z- conformation is adopted by flipping of the base pairs upside down. Triple stranded nucleic acid have also been found to exist in DNA. A typical example is the formation of triple stranded DNA as an intermediate in the action of *E.Coli* recombination enzyme Rec A (Camerine-Otero and Hseih, 1993). The X-ray diffraction pattern of polynucleotides such as polyinosinic and polyguanylic acid indicate that the strands are parallel to each other with a three or four fold symmetry axis along the fibre (Rich, 1958; Zimmerman *et al.,* 1975). Other conformation includes the cruciform structure, widely found in the eukaryotic genome (Wilson and Thomas, 1974; Lu *et al.,* 1992) and quadruple-stranded nucleic acids (Rich, 1958).

Antigenicity of DNA

The antigenicity of nucleic acids has received considerable attention, since the first report of anti-DNA antibodies in sera of patients with systemic lupus erythematosus (SLE) in 1957 and identification of DNA as the reactant of these antibodies (Cepelline *et al.,* 1957). In the early 1960's, methods were developed for the experimental induction of antibodies to nucleic acids (Levine *et al.,* 1960; Erlanger and Beiser 1964; Plescia *et al.,* 1964). Immunization of experimental animals with denatured DNA, synthetic nucleic acid polymers like poly(dT), poly(dC), poly(dA), poly(dI), poly(G), double stranded

RNA, 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).

The DNA molecule has several unique features, which have a direct bearing on its immune reactions. In nature it exists in different conformational forms including both right handed (A- and B-DNA) and left handed helix (Z-DNA) (Setlow, 1992). DNA is a multi determinant polymer and its polyanionic nature results in a hydrophobic core of stacked bases and a sugar phosphate polyanionic backbone. The phosphate residues make DNA a notoriously sticky antigen.

While native B-DNA is non-immunogenic, the left-handed Z-DNA is a potent immunogen (Lafer *et al.,* 1981; Madaio, 1984) and induces selective and specific antibodies (Zarling *et al.,* 1984). Those DNA structures that differ significantly from B-DNA are much stronger immunologic stimuli than nDNA (Anderson *et al.,* 1988a). Purified calf thymus DNA modified with drugs, hormones, free radicals etc. has been reported to induce antibodies against the modified DNA (Alam and Ali *et al.,* 1992; Arif *et al.,* 1994; Moinuddin and Ali, 1994; Arjumand *et al.,* 1995; Ara and Ali, 1995). Polynucleotides that differ from B-conformation are much stronger immunogens (Braun and Lee; 1988), while polynucleotides of B-conformation become immunogenic after modification with furocoumarins (Arif and Ali, 1996).

The reversal in handedness of double stranded helical DNA accompanying B to Z-transition is one of the most dramatic examples of DNA polymorphism (Pietrasanta *et al.,* 1994). A-like DNA is characterized by more base pairs per turn of the helix and a wider minor groove than B-like DNA. A-like DNA is not associated with the living system and is generally formed under conditions of low water content (Saenger, 1984). It has been found out that DNA in some *Bacillus* species is induced in A-like conformation (Setlow, 1992).

Mammalian DNA elicit poor immune response to single stranded DNA and fails to induce antibodies against native or double stranded DNA, the serologic marker of SLE (Madaio *et al.,* 1984). However, studies have shown that DNA complexes with synthetic peptide Fus-I can induce anti-dsDNA response in mice (Desai *et al.,* 1993). This led to the conclusion that antigen drive in lupus involves either a substance other than DNA or

DNA in a form (e.g. nucleosomes) that is not readily mimicked by artificial complexes (Burlingame *et al.*, 1993, Mohan *et al.*, 1993). Since the antibody reactivity of both single and double stranded forms appears independent of DNA species origin, these findings suggest recognition of conserved conformational determinants (e.g. helical backbone of B-DNA) and a lack of DNA sequences microheterogeneity on antigenicity (Stollar, 1975; Pisetsky, 1993). Anti-DNA antibodies may also result by autoimmunization with chromatin, rather than native DNA (Theofilopoulos, 1995).

Immunization of animals with nucleic acid-MBSA complexes resulted in antibodies that reacted with free denatured DNA, but not with B-conformation of DNA. They also showed cross-reactivity with denatured DNA from widely different species. There is compelling evidence that bacterial DNA, in contrast to mammalian DNA, induces a variety of responses in both normal humans and animals (Pisetsky, 1996). Antibodies to bacterial DNA in normal human sera are predominantly of IgG2 subclass, whereas, lupus anti-DNA antibodies are mainly IgG1 and IgG3. Animals following immunization with bacterial DNA induce antibodies showing negligible cross-reactivity with mammalian DNA (Gilkeson *et al.*, 1991). Low molecular weight DNA was found in the DNA-anti DNA complexes of lupus patients and is enriched in CG contents (Sano *et al.*, 1983). The CG rich DNA region has the tendency to form Z-DNA, which is highly immunogenic.

The immunologic potential of DNA has immediate relevance in the therapeutic arena. DNA vaccine seems to be a broadly applicable technique for generating protective immune response against viral, parasitic and tumour antigens in animal ranging from mice to non-human primates. DNA vaccine research is providing new insights into some of the basic immunological mechanisms of vaccination such as antigen presentation, the role of effector cells and immunoregulatory factors. In addition, DNA vaccines help to manipulate the immune system in situations where the response to agents is inappropriate or ineffective. The study of the potential deleterious effect of DNA vaccines leads to further knowledge regarding the relationship between bacterial DNA and the immune system, as well as its potential application for the study of neonatal tolerance and autoimmunity (Mor and Eliza, 2001).

Free radicals

A free radical may be defined as any chemical species that contains one or more unpaired electrons and is capable of independent existence. They are extremely reactive, hence their half lives are very short and steady state concentrations quite low (Freeman, 1984; Halliwell and Gutteridge, 1984; 1989; Cadenas, 1989). They can be formed by homolytic cleavage, heterolytic cleavage or by the loss of single electron from a molecule. As such, free radicals can be formed in three ways:

(i) By the homolytic cleavage of a covalent bond of a molecule, with each fragment having an unpaired electron (Von Sonntag, 1987).

(ii) By the loss of a single electron from a molecule.

(iii) By the addition of a single electron to a molecule.

The latter, electron transfer, is more common process in biological systems than homolytic fission, which generally requires high energy input from either high temperatures, UV light or ionising radiations. Free radicals can be positively charged, negatively charged or electrically neutral. The most important free radicals in biological systems are radical derivatives of oxygen known as reactive oxygen species (ROS). ROS not only includes oxygen-centered radicals such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$), but also non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and ozone (O_3). The reactivity of ROS is due to the unstable electronic configuration of these radicals.

In living cells, ROS are produced continuously during normal aerobic metabolism (Halliwell and Aruoma, 1991) as well as in many pathologic conditions such as tissue ischaemia, cancer, inflammation and degenerative diseases (Halliwell and Gutteridge, 1984; Vuilaume, 1987). The autooxidation of certain small cytoplasmic molecules such as catecholamine, flavins and the activity of some enzymes (xanthine oxidase and aldehyde dehydrogenase) have been known to produce ROS, *in vivo* (Fridovich, 1983). Besides these, the metabolism of arachidonic acid and respiratory burst from phagocytes also give rise to ROS (Halliwell and Gutteridge, 1989).

It has been demonstrated that iron, in presence of H_2O_2 and ascorbate generates $^{\bullet}OH$ which causes cleavage of proximate DNA. Besides these endogenous sources, ROS are also generated by a number of exogenous sources such as inhaled smoke, polluted air, certain ingested food and a variety of other carcinogens. The low wavelength ionising radiation (e.g. γ and X-ray) and ultraviolet (UV) light are also important exogenous sources of ROS (Davies, 1987; Epe, 1991). Hydroxyl radical is also known to be generated by UV (254 nm) irradiation of H_2O_2 (Ara and Ali, 1992; 1993).

Superoxide anion ($O_2^{\bullet -}$) *is formed by the single electron transfer to ground state* dioxygen. During mitochondrial respiration 1-5% of oxygen is converted to $O_2^{\bullet -}$ (Freeman and Crapo, 1982; Cross *et al.,* 1987). Superoxide radical shows limited reactivity, but in aqueous solution it reacts with itself producing hydrogen peroxide and molecular oxygen in presence of enzyme superoxide dismutase (Halliwell, 1994). The protonated form of $O_2^{\bullet -}$, called perhydroxyl radical (HO_2), is more reactive than $O_2^{\bullet -}$ itself (Halliwell and Gutteridge, 1989).

Molecular oxygen in electronic singlet state is a powerful oxidant. Only one type of living cell, the human eosinophil, produces detectable quantities of singlet oxygen (Kanofsky *et al.,* 1988). Superoxide radical interacts with (H_2O_2) to generate $^1O_2$ molecule which is also generated predominantly in photosensitization reactions. It shows high reactivity towards biomolecules. Recently it has been shown that UV-irradiation of certain antibiotics (Nalidixic acid, amphotericin-B, cephradine, cefazolin, nafcillin etc.) leads to the generation of singlet oxygen (Pandey *et al.*, 2002).

Much of the toxicity of free radicals *in vivo* is thought to result from reaction of $^{\bullet}OH$, which can be produced in cells by metal ion dependent conversion of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide or by interaction of ionizing radiation with cellular water (von Sonntag, 1987). Homolytic fission of O-O bond in hydrogen peroxide by heat or ionizing radiation produces two $^{\bullet}OH$ radicals. A simple mixture of H_2O_2 and iron (II) salt also forms $^{\bullet}OH$ as was first observed by Fenton in 1894

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

and the second, by the interaction of superoxide anion with H_2O_2 through the Haber-Weiss reaction (Haber and Weiss, 1934).

TABLE – 1

Some characteristics of reactive oxygen derivatives

Species	Chemical symbol	Origin	Properties	Protectors
Super-oxide anion	$O_2^{\cdot -}$	$O_2 + e^-$	Good reductant, poor oxidant	SOD
Singlet oxygen	$^1O_2$	$^3O_2$, peroxidation	Powerful oxidizing agent	Vit. C, Vit. E, β-carotene
Hydroxyl radical	$^{\cdot}OH$	H_2O_2, H_2O	Extremely reactive, very low diffusion distance	Antioxidants
Hydrogen peroxide	H_2O_2	$O_2^{\cdot -}$ bio-generation	Oxidant, high diffusion capability	Catalase, glutathione peroxidase

Adapted from Simic *et al.* (1989) and Yu (1994).

$$O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + H_2O + {}^{\bullet}OH$$

Hydrogen peroxide though not a free radical, is harmful to the cell because of its ability to cross biological membranes and produce the highly reactive hydroxyl radical ($^{\bullet}OH$) (Ward *et al.*, 1987; Pryor and Church, 1991). Superoxide and H_2O_2 though less reactive than $^{\bullet}OH$ have a longer half-life and react with molecules far away from their site of production (Halliwell and Gutteridge, 1984; Pryor, 1986).

Oxidative DNA damage and repair

Oxidative DNA damage is produced when reactive oxygen species are generated in cells by normal aerobic metabolism or by exogenous source such as ionising radiation or carcinogenic compounds, which may be implicated in mutagenesis, carcinogenesis and aging (Halliwell and Gutteridge, 1990). The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination and deamination (Totter *et al.*, 1980). Conversion of guanine to 8-hydroxyguanine (8-OHG), a frequent product of ROS attack has been found to alter the enzyme-catalyzed methylation of adjacent cytosines (Weitzman, 1994), thus providing a link between oxidative DNA damage and altered methylation patterns.

The chemistry of DNA damage by several ROS has been well characterized *in vitro* (von Sonntag, 1987). Different ROS affect DNA in different ways, e.g. H_2O_2 do not react at all with DNA bases. Hydroxyl radical generates a multiplicity of products from all four DNA bases and this pattern appears to be a diagnostic fingerprint of $^{\bullet}OH$ attack. By contrast $^1O_2$ selectively attacks guanine (Van den Akker *et al.*, 1994). The most commonly produced base lesion, and the one most often measured as an index of oxidative DNA damage, is 8-OHG. It is sometimes measured as the nucleoside 8-hydroxydeoxyguanosine (8-OHdG) (Ames, 1989). Superoxide is capable of inducing strand breaks (Sah *et al.*, 1995).

Reduction of DNA can also lead to the ring opening product 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Dizdaroglu, 1991). Other abundant oxidatively modified purine and pyrimidines include 7,8-dihydro-8-oxoadenine (8-oxo-dA), 2-hydroxyadenine, Fapyadenine, 5-hydroxymethyluracil, 5-hydroxycytosine, cytosine

glycol and thymine glycol. In addition, a large number of other modification of bases and sugars have been identified (Dizdaroglu, 1991; 1994; Kamiya *et al.*, 1995).

Mitochondrial DNA (mtDNA) has been found to be especially vulnerable to attack by oxygen free radical (Driggers *et al.*, 1996). Oxidative damage to cellular DNA can be mutagenic and may, therefore, play an important role in tumorigenesis. It has been shown that 8-OHdG is mutagenic, causing GC→AT base pair substitutions (Kuchino *et al.*, 1987) and can result in activation of the human C-Ha-ras-Igene (oncogene) (Kamiya *et al.*, 1992).

DNA repair is an immediate response after damage and several repair pathways are likely to be elicited when cells are exposed to oxygen species (Sage, 1993). The nucleotide excision repair system of *E.Coli* Uvr ABC, however, is not pivotal for handling oxidative damage in contrast to its crucial role in the repair of structurally distorting lesions, such as pyrimidine photodimers and some carcinogenic adducts (Sancer and Tang, 1993). Unrepaired DNA or inaccurate repair of damage can lead to cell death (Jackson, 1996).

In vivo oxidative DNA damage repaired continuously by a variety of enzymes. Strand breaks are annealed and modified bases are excised as such or as nucleotides (Ramotar and Demple, 1993; Demple and Harrison, 1994). DNA glycosylases excise bases and subsequently phosphodiester bonds on each side of the basic site are incised by endonucleases allowing the insertion of an intact nucleotide. The formamidopyrimidine-DNA glycosylase enzyme (Fpg; mut M) in *E.Coli* repairs the oxidized purines 8-oxoguanine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine and to lesser extent the corresponding adenine derivatives of base excision (Boiteux *et al.*, 1992). The formamidopyrimidine DNA glycosylase protein appear to repair 8-oxodG in non coding and actively transcribed mammalian DNA sequences with equal efficiency (Bohr *et al.*, 1995).

Superoxide anion radical

One electron reduction of oxygen leads to the formation of superoxide anion radical (Florence, 1990; Harris, 1992). The superoxide anion radical ($O_2^{\bullet -}$) is considered

to be a highly toxic entity in many biological systems (Oberly, 1982; Rotelio, 1986; von Sonntag, 1987; Harman, 1993). It is formed in normal metabolism as well as through the action of many drugs, poisons and radiation. It is also involved in radiation damage, DNA damage, phagocytosis, ageing, cancer etc. Since, superoxide dismutase (SOD) is specific for $O_2^{\cdot -}$ as substrate, it follows that it must be a toxic species. Superoxide dismutases (SODs) are metalloenzymes that catalyse very efficiently the dismutation of superoxide ions into oxygen and hydrogen peroxide. It has been widely recognized that these enzymes, which are present in almost all living organisms provide a defence system that is essential for their survival under aerobic conditions. The deleterious role of superoxide during ischaemia (McCord, 1988), which occurs in organ transplantation and many surgical interventions and during heart and brain events, suggests that SOD may have a potential clinical use.

Superoxide anion is generated most frequently by the xanthine-xanthine oxidase system (Greenwald, 1985). Xanthine oxidase produces $O_2^{\cdot -}$ when it oxidizes aerobically xanthine to urate. The exogenous chemical sources of $O_2^{\cdot -}$ that can conveniently be used to assess the genotoxic potential of oxygen metabolite include:

(i) Xanthine oxidase acting aerobically on xanthine or hypoxanthine, which produces a steady flux of superoxide radical.

(ii) Potassium superoxide (KO_2), which dissolves in water with the liberation of superoxide anion radical.

Superoxide anion is the only species generated by these two systems. However, H_2O_2 is also immediately formed by spontaneous or catalyzed dismutation of $O_2^{\cdot -}$. Both exogenous sources of $O_2^{\cdot -}$ have been shown to be genotoxic, which is attributed to its ability to damage chromosomal DNA (Emrit *et al.*, 1982). Interestingly, chromosomal breakage is observed in several autoimmune diseases such as lupus erythematosus, rheumatoid arthritis and ulcerative colitis (Emerit, 1986). Therefore, it is possible that superoxide radical produced in excessive amount might be responsible for DNA damage in autoimmune diseases. The currently persisting dominant view of $O_2^{\cdot -}$ toxicity is that it damages cells only indirectly, by giving rise to hydroxyl radical (Halliwell and

Gutteridge, 1992). However, the seminal work of Fridovich (1986) has demonstrated that $O_2^{\cdot -}$ itself can exert direct deleterious effects in biological systems. A number of enzymatic targets of superoxide have been identified. For example, *in vitro* inactivation of ribonucleotide reductase, the key enzyme providing the deoxyribonucleotides required for DNA synthesis is lethal for organisms (Gaudu, 1996).

Superoxide chemistry differs when the reaction is carried out in organic solvents and in aqueous solution. In nonpolar environments $O_2^{\cdot -}$ is a powerful base (proton acceptor), nucleophile and reducing agent (Sawyer and Valentine, 1981; Frimer, 1982). It can also act as an oxidizing agent, but this ability is only seen with compounds that can donate H^+ ions such as catechol, ascorbate or α-tocopherol (Vitamin-E). In aqueous solutions, the basic properties and nucelophilicity of $O_2^{\cdot -}$ are greatly reduced, as is its oxidizing capacity and it acts as a reducing agent. For example, it reduces Fe(III) ions at the active site of cytochrome C and Cu(II) ions at the active site of plastocyanin. Any reaction undergone by $O_2^{\cdot -}$ in aqueous solution will be in competition with the dismutation reaction (Halliwell and Gutteridge, 1984). Superoxide radical can reduce many metal cations by electron transfer.

$$Fe^{+3} + O_2^{\cdot -} \longrightarrow Fe^{+2} + O_2$$

Analogous reactions occur with other cation (Mn^{+3}, Cu^{+2}), chelated iron and many enzymes (Valentine, 1979; Wesser *et al.*, 1981). Several workers (Winterbourn, 1979; 1981; Fee, 1982) have argued that this can not occur *in vivo* because the concentration of other biological reducing agents would be greater than that of $O_2^{\cdot -}$.

Sources of superoxide

Xanthine oxidase has been proposed to be an important source of oxygen derived free radicals in reperfused tissue (Granger *et al.*, 1981; Chambers *et al.*, 1985; McCord, 1985; Hearse *et al.*, 1986). Nascent xanthine oxidase exists as a dehydrogenase using NAD^+ as an electron acceptor during the oxidation of hypoxanthine and xanthine (Batteli *et al.*, 1972).

The production of superoxide by the mitochondria has been known for nearly three decades (Boveris *et al.*, 1976; Turrens and Boveris, 1980). The rate of superoxide production by the mitochondria increases when the concentration of oxygen is increased or the respiratory chain becomes largely reduced (Turrens *et al.*, 1982). Mitochondria produce $O_2^{\cdot -}$ anion at two sites in the electron transport chain. The first site is the ubiquinone to cytochrome C_1 step, which passes through the intermediate ubisemiquinone (Boveris *et al.*, 1976; Cadenas *et al.*, 1977; Turrens *et al.*, 1985). The second site of superoxide anion formation is the NADH dehydrogenase (Turrens and Boveris, 1980).

The activated NADPH oxidase of phagocytic cells is a potentially large and significant source of free radicals in stimulated neutrophils. After initiation of the respiratory burst, more than 90% of the consumed oxygen can be accounted for by the generation of superoxide. Production of $O_2^{\cdot -}$ and H_2O_2 by neutrophils is enhanced after the cells adhere to surfaces or after being primed with a chemical stimulus (Dahinder *et al.*, 1983).

Biological effects of the superoxide radical

The superoxide radical, $O_2^{\cdot -}$, is formed in all aerobic organisms and systems generating it have been observed to kill bacteria, inactivate viruses, damage enzymes and membranes, and destroy animal cells in culture (Fridovich, 1978; Halliwell, 1981). Bovine liver catalase is inactivated by $O_2^{\cdot -}$ and H_2O_2 produced by the aerobic xanthine oxidase reaction and this was completely prevented by SOD (Rister and Baehner, 1976).

Numerous studies of the effects of a $O_2^{\cdot -}$ flux upon erythrocytes have been reported. 1,4-Napthoquinone-2-sulphonate reacts with oxyhemoglobin yielding methemoglobin plus $O_2^{\cdot -}$ and can be used to increase $O_2^{\cdot -}$ production in erythrocytes (Goldberg and Stern, 1976). Studies have implicated both $O_2^{\cdot -}$ and H_2O_2 in the lung damage caused by hyperoxia. There is one report in which $O_2^{\cdot -}$ is clearly shown to exert an effect, independent of H_2O_2. Johnson *et al.* (1981) instilled enzymatic sources as well as scavengers of $O_2^{\cdot -}$ and H_2O_2, directly into the airways of rat lung xanthine oxidase plus

xanthine, used as a source of $O_2^{\cdot -}$ and H_2O_2, caused damage which was prevented by SOD, but not by catalase.

A number of molecules also react with $O_2^{\cdot -}$. Some of these such as epinephrine (McCord and Fridovich, 1969), bilirubin (Robertson and Fridovich, 1982), biliverdin (Galliani *et al.*, 1984), α-tocopherol and water soluble analogues thereof are clearly biologically relevant (Ozawa, 1985).

Singlet oxygen

Oxygen in the triplet state (singlet oxygen) is a potent biological toxin. It oxidatively damages a variety of biological molecules including DNA (Foote, 1981; Straight and Spikes, 1985; Wefers *et al.*, 1987), inactivates viruses (Wefers *et al.*, 1987), and, kills both bacteria and mammalian cells (Dahl *et al.*, 1987; Dahl, 1991). Singlet oxygen has been found to be a common mediator of photochemical damage in both plants and animals. The lifetime of $^1O_2$ within the living cell is very short, of the order of 0.1 microseconds, while in water and organic solvents it has a relatively longer half life (Moan *et al.*, 1979; Firey *et al.*, 1988; Patterson *et al.*, 1990; Moan and Berg, 1991; Baker and Kanofsky, 1992).

Production of singlet oxygen in biological systems

Singlet oxygen is not a direct product of the enzymatic reactions, but rather a secondary reaction product. The two most carefully studied mechanisms for the production of singlet oxygen by enzymatic systems are the reaction of hydrogen peroxide with hypohalous acid:

$$H_2O_2 + HOX \longrightarrow H^+ + X^- + H_2O + O_2\ (^1\Delta g) \qquad \text{(i)}$$

(where X is chlorine or bromine) and the reaction between two peroxyl radicals:

$$2RR'HCOO \longrightarrow RR'CO + RR'CHOH + O_2\ (^1\Delta g) \qquad \text{(ii)}$$

In both these mechanism, two highly reactive species must come together to make singlet oxygen. Indeed, only one type of living cell, the human eosinophil, produces detectable quantities of singlet oxygen (Kanofsky *et al.*, 1988).

In human eosinophils, several factors contribute to the efficiency of singlet oxygen generation. First, these cells have an extremely active respiratory burst, generating 20 fmol min^{-1} $cell^{-1}$ of superoxide anion (Kanofsky *et al.*, 1988). Second, eosinophils generate hypobromous acid rather than hypochlorous acid (Weiss *et al.*, 1986). The formation of singlet oxygen by human eosinophils is only 0.4% of the available oxygen (Kanofsky *et al.*, 1988). Kanofsky and Sima (1991) found that ozone reacts with certain biological molecules to generate singlet oxygen but not all biomolecules react with ozone to generate singlet oxygen.

Photochemical generation of singlet oxygen in biological systems

Singlet oxygen is believed to be the major mediator of biological damage from a number of photosensitizers, including porphyrins, phthalocyanines and halogenated fluorescein. Singlet oxygen is produced by a reaction sequence called a Type II mechanism:

$$p + h\lambda \rightarrow p^* \quad \text{(i)}$$

$$^1p^* \rightarrow {}^3p^* \quad \text{(ii)}$$

$$^3p^* + O_2 \sum g^- \rightarrow P + O_2 (^1\Delta g) \quad \text{(iii)}$$

Here p is ground state photosensitizer, $^1p^*$ is photosensitizer in an excited singlet state, and $^3p^*$ is photosensitizer in an excited triplet state. High oxygen concentration favours type II mechanism.

Biological consequences of $^1O_2$-modified DNA

The activated oxygen species, including the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and singlet oxygen can be generated by the incomplete reduction of oxygen to water during respiration, by exposure to radiation, light, metals, redox active drugs or by release from stimulated macrophages (Sies, 1986), because many photosensitizers are biological in origin, and light and oxygen are also usually present, $^1O_2$ is a common product in living cells. Allen *et al.*, (1972) were the first

to suggest that neutrophils might produce toxic quantities of $^1O_2$ during the respiratory burst via a reaction scheme catalyzed by the lysosomal myeloperoxidase.

$$H_2O_2 + X^- + H^+ \xrightarrow{\text{Myeloperoxidase}} HOX + H_2O \qquad \text{(i)}$$

$$H_2O_2 + HOX \longrightarrow H_2O + H^+ + X^- + O_2\ (^1\Delta g) \qquad \text{(ii)}$$

where X is a halide or a pseudohalide ion (SCN^-). Singlet oxygen behaves like a strong electrophile in solution and reacts avidly with molecules possessing regions of high electron density, hence, it is clear that oxidative damages to biomolecules mediated by $^1O_2$ are rather frequent and DNA, protein or lipids are all at risk (Foote, 1976).

(a) Reaction with DNA

In 1989, Floyd and co-workers showed that the photosensitized action of methylene blue leads to the effective formation of 8-hydroxyguanine (8-oxodG) in DNA. Other photosensitizers known to produce higher yields of $^1O_2$, such as Rose Bengal (Floyd *et al.*, 1990) and hematoporphyrin D (Floyd *et al.*, 1990) are also found to lead to the formation of 8-oxodG, reinforcing the idea that this lesion is formed by a reaction between dG and singlet oxygen. Epe and co-workers (1988) found out that the DNA damage profile is completely different from the one caused by hydroxyl radicals. Single strand breaks account for less than 5% of the total DNA damage and originate probably from superoxide anion formed during endoperoxide decomposition. Contrary to the idea that singlet oxygen cannot lead to the direct breakage of the sugar – phosphate backbone of DNA. Because of its action at the DNA base level and may be on the sugar phosphate backbone, singlet oxygen should be considered as a DNA damaging agent when it is generated in the DNA vicinity. The results of Piette *et al.* (1984) clearly indicate that when guanine residues oxidized by singlet oxygen are not removed from DNA they are capable of blocking DNA replication, which explains biological properties like the transforming or transfecting abilities of DNA that are altered by lesions.

(b) The cytotoxic action of singlet oxygen

There are several lines of evidence supporting the idea that singlet oxygen is one of the major cytotoxic species to eukaryotic cells (Eisenberg *et al.*, 1984; Dubbelman *et*

al., 1988), bacteria (Epe *et al.*, 1989) and viruses (Houba-Herin *et al.*, 1982). It has been shown that oxidation of the genetic material by singlet oxygen leads to an important loss of the infectivity as DNA replication is strongly impaired in the infected cells (Piette *et al.*, 1984) or injection of DNA molecules after phage adsorption does not occur (Bryant and King, 1984). Ito (1974) and Kobayashi *et al.* (1976) have shown that damages caused in the nucleus by $^1O_2$ could be, to some extent, responsible for cell inactivation.

(c) The mutagenic and genotoxic effects of singlet oxygen

Some evidence showing that $^1O_2$ can promote mutagenic effects has been provided from a variety of organisms ranging from yeast (Ito and Kobayashi, 1977) and bacteria (Gutter *et al.*, 1977) to viruses (Piette *et al.*, 1978). The involvement of $^1O_2$ in mutagenesis has been demonstrated by the effectiveness of the reaction, which can be reduced by the presence of singlet oxygen quenchers or enhanced by using a deuterated medium (Ito, 1974; 1978). It has been demonstrated that guanine oxidation products induced by singlet oxygen constitute premutational lesions if they are not repaired Decuyper-Debergh *et al.* (1987). The unreparied quanine oxidation products by singlet oxygen can be mutagenic leading to G → T transversions (Kuchino *et al.*, 1987; Wood *et al.*, 1990). Moan *et al.* (1980) have demonstrated that hematoporphyrin and visible light can induce genotoxic responses in human NIHK 3025 cells, observed as sister chromatid exchanges and single-strand breaks visualized after treatment with alkali.

(d) Repair of DNA damage induced by singlet oxygen

DNA modifications induced either by methylene blue photosensitization or by chemically generated singlet oxygen have been shown by Müller *et al.* (1990) to be recognized and incised by repair endonucleases present in crude bacterial cell extract and it appears that only a small fraction of the incised modification are sites of base loss (A-P sites) sensitive to exonuclease III, endonuclease IV or to the UV-endonuclease from *M. luteus*. Experiments with purified FPG (formamidopyrimidine-DNA glycosylase) confirm that this enzyme is responsible for recognition of singlet oxygen induced DNA base oxidation. Czeczot *et al.* (1991) conclude that DNA base modifications (most

probably 8-oxodG) induced by the photosensitization reaction mediated by methylene blue are repaired by FPG protein and Uvr ABC endonuclease. In view of the wide spread distribution of 8-oxodG in cellular DNA, the demonstrated miscoding and mutagenic properties of this lesion and the existence of a bacterial gene coding for enzyme involved in its repair, Tchou *et al.* (1991) propose that 8-oxodG is the primary physiological substrate for a constituent glycosylase found in bacteria and probably in mammalian cells.

Singlet oxygen induces at least four different DNA base modifications constituting premutational lesions. If not repaired by glycosylase or endonuclease, these DNA damages promote (i) loss of transformation efficiency; (ii) partial inhibition of DNA replication and (iii) G to T transversions. Cytogenetic effects such as sister chromatid exchanges in human lymphocytes can also be observed, (summarized in Fig. 1.)

Recently it has been demonstrated that $^1O_2$ is a powerful antithrombotic agent through inhibition of coagulation and activation of fibrinolysis (Stief and Fareed, 2000). The studies of Stief *et al.* (2001) have shown that $^1O_2$ generator such as chloramine inhibits and reverses platelet aggregation. Singlet oxygen like the prooxidant nitric oxide has been shown to inhibit platelet function (Salvemini *et al.*, 1989; Stief *et al.*, 2000). Photosensitization reaction of drugs leading to the formation of reactive oxygen species under ultraviolet radiation (UVR) can cause tissue injury, resulting in damage to various cellular macromolecules. Pandey *et al.* (2002) have evaluated the UV-radiation induced singlet oxygen generation potential of selected drugs and concluded that synergistic action of both can lead to undesirable phototoxic responses. Kim *et al.* (2001) have shown that singlet oxygen-mediated damage to SOD and catalase may result in the perturbation of cellular antioxidant defence mechanism and subsequently lead to a pro-oxidant state.

Biological antioxidant defences

Under physiological conditions, aerobic organisms consume nearly 98% of molecular oxygen at cytochrome a_3 of the mitochondrial respiratory chain (Chance *et*

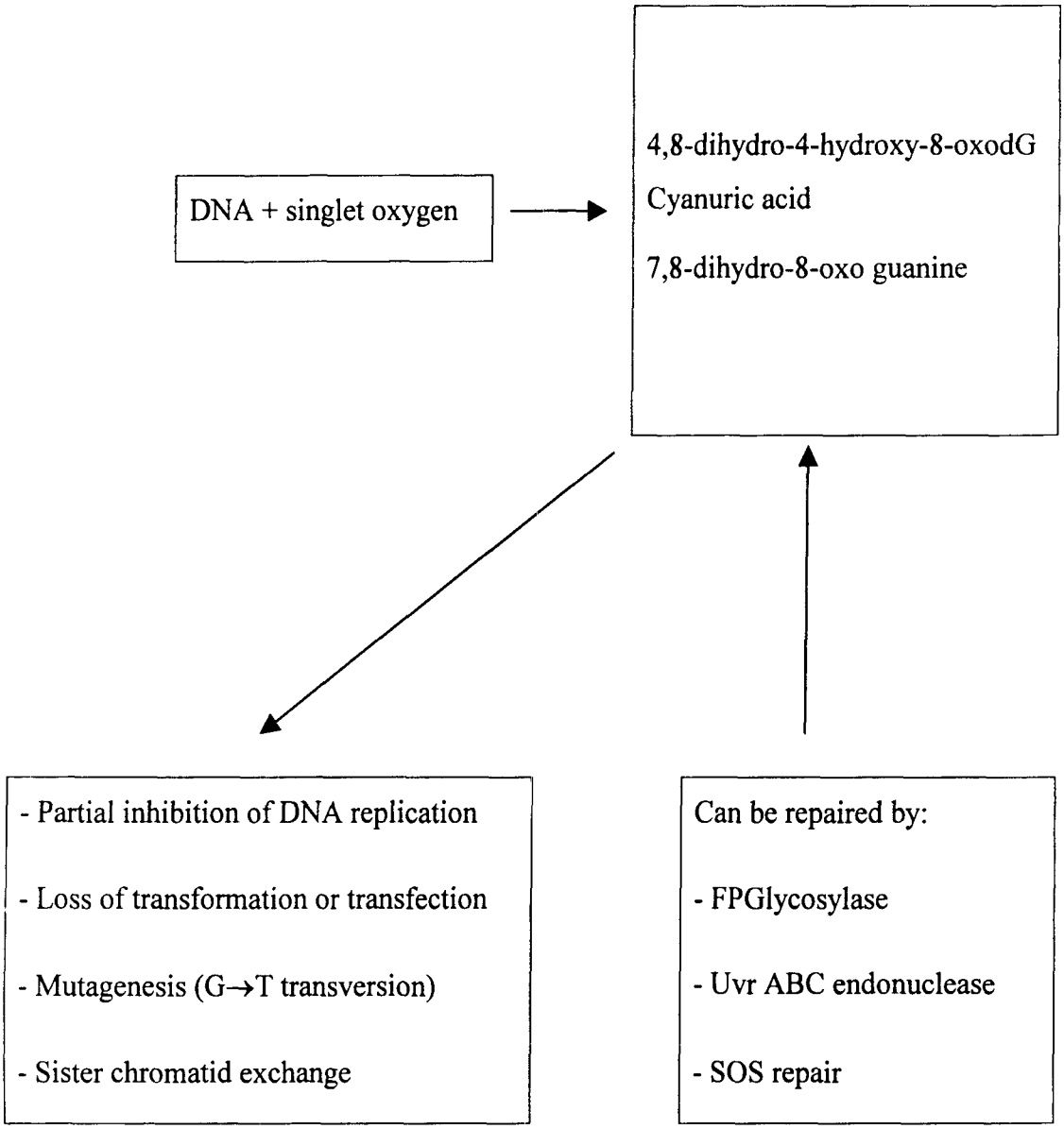

Fig. 1. Biological consequences associated with DNA oxidation mediated by singlet oxygen

Adapted from J. Piette 1991, J. Photochem. B: Biol., 11: 241-260.

al., 1979). During this process, dioxygen is reduced by four electrons, with no release of partially reduced oxygen intermediate (Malmstrom, 1982). Nevertheless, there is a fraction of O_2 (1-2%) not consumed at cytochrome a_3 which is mono- or divalently reduced to $O_2^{\bullet -}$ or H_2O_2. These molecules can attack and irreversibly damage a diverse spectrum of biomolecules including proteins, phospholipids, nucleic acids and sugars.

Aerobic organisms have potent antioxidant defences whose role is to neutralize and minimize the potentially cytotoxic and genotoxic effects of reactive oxidants. The defences may be primary (that directly decompose or scavange H_2O_2 and $^{\bullet}OH$) or secondary (which consist of the repair mechanisms that act on biomolecule that have undergone oxidative damage). Enzymatic and non-enzymatic antioxidants involved in cytotoxicity have been summarised in Tables 2 and 3.

Autoimmunity

Autoimmunity is a state wherein the host mounts an immune response to self. Autoimmune state includes at least four possible permutations (Brickman and Shoenfeld, 2001). One type of autoimmune state is beneficial to the normal host. A second autoimmune state is beneficial to abnormal hosts such as the immune responses to transformed cells that occur in patients suffering from malignancies or from intracellular infections (Shoenfeld, 1996). A third possibility is the detrimental autoimmune response in the abnormal host exemplified by the paraneoplastic syndrome (Lernmark, 1997). The immune response to malignant cells cross react with normal tissue antigens causing an autoimmune disorders. The fourth autoimmune state is the detrimental immune response in an otherwise normal host which results in a classic autoimmune, clinico-pathologic condition such as lupus, diabetes or thyroiditis.

The association of certain serotype of MHC antigens with autoimmune diseases has been the subject of extensive investigation. MHC molecules play a key role in the presentation of self antigens to T-cell precursors maturing in the thymus during which autoreactive clones are eliminated through apoptosis. Hence, MHC antigens determine the shaping of the T cell repertoire released to the periphery. A failure of this thymic selection could lead to autoimmune diseases. A particular MHC molecule might delete T

TABLE – 2

Antioxidant enzymes involved in cytotoxicity

Enzyme	Reaction and major role
SOD	$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$; scavenging of superoxide radicals
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$ Breakdown of hydrogen peroxide
Glutathione peroxidase	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ Decomposition of hydrogen peroxide

Adapted from Kakkar, P. and Viswanathan, P.N. (1992) J. Sci. Indust. Res. Vol. 51: 802-809.

TABLE – 3

Non-enzymatic antioxidants involved in cytotoxicity

Compound / anti-oxidant	Effect
Ascorbic acid	Reducing agent
α-Tocopherol	Inhibits chain reaction of lipid peroxidation
Carotenes	Scavenge $^1O_2$
	Quench $^1O_2$
Thiols	Reducing agent
Tryptophan	$^1O_2$, $^{\bullet}OH$ scavenger
Histidine	$^1O_2$, $^{\bullet}OH$ scavenger
Methionine	$^{\bullet}OH$ scavenger
Uric acid	$^1O_2$, $^{\bullet}OH$ scavenger in blood plasma
Albumin	Binding of copper ions, scavenger of HOCl.

Adapted from Kakkar, P. and Viswanathan, P.N. (1992) J. Sci. Indust. Res. Vol. 51: 802-809.

cells that normally suppress autoimmune responses in the periphery or alternatively, might allow autoimmune T cell receptor to escape thymic deletion (Mellins, 1992).

According to Botazzo *et al.* (1983), an environmental trigger, either a microbe or a toxin, leads to tissue damage which releases antigen from sites that were previously sequestered from the immune system. Recently it has been shown by Leadbetter *et al.* (2002) in a mouse model of systemic autoimmune disease, the simultaneous activation of cell surface antigen receptors and another Cell-like receptor, TLR9, causes a particular sub-class of self-immunoglobulin IgG 2a, to be recognized by B cells as if it were a pathogen. This triggers T cell-independent proliferation of the B cells.

Factors associated with autoimmune disease

Inspite of the enormous progress in the knowledge of immune reactions, the cause of autoimmunity is still unknown. Many theories have been proposed to explain the origin of autoimmune response. Based on various theories, the possible factors are as follows:

(a) Genetic factors

Autoimmune diseases show a highly significant familial predisposition. Relatives of patients are known to be at high risk for developing the diseases. A majority of genes, which have been associated with autoimmune diseases map within the HLA region. The MHC is comprised of three functionally distinct and polymorphic gene clusters: classes I, II and III. Most of the MHC association with SLE, involve class II (HLA-DR and DQ alleles) and class III alleles especially null or deficiency alleles of the fourth component (C_4) of complement (Bias *et al.,* 1986; Goldstein and Arnett, 1987; Braun and Zachary, 1988; Arnett and Moulds, 1991; Deodhar, 1992).

(b) Non-MHC genetic factors

In both animals and humans, autoimmune diseases have been linked to non-MHC genetic factors. Fcγ receptor phenotypes (Oh *et al.,* 1999), DNAse I (Walport, 2000) and serum amyloid protein (Sap) genes (Walport, 2000) as well as several apoptosis

associated genes (Oh, 1999) are few of the non-MHC factors suspected of predisposing to autoimmune diseases.

(c) Hormonal factors

Hormones are a significant factor in the development of autoimmune disorders (Lahita, 1999; Ahmed, *et al.*, 1999). Patients born with Klinefelter's syndrome (47, XXY), a feminising genotype, also have an unusually high prevalence of lupus (Neu and Roy, 1969). Animal models of autoimmune diseases show similar predilections. For example, litter born to NZB mice crossed with NZW mice develop a lupus like illness. Hormonal manipulation of NZB/NZW F1 mice effect their disease development such that femininisation worsens disease while masculinisation slows diseases progression (Talal, 1978). Additional hormones including progesterone (Van Vollenhoven, 1994) and prolactin (Walker *et al.*, 1998) appear to have immunoregulatory properties.

(d) Environmental factors

Environmental factors have been implicated in autoimmune diseases including infectious agents, medications, chemicals, toxins, and ultraviolet light (Moar *et al.*, 1998; Saraux *et al.*, 1999). Ultraviolet light is known to trigger lupus (McGrath, 1999). D-penicillamine a medication formerly used to treat several immune diseases including RA, scleroderma and primary biliary cirrhosis has been implicated in the development of autoimmune diseases such as lupus and glomerulonephritis (Brik *et al.*, 1995). Malignancies are more common in most autoimmune disease than in the general population (i.e. lymphomas are 30-40 times more common in primary Sjogren's syndrome) (Ramos-Casals *et al.*, 2000) and, conversely, autoimmune manifestations are a relatively common paraneoplastic manifestation (i.e. vasculitis, sensory neuronopathy or autoimmune encephalomyelitis) (Graus *et al.*, 1986).

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease involving both humoral and cellular aspects of the innate and acquired immune systems

and is characterized by autoantibodies with a spectrum of specificities that participate in disease pathogenesis (Kimberly, 2001). Lupus occurs worldwide and affects females more commonly than males (10:1) (Alarcon *et al.,* 1999). SLE can affect diverse organ systems, including the skin, joints, brain, lungs and kidneys. The disease is characterized by immune dysregulation, leading to high-level autoantibody production, immune complex deposition and vasculitis (Rothfield, 1985). SLE is episodic in its activity and variable in severity. The clinical manifestations include fever, an erythematosus 'butterfly' rash across the face, lesions of discoid lupus or a vasculitic rash, polyarthralgia and arthritis, polyserositis (especially pleurisy and pericarditis), anemia, thrombocytopenia, renal, neurologic and cardiac abnormalities. One of the impediments in understanding human systemic lupus has been its marked heterogeneity (Steinberg *et al.,* 1991; Steinberg, 1992).

The immune system plays a crucial role in the pathogenesis of both active inflammatory and non-inflammatory mechanisms of organ damage in SLE. Nucleosomes, apoptotic material and efficient pathways for routine, non-immunogenic clearance appear pivotal in pathogenesis of SLE (Kimberly, 2001). The etiology of SLE is complex, involving both environmental and genetic factors and, likely a synergistic relationship between the two. Environmental triggers such as Epstein-Barr virus (James *et al.,* 1997), human T cell lymphotropic virus (Brand *et al.,* 1999), hormonal and chemical exposure (Cooper *et al.,* 1998), support a gene-environment interaction, and one cohort-comparison study supports a gene-gene interaction (Mehrian *et al.,* 1998). SLE is highly variable among individual patients and, to some extent, between ethnic group since certain disease manifestations seem to cluster within racial populations whereas others do not (Petri, 1998).

The genetic hypothesis in SLE is also supported by the high incidence of SLE in patients with certain complement deficiencies (C1, C2 and C4) and associations of disease and autoantibody production with HLA class II alleles (Arnett, 1997). Polymorphisms in low-affinity IgG (fcγ) receptors, which are important for the clearance of immune complexes, are also implicated in the pathogenesis of lupus (Salmon *et al.,* 1996; Wu *et al.,* 1997). Autoantibodies in family members and their relatives are not

directed against the same nuclear antigens. A familial aspecific dysfunction of the B-lymphocyte is the most likely explanation for autoantibody production in SLE (Michiel *et al.*, 2001). Monozygotic, but not dizygotic twins have high concordance rates for SLE (Miles and Isenberg, 1993), indicating that genes play an important role in susceptibility. Presence of autoantibodies against nuclear antigens is more frequent among non-affected family members of patients with SLE than in the general population. This familial aggregation suggests that presence of autoantibodies is under genetic control (Llorente *et al.*, 1995).

It has been proposed that IL-10 a pleiotropic cytokine has a key role in the pathophysiology of autoimmune disorders such as SLE and the autoimmune lymphoproliferative syndrome (Llorente *et al.*, 1994; Fuss *et al.*, 1997). It appears to be important in the immune dysfunction seen in SLE patients and their relatives. Increased production of IL-10 has been found both *in vitro* and *in vivo* in patients with SLE (Llorente *et al.*, 1993; Llorente *et al.*, 1994; Houssiau *et al.*, 1995; Al-Janadi *et al.*, 1996; Liu and Jones, 1998). IL-10 together with other type 2 cytokines such as IL-6 facilitates antibody production (Linker *et al.*, 1991; Spronk, 1992; Llorente *et al.*, 1993; Al-Janadi *et al.*, 1993).

Recent studies suggest that the Cr2 gene, which encodes for complement receptor CR1 and CR2, is important in disease susceptibility. Xiaobo *et al.* (2002) demonstrate that abnormalities in CR1 and CR2 may be linked to the production of autoantibodies by modifying the effect of other systemic lupus erythematosus susceptibility genes. Phenotypic expression of other disease manifestations need additional Cr2-independent genetic factors (Xiaobo *et al.*, 2002). CR1 expression is reduced on erythrocytes, polymorphonuclear leukocytes and B lymphocytes in patients with SLE, autoimmune hemolytic anemia, and Sjogren's syndrome (Wilson *et al.*, 1986; Fyfe *et al.*, 1987). Expression of CR2 on cells is also reduced in patients with SLE (Prodeus *et al.*, 1998). Xiaobo *et al.*, (2002) have reported that mutations in the Cr2 gene, especially when combined with mutations in the *Fas* gene, increase the production of autoantibodies. Polymorphisms in MHC class II genes have been found to associate with susceptibility

for SLE, suggesting that specific MHC-restricted immune responses are involved (Hahn, 1997).

CD_4^+ T cell reactive with autoantibody V_H-region peptides play an important role in SLE (Waisman *et al.,* 1997). T cells from SLE patients are activated by peptides derived from anti-DNA Ig peptides are not the only source of the determinants for anti-DNA antibody production (Williams, 1995; Linker-Israeli *et al.,* 1996). Nucleosome-autoreactive T cells also augment anti-DNA production (Mohan and Datta, 1995; Shi *et al.*, 1998). It is likely that determinants from several different antigens can activate autoreactive T cell help for pathogenic anti-DNA antibody production in SLE.

Bijl *et al.* (2002) have concluded that IgG1 and IgG2 subclass antibodies to nucleohistone and to dsDNA are the predominant subclasses found in plasma of lupus patients with renal disease. The frequent occurrence of a rise in IgG2 antinucleohistone and IgG1 anti-dsDNA antibodies in patients prior to a renal relapse suggests that, besides IgG1 subclass autoantibodies, IgG2 subclass antibodies to nucleohistone have a particular pathophysiological role in lupus nephritis. Heyman *et al.* (2002) have suggested that immune liver disease, mainly autoimmune cholangiopathy, may be an important clinical manifestation of SLE.

Anti-DNA antibodies are heterogeneous showing polyspecificity and cross reactivity. Monoclonal antibodies, sera from SLE patients and lupus prone animal models show that reactivity may be greater towards denatured forms of DNA (Stollar *et al.,* 1986). These anti-DNA antibodies recognize epitopes present not only on nucleic acids (Andrzejewski *et al.,* 1980; Swanson *et al.*, 1996), but also on nucleoproteins (Stollar, 1981), proteins (Pisetsky *et al.,* 1985; Wong *et al.,* 1995; Tsuzaka *et al.,* 1996), cell membrane structure (Jacob *et al.,* 1986), polysaccharides (Kashihara *et al.,* 1993), phospholipids (Tiikainem *et al.,* 1991), erythrocytes, leucocytes, platelets and neurons. This polyspecificity of anti-DNA autoantibodies could be due to variable binding sites in individual immunoglobulin or to recurrent epitopes in different antigens. The polyreactivity of anti-DNA autoantibodies suggest that DNA itself may not be the immunogen responsible for what are known as anti-DNA antibodies. Thus, the exact antigen responsible for anti-DNA autoantibody production remains an enigma.

Apoptosis or programmed cell death is an ongoing process which allows for removal of T and B lymphocytes expressing inappropriate receptors, cells which have been inappropriately stimulated, as well as damaged or senescent cells (Brickman and Shoenfeld, 2001). It also plays an important role in the down regulation of cells, which have already undergone appropriate activation and proliferation, a process referred to as activation-induced cell death (Brickman and Shoenfeld, 2001). During the apoptotic process, DNA and histones associated with nucleosomes are extruded, intranuclear and cellular protein fragments are released by activated caspases, protected intracellular antigens are sequestered in apoptotic surface blebs and the bilipid cell membrane is flipped inside out exposing phospholipids, including phosphotidyl serine, generally hidden from the immune system (Bordron, 1998; Mountz and Zhou, 2001).

In SLE, greater emphasis involve pathogenic concept, mainly focuses on a dysregulation of programmed cell death, giving the immune system the chance to form autoantibodies against nuclear constituents like double-stranded DNA or ribonucleoproteins (RNP) (Lorenz, 2001). During apoptotic breakdown, many nuclear constituents are post-translationally modified, possibly altering antigenicity. Potential autoantigens might be sequestered during apoptosis and become accessible for immuno-competent cells. There might be two major mechanisms which could lead to an increased amount of circulating nuclear antigens in SLE patients: an increased amount of apoptosis or a decreased clearance of apoptotic cell material appears to an important etiopathogenic event in SLE (Lorenz, 2001). Lymphocytes of SLE patients with a bacterial infection do have an increased amount of spontaneously occurring apoptotic cell death within peripheral blood mononuclear cell (Lorenz *et al.*, 1997) which could account for the clinical observation, that a flare of SLE could follow an infectious episode (Kreig, 1995). Emlen *et al.* (1994) have demonstrated that the rate of apoptosis of lymphocytes derived from SLE patients was 2-3 times faster than that seen in lymphocytes from normal controls or patients with rheumatoid arthritis.

In the case of SLE patients, the impaired clearance of apoptotic cells resulting in an accumulation of late apoptotic and secondary necrotic cells including oligonucleosomes might lead to an activation of autoreactive T cells with subsequent

anti-dsDNA antibodies production (Voll *et al.,* 1997). It is well established that affinity maturation, memory formation and isotype switch are T cell dependent immune processes. The similarity of anti-dsDNA response in murine and human SLE strongly support the hypothesis of the involvement of T helper cells in the pathogenesis (Lorenz, 2001).

Oxygen derived free radicals are known to be involved in the pathobiology of SLE, particularly in respect of processes leading to the formation of pathological anti-DNA antibodies. It has been suggested that activated phagocytic cells release ROS (Allan *et al.,* 1988), which penetrate cellular membrane, interact with nuclear DNA and lead to its modification (Bashir *et al.,* 1993). Such modification of DNA increases its immunogenicity, thus trigerring production of anti-DNA autoantibodies (Blount *et al.,* 1992).

Stryer (1988) have shown that mitochondrial hyperpolarization is a likely cause of increased ROS production, and may be ultimately responsible for increased spontaneous cell death in patients with SLE. Lunec *et al.* (1994) have suggested that SLE patients cannot efficiently remove 8-hydroxydeoxyguanine (8-OHdG) from cellular DNA, which may result in cell death and release of oxidized DNA. In addition, they have shown that denaturation of dsDNA by ROS results in an increased binding of anti-DNA antibodies present in sera from SLE patients (Lunec *et al.,* 1989) and that ROS modification of human DNA produces a more discriminating antigen for the diagnosis of SLE (Blount *et al.,* 1990; Alam *et al.,* 1993). ROS-damaged DNA may, therefore, play a significant role in the generation of immune complexes which are of recognized importance in the pathogenesis of this disease.

There is an increasing evidence to suggest a link between autoimmune diseases and cancer (Sela and Shoenfeld, 1988; Cash and Klippel, 1991). Petterson *et al.* (1992) have shown that cancer, especially lymphoma and soft tissue sarcoma, is more common in patients with SLE than in general population. Patients who develop SLE later in life seemed most susceptible to cancer, which obviously reflect the higher incidence of cancer in older people (Petterson *et al.,* 1992).

Cancer

Carcinogenesis is the malignant transformation of a cell or group of cells (Farber and Camerson, 1980; Potter, 1983; Farber, 1984). This multistep process can be divided into two main stages, initiation and promotion (Boutwell *et al.*, 1982; Perera, 1991). The initiation phase is caused by single exposure to a carcinogenic agent and involves an irreversible modification of the genetic material of the cells. On the other hand, promotion requires multiple exposure to the promoters to alter gene expression and produce a tumour. In contrast to initiation, the promotion stage is reversible (Cerutti, 1985; Demple, 1990; Martinez-Cayuela, 1995).

As with other chronic diseases, cancer too has a multifactorial aetiology which includes both genetic and environmental factors (Gourley *et al.*, 1992). The environmental factors are responsible for 80% to 90% of all human cancers. Genetic influence, though long been suspected in incidence of cancer, is less conspicuous and more difficult to identify (Clemens, 1991). There is probably a complex inter-relationship between hereditary susceptibility and environmental carcinogenic stimuli in the causation of a number of cancers.

Among the environmental factors, consumption of tobacco in various forms (e.g. smoking, chewing) is the major cause of cancer of lung, larynx, mouth, pharynx, bladder, pancreas and probably kidney (WHO, 1983). Alcohol consumption is associated with cancer of oesophagus, liver and rectum (Kabat *et al.*, 1986; Eskelson *et al.*, 1993). Dietary factors such as high fat diet, beef consumption, food additives and contamination have been related to cancer (Ames, 1983).

It is a well-known fact that viruses can cause tumors. The genes implicated in malignancy are often altered forms of human genes. Often, viruses activate host genes that are usually quiescent i.e. the activation of proto-oncogenes into oncogenes, the products of which when altered or expressed contribute to malignancy. Apart from viruses, mutations too can convert proto-oncogenes into carcinogenic oncogenes. Human c-oncogene was found to be mutated, rearranged or unusually active in many viral and non-viral tumors. Retroviruses lacking *c-onc* gene are also oncogenic, giving rise to

tumors more slowly than those with *v-onc* genes (Weiss, 1986). Chromosome translocations in human tumor cells brings *onc* genes into juxtaposition with active regions for gene expression leading to their constitutive expression (Klein and Kelin, 1984) and an over expression of the same. Cellular *ras* genes become oncogenic by point mutation resulting in a single codon change (Marshall, 1985). Such minimal change in *ras* can lead to cell transformation in established cell lines.

Role of ROS in cancer

The complex series of cellular and molecular changes that occur through the development of cancer can be mediated by a diverse endogenous and exogenous stimuli (Frenkel, 1992). DNA appears most important in tumor biology since it is firmly established that cancer is a genetic disease (Cotran *et al.*, 1989) and DNA damage is thought to be involved in all stages of carcinogenesis (Halliwell and Gutteridge, 1989; Vile and Morris, 1992; Olinski, 1993).

Any agent capable of chemically modifying DNA in cell could be carcinogenic. Thus, reactive oxygen species known to cause oxidative damage to DNA are potent carcinogens (von Sonntag, 1987a; Halliwell and Aruoma, 1991; Dizdaroglu, 1992; Cerutti *et al.*, 1994; Loft and Poulsen, 1996). Although chromatin may offer some protection against oxidative damage to DNA (Ljungman and Harawalt, 1992). ROS acts at several steps in multistage carcinogenesis (Lunec, 1990; Clayson *et al.*, 1994; Ames *et al.*, 1995; Drexer *et al.*, 1996; Pryor, 1997).

When the mutations caused by ROS relate to critical genes such as oncogenes or tumor suppressor genes, initiation and/or progression of cancer may result (Loft and Poulsen, 1996). With respect to cancer, DNA is considered to be the most important target of ROS (Feig *et al.*, 1994; Ames *et al.*, 1995). Oxidative damage to DNA includes a range of specifically oxidized purines and pyrimidines as well as alkali labile sites and strand breaks, formed directly or by repair process (Dizdaroglu, 1994; Breen and Murphy, 1995).

Although, ROS damage all four bases in DNA, mutation are usually related to modification of GC base pairs (Retel *et al.*, 1993). The most abundant of the lesions is 8-

oxo-7, 8-dihydro-2-deoxyguanosine (8-oxodG) (Halliwell and Aruoma, 1993; Toyokuni *et al.*, 1994). 8-oxodG is most mutagenic, resulting in G to T transversions (Kuchino *et al.*, 1987; Floyd, 1990; Shibutani *et al.*, 1991). The generation of 8-oxodG may account for the formation of G → T transversion through mispairing with adenine. Besides misparing, the oxidative products of DNA bases may function in mutagenesis through other mechanisms including abnormal interactions with the DNA replicative machinery (Feig and Loeb, 1993; Weitzman *et al.*, 1994). Oxidative stress has been implicated as an important factor in metastasis, notably because it results in a loss of cell adhesion which is the pre-requisite for cellular detachment and host tissue invasion (Fidler and Nicolson, 1991; Kundu *et al.*, 1995).

DNA damage *in vivo* is constantly repaired by a variety of enzymes. Strand breaks are annealed and modified bases are excised out as such or as nucleotides (Demple and Harrison, 1994). Defects in DNA repair systems are primarily responsible for several human diseases and some of these have been shown to increase cancer risk and increased levels of oxidative DNA damage.

The role of ROS in the development of cancer in humans is further supported by the presence of oxidative DNA products in cancer tissue. The concentration of 8-oxodG, expressed as the number of molecules/10^5 bases, in normal versus cancerous tissue was found to be 7.3 versus 23 in lung, 0.94 versus 5.1 in stomach, 3.1 versus 9.2 in ovary and 2.7 versus 4.4 in colon cancer (Malins and Haimanot, 1991; Olinski *et al.*, 1992; Malins *et al.*, 1993). The level of 8-oxodG was much higher in invasive ductal carcinoma of female breast than in any other type of cancer (Malins and Haimanot, 1991; Olinski *et al.*, 1992). Thus, oxidative DNA base damage products are generally higher in cancerous tissue.

Prostrate cancer is characteristically diagnosed in old age or at autopsy and it contributes to the third most frequent cancer related cause of death in males in the United States. The paucity of known chemical agents associated with prostrate cancer and the absence of any apparent environmental or occupational risk factors suggests that prostrate cancer is associated with endogenous cellular processes (Feig *et al.*, 1994). The most reasonable candidate for endogenously formed genotoxins that accumulate in later life

are the reactive oxygen species (Feig *et al.*, 1994). Chronic hepatitis is associated with the presence of inflammatory cells, presumably generating ROS. The 8-oxodG content in liver with chronic hepatitis has been found to be significantly higher than the content in normal liver (Shimoda *et al.*, 1994). Oxygen free radicals are also implicated in the mechanism of gastric (Salim, 1992a) and colonic cancers (Salim, 1992b).

Objectives of the present study

Systemic lupus erythematosus (SLE) is an autoimmune disease of indeterminate etiology that predominantly afflicts women in the childbearing age. Anti-DNA antibodies play a very important role in the clinical manifestations of SLE, especially nephritis, most likely by immune complex deposition. The central role of anti-DNA antibodies in SLE has led to intense investigation into the mechanism underlying this response, in both patients as well as inbred mice developing lupus like illnesses. The review of literature makes it evident that oxidative DNA damage is an important carcinogenic factor and plays a vital role in the pathogenesis of both SLE and cancer.

In the present study, commercially obtained double stranded calf thymus DNA was purified free of proteins and subjected to digestion with micrococcal nuclease to obtain 200 bp fragments. These fragments were modified with singlet oxygen and superoxide anion radical generated by riboflavin and 365 nm UV light.

The $^1O_2$-$O_2^{\cdot -}$-DNA fragments were characterised by various physiochemical techniques and employed in competitive assay to delineate the antigen binding characteristics of induce antibodies against $^1O_2$-$O_2^{\cdot -}$-DNA. The free radical modifications on DNA were studied by UV spectroscopy, thermal denaturation studies, nuclease S1 digestibility and fluorescence spectroscopy.

Antigenicity of $^1O_2$- $O_2^{\cdot -}$-DNA was probed by inducing antibodies in rabbits. $^1O_2$-$O_2^{\cdot -}$-DNA was found to be highly immunogenic as assessed by direct binding ELISA. The specificity of induced antibodies was evaluated by competition ELISA and gel retardation assay.

Anti $^1O_2$-$O_2^{\cdot -}$-DNA antibodies were used as probes for immunochemical detection of oxidative damage in DNA isolated from various cancer patients. Alternatively, circulating antibodies in sera of cancer and SLE patients were characterized for their binding towards native and $^1O_2$-$O_2^{\cdot -}$-DNA.

MATERIALS

Calf thymus DNA, nuclease S1, micrococcal nuclease, riboflavin, superoxide dismutase, bovine serum albumin, agarose, anti-human/anti-rabbit IgG-alkaline phosphatase and FITC conjugates, ethidium bromide, Coomassie Brilliant Blue G-250 and R-250, sodium dodecyl sulphate, Freund's complete and incomplete adjuvants, methylated bovine serum albumin, Tween-20, Triton X-100, nitroblue tetrazolium, sodium azide, para-nitrosodimethyl aniline were purchased from Sigma Chemical Company, U.S.A. Protein-A agarose from Genei, synthetic polynucleotides and Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. EDTA (disodium salt), chloroform and isoamyl alcohol were from Qualigens, India.

Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from NUNC, Denmark. QIAamp blood midi kit from Qiagen, p-nitrophenyl phosphate and Folin-Ciocalteau reagent were obtained from Centre for Biochemical Technology, New Delhi. Acrylamide, ammonium persulphate, bisacrylamide, N,N,N',N'-tetramethylethylene diamine (TEMED) were from Bio-Rad Laboratory U.S.A. EDTA (disodium salt), hydrogen peroxide, chloroform, isoamyl alcohol, methanol, glacial acetic acid were from Qualigens, India. Diphenylamine and ethanol were chemically pure. All other chemicals were of highest analytical grade available.

Equipments

Shimadzu UV-240 Spectrophotometer equipped with thermo-programmer and controller unit, ELISA microplate reader, Elico pH meter model L1-120, ultraviolet lamp (Vilber Lourmat, France), agarose gel electrophoresis assembly (GNA-100); Beckman ultracentrifuge, Avanti 30 table top high speed refrigerated centrifuge (Beckman, U.S.A.); polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.) and fluorimeter (Hitachi, Japan) were the major equipments used in this study.

Collection of sera

SLE sera were obtained from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi, showing high titre anti-DNA antibodies and fulfilled the American College of Rheumatology criteria for the diagnosis of SLE (Arnett *et al.*, 1988). Normal human sera were obtained from healthy individuals and stored in small aliquots at -20°C. Sera of cancer patients proven with histopathological diagnosis were obtained from J.N. Medical College Hospital A.M.U., Aligarh. Sera were decomplemented by heating at 56°C for 30 min and stored in aliquots at -20°C.

METHODS

Purification of calf thymus DNA

Commercially obtained calf thymus DNA was purified free of proteins and single stranded regions as described by Ali *et al.* (1985). DNA (2 mg/ml) was dissolved in 0.1 X SSC buffer (15 mM sodium citrate and 150 mM sodium chloride, pH 7.3) and then mixed with an equal volumes of chloroform-isoamyl alcohol (24:1) in a stoppered container and gently mixed occasionally for 1 hr. The aqueous layer containing DNA was separated from the organic layer and re-extracted with chloroform-isoamyl alcohol. The DNA was precipitated with two volumes of cold absolute ethanol and collected on a glass rod. After drying in air, the DNA was dissolved in acetate buffer (30 mM sodium acetate containing 30 mM zinc chloride, pH 5.0) and treated with nuclease S1 (150 units/mg DNA) at 37°C for 30 min to remove single stranded regions. The reaction was stopped by adding one-tenth volume of 200 mM EDTA, pH 8.0. The nuclease S1 treated DNA was extracted twice with chloroform-isoamyl alcohol and finally precipitated with two volumes of cold ethanol. The precipitate was dissolved in phosphate-buffered saline (PBS) (10 mM sodium phosphate containing 150 mM sodium chloride), pH 7.4.

Fragmentation of purified native calf thymus DNA

Micrococcal nuclease was used to digest purified DNA to obtain smaller fragments (Ali *et al.*, 1985). Purified DNA (2 mg/ml) in 6 mM Tris, 100 mM NaCl and 2 mM $CaCl_2$, pH 8.0 was treated with 0.25 units of micrococcal nuclease per mg DNA at 37°C for 6 min. The reaction was stopped by adding one-tenth volume of 200 mM EDTA, pH 8.0. The mixture was extracted twice with chloroform-isoamyl alcohol and fragmented DNA was precipitated with cold absolute ethanol and dissolved in 30 mM acetate buffer, pH 5.0 containing 30 mM $ZnCl_2$. The DNA was digested with nuclease S1 (150 units/mg DNA) at 37°C for 30 min. The reaction was stopped with one-tenth volume of 200 mM EDTA, pH 8.0. The digested DNA fragments were extracted twice with chloroform-isoamyl alcohol and precipitated with cold absolute ethanol. The DNA fragments were then dissolved in TBS (0.01 M Tris, 0.15 M NaCl) pH 8.0 and separated on the basis of size by gel filtration on Sepharose 4B column (46.0 cm x 1.2 cm) equilibrated with TBS. Fractions of 4 ml were collected. The size of the DNA fragments was determined by PAGE.

Polyacrylamide gel electrophoresis

Nucleic acid samples were subjected to PAGE under non-denaturing conditions as described by Laemmli (1970). The following stock solutions were prepared:

(a) Acrylamide-bisacrylamide (30:0.8)

30 gm acrylamide and 0.8 gm bisacrylamide was dissolved in distilled water to a final volume of 100 ml. The solution was filtered and stored at 4°C in an amber colored bottle.

(b) Resolving gel buffer

36 gm Tris was dissolved in 48 ml of 1 N HCl, pH adjusted to 8.8 and final volume made upto 100 ml with distilled water.

(c) Electrode buffer

The electrophoretic buffer used was TAE, pH 7.9 (40 mM Tris, 1.14 ml glacial acetic acid and 1 mM EDTA).

(d) Procedure

The PAGE assembly was set up and the glass plates separated by 1.5 mm thick spacer were sealed with 1% agarose from the sides and bottom. The non-denaturing gel was prepared and poured between the glass plates and allowed to polymerize at room temperature. Nucleic acid samples were mixed with one-tenth volume of 'stop mix' (30% Ficoll, 0.025% xylene cyanol FF and 500 mM EDTA in 10 times concentrated TAE buffer) and applied onto the gel. The gel was electrophoresed for 8-10 hr at room temperature at 80 volts, stained with ethidium bromide (0.5 μg/ml) and visualised under UV light.

Recipe for 7.5% non-denaturing PAGE

Acrylamide-bisacrylamide	10.0 ml
Resolving gel buffer	5.0 ml
1.5% ammonium persulphate	1.5 ml
Distilled water	23.5 ml
TEMED	20.0 μl

Polyacrylamide gel electrophoresis for proteins

Polyacrylamide slab gel electrophoresis was performed under denaturing conditions as described by Laemmli (1970). The following stock solutions were prepared:

(a) Acrylamide-bisacrylamide (30:0.8)

A stock solution was prepared by dissolving 30 gm of acrylamide and 0.8 gm bisacrylamide in distilled water to a final volume of 100 ml.

(b) Resolving gel buffer

A stock solution was prepared by dissolving 36.3 gm Tris base in 48.0 ml of 1N HCl. The contents were mixed, pH adjusted to 8.8 and the final volume brought to 100 ml with distilled water.

(c) Stacking gel buffer

6.05 gm Tris was dissolved in 40 ml distilled water, pH titrated to 6.8 with 1N HCl and the final volume adjusted to 100 ml with distilled water.

(d) Electrode buffer

3.03 gm Tris, 14.4 gm glycine and 1.0 gm SDS were dissolved in distilled water, pH adjusted to 8.3 and final volume made up to one litre.

(e) Procedure

Glass plates, separated by 1.5 mm thick spacer were sealed with 1% agarose. The resolving gel mixture was prepared by mixing the components in the appropriate volume and poured into the space between the glass plates leaving sufficient space at the top for the stacking gel. After the polymerization of separating gel, stacking gel mixture was poured and allowed to solidify. Protein samples containing 10% glycerol and 0.002% bromophenol blue were applied and electrophoresis was carried out at 60V for 6-8 hrs. Staining of the gel was achieved with 0.1% Coomassie Brilliant Blue R-250 (in 25% iso-propanol and 10% glacial acetic acid). Destaining was carried out in a 10% mixture each of acetic acid and methanol.

Recipe for 7.5% SDS-PAGE

Solutions	Stacking gel	Resolving gel
Acrylamide-bisacrylamide	1.25 ml	7.5 ml
Stacking gel buffer	2.5 ml	-
Resolving gel buffer	-	3.75 ml
Distilled water	5.65 ml	16.95 ml
10% SDS	0.1 ml	0.3 ml
1.5% ammonium persulphate	0.5 ml	1.5 ml
TEMED	0.75 µl	15.0 µl

Determination of DNA concentration

DNA concentration was estimated colorimetrically by the method of Burton (1956) using diphenylamine reagent.

(a) Crystallization of diphenylamine

Diphenylamine (2 g) was dissolved in 200 ml boiling hexane. After adding 0.5 g of activated charcoal, the hot mixture was filtered through Whatman No 1 filter paper and the filtrate was kept overnight at 4°C and dried at room temperature before use.

(b) Preparation of diphenylamine reagent

750 mg of recrystallized diphenylamine was mixed with 50 ml of glacial acetic acid and 0.75 ml concentrated sulphuric acid. The reagent was prepared fresh before use.

(c) Procedure

One ml of DNA sample was mixed with 1.0 ml of 1N perchloric acid and incubated at 70°C for 15 min. 100 µl of 5.43 mM acetaldehyde was added followed by 2.0 ml of freshly prepared diphenylamine reagent. The contents were mixed and incubated at room temperature for 16-20 hr. Absorbance was read at 600 nm and the

concentration of DNA in unknown samples was determined from a standard plot of purified calf thymus DNA.

Determination of protein concentration

Protein was estimated by the methods of Lowry *et al.* (1951) and Bradford (1976).

Protein estimation by Folin's-phenol reagent

The protein estimation by this method utilizes alkali (to keep the pH high), Cu^{2+} ions (to chelate proteins) and tartarate (to keep the Cu^{2+} ions in solution at high pH).

(a) Folin-Ciocalteau's reagent

The reagent was purchased from Centre for Biochemical Technology, New Delhi and diluted 1:4 with distilled water before use.

(b) Alkaline copper reagent

The components of alkaline copper reagent were prepared as follows:

(i) Two percent sodium carbonate, 100 mM sodium hydroxide.

(ii) 0.5 percent copper sulphate in 1 percent sodium potassium tartarate.

The working reagent was prepared fresh before use by mixing components (i) and (ii) in the ratio of 50:1.

(c) Procedure

To 1 ml of protein sample was added 5 ml of alkaline copper reagent and incubated for 10 min at room temperature. 1 ml of working Folin-Ciocalteau's reagent was added and the tubes were read at 660 nm after 30 min. The concentration of protein in unknown sample was determined from a standard plot of bovine serum albumin.

Protein estimation by dye-binding method

This assay is based on color change when Coomassie Brilliant Blue G-250 in acidic medium, binds strongly to protein hydrophobically and at positively charged

groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color is observed (λ_{max}-595 nm).

(a) Dye preparation

100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre and filtered through Whatman No. 1 filter paper to remove undissolved particles.

(b) Protein assay

To 1 ml of solution containing 10-100 μg protein was added 5 ml of dye solution and contents mixed by vortexing. The absorbance was read at 595 nm after 5 min against a reagent blank.

Modification of calf thymus DNA by free radicals

Purified calf thymus DNA was modified by superoxide anion radical and singlet oxygen. Superoxide anion radical was detected by photosensitized reduction of nitroblue tetrazolium (NBT), leading to the formation of a blue colored product, nitroblue formazan (Nakayama et al, 1983). Production of superoxide radical was confirmed by monitoring the inhibition of formation of blue colored product in the presence of superoxide dismutase (SOD).

Formation of singlet oxygen was measured in aqueous solution by monitoring the bleaching of p-nitrosodimethylaniline (pRNO) (Kraljic and Moshni, 1978). Production of singlet oxygen was confirmed by monitoring the bleaching of pRNO in presence of sodium azide (NaN_3), a specific quencher of singlet oxygen.

DNA fragments of around 200 bp were modified by the method of Naseem *et al.* (1988). A total volume of 3.0 ml contained, 100 μg/ml DNA, 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.06% Triton X-100 and 40 μM riboflavin. Immediately after mixing, the reaction was carried out in the presence of UV light (365 nm) at room temperature. The samples were dialyzed extensively to remove riboflavin and Triton X-100.

Spectroscopic analysis

(a) The ultraviolet spectra of modified and unmodified 200 bp DNA were recorded in the wavelength range of 200-400 nm on a Shimadzu UV-240 spectrophotometer.

(b) The modification incurred on DNA fragments was also analyzed by UV-difference spectroscopy.

(c) Fluorescence emission spectroscopy of native and modified 200 bp DNA samples using ethidium bromide was performed on a fluorimeter.

Absorption – temperature scan

Thermal denaturation analysis of nucleic acids was performed in order to ascertain the degree of modification incurred on the nucleic acids by determining mid point melting temperature (Tm). Native and modified samples were subjected to heat denaturation on a Shimadzu UV-240 spectrophotometer coupled with a temperature programmer and controller assembly (Hasan and Ali, 1990). All the samples were melted from 30°C to 95°C at a rate of 1.5°C / min after 10 min equilibration at 30°C. The change in absorbance at 260 nm was recorded with increasing temperature. Percent denaturation was calculated as follows:

$$\text{Percent denaturation} = \frac{A_T - A_{30}}{A_{max} - A_{30}} \times 100$$

Where, A_T = Absorbance at a temperature T°C.

A_{max} = Final maximum absorbance on the completion of denaturation (95°C).

A_{30} = Initial absorbance at 30°C.

Nuclease S1 digestibility

Native and modified DNA were characterized by nuclease S1 digestibility (Matsuo and Ross, 1987). One microgram each of native and modified DNA in acetate buffer (30 mM each of sodium acetate and zinc chloride, pH 5.0) were treated with nuclease S1 (20 units/μg DNA) for 30 min at 37°C. The reaction was stopped by adding

one-tenth volume of 200 mM EDTA, pH 8.0. The digested and control samples were subjected to agarose gel electrophoresis.

(a) Gel preparation

Agarose (1%) in TAE buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM EDTA) was dissolved by heating. The solution was cooled to about 50°C and then poured into gel tray and allowed to solidify at room temperature.

(b) Sample preparation and loading

Native and modified DNA samples treated with nuclease S1 were mixed with one-tenth volume of sample buffer (0.125% bromophenol blue, 30% Ficoll 400, 500 mM EDTA in 10X electrophoresis buffer). The samples were loaded in the wells and electrophoresed for 2 hr at 30 mA. The gels were stained with ethidium bromide (0.5 μg/ml), viewed by illumination under UV light and photographed.

Immunization schedule

Native and modified DNA (100 μg) were complexed with an equal volume (w/w) of methylated BSA and emulsified with an equal volume of complete Freund's adjuvant and injected intramuscularly in female rabbits. Subsequent injections were given in incomplete Freund's adjuvant. Each animal received a total of 800 μg of antigen in the course of 8 injections. Blood was collected from marginal vein of the ear, serum was separated and decomplemented by heating at 56°C for 30 minutes. Pre-immune serum was collected prior to immunization. The sera were stored in small aliquots at -20°C with 0.1% sodium azide as preservative.

Isolation of IgG by Protein-A agarose

Serum IgG was isolated by affinity chromatography on Protein-A agarose column. Serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to column (12 x 45 mm) equilibrated with the same buffer. The wash through was recycled 2-3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding, 1976) and

neutralized immediately with 1 ml of 1M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.4 OD_{280} = 1.0 mg IgG/ml. The isolated IgG was dialyzed against PBS, pH 7.4 and stored at -20°C with 0.1% sodium azide.

Immunological detection of antibodies

Level of antibodies was evaluated by immunodiffusion, enzyme linked immunosorbent assay and gel retardation assay.

(a) Immunodiffusion

Immunodiffusion (ID) was carried out by Ouchterlony's double diffusion system. Six ml of 0.4% molten agarose in PBS containing 0.1% sodium azide was poured on to a glass petridishes and allowed to solidify at room temperature. Wells of 5 mm diameter were cut into hardened gel and an appropriate concentration of antigen and antibody was placed in the wells. The petridishes were allowed to stand in a moist chamber at room temperature for 48-72 hr. The gels were washed with 5% sodium citrate to remove non-specific precipitin lines. The result was analyzed visually.

(b) Enzyme linked immunosorbent assay

The following reagents were prepared in distilled water and used in enzyme immunoassay.

(i) Buffers and reagents

Tris buffered saline (TBS)

10 mM Tris, 150 mM NaCl, pH 7.4

Tris buffered saline-Tween 20 (TBS-T)

20 mM Tris, 144 mM NaCl, 2.68 mM KCl

pH 7.4, containing 500 µl Tween 20/L.

Carbonate-bicarbonate buffer

15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6, containing 2 mM magnesium chloride.

Citrate-phosphate buffer

50 mM citric acid, 50 mM Na_2HPO_4, pH 5.0.

Substrate

500 μg p-nitrophenyl phosphate (p-NPP)/ml carbonate-bicarbonate buffer, pH 9.6.

(ii) Procedure

Antibodies were detected by ELISA using polystyrene microtitre plates as solid support (Aotsuka *et al.*, 1979). One hundred microlitre of 2.5 μg/ml antigen in TBS, pH 7.4 was coated in test wells of microtitre plates, incubated for 2 hr at 37°C and overnight at 4°C. The antigen coated wells were washed three times with TBS-T to remove unbound antigen. Unoccupied sites were blocked with 150 μl of 1.5% BSA in TBS for 4 - 5 hrs at room temperature. The plates washed once with TBS-T and antibody (100 μl/well) to be tested, were diluted in TBS and added to each well. After 2 hr incubation at 37°C and overnight at 4°C, the plates were washed four times with TBS-T and an appropriate anti-immunoglobulin alkaline phosphatase conjugate was added to each well. After incubation at 37°C for 2 hr, the plates were washed four times with TBS-T and three times with distilled water and developed using p-nitrophenyl phosphate substrate respectively. The absorbance was recorded at 410 nm on an automatic microplate reader. Each sample was run in duplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as a mean of A_{test} -$A_{control}$.

Competition ELISA

The antigenic specificity of the antibodies was determined by competition ELISA (Hasan *et al.*, 1991). Varying amount of inhibitors (0-20 μg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated at room temperature for 2 hr and overnight at 4°C. The immune complex thus formed was coated in the wells instead of the serum. The remaining steps were the same as in direct binding ELISA.

Percent inhibition was calculated using the formula

$$\text{Percent inhibition} = 1 - \frac{A_{inhibited}}{A_{uninhibited}} \times 100$$

Band shift assay

For the visual detection of antigen-antibody binding and immune complex formation, gel retardation assay was performed (Sanford *et al.*, 1988). A constant amount of antigen (native and modified DNA) was incubated with varying amounts of IgG in PBS, pH 7.4 for 2 hr at 37°C and overnight at 4°C. One-tenth volume of 'stop mix' dye was added to the mixture and electrophoresed on 1% agarose for 2 hr at 30 mA in TAE (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA) buffer, pH 7.9. The gels were stained with ethidium bromide (0.5 μg/ml), visualized under UV light and photographed.

Isolation of human-DNA

Human DNA was isolated from whole blood using QIAamp Blood Midi Kit supplied by QIAGEN, U.S.A. Blood was collected in presence of anticoagulant (EDTA).

Procedure

To 2 ml of whole blood was added 200 μl protease stock solution and 2.4 ml buffer AL. Contents were mixed by vortexing and incubated at 70°C for 10 min. Two ml ethanol (95-100%) was added the contents were again mixed by vortexing. Half of the solution (~3.3 ml) was transferred into the Midi column with centrifugation tube and was spinned at 3000 rpm for 3 min. The filtrate was discarded. The remaining half of the sample was transferred to the same Midi Column and the whole procedure repeated. The Midi column was washed with 2 ml of buffer AW1 and centrifuged at 5000 rpm for 1 min. Two ml of buffer AW2 was added and the column centrifuged again at 5000 rpm for 15. The Midi column was placed in a fresh 15 ml centrifuge tube. 300 μl of buffer AE was added onto the Midi column, incubated at room temperature for 5 min and centrifuged at 5000 rpm for 5 min. This step was repeated once with 300 μl buffer AE. The pooled filtrate contain human DNA. The absorbance of DNA solution was read at 260 nm and 280 nm to ascertain its purity and concentration.

Photochemical generation of superoxide anion radical and singlet oxygen species

Singlet oxygen and superoxide anion radicals were generated by illumination of riboflavin with UV light (365 nm). The generation of superoxide anion was monitored by nitroblue tetrazolium (NBT) assay. In order to optimize the superoxide generation, the time of illumination and dose dependent response of riboflavin and Triton X-100 was studied. The results showed an increase in absorbance at 560 nm with increasing time period of illumination. It is evident from the curve that 20 min of illumination of UV light caused optimum generation of superoxide radical (Fig. 2). Similarly, dose dependent experiments were carried out with varying amounts of riboflavin (Fig. 3) and Triton X-100 (Fig. 4). The ability of riboflavin to form singlet oxygen ($^1O_2$) was determined by monitoring the bleaching of pRNO (Joshi, 1985; Fig. 5).

The generation of these radicals was further confirmed by the use of their respective quenchers i.e. SOD for superoxide anion radical ($O_2^{\cdot -}$) and sodium azide for singlet oxygen ($^1O_2$). The evidence for the formation of ($^1O_2$) was obtained by examining the progress of the reaction in presence of sodium azide (NaN_3), a specific quencher for $^1O_2$. A near complete inhibition of $^1O_2$ production was observed (Fig. 6). Dose dependent experiment was carried out with varying concentration of NaN_3 (0, 10, 15, 25, 50, 75, 100 mM). Maximum inhibition was obtained at 100 mM (Fig. 7). Superoxide dismutase was used to further confirm the production of superoxide anion radical. No inhibition in the formation of blue coloured product (nitroblue formazan) was observed due to the presence of singlet oxygen in the system (Fig. 8). Inhibition in the formation of nitroblue formazan was observed when both SOD and NaN_3, quencher of singlet were used (Fig. 9).

Commercially available calf thymus DNA was purified free of proteins, RNA and single stranded regions and digested with micrococcal nuclease to obtain DNA fragments. The digested DNA was subjected to gel filtration on Sepharose 4B column (Fig. 10). Alternate fractions on PAGE resulted in DNA of varying size (Fig. 10 inset). The fraction(s) of 200 bp (average size) was selected for these studies.

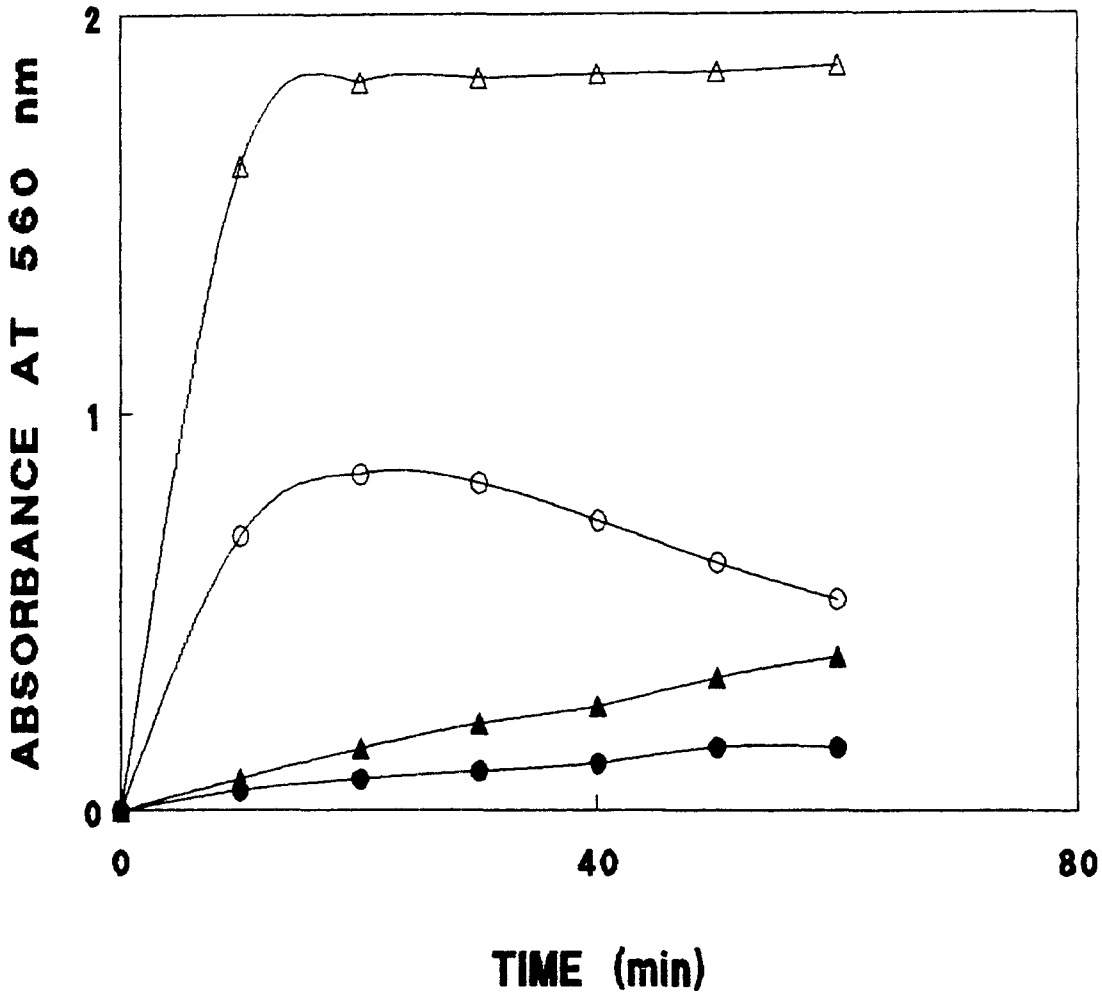

Fig. 2. Time dependent generation of superoxide ($O_2^{\bullet -}$) anion radical by riboflavin in light (Δ), in dark (●), without Triton X-100 (O), without riboflavin (▲).

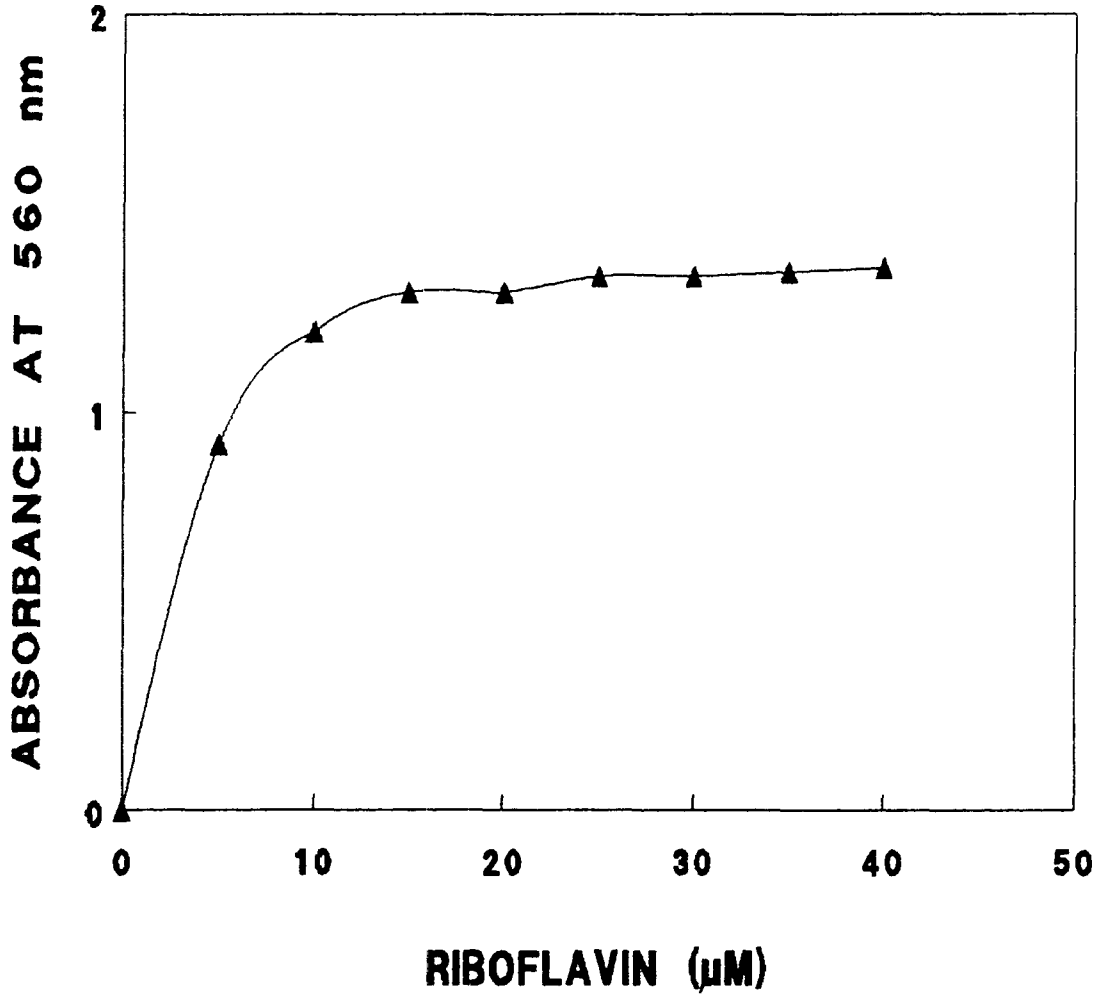

Fig. 3. Superoxide anion radical generation under varying concentrations of riboflavin.

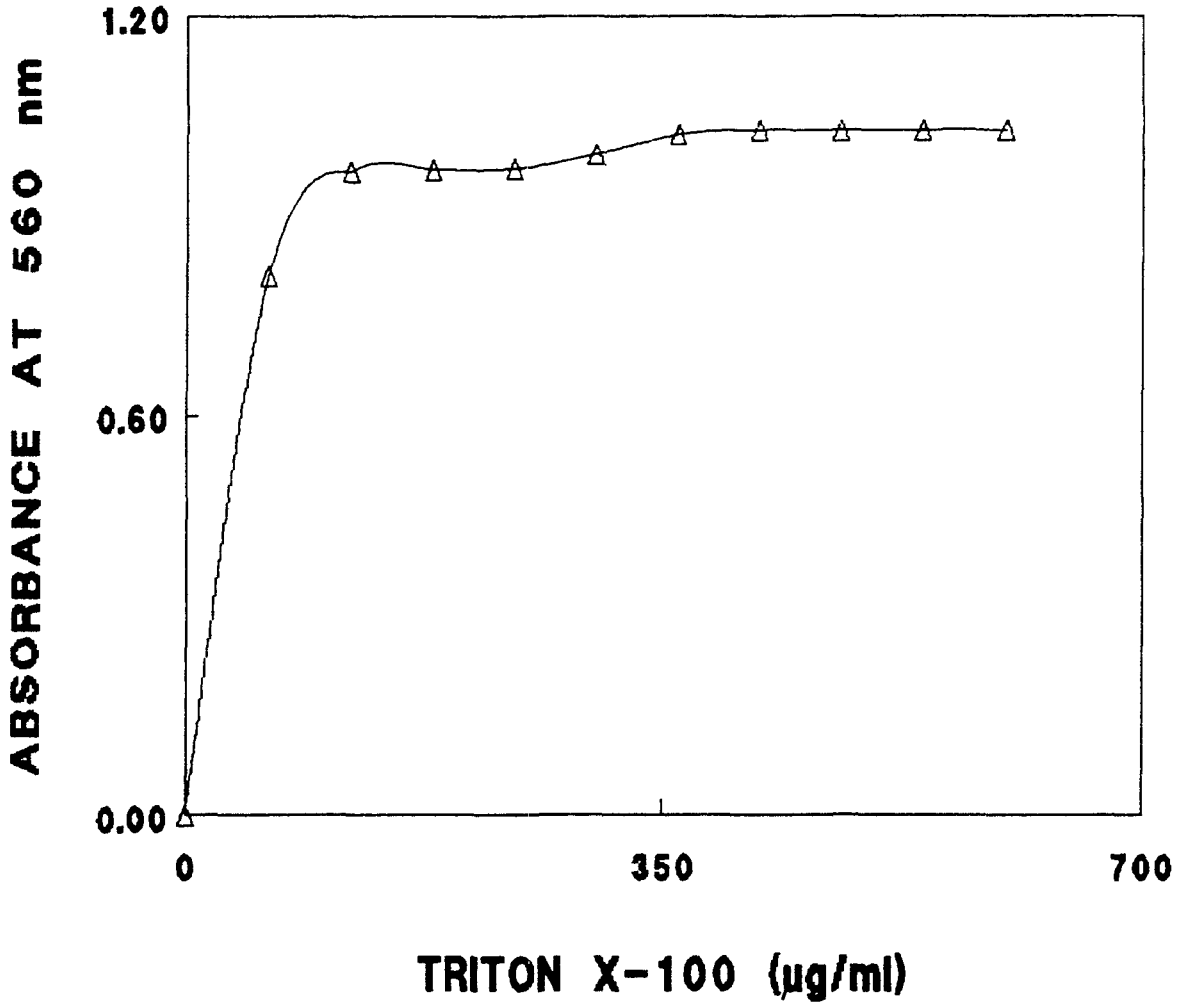

Fig. 4. Superoxide anion radical generation under varying concentrations of Triton X-100.

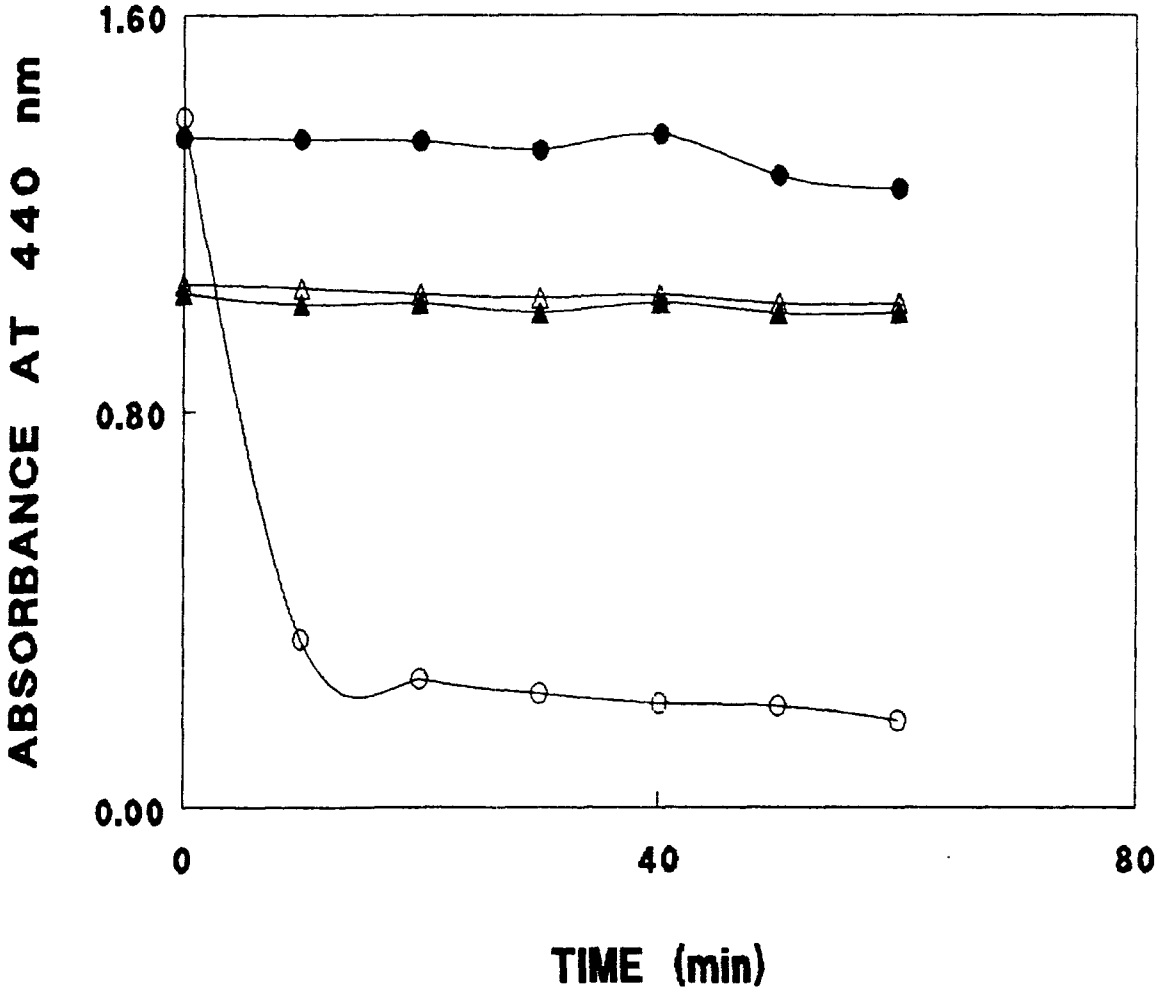

Fig. 5. Time dependent production of singlet oxygen ($^1O_2$) by riboflavin in light (○), riboflavin in dark (●), pRNO alone in light (△), pRNO alone in dark (▲).

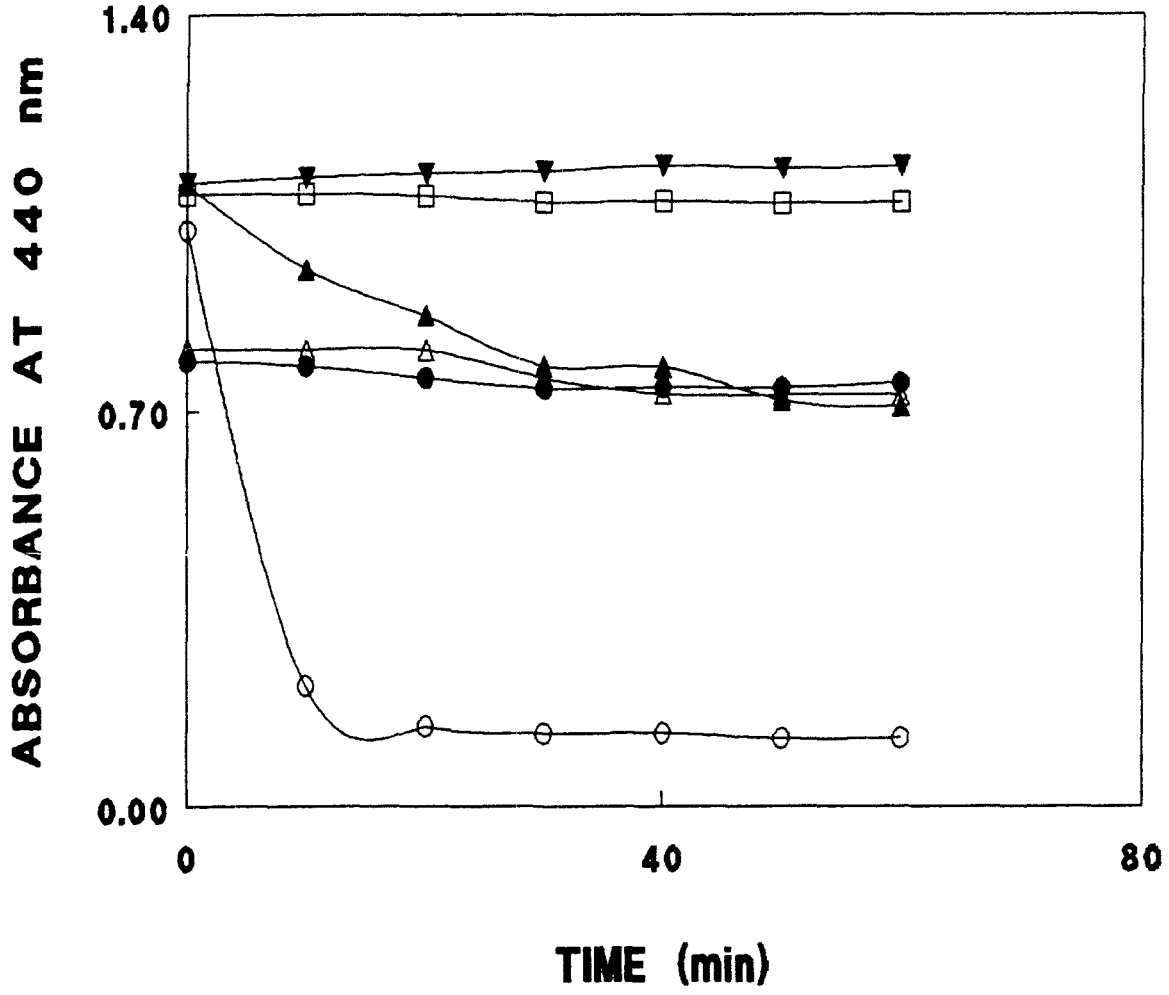

Fig. 6. Effect of sodium azide (NaN_3) on generation of $^1O_2$ by riboflavin in light (○) and in dark (□); with pRNO in light (△), and in dark (●); with NaN_3 in light (▲) and in dark (▼).

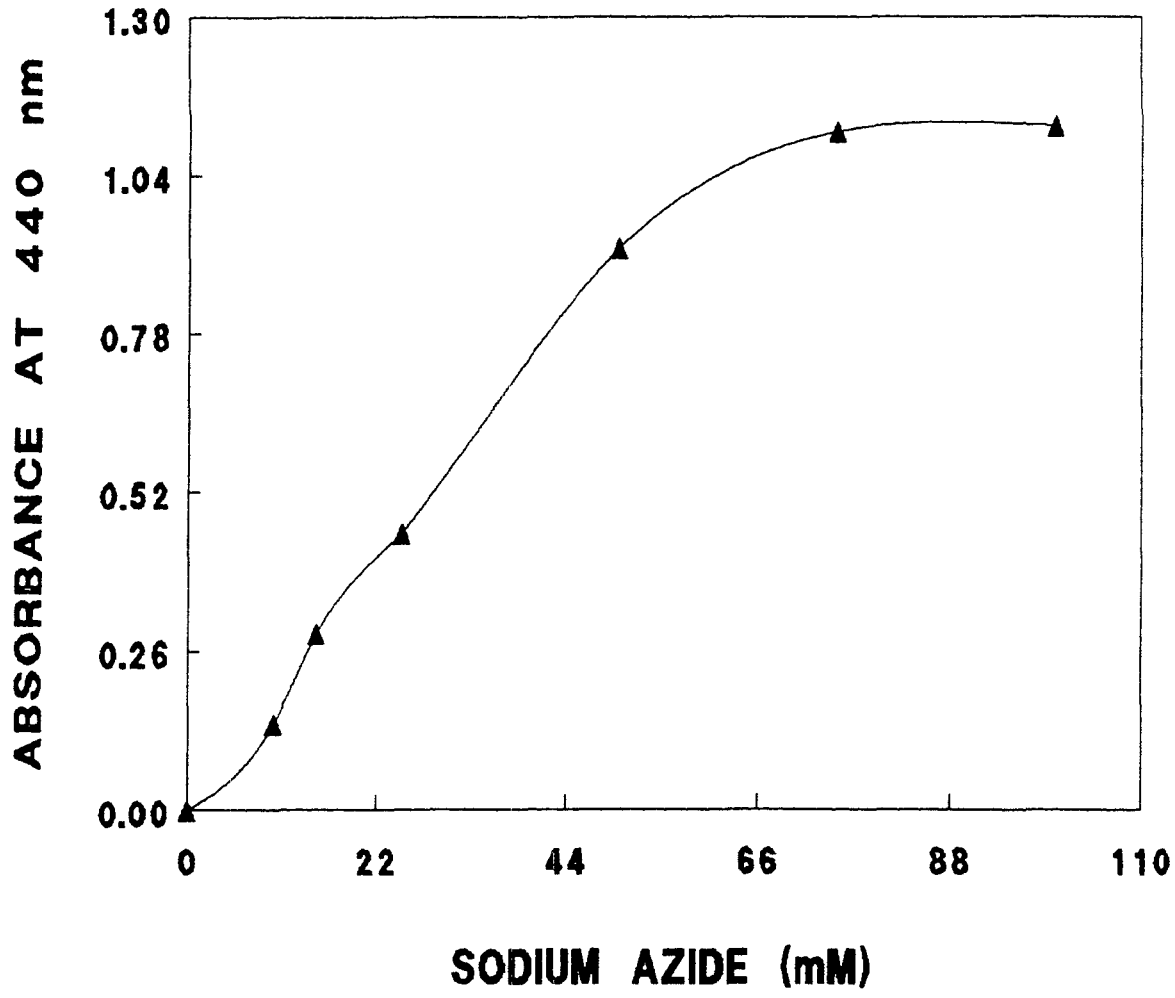

Fig. 7. Quenching of $^1O_2$ with increasing concentrations of sodium azide.

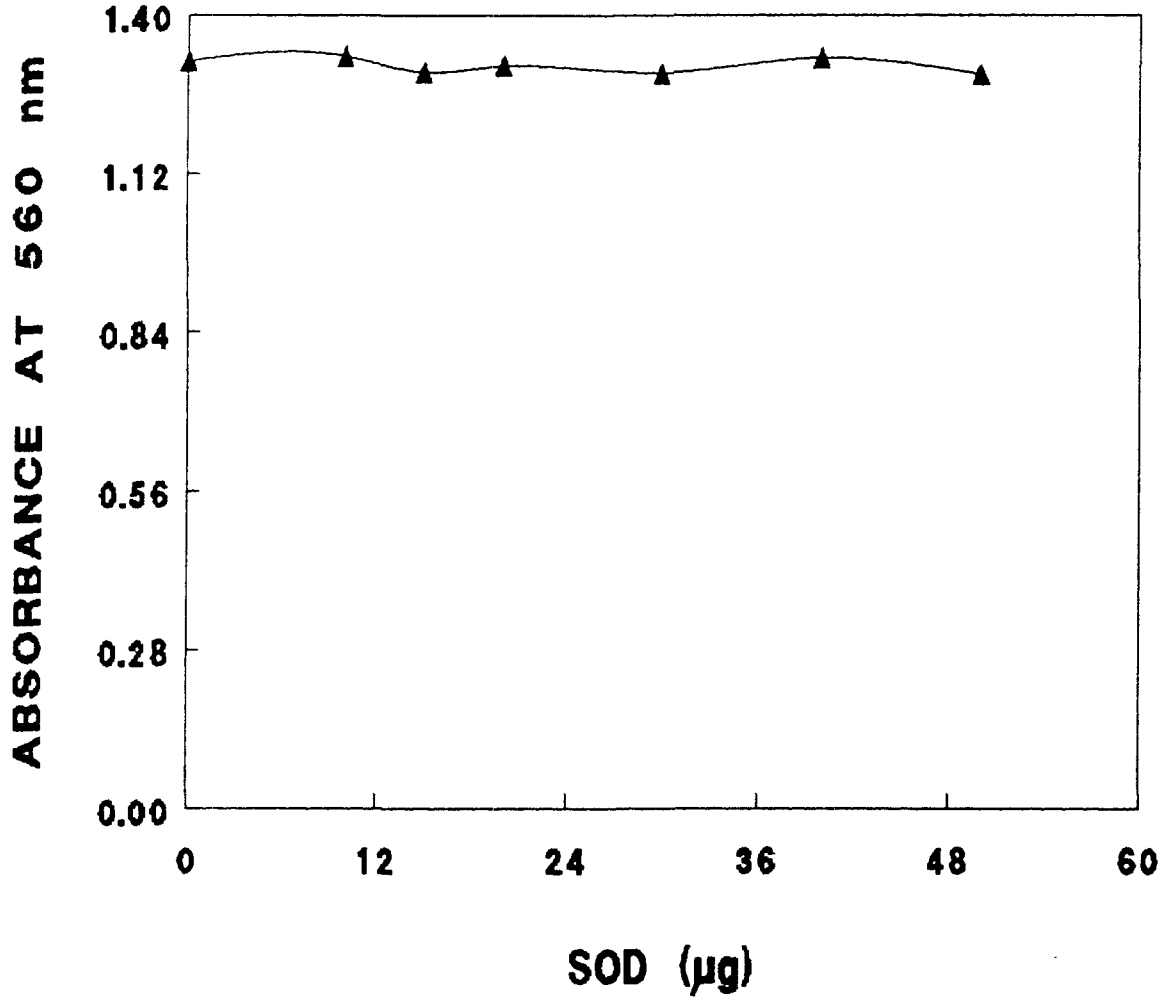

Fig. 8. Quenching of $O_2^{\cdot -}$ with increasing concentrations of SOD.

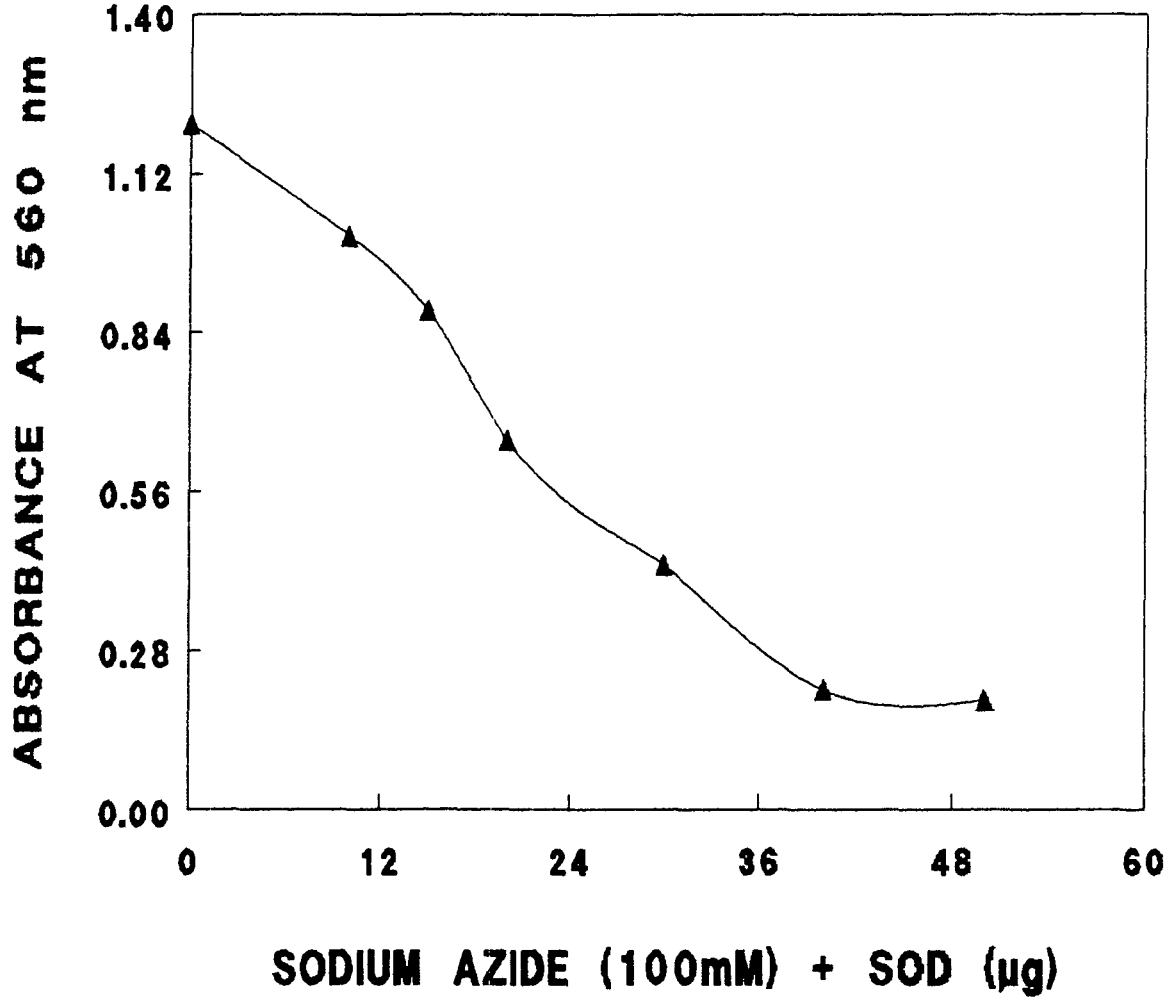

Fig. 9. Quenching of $^1O_2$ and $O_2^{\bullet -}$ with increasing concentrations of SOD, keeping NaN_3 constant.

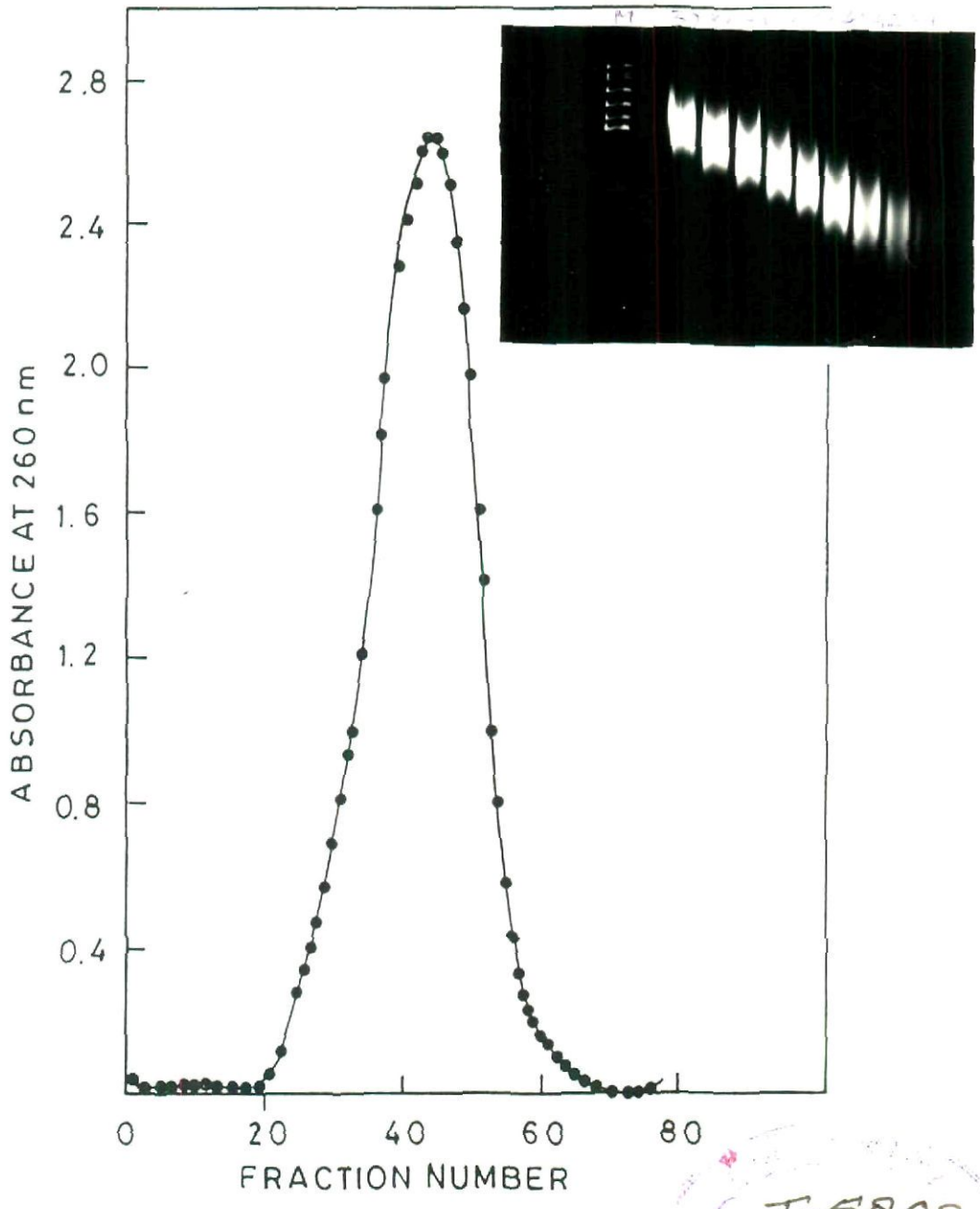

Fig. 10. Elution profile of micrococcal nuclease digested calf thymus DNA on Sepharose 4B column. **Inset**: Polyacrylamide gel electrophoresis of fractionated DNA. Lanes (3-10) represent alternate fractions from 30-44, while lanes 1 contained 100 bp DNA ladder as marker.

Modification of DNA by superoxide anion radical and singlet oxygen species

DNA fragments of average size of 200 bp in 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 0.06% Triton X-100, 40 μM riboflavin was illuminated for 1 hr under 365 nm UV light at room temperature. The $^1O_2$-$O_2^{\cdot -}$-DNA samples were dialyzed against PBS, pH 7.4 to remove riboflavin and Triton X-100.

Characterization of $^1O_2$-$O_2^{\cdot -}$-DNA

Figure 11 shows the UV absorption spectra of superoxide radical and singlet oxygen species modified DNA with characteristic hyperchromicity. The spectra shows a shift of approximately 10 nm towards longer wavelength side at both λmax and λmin for $^1O_2$-$O_2^{\cdot -}$-DNA relative to native DNA. The percent hyperchromicity was 30% in 1 hr illuminated sample.

The modifications incurred on nDNA fragments was also analyzed by UV difference spectroscopy (Fig. 11 inset). The spectral curve exhibited an appreciable negative inversion in absorption between 240-260 nm, followed by increased absorption at 270 nm. Moreover the spectral curve also exhibited a shoulder at around 290 nm. Elimination of the characteristic 260 nm peak is indicative for the loss of the double helical structure of DNA. These changes in the curve are also indicative of the possible generation of single stranded regions and ring opening of the nucleic acid bases as a consequence of the modification.

Native and $^1O_2$-$O_2^{\cdot -}$-DNA was used to record fluorescence spectra. The decrease in the fluorescence intensity was observed probably indicating the generation of strand breaks due to lesser amount of ethidium bromide intercalation in modified DNA as compared to native DNA (Fig. 12).

DNA was modified in presence and absence of NaN_3. Figure 13 shows the UV absorption spectra of native DNA, $^1O_2$-$O_2^{\cdot -}$-DNA and $^1O_2$-$O_2^{\cdot -}$-DNA in presence of NaN_3. Approximately 50% quenching in the absorbance was observed.

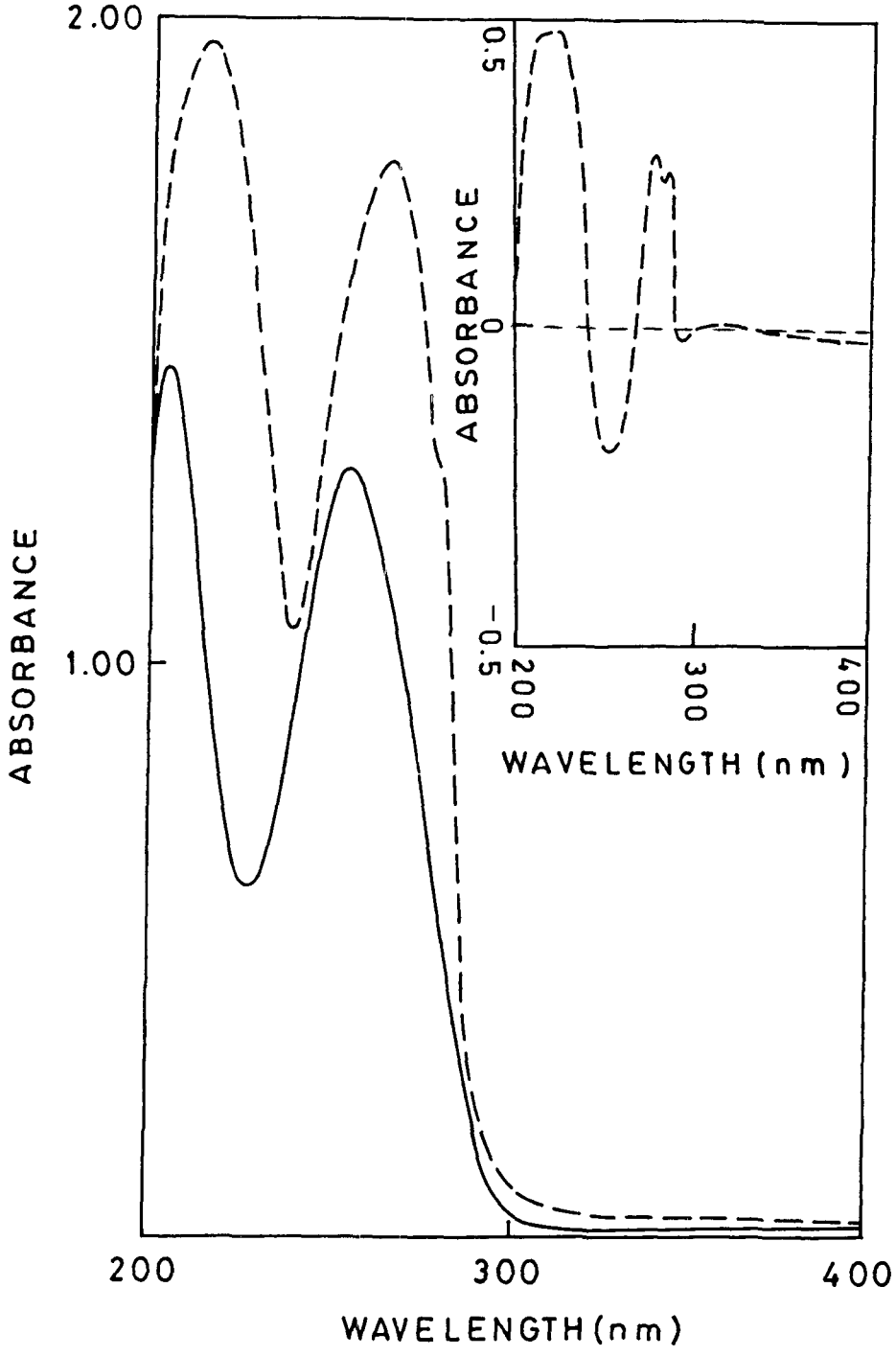

Fig. 11. Ultraviolet absorption spectra of 200 bp native DNA and $^1O_2$-$\dot{O}_2^-$-DNA. Native DNA (—), $^1O_2$-$\dot{O}_2^-$-DNA (----). **Inset**: Ultraviolet difference spectroscopical scanning of $^1O_2$-$\dot{O}_2^-$-DNA, native DNA served as control.

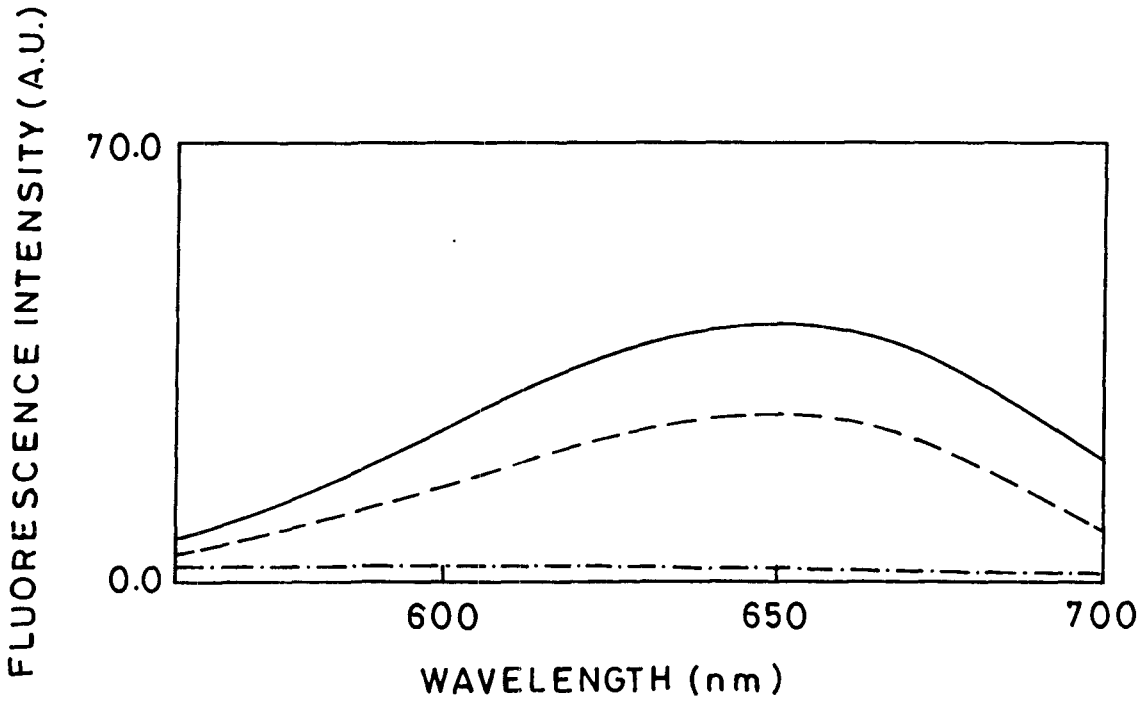

Fig. 12. Fluorescence emission spectra of EtBr (·····), native DNA (—) and $^1O_2$-$O_2^{\bullet-}$-DNA (---). The samples were excited at 300 nm.

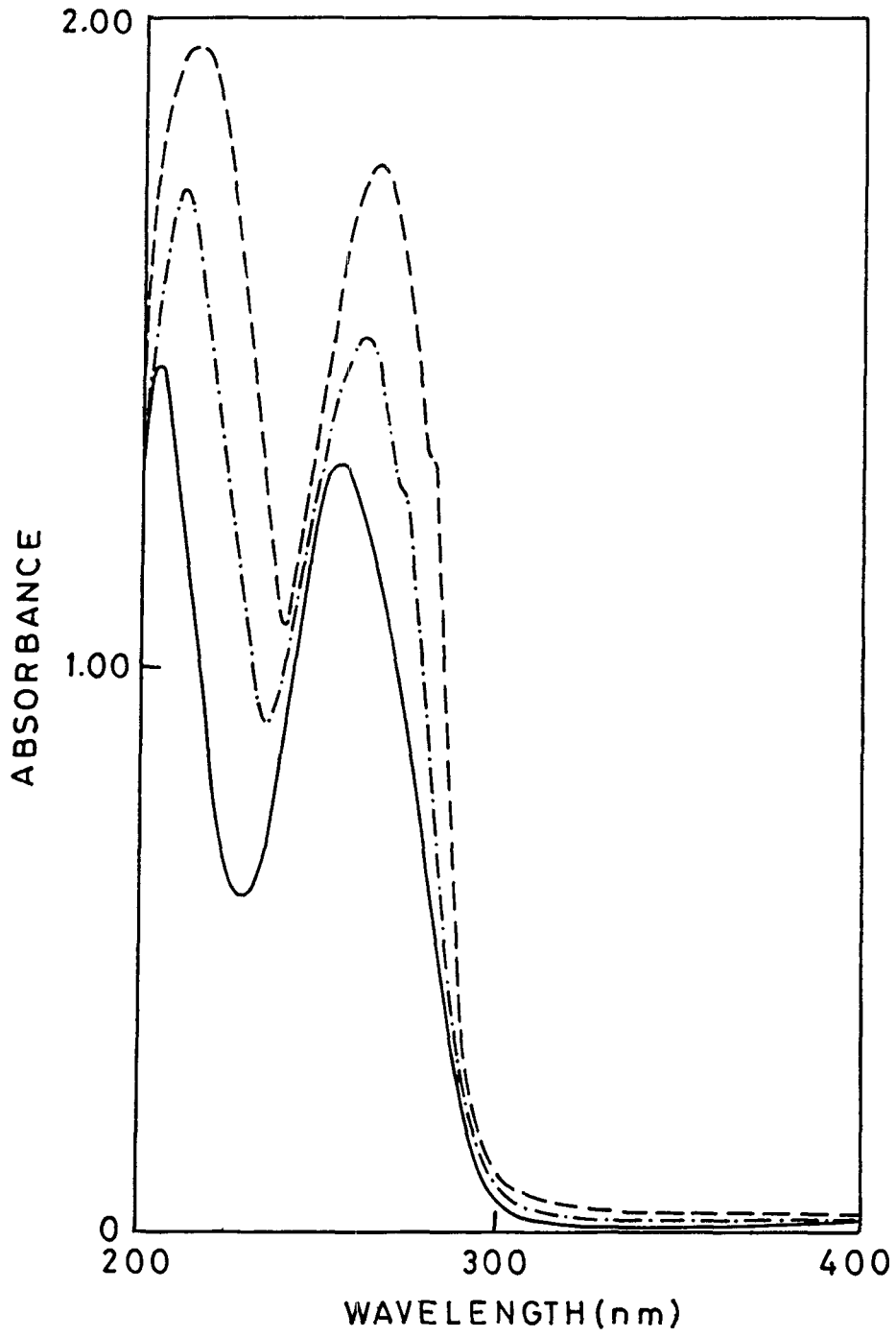

Fig. 13. UV absorption spectra of 200 bp DNA modified in presence of sodium azide (100 mM), a specific quencher of $^1O_2$. 200 bp DNA (—), modified 200 bp DNA (---), modified DNA in presence of NaN_3 (-·-·-).

Thermal melting of $^1O_2$-$O_2^{\cdot-}$-DNA

Thermally induced transitions were measured spectrophotometrically at 260 nm by heating nucleic acid samples at a rate of 1.5°C per min. Melting curves were recorded at temperatures from 30°C to 95°C. Increase in UV absorption at 260 nm was taken as a measure of denaturation. Figure 14 shows the thermal denaturation profile of native and $^1O_2$-$O_2^{\cdot-}$-DNA. The melting temperature of native DNA at which 50% of the double helical structure is lost was found to be 86°C, while in case of $^1O_2$-$O_2^{\cdot-}$-DNA it was found to be 78°C. The results exhibit a net decrease of 8°C in the Tm value for the $^1O_2$-$O_2^{\cdot-}$-DNA when compared to its unmodified native conformer. These findings indicate, therefore, that the decrease in Tm to the extent of 8°C is primarily due to structural alteration of DNA which occurs upon generation of single strand breaks and base modifications. The percent hyperchromicity and the values of native and $^1O_2$-$O_2^{\cdot-}$-DNA are shown in Table 4.

Nuclease S1 digestibility

Native and $^1O_2$-$O_2^{\cdot-}$-DNA were digested with nuclease S1 (20 units/μg DNA) for 30 min. The resulting DNA sample was subjected to agarose gel electrophoresis. The controls were the unmodified DNA samples with and without nuclease S1 treatment. The results showed substantially decreased intensity in case of S1 treated $^1O_2$-$O_2^{\cdot}$ -DNA compared to non-S1 treated control. However, some loss in fluorescence intensity was observed in unmodified native DNA (Fig. 15).

Antigenicity of $^1O_2$-$O_2^{\cdot-}$-DNA

The antigenicity of the $^1O_2$-$O_2^{\cdot-}$-DNA was probed by inducing antibodies in rabbit. The induction of antibodies and their specificity was assayed by direct binding and competition ELISA. The binding of these antibodies to the immunogen and native DNA was further substantiated by band shift assay.

The antibodies raised against $^1O_2$-$O_2^{\cdot-}$-DNA was found to be non-precipitating in immunodiffusion. Direct binding ELISA was used to characterize the immune response

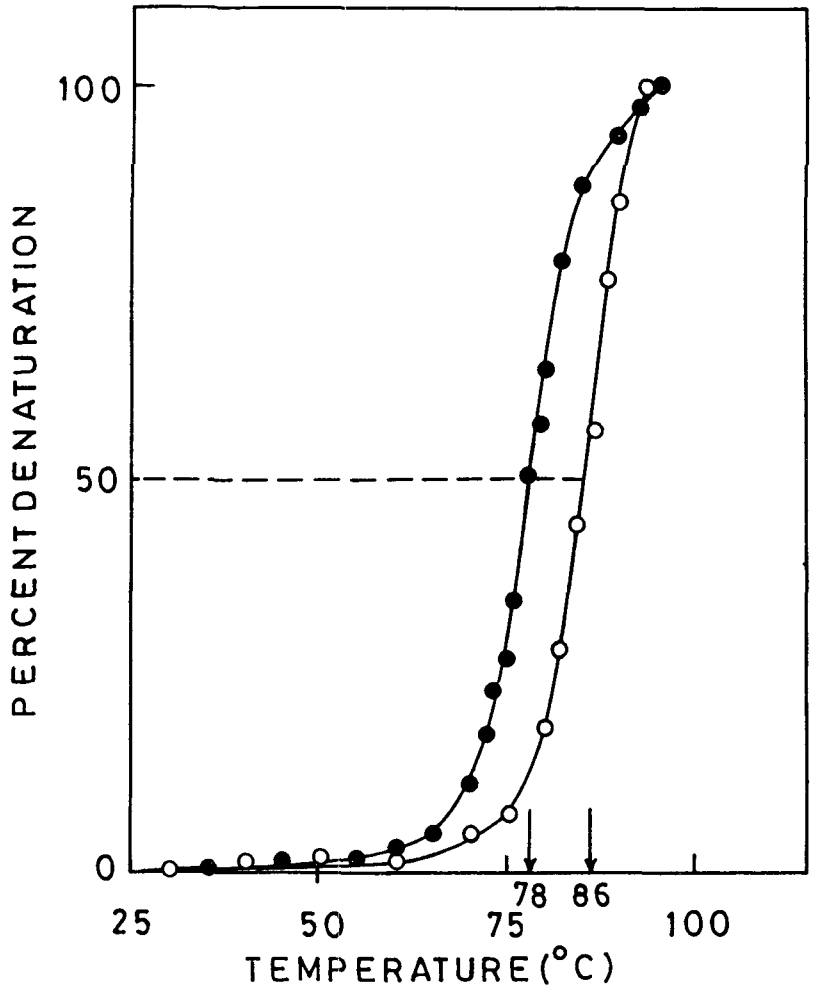

Fig. 14. Thermal melting profile of native DNA (O) and $^1O_2$-$\dot{O}_2^-$-DNA (●).

TABLE – 4

Ultraviolet and thermal denaturation characteristics of native and $^1O_2$-$O_2^{\bullet-}$-DNA under identical experimental condition

Parameters	Native DNA	$^1O_2$-$O_2^{\bullet-}$-DNA
Absorbance ratio (A_{260} / A_{280})	1.8	1.6
Percent hyperchromicity at 95°C	36.7	17.2
Melting temperature (Tm), °C	86.0	78.0
Onset of duplex melting, °C	65.0	58.0

Fig. 15. Nuclease S1 digestibility of native and $^1O_2$-$O_2^{\bullet-}$-DNA. Lane 1 contained native DNA, while lane 2 contained native DNA treated with nuclease S1. Lane 3 contained $^1O_2$-$O_2^{\bullet-}$-DNA, while lane 4 contained $^1O_2$-$O_2^{\bullet-}$-DNA treated with nuclease S1. Electrophoresis was carried out on 1% agarose gel for 2 hr at 30 mA.

in rabbits following immunization with $^1O_2$-$O_2^{\cdot-}$-DNA. The anti-serum showed a titre of 1:25600 (Fig. 16). The antibodies against native DNA complexed with methylated BSA was also raised in rabbits. The results evaluated by direct binding ELISA showed negligible binding. Preimmune serum as control did not show appreciable binding with the immunogen. The specificity of the induced antibodies for antigenic determinants on $^1O_2$-$O_2^{\cdot-}$-DNA was evaluated by competitive binding assays. A maximum of 70% inhibition in antibody binding was recorded at an inhibitor ($^1O_2$-$O_2^{\cdot-}$-DNA) concentration of 20 μg/ml (Fig. 17).

(a) Purification and binding characteristics of immune IgG

Immunoglobulin G was isolated from preimmune and immune rabbit serum by affinity chromatography on Protein- A Agarose column (Fig. 18). The purity of the IgG was evaluated by SDS-polyacrylamide gel electrophoresis in absence of a reducing agent. The purified IgG migrated as a single band upon electrophoresis (Fig. 18 inset).

Direct binding ELISA of the purified IgG showed a strong reactivity with the immunogen (Fig. 19). Preimmune IgG as negative control showed negligible binding to $^1O_2$-$O_2^{\cdot-}$-DNA.

(b) Antigenic specificity of anti-$^1O_2$-$O_2^{\cdot-}$-DNA antibodies

The anti-$^1O_2$-$O_2^{\cdot-}$-DNA antibodies exhibited a broad spectrum of reactivity as demonstrated by inhibition assay using the immunogen, nucleic acids, synthetic polynucleotides, chondroitin sulphate, cardiolipin and chromatin as inhibitors. A maximum of 84.5% inhibition of anti-$^1O_2$-$O_2^{\cdot-}$-DNA IgG with immunogen as an inhibitor was observed (Fig. 20). Fifty percent inhibition was achieved at an inhibitor concentration of 1.9 μg/ml. Competition experiments with native DNA showed considerable inhibition in antibody activity. Pre-incubation of immune antibody with native DNA inhibited its binding to $^1O_2$-$O_2^{\cdot-}$-DNA by 47.8% at 20 μg/ml (Fig. 20). ROS-DNA, 200 bp DNA showed a maximum inhibition of 50.25%, 32.3% at 20 μg/ml, respectively (Fig. 20).

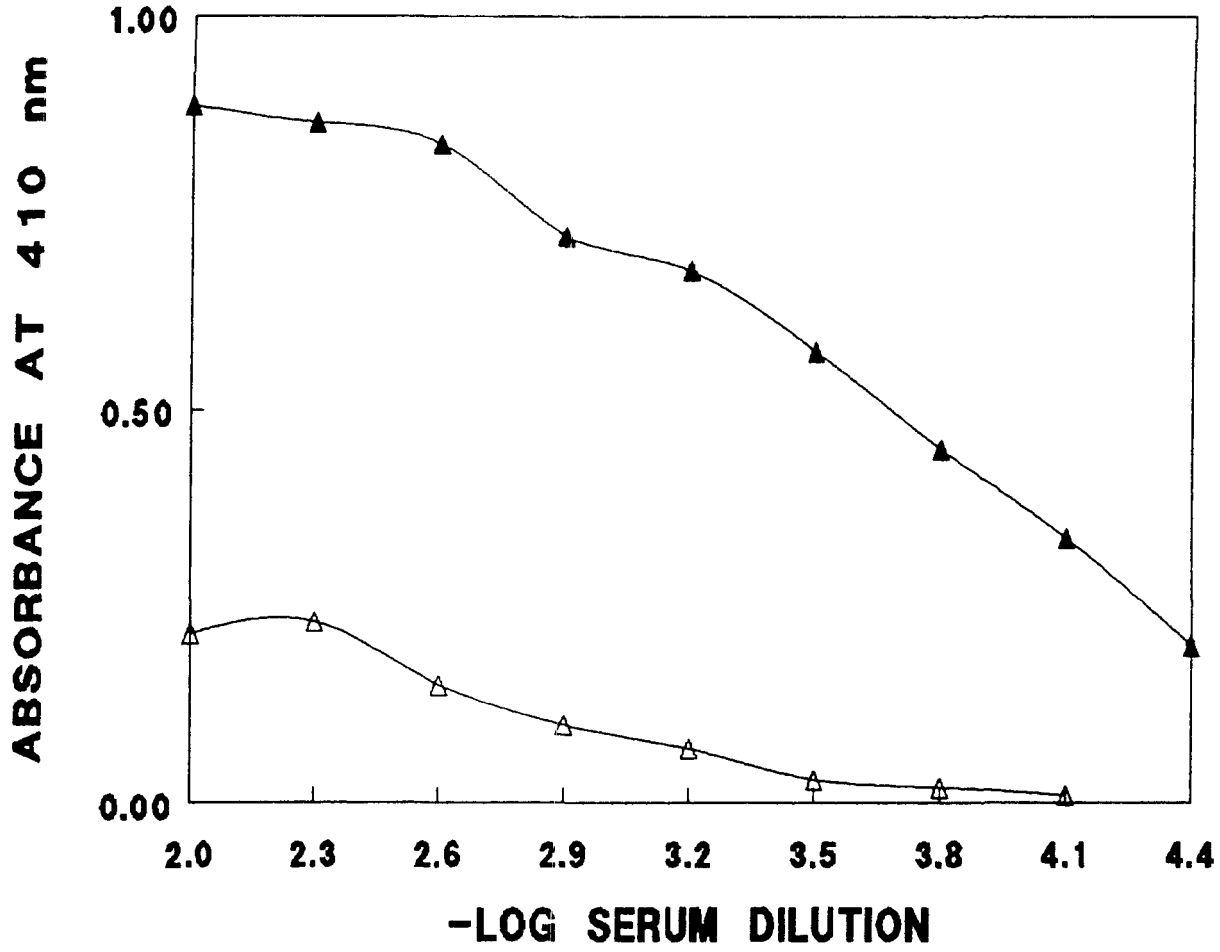

Fig. 16. Direct binding ELISA of $^1O_2$-$O_2^{\bullet-}$-DNA with preimmune (△) and immune sera (▲). Microtitre plate was coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 μg/ml).

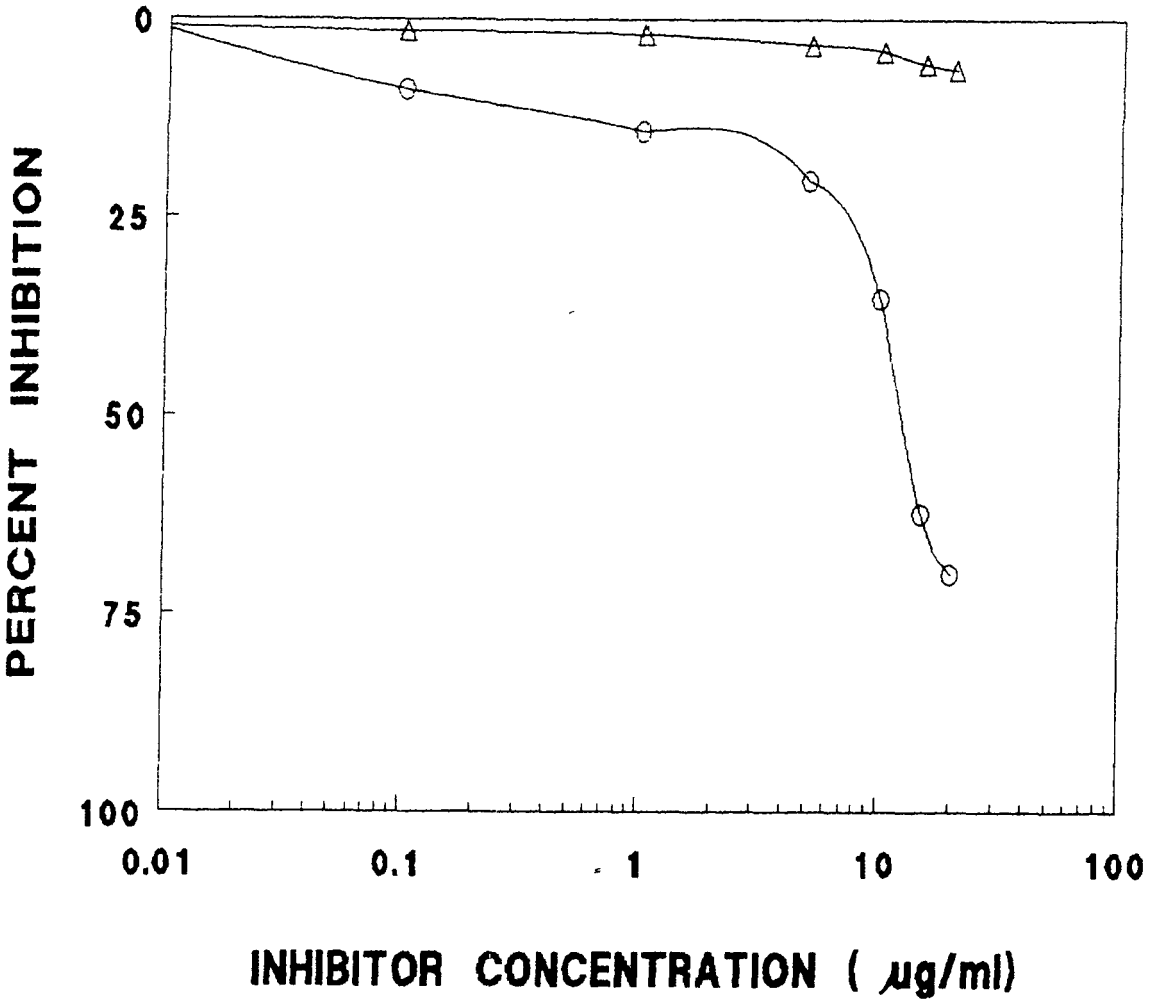

Fig. 17. Inhibition ELISA of immune (O) and preimmune (△) serum antibodies with $^1O_2$-$O_2^{\bullet-}$-DNA. The microtitre plate was coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and the serum dilution was 1:100.

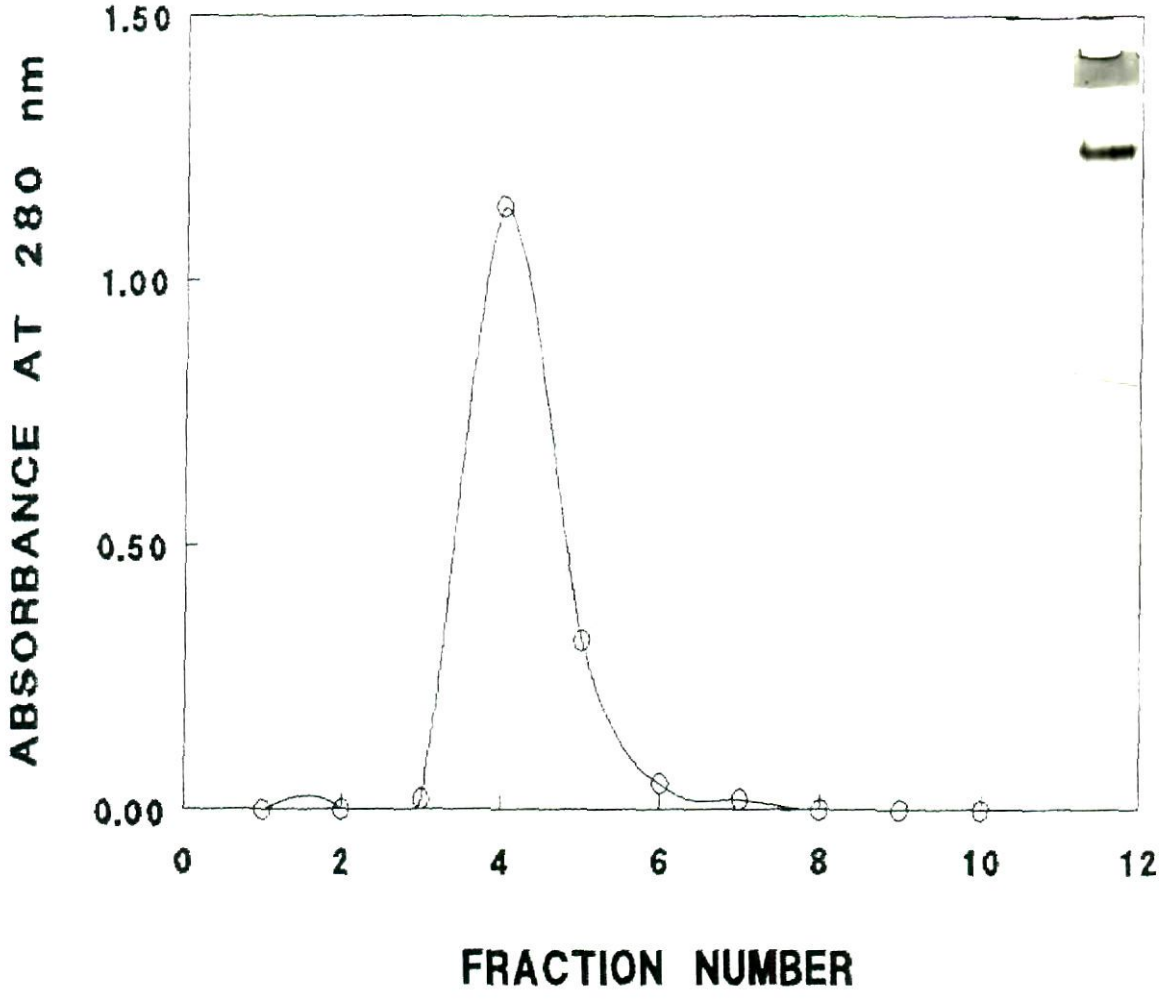

Fig. 18. Elution profile of anti-$^1O_2$-$O_2^{\bullet-}$-DNA IgG on Protein – A Agarose column. **Inset**: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.

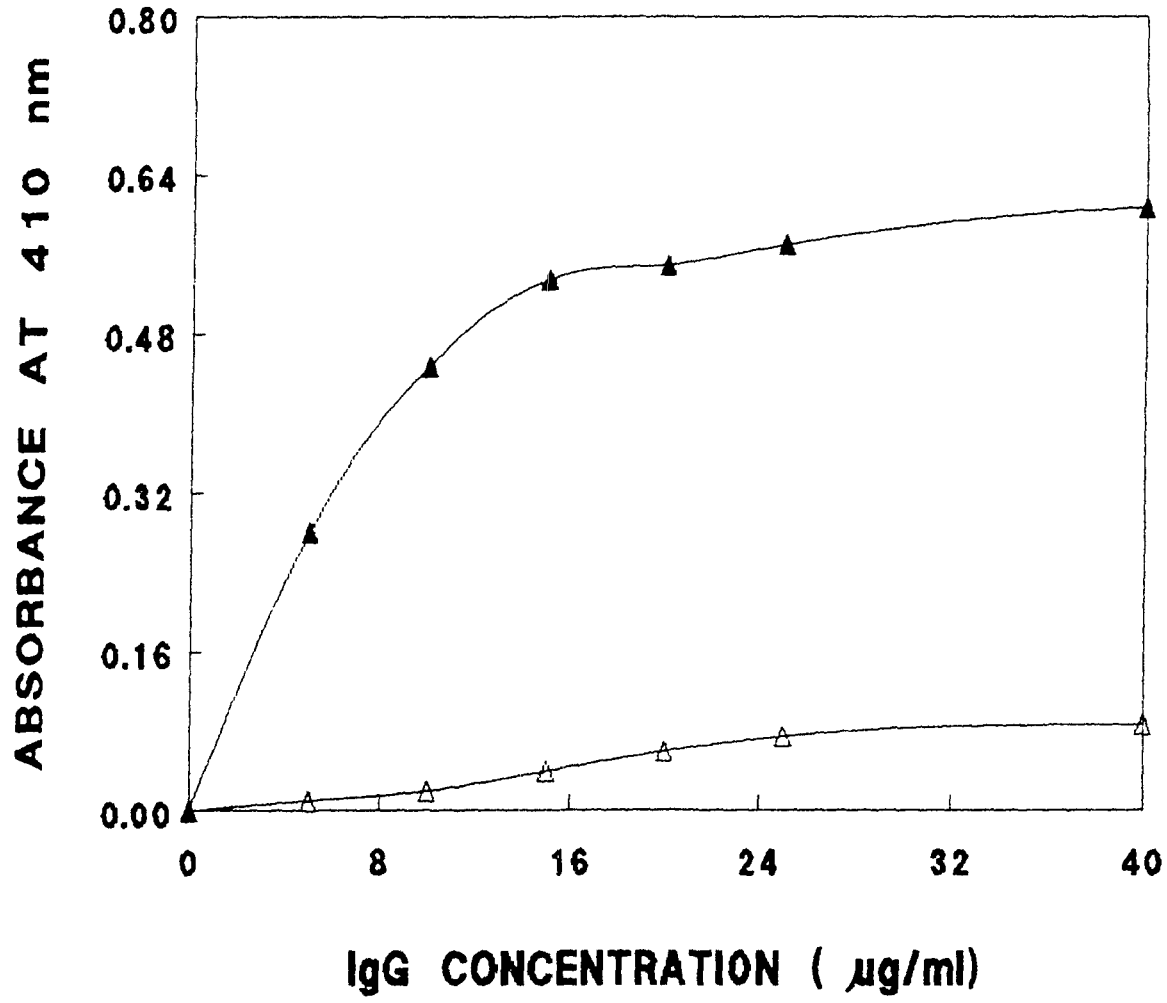

Fig. 19. Direct binding ELISA with affinity purified preimmune (△) and immune IgG (▲) with $^1O_2$-$O_2^{\bullet-}$-DNA. The microtitre plate was coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml).

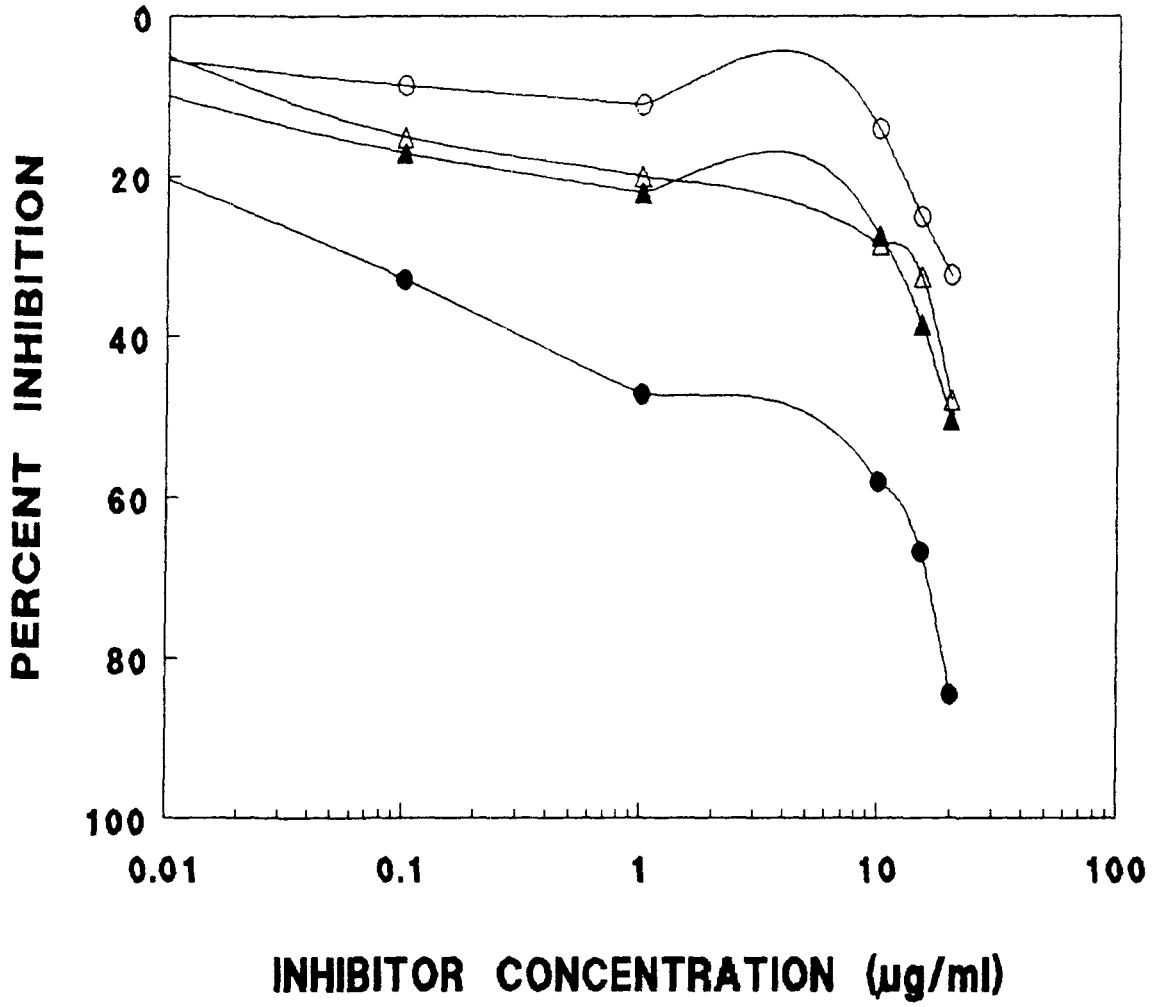

Fig. 20. Inhibition of immune IgG binding to $^1O_2$-$O_2^{\cdot-}$-DNA. The competitors were $^1O_2$-$O_2^{\cdot-}$-DNA (●), native calf thymus DNA (△), 200 bp DNA (○), $^{\cdot}OH$-modified DNA (▲). The microtitre plate was coated with $^1O_2$-$O_2^{\cdot-}$-DNA (2.5 µg/ml).

Native chromatin, ROS-chromatin, cardiolipin and chondroitin sulphate were used as inhibitors of antibody activity. Native chromatin and ROS-chromatin showed a maximum inhibition of 7.9% and 12.5% respectively. Cardiolipin and chondroitin sulphate inhibited antibody binding to antigen to the extent of 37% and 48%, respectively (Fig. 21).

To further define the structural determinants recognized by anti-$^1O_2$-$O_2^{\bullet -}$-DNA antibodies, their interactions with various synthetic polymers were studied. Poly(dA-dU).poly(dA-dU) showed maximum inhibition of 12.7% at a concentration of 20 μg/ml, whereas, poly(dI-dC).poly(dI-dC) and poly(dA-dT).poly(dA-dT) showed maximum inhibitions of 38.7% and 44.4%, respectively (Fig. 22). Table 5 summarises the results of the inhibition studies of the anti-$^1O_2$-$O_2^{\bullet -}$-DNA antibodies with various inhibitors.

Band shift assay

The visual detection of antigen antibody interaction was performed by band shift assay. Constant amounts of native and $^1O_2$-$O_2^{\bullet -}$-DNA were incubated with varying amounts of immune IgG for 2 hr at room temperature and overnight at 4°C. The resulting immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA. Figure 23 shows the binding of IgG to $^1O_2$-$O_2^{\bullet -}$-DNA. As clearly evident, with an increase in the amount of IgG, there was an increase in the formation of high molecular weight immune complexes, which resulted in retarded mobility with a subsequent decrease in the fluorescence intensity of antigen.

Binding characteristics of human anti-DNA autoantibodies to $^1O_2$-$O_2^{\bullet -}$-DNA

The binding pattern of SLE anti-DNA autoantibodies to native and $^1O_2$-$O_2^{\bullet -}$-DNA was determined by direct binding ELISA. All the 24 sera showed appreciable binding to native and $^1O_2$-$O_2^{\bullet -}$-DNA. Preferentially high binding was observed in case of $^1O_2$-$O_2^{\bullet -}$-DNA. Figures 24 and 25 shows the enhanced binding to SLE anti-DNA autoantibodies (1:100 serum dilution) with $^1O_2$-$O_2^{\bullet -}$-DNA as compared to native DNA. With normal human sera no appreciable binding was observed.

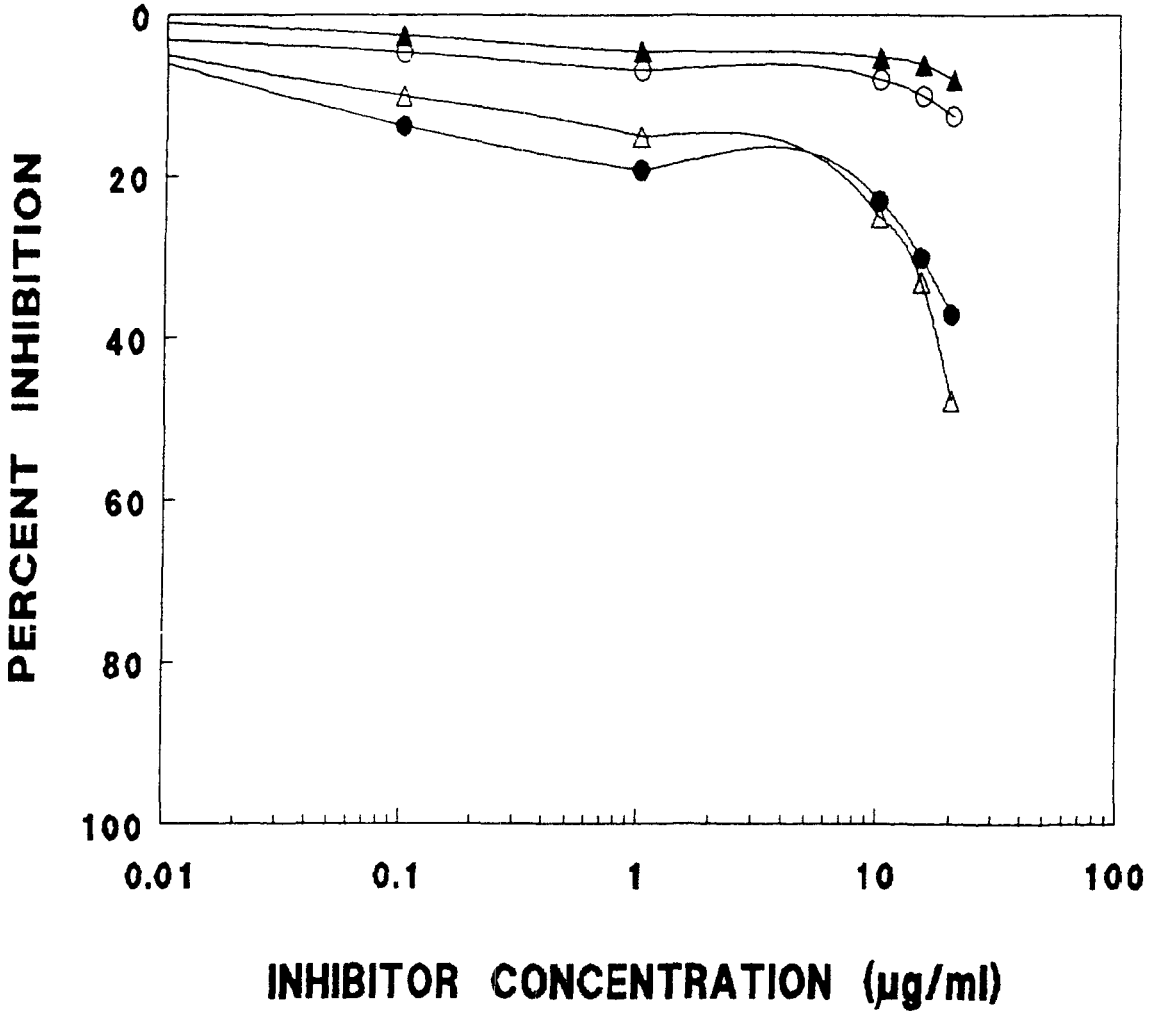

Fig. 21. Inhibition of immune IgG binding to $^1O_2$-$O_2^{\bullet -}$-DNA. The competitors were cardiolipin (●), chondroitin sulphate (△), chromatin (▲), $^{\bullet}$OH-modified chromatin (O). The microtitre plate was coated with $^1O_2$-$O_2^{\bullet -}$-DNA (2.5 µg/ml).

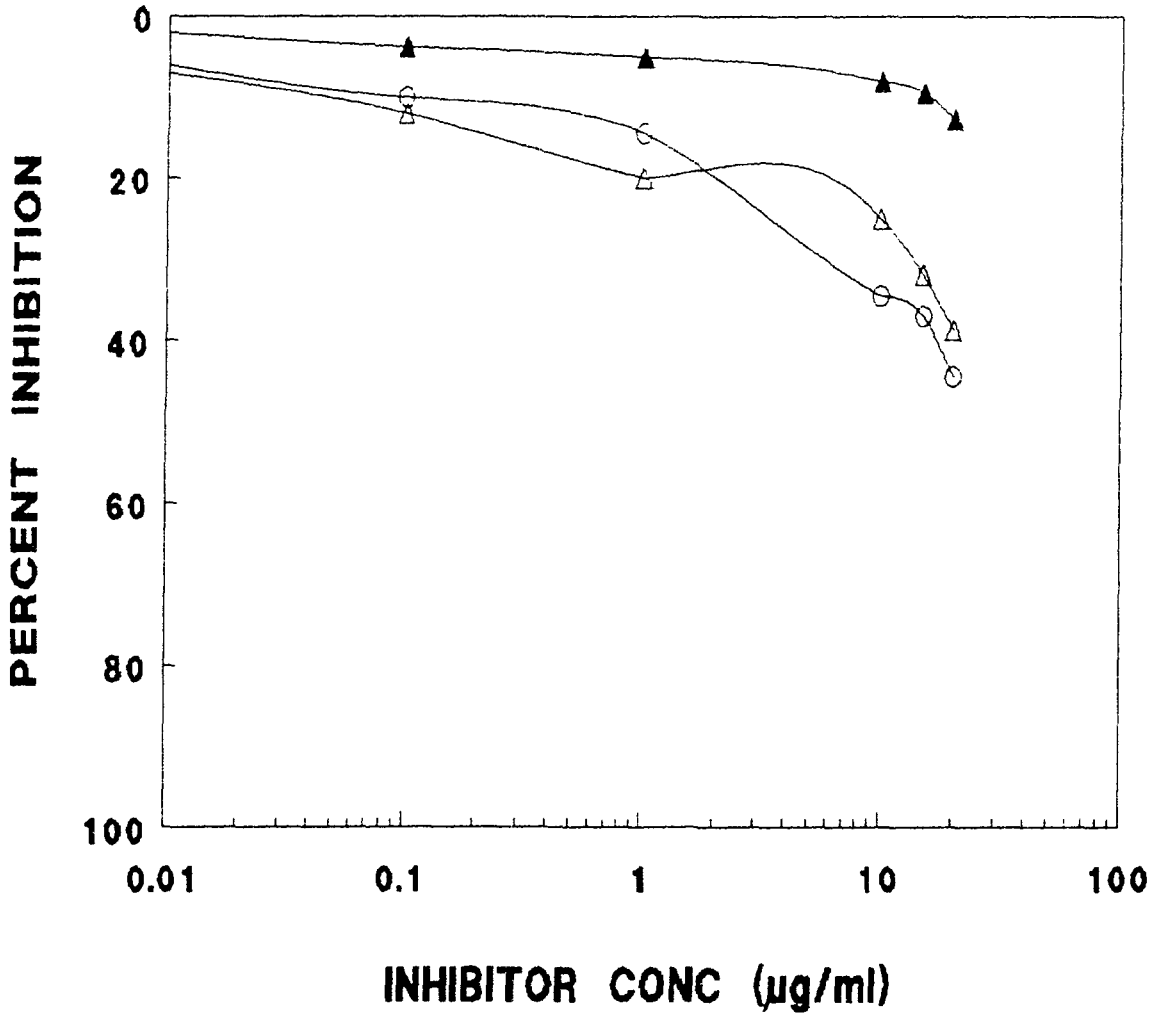

Fig. 22. Inhibition of immune IgG binding to $^1O_2$-$O_2^{\bullet-}$-DNA. The competitors were poly(dI-dC).poly(dI-dC) (△), poly(dA-dU).poly(dA-dU) (▲), poly(dA-dT).poly(dA-dT) (O). The microtitre plate was coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml).

TABLE – 5

Antigenic specificity of $^1O_2$-$O_2^{\cdot-}$-DNA IgG

Inhibitor	Maximum % inhibition at 20 μg/ml
$^1O_2$-$O_2^{\cdot-}$-DNA	84.5
Native DNA	47.8
200 bp DNA	32.3
ROS-200 bp DNA	50.25
Poly (dA-dU). poly (dA-dU)	12.7
Poly (dI-dC). poly (dI-dC)	38.7
Poly (dA-dT). poly (dA-dT)	44.4
Chondroitin sulphate	48.0
Cardiolipin	37.0
Native chromatin	7.9
ROS modified chromatin	12.5

The microtitre plates were coated with modified DNA (2.5 μg/ml).

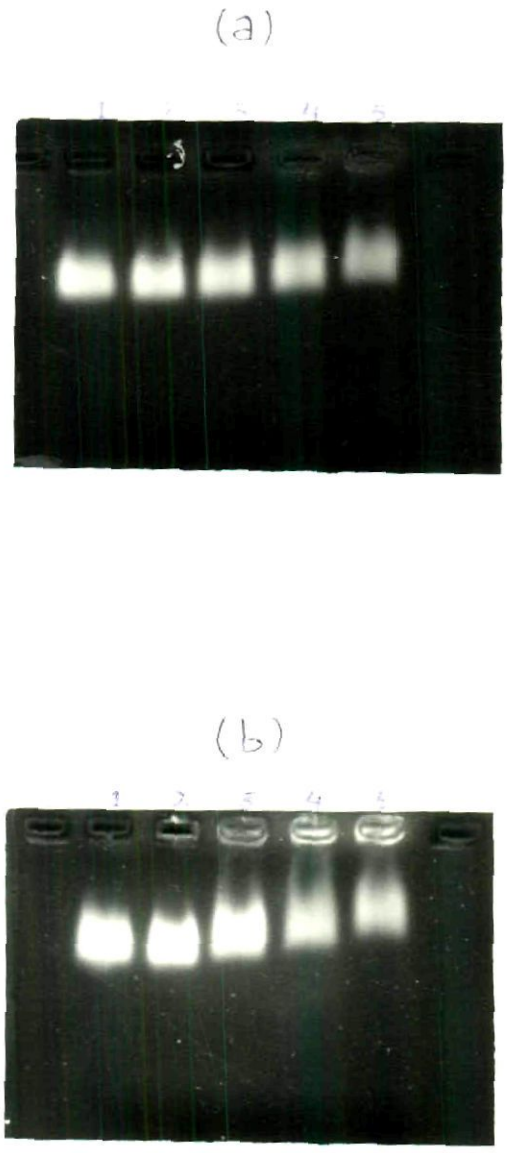

Fig. 23. Band shift assay of anti-$^1O_2$-$O_2^{\bullet-}$-DNA IgG binding to (a) native DNA and (b) $^1O_2$-$O_2^{\bullet-}$-DNA. Native DNA and $^1O_2$-$O_2^{\bullet-}$-DNA (1μg) each were incubated with 10,20,40 and 60 μg of IgG for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose gel for 2 hr at 30 mA. Lane 1, contains native or $^1O_2$-$O_2^{\bullet-}$-DNA, while lanes 2,3,4, and 5 contain native or $^1O_2$-$O_2^{\bullet-}$-DNA with increasing concentrations of IgG.

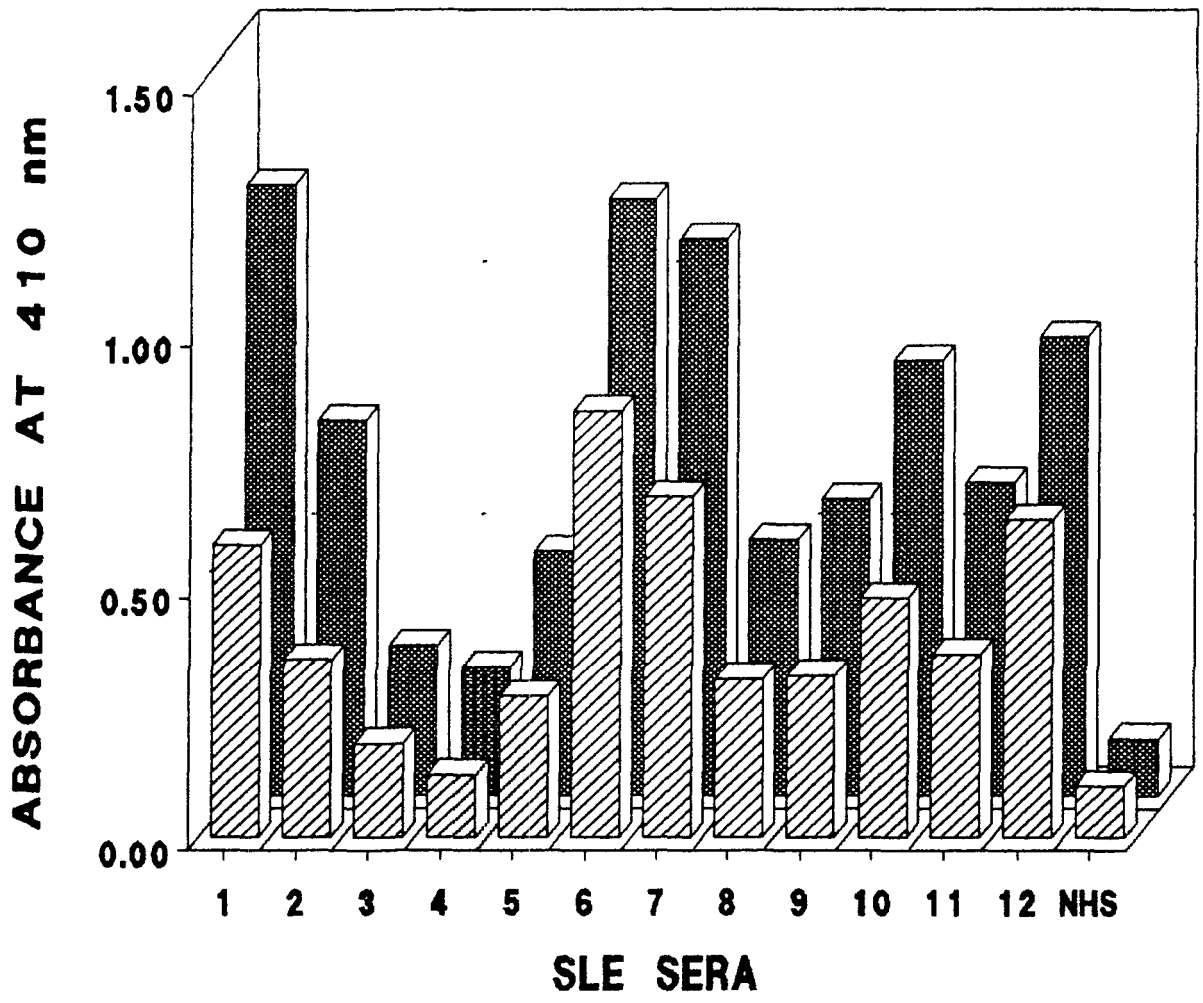

Fig. 24. Direct binding ELISA of SLE sera to native DNA (▨), and $^1O_2$-$O_2^{\bullet}$ -DNA (▩). Normal human sera (NHS) served as negative control. Serum dilution was 1:100.

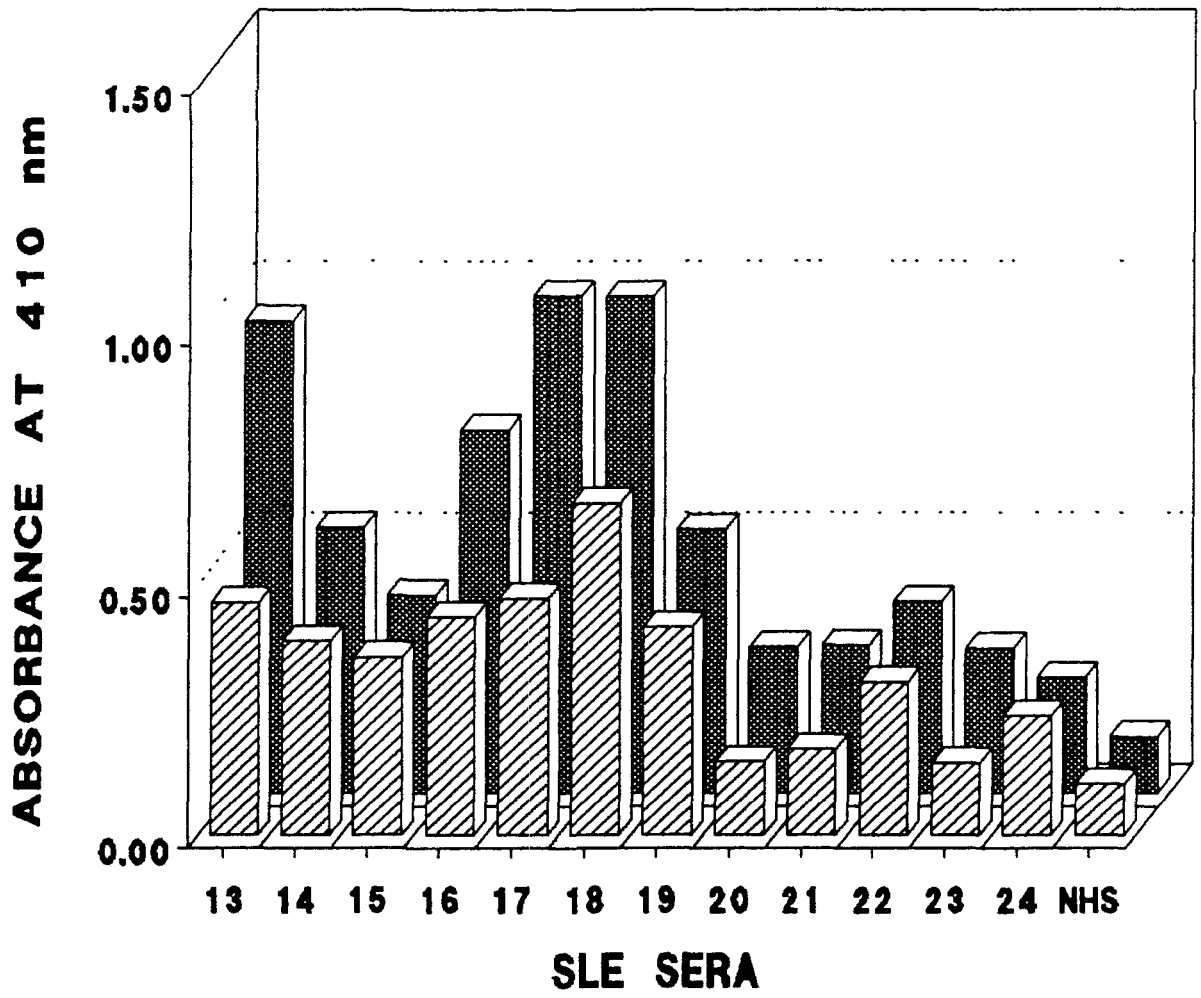

Fig. 25. Direct binding ELISA of SLE sera to native DNA (▨), and $^1O_2$-$O_2^{\bullet -}$-DNA (▩). Normal human sera (NHS) served as negative control. Serum dilution was 1:100.

Antigenic specificity of SLE autoantibodies

Competition ELISA was also performed to further evaluate the specificity of each SLE sera for native and $^1O_2$-$O_2^{\bullet-}$-DNA (Figs 26-37). The antibody was incubated with increasing concentrations (0-20 μg/ml) of inhibitors (native or $^1O_2$-$O_2^{\bullet-}$-DNA). Out of 24 SLE sera tested $^1O_2$-$O_2^{\bullet-}$-DNA showed higher degree of recognition by 17 sera and inhibited their activity to a maximum of 77%. On the other hand native DNA was found to be less reactive for the same samples showing a maximum inhibition of 50%. However, one sample showed greater recognition towards native DNA as compared to $^1O_2$-$O_2^{\bullet-}$-DNA. The results of this study, summarised in Table 6 shows $^1O_2$-$O_2^{\bullet-}$-DNA as a better inhibitor for 23 SLE sera.

Purification of SLE and cancer sera IgG

SLE and cancer sera IgG were purified by affinity chromatography on Protein- A Agarose. Protein A has been known to bind IgG from most of the mammalian species. The purified IgG eluted in a single symmetrical peak (Fig. 38). Their purity was checked by SDS-PAGE under non-reducing conditions which resulted in a single band (Fig. 38 inset).

Band shift assay

Band shift assay was employed to visualize the interaction of native and $^1O_2$-$O_2^{\bullet-}$-DNA with SLE IgG. A constant amount (1 μg) of antigen was incubated with varying amounts of SLE IgG (0-80 μg) for 2 hr at 37°C and overnight at 4°C. These immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA. An increase in the IgG concentration caused an increase in the immune complex formation which resulted in a relative increase in the molecular weight and consequently retarded mobility (Fig. 39).

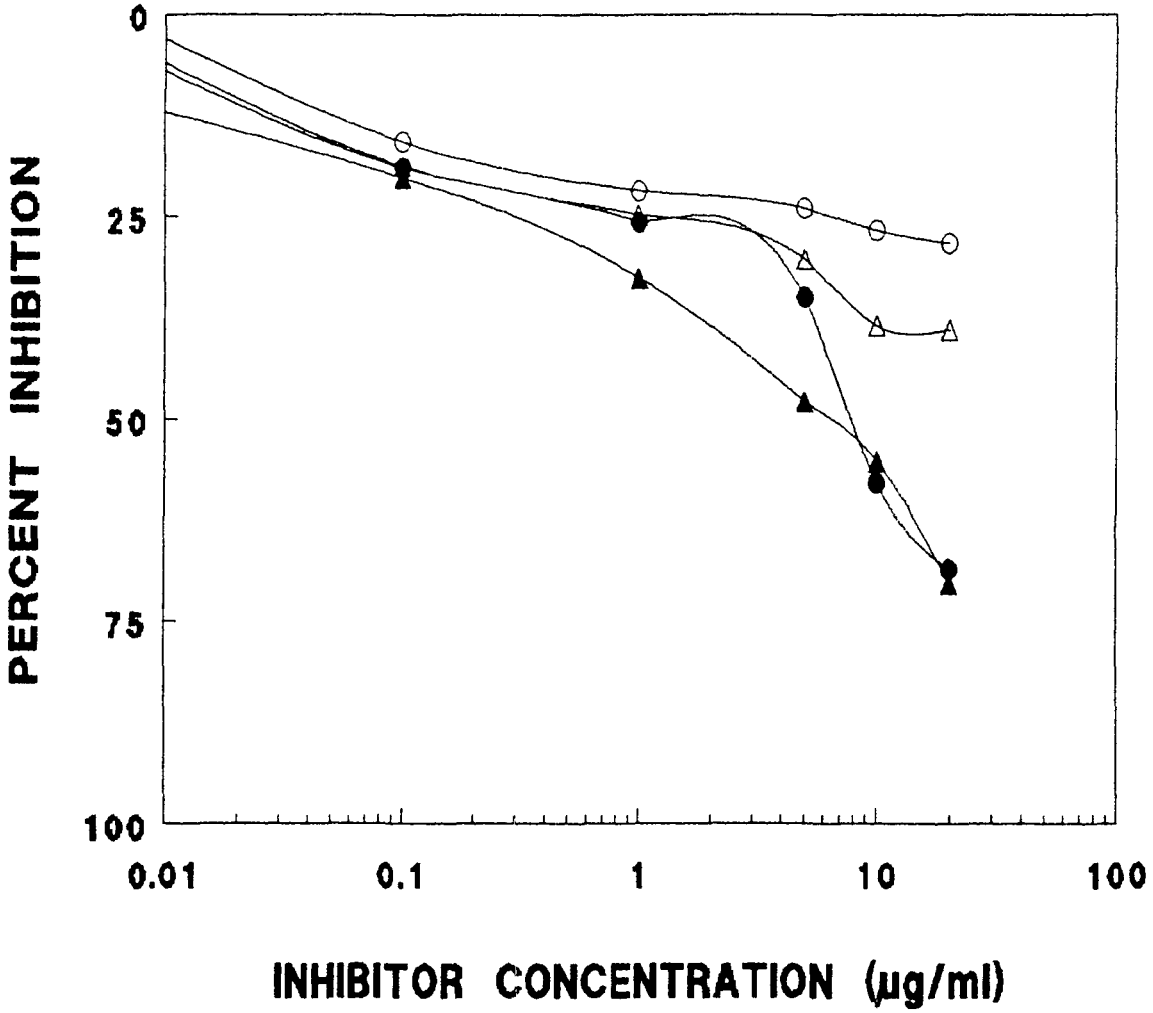

Fig. 26. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (1 and 2). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

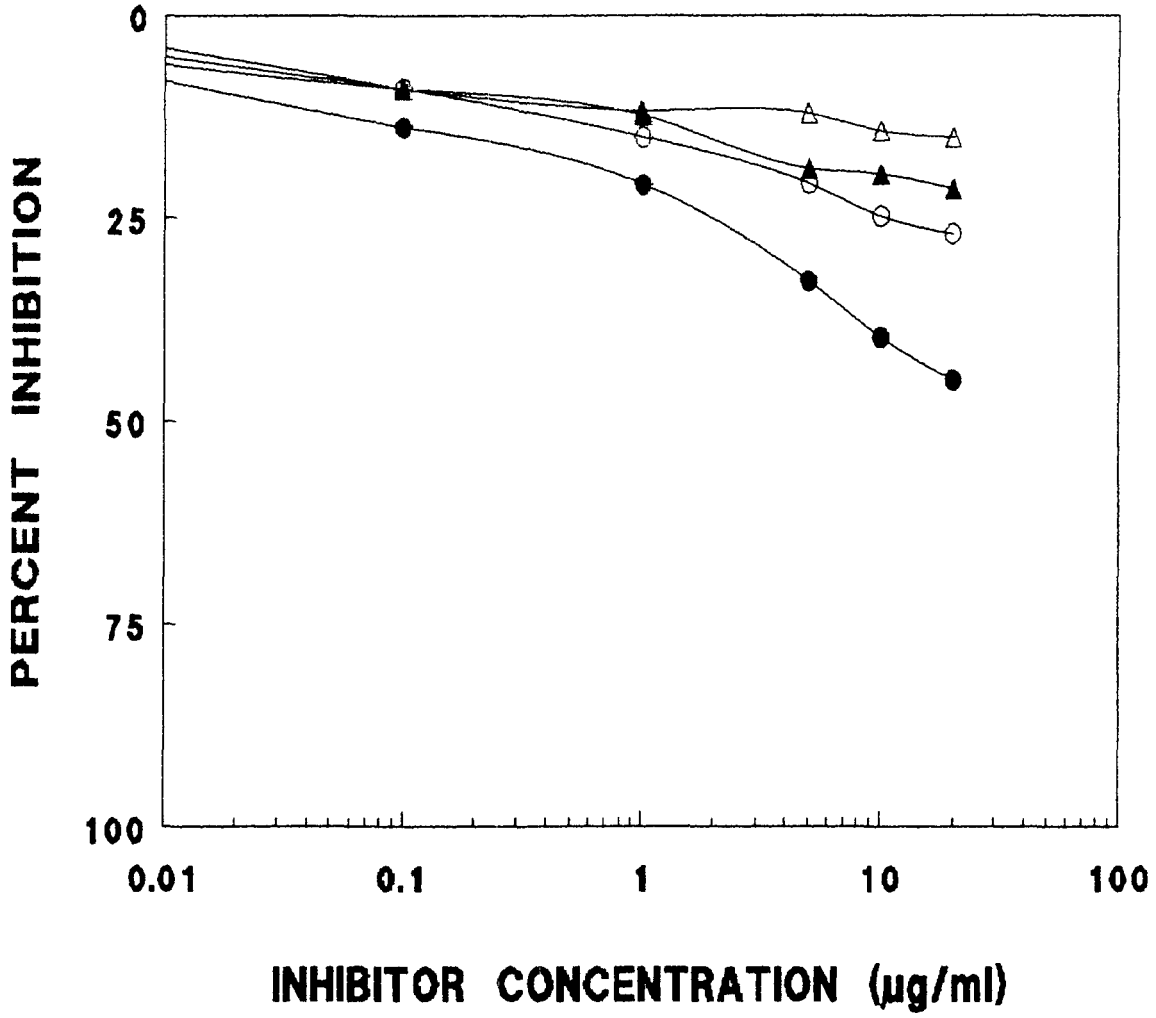

Fig. 27. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (3 and 4). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

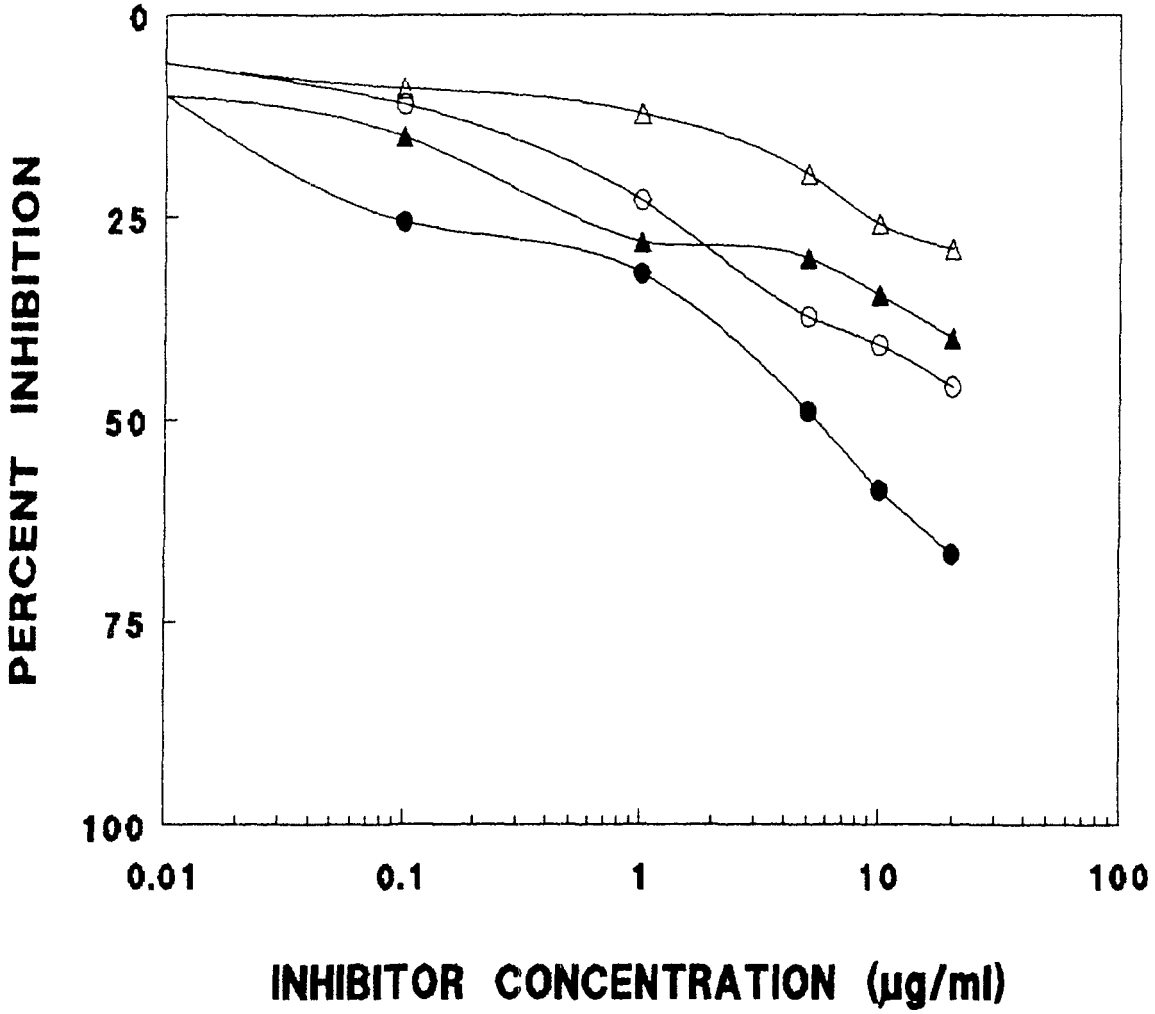

Fig. 28. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (5 and 6). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

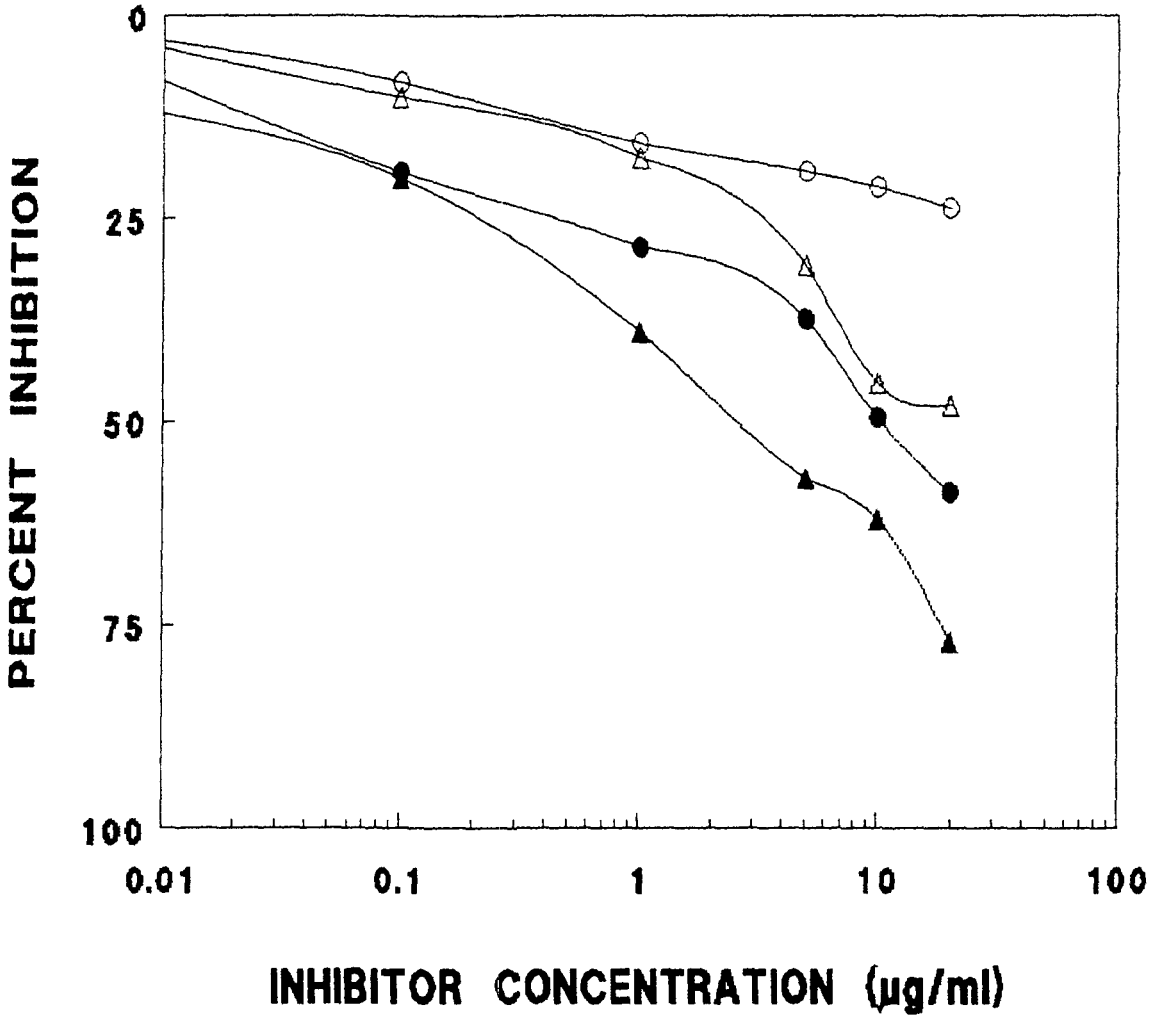

Fig. 29. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (7 and 8). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\cdot-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\cdot-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

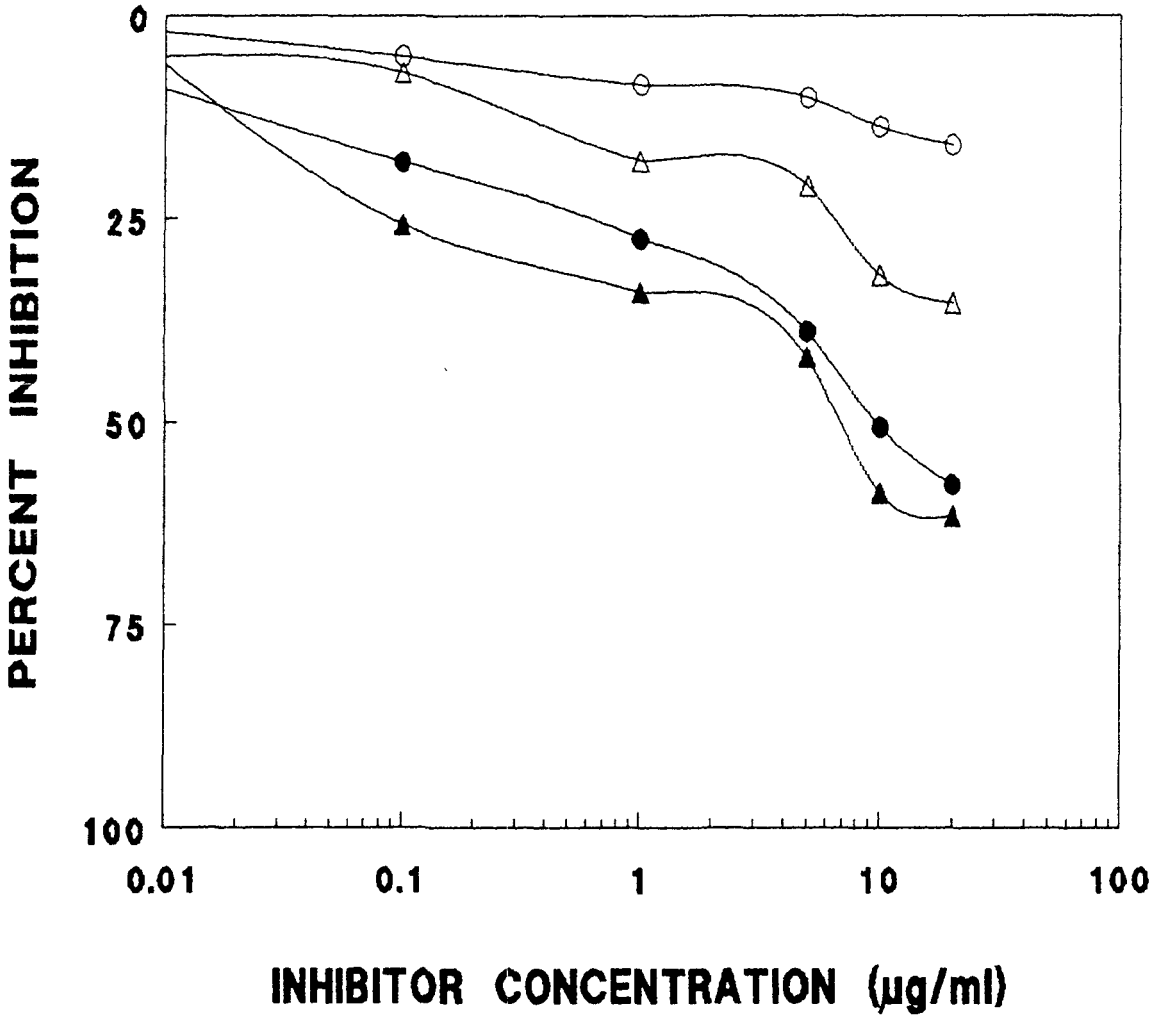

Fig. 30. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (9 and 10). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

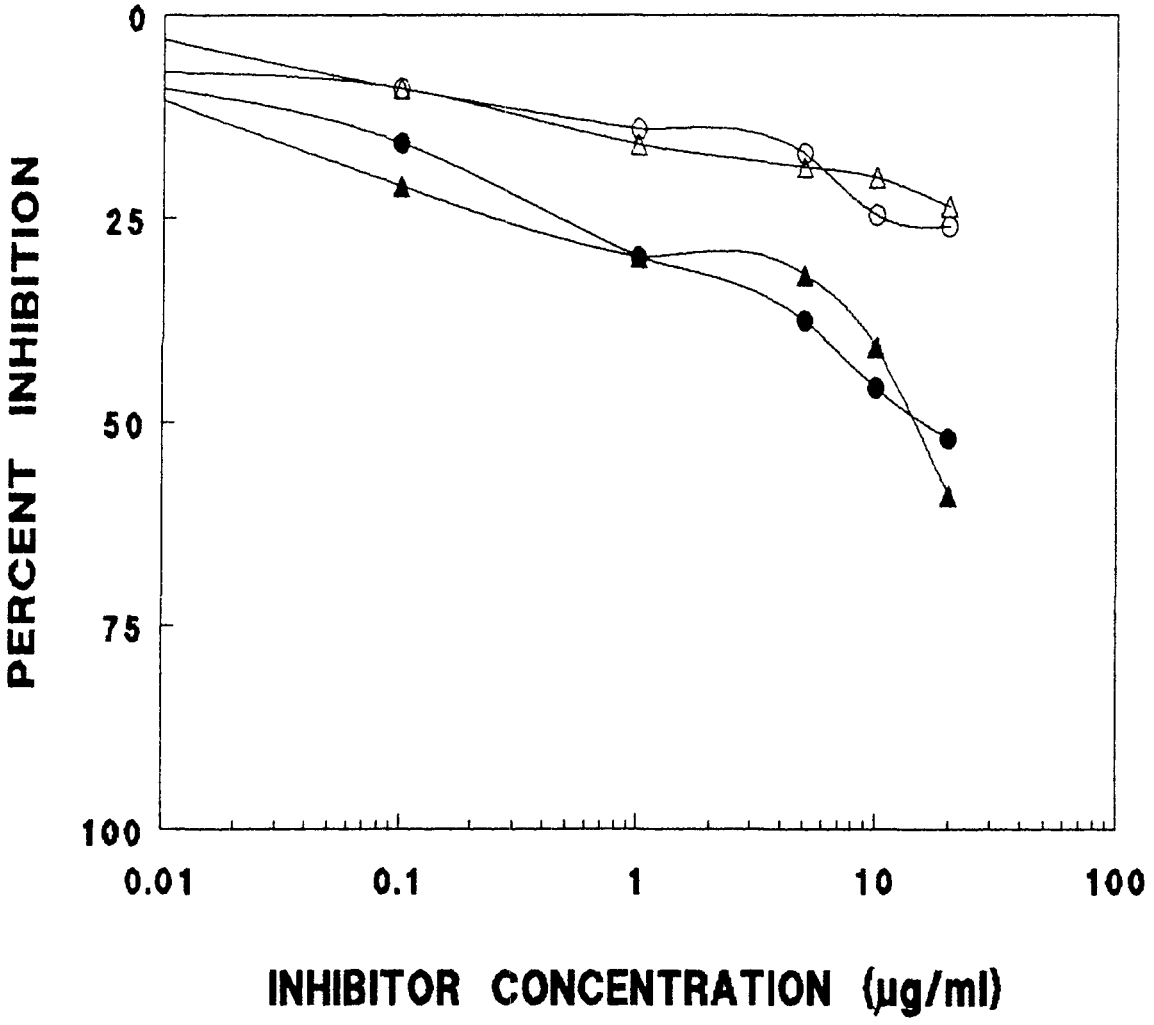

Fig. 31. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (11 and 12). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet -}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet -}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

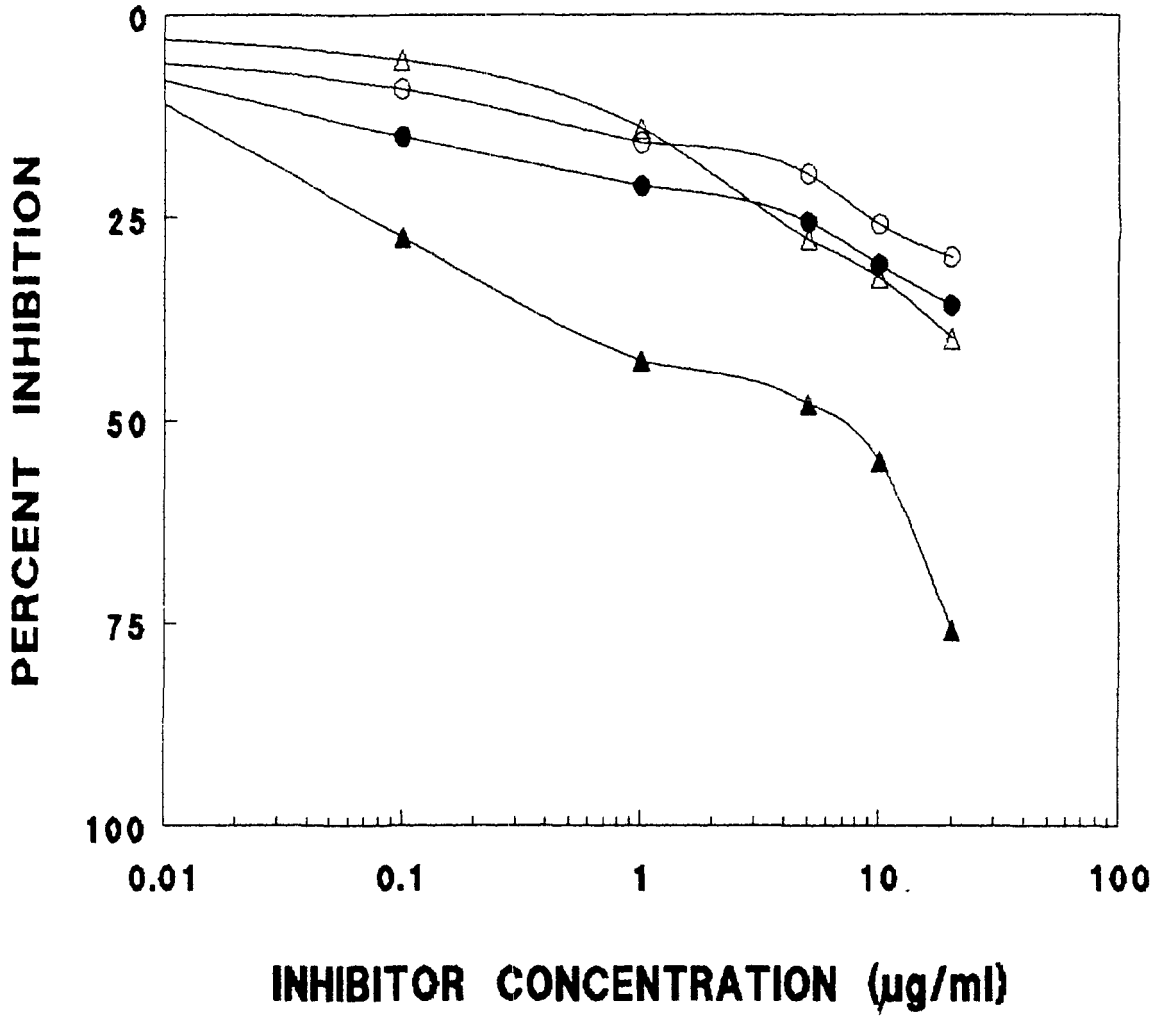

Fig. 32. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (13 and 14). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

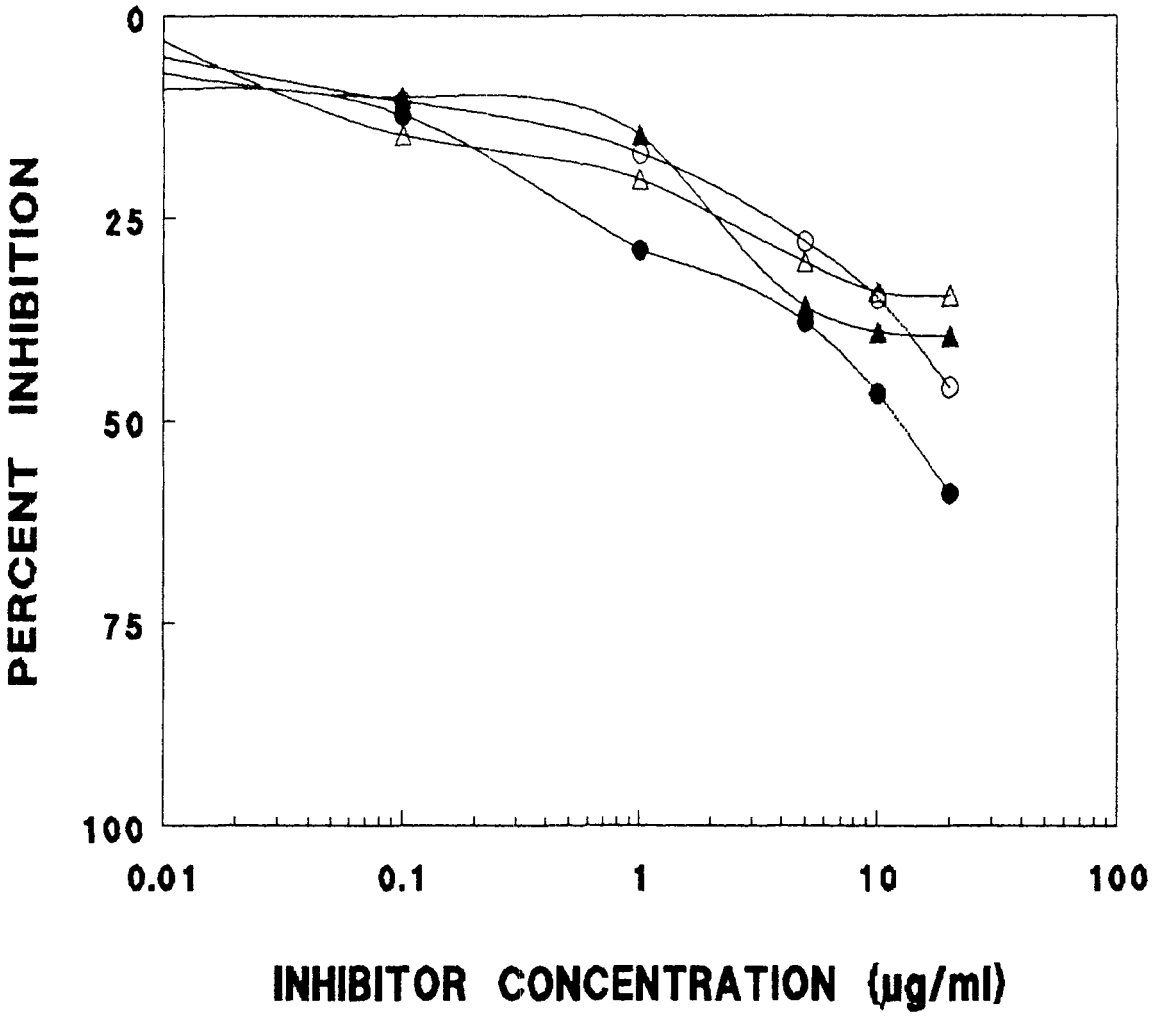

Fig. 33. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (15 and 16). The inhibitors used were native DNA (△,○), and $^1O_2$-$\dot{O}_2^-$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$\dot{O}_2^-$-DNA (2.5 µg/ml) and serum dilution was 1:100.

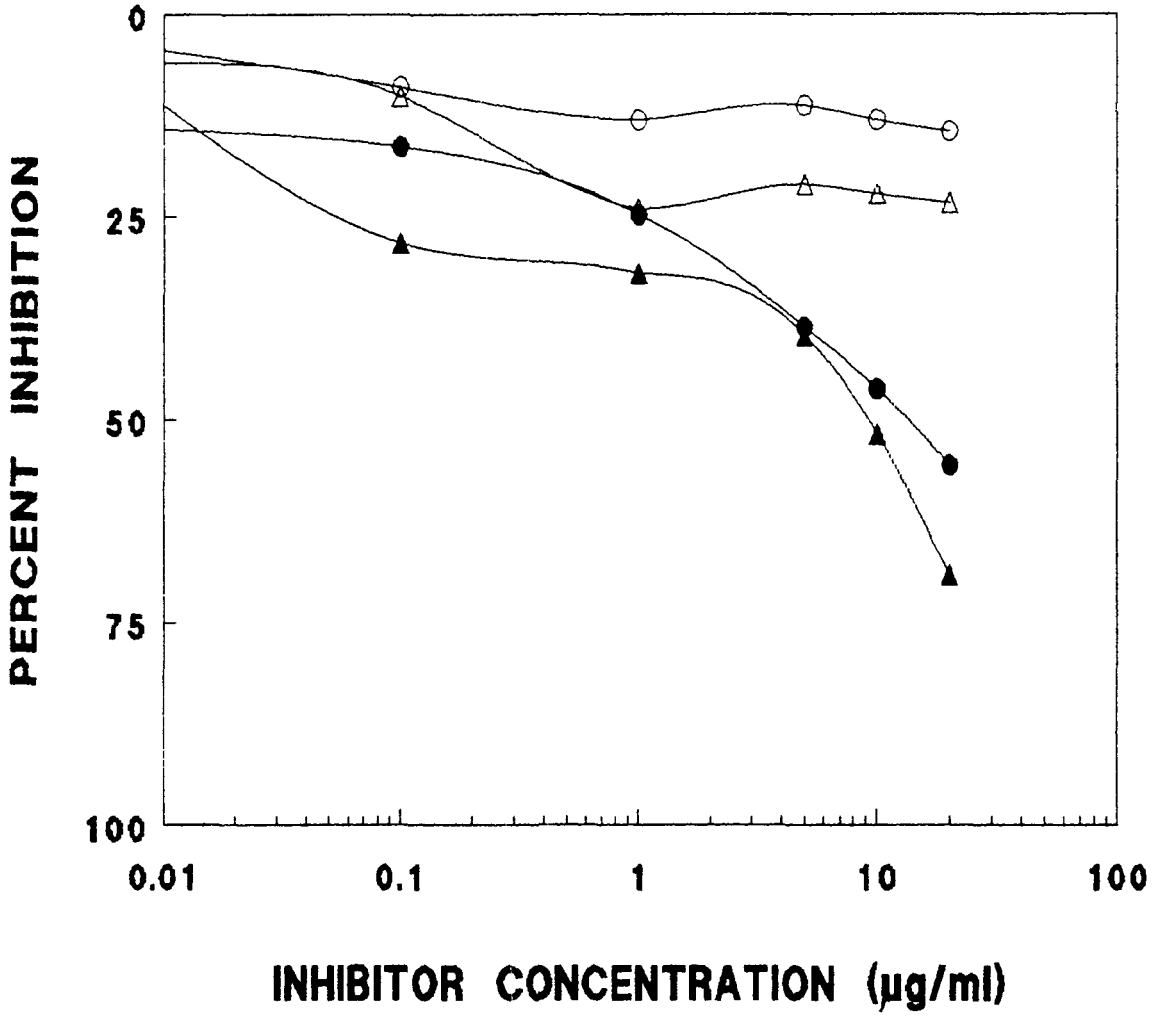

Fig. 34. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (17 and 18). The inhibitors used were native DNA (△,○), and $^1O_2$-$\dot{O}_2^-$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$\dot{O}_2^-$-DNA (2.5 µg/ml) and serum dilution was 1:100.

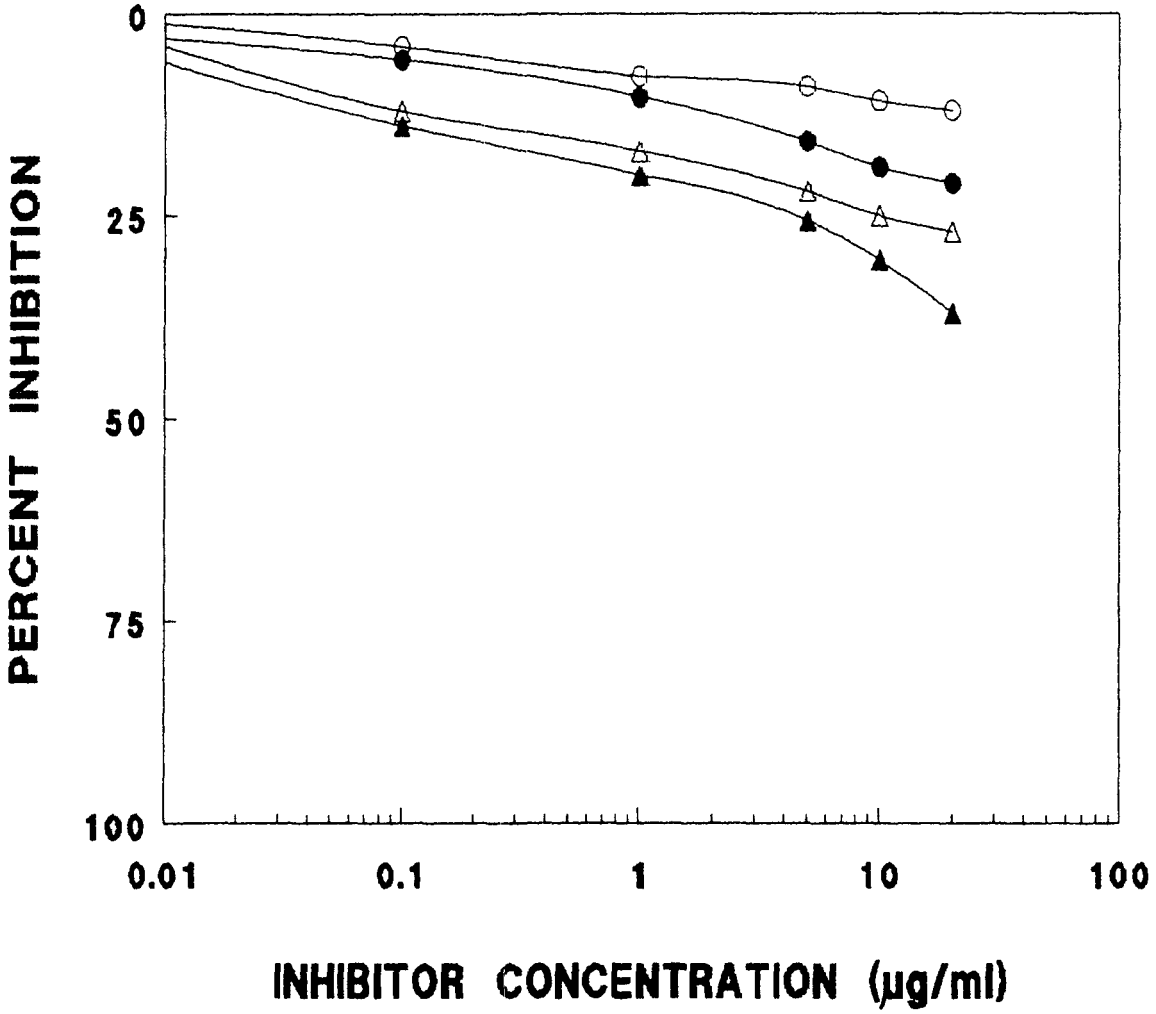

Fig. 35. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (19 and 20). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

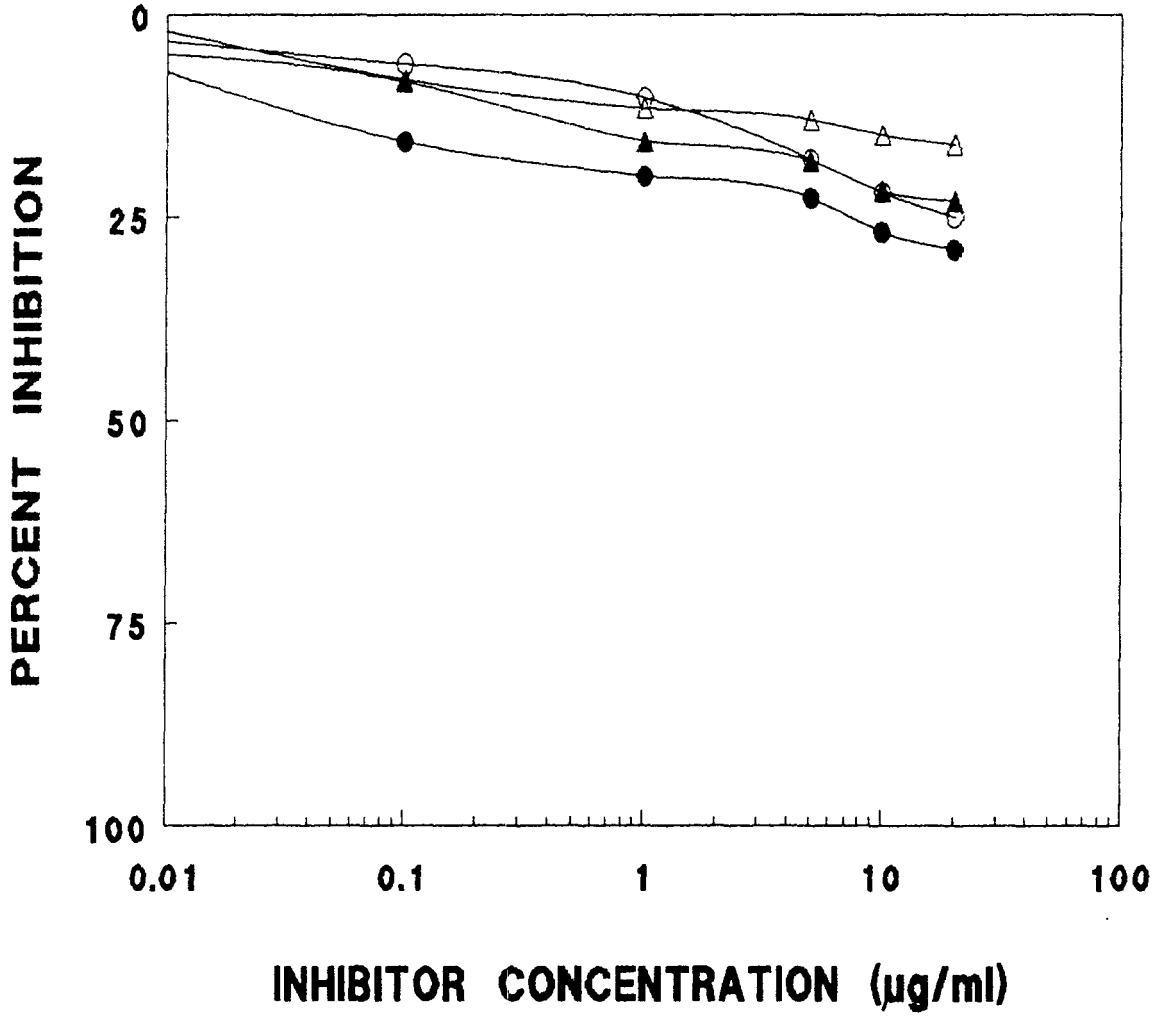

Fig. 36. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (21 and 22). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

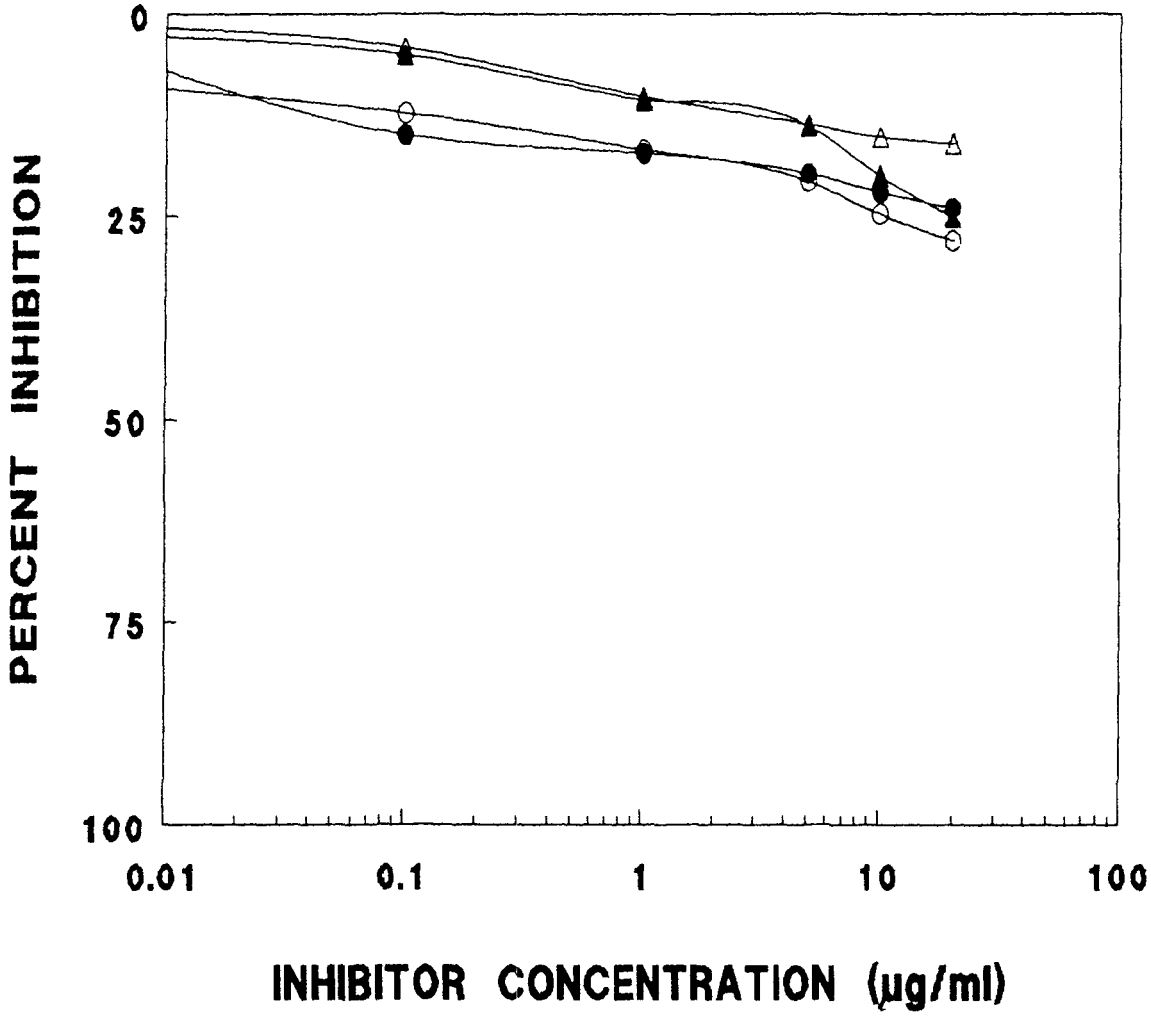

Fig. 37. Inhibition ELISA of SLE anti-DNA autoantibodies from SLE sera (23 and 24). The inhibitors used were native DNA (△,○), and $^1O_2$-$O_2^{\bullet -}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet -}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

TABLE – 6

Competitive inhibition data of SLE sera

Sera no.	Maximum percent inhibition at (20 μg/ml)	
	Native DNA	$^1O_2$-$O_2^{\bullet -}$-DNA
1	39.0	70.1
2	28.3	68.6
3	15.0	21.4
4	27.0	45.0
5	29.0	40.0
6	46.0	66.5
7	48.0	77.0
8	23.7	58.7
9	35.4	61.6
10	16.0	57.7
11	23.5	59.0
12	26.0	52.0
13	50.0	75.9
14	30.0	41.5
15	34.7	39.7
16	46.0	59.0
17	23.3	69.1
18	14.4	55.6
19	27.0	37.0
20	12.0	21.0
21	16.0	23.0
22	25.0	29.0
23	16.0	25.0
24	28.0	24.0

Microtitre plates were coated with $^1O_2$-$O_2^{\bullet -}$-DNA (2.5 μg/ml).

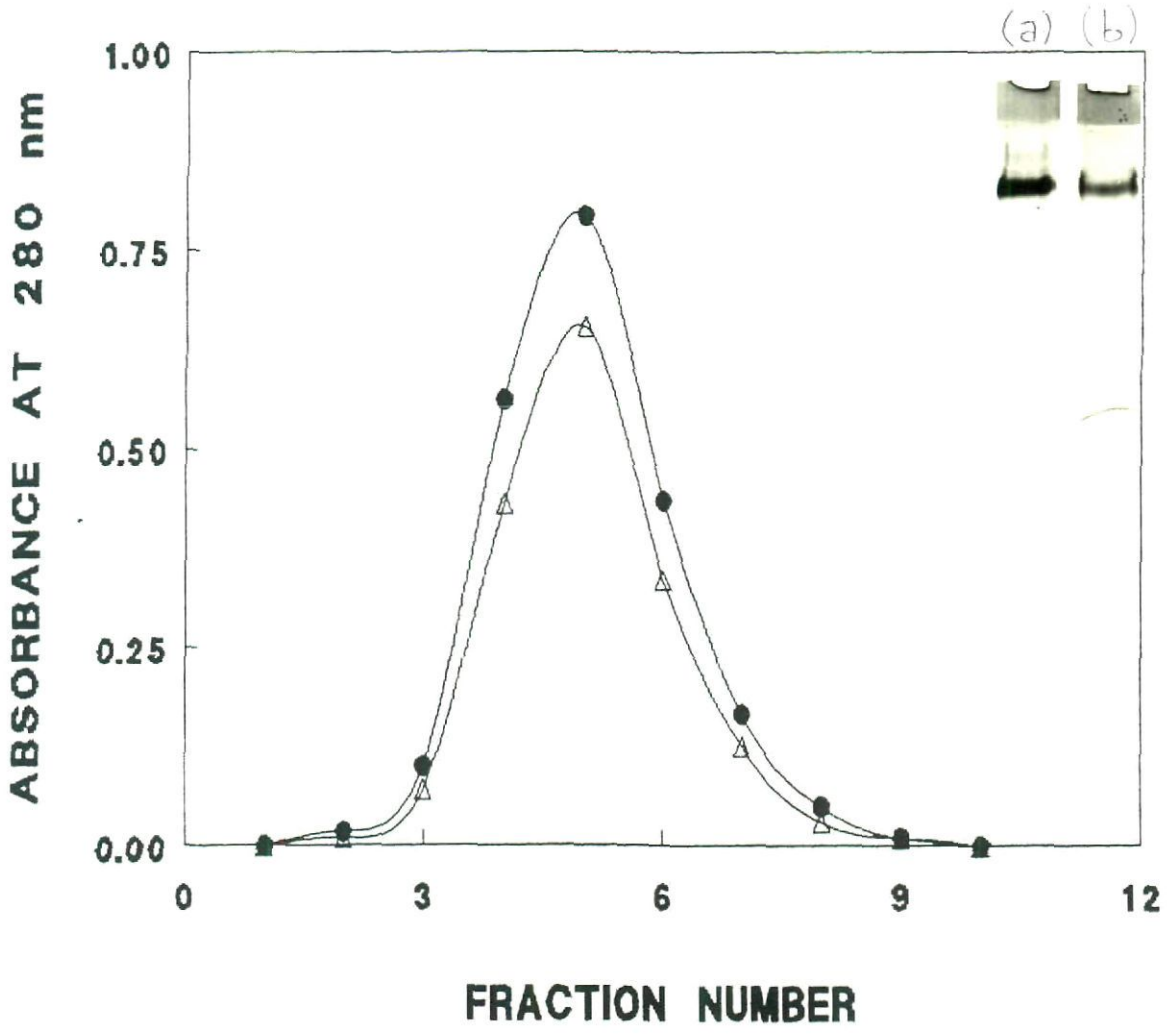

Fig. 38. Elution profile of SLE (●) and lung cancer IgG (△) on Protein–A Agarose column. **Inset**: Purity of **(a)** isolated SLE IgG and **(b)** cancer IgG on 7.5% SDS-PAGE.

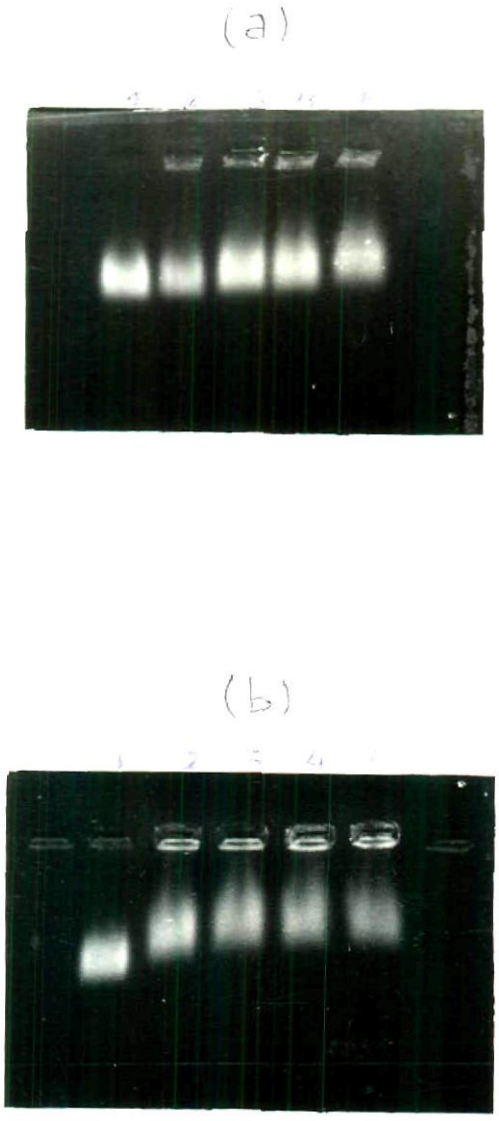

Fig. 39. Band shift assay of SLE IgG binding to (a) native DNA and (b) $^1O_2$-$O_2^{\bullet -}$-DNA. Native DNA and $^1O_2$-$O_2^{\bullet -}$-DNA (1μg) each were incubated with 10,20,40 and 80 μg of IgG for 2 hr at 37°C and overnight at 4°C. Electrophoresis was carried out on 1% agarose gel for 2 hr at 30 mA. Lane 1 contains native or $^1O_2$-$O_2^{\bullet -}$-DNA, while lanes 2,3,4, and 5 contain native or $^1O_2$-$O_2^{\bullet -}$-DNA with increasing concentrations of SLE IgG.

Recognition of native and modified antigens by cancer antibodies

The study comprised 30 sera from patients suffering from cancer of different organ and organ systems. Sera from normal, healthy individuals served as control. Cancer sera were obtained after careful clinical examination of patients with histopathological diagnosis attending J.N. Medical College Hospital, A.M.U., Aligarh. The binding of circulating antibodies in cancer sera (at 1:100 serum dilution) with native and $^1O_2$-$O_2^{\cdot-}$-DNA was assessed by direct binding ELISA. All the sera tested showed higher recognition to $^1O_2$-$O_2^{\cdot-}$-DNA (Fig. 40). The binding specificity of antibodies in cancer sera were analysed by competitive binding assay (Table 7). The samples contained 5 sera of lung cancer. All the sera showed greater recognition of $^1O_2$-$O_2^{\cdot-}$-DNA than native DNA. The maximum inhibition observed was 60.8%, 61.3%, 65.3%, 66.8% and 70.7% with $^1O_2$-$O_2^{\cdot-}$-DNA (Fig. 41). The results indicated higher binding of antibodies in lung cancer sera towards $^1O_2$-$O_2^{\cdot-}$-DNA than native DNA.

Five sera were collected from patients with cancer of urinary bladder. All five sera showed higher inhibition of 59.3%, 52.6%, 55.4%, 55.4%, 68.75% with $^1O_2$-$O_2^{\cdot-}$-DNA over native DNA (Fig. 42). Four sera were from patients suffering from breast cancer. All sera showed greater inhibition with $^1O_2$-$O_2^{\cdot-}$-DNA 59%, 46%, 50.7%, 53.6%, where as, with native DNA all four sera showed inhibition below 40% (Fig. 43).

Among four sera from cancer of head and neck, two sera showed higher inhibition of 65% and 67.2% with $^1O_2$-$O_2^{\cdot-}$-DNA while two sera showed a moderate inhibition of 45% and 48.5% (Fig. 44).

Four sera were collected from patients suffering from oral carcinoma. Among them, two sera showed 69.6% and 65.6% inhibition with $^1O_2$-$O_2^{\cdot-}$-DNA and one sera showed greater inhibition with native DNA 46% and 40% inhibition with $^1O_2$-$O_2^{\cdot-}$-DNA while one sera showed moderate inhibition with $^1O_2$-$O_2^{\cdot-}$-DNA 48% (Fig. 45).

Four sera from gall bladder cancer showed 39.66%, 42.8%, 45% and 41% inhibition with $^1O_2$-$O_2^{\cdot-}$-DNA and 20.8%, 25.6%, 27% and 28% inhibition with native DNA (Fig. 46).

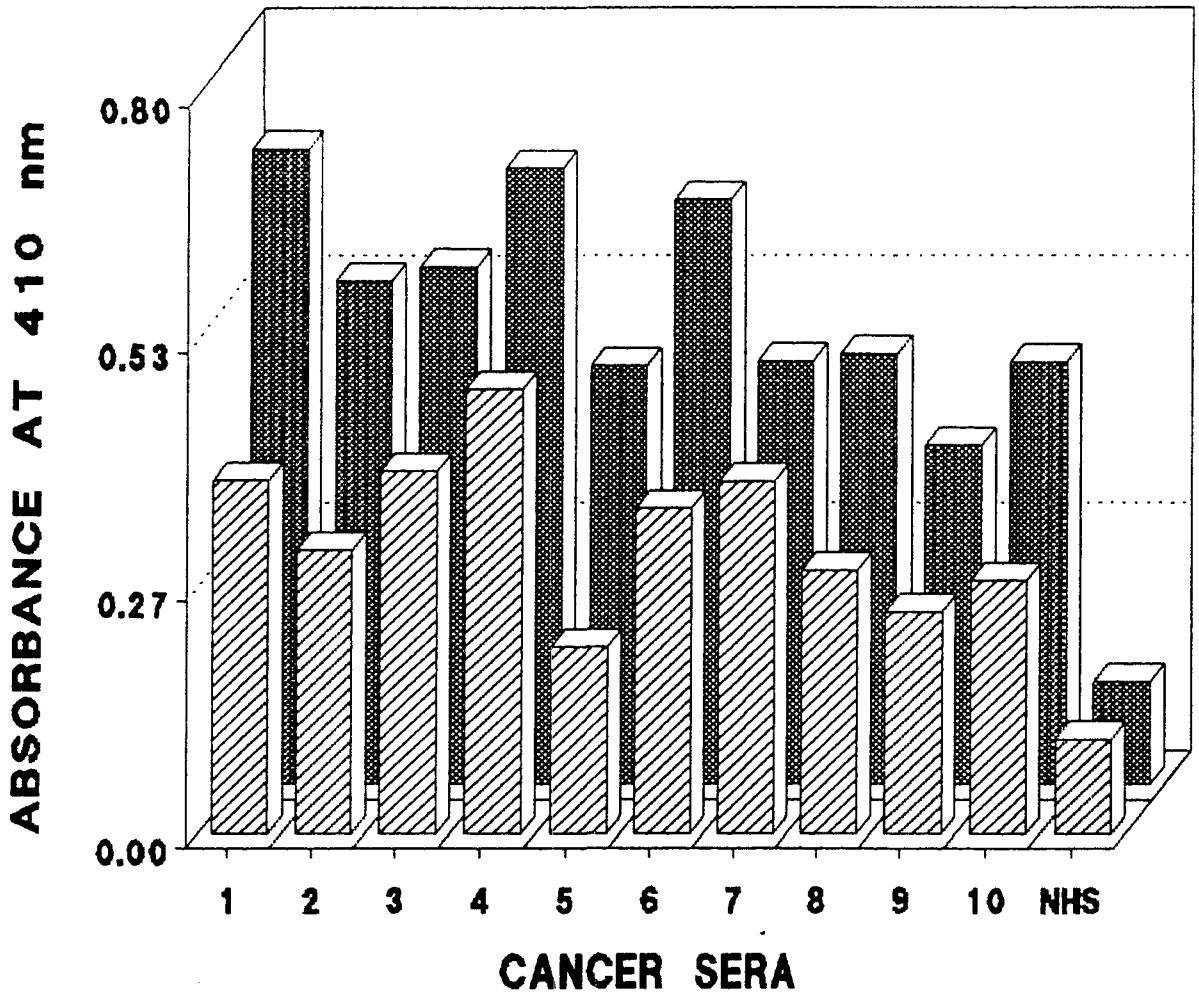

Fig. 40. Binding of various cancer sera to native (▨) and $^1O_2$-$O_2^{\bullet-}$-DNA (▩). Normal human sera (NHS) serve as negative control. The histograms show mean absorbance values for binding of NHS and sera from patients with cancer of lung (1), urinary bladder (2), breast (3), head and neck (4), oral (5), gall bladder (6), prostrate (7), vulva (8), CML (9), Hodgkin's (10).

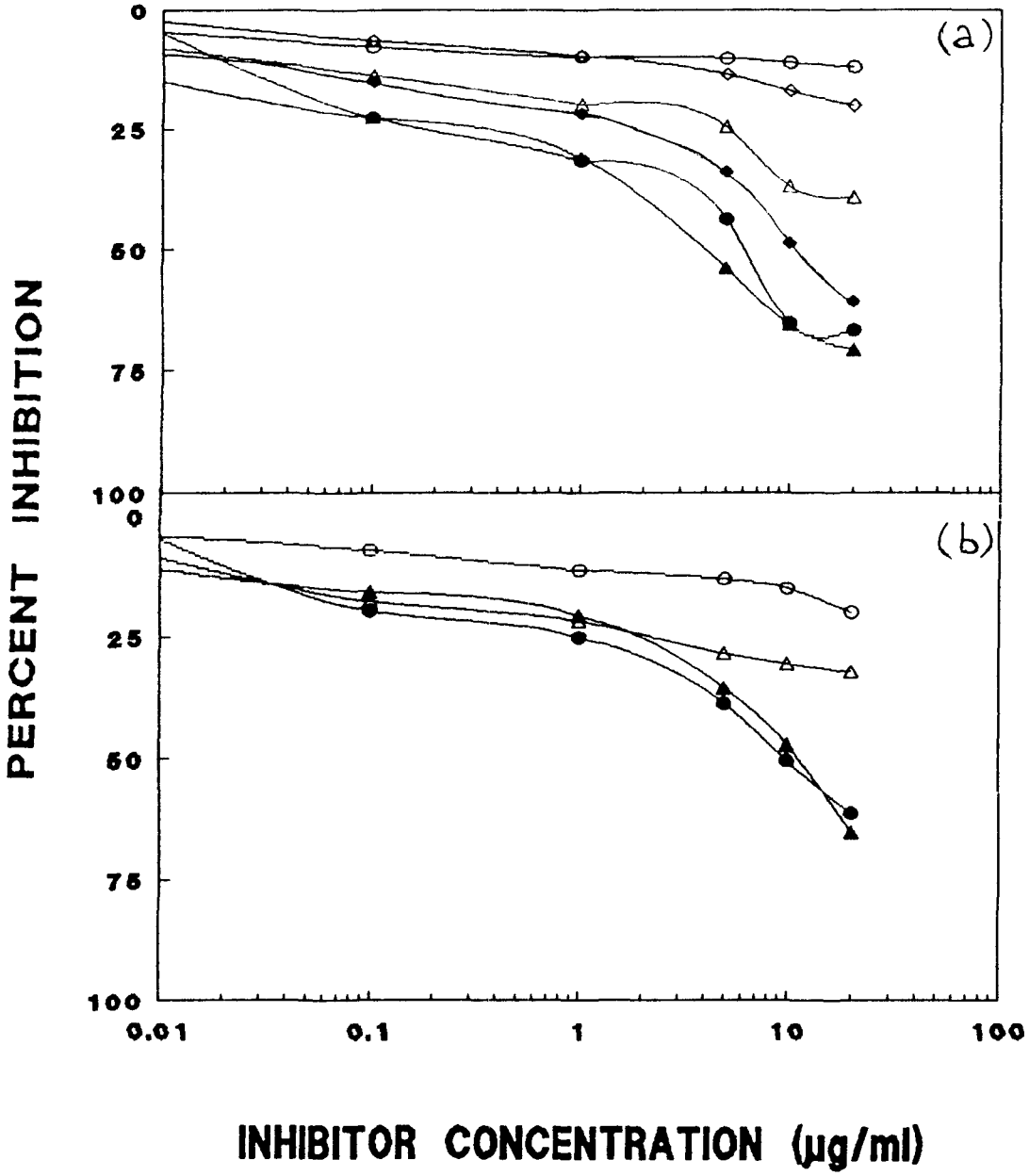

Fig. 41. Detection of autoantibodies against native and $^1O_2$-$\dot{O}_2^-$-DNA, in the sera of patients with cancer of lung. (a) Cancer sera 1,2 and 3 by native DNA (△,○,◇), and $^1O_2$-$\dot{O}_2^-$-DNA (▲,●,◆). (b) Cancer sera 4 and 5 by native DNA (△,○) and $^1O_2$-$\dot{O}_2^-$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$\dot{O}_2^-$-DNA (2.5 µg/ml) and serum dilution was 1:100.

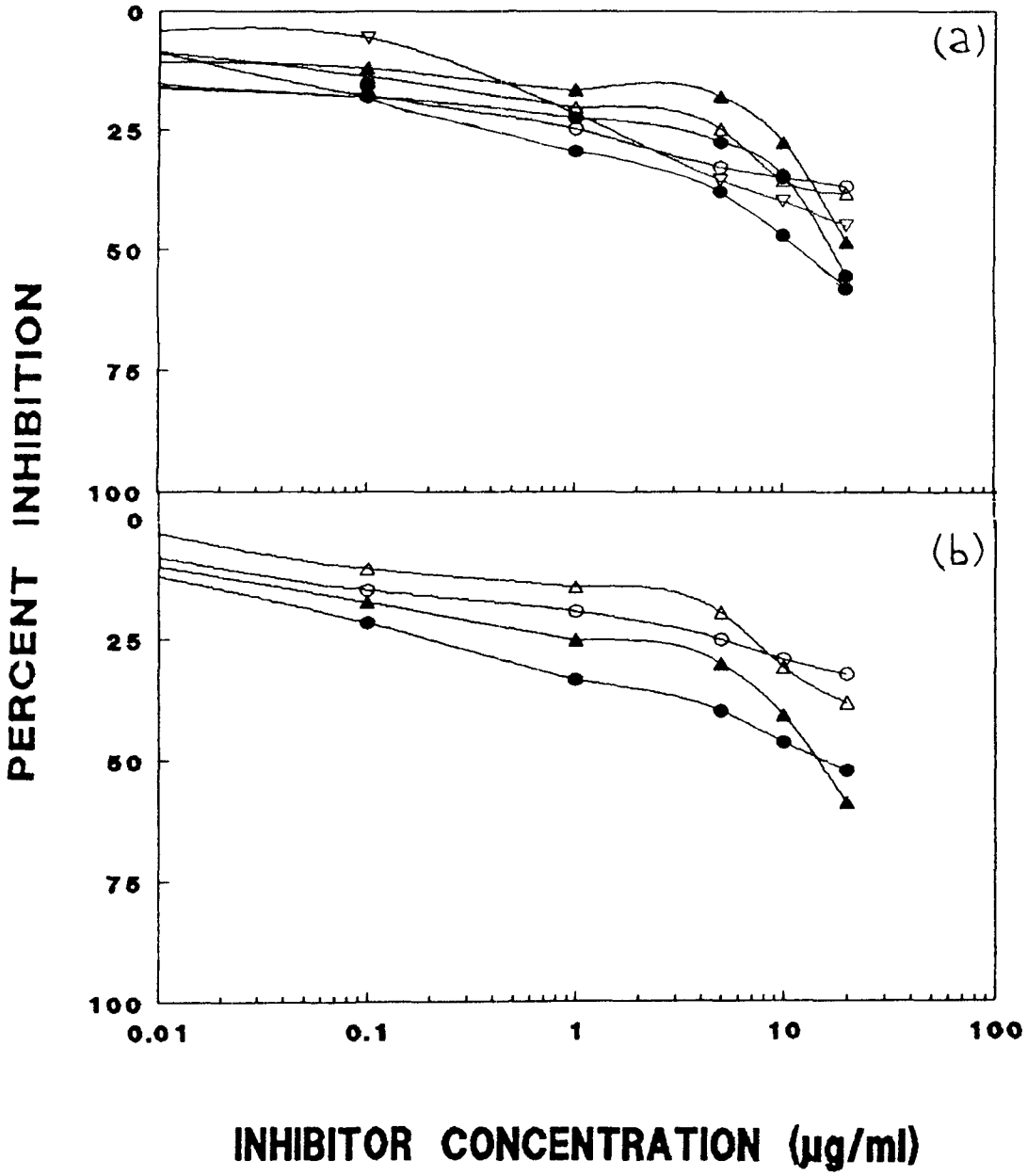

Fig. 42. Detection of autoantibodies against native and $^1O_2$-$O_2^{\cdot-}$-DNA, in the sera of patients with cancer of urinary bladder. (a) Cancer sera 1,2 and 3 by native DNA (△,○,▽), and $^1O_2$-$O_2^{\cdot-}$-DNA (▲,●,▼). (b) Cancer sera 4 and 5 by native DNA (△,○) and $^1O_2$-$O_2^{\cdot-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\cdot-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

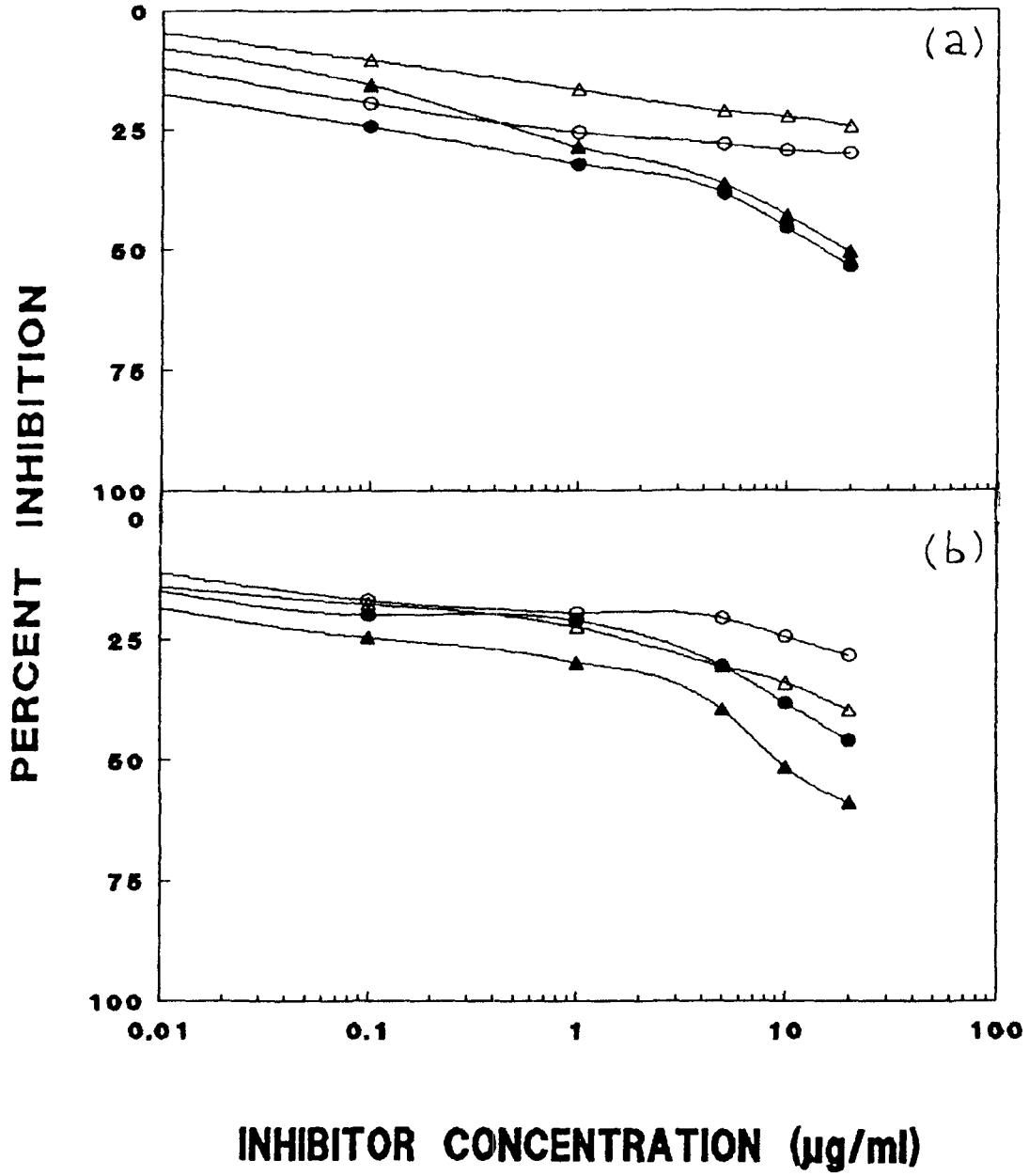

Fig. 43. Detection of autoantibodies against native and $^1O_2$-$O_2^{\bullet-}$-DNA, in the sera of patients with breast cancer. (a) Cancer sera 1 and 2 by native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). (b) Cancer sera 3 and 4 by native DNA (△,○) and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

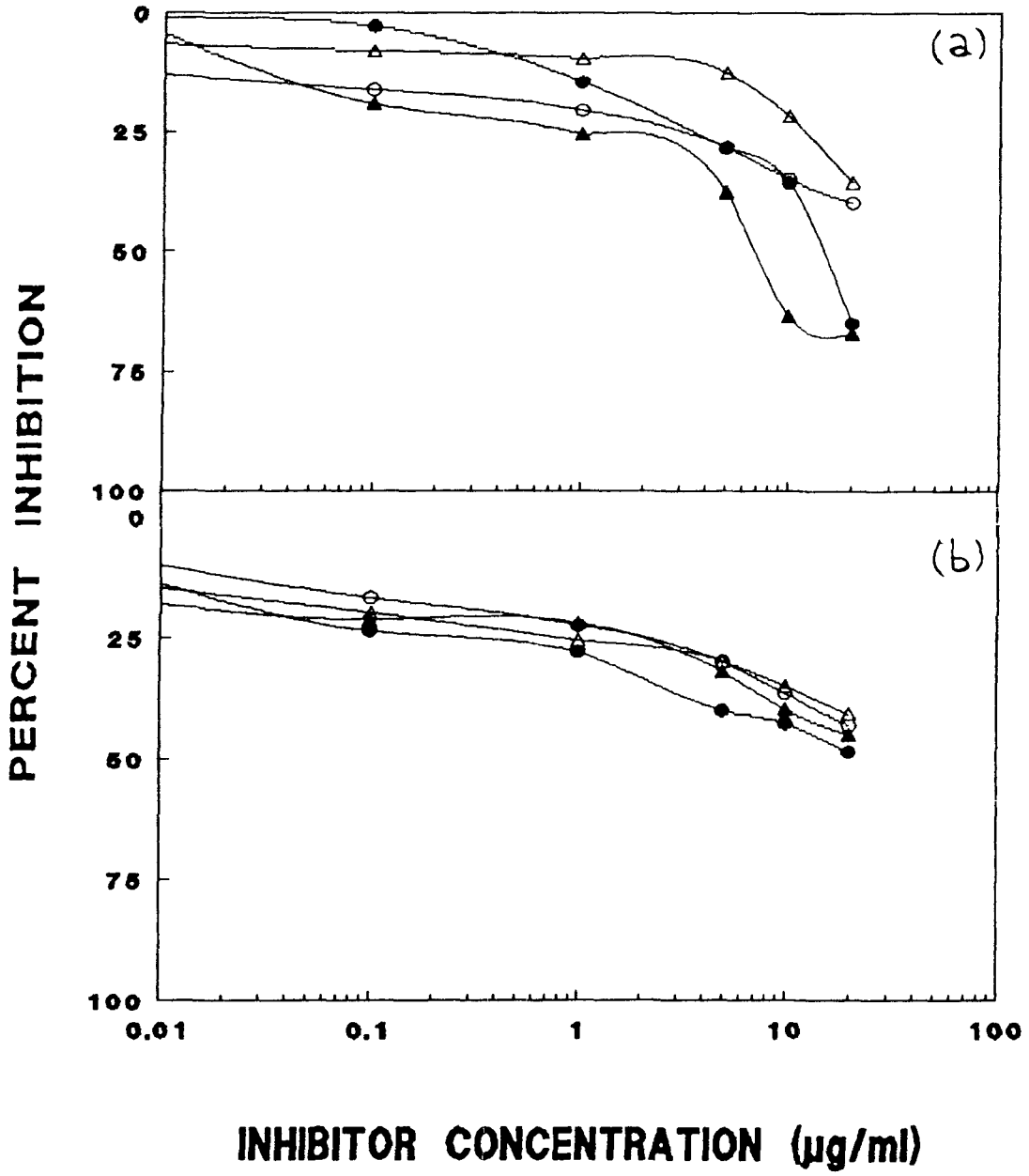

Fig. 44. Detection of autoantibodies against native and $^1O_2$-$O_2^{\bullet-}$-DNA, in the sera of patients with cancer of head and neck. (a) Cancer sera 1 and 2 by native DNA (△,○), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). (b) Cancer sera 3 and 4 by native DNA (△,○) and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

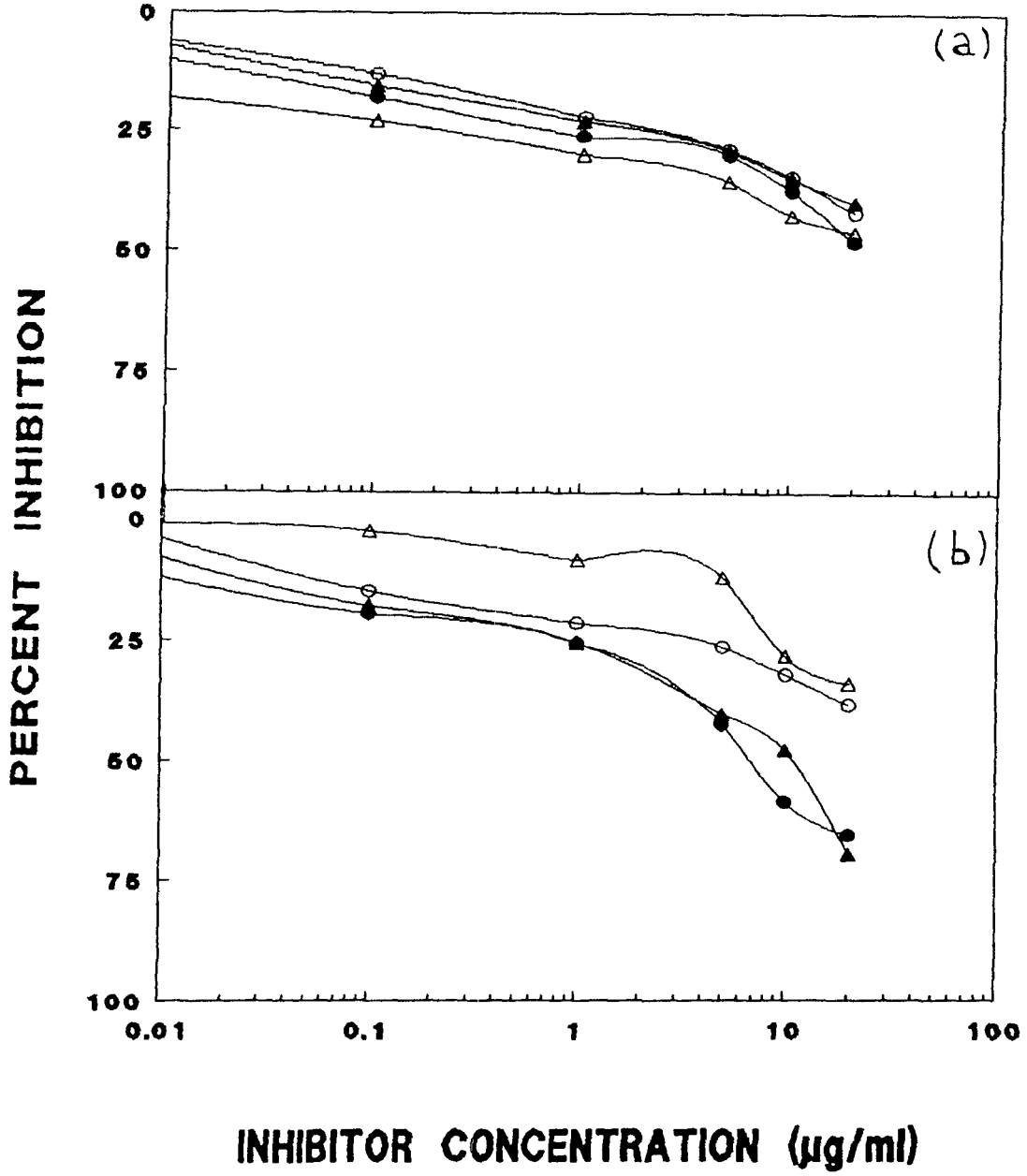

Fig. 45. Detection of autoantibodies against native and $^1O_2$-$O_2^{\bullet-}$-DNA, in the sera of patients with oral cancer. (a) Cancer sera 1 and 2 by native DNA (△,O), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). (b) Cancer sera 3 and 4 by native DNA (△,O) and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

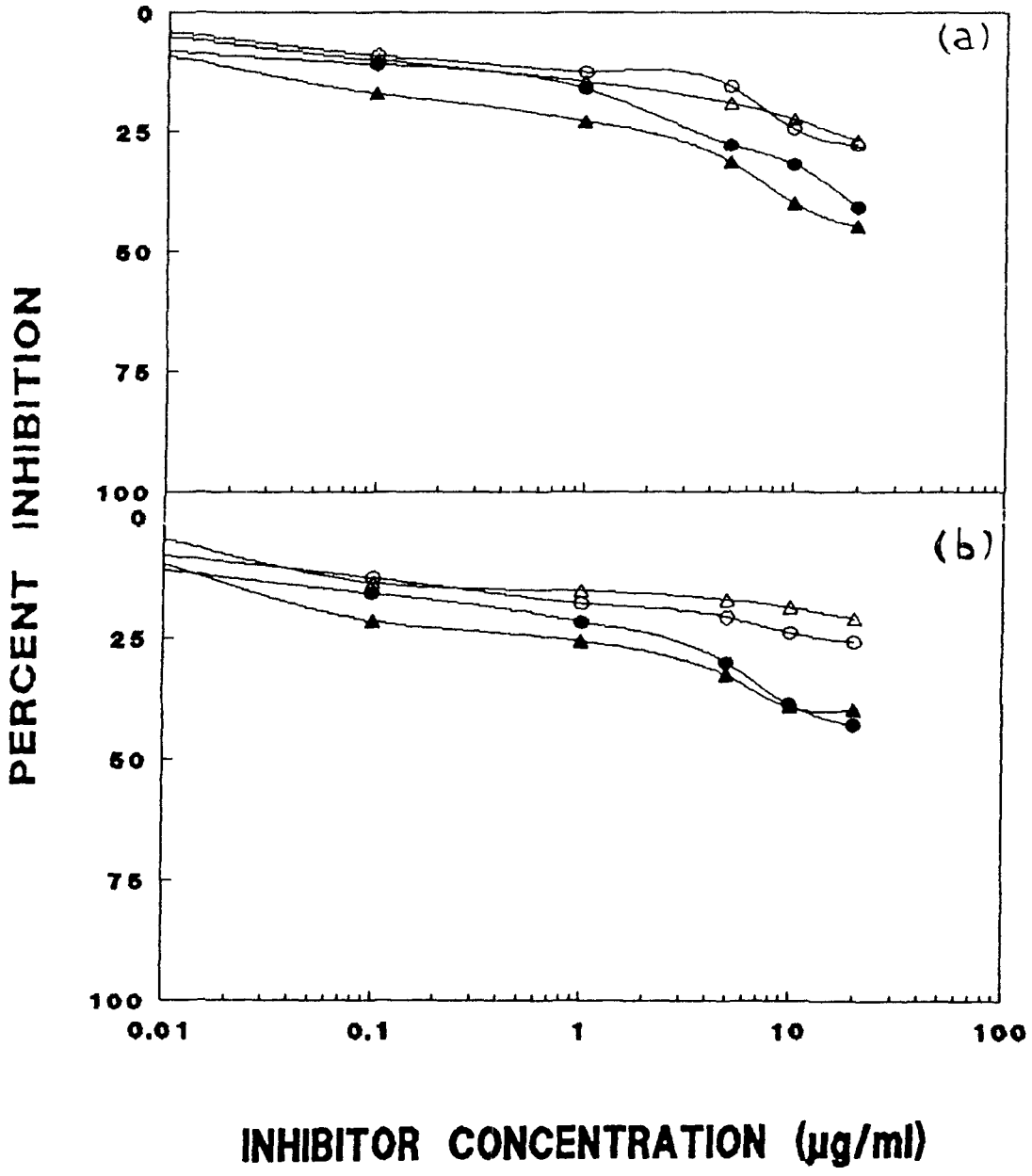

Fig. 46. Detection of autoantibodies against native and $^1O_2$-$O_2^{\cdot-}$-DNA, in the sera of patients with cancer of gall bladder. (a) Cancer sera 1 and 2 by native DNA (△,O), and $^1O_2$-$O_2^{\cdot-}$-DNA (▲,●). (b) Cancer sera 3 and 4 by native DNA (△,O) and $^1O_2$-$O_2^{\cdot-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\cdot-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

Four sera were collected from patients suffering from prostrate cancer. Three sera showed maximum inhibition of 25.8%, 37%, 45.4% with $^1O_2$-$O_2^{\cdot-}$-DNA and one sera showed almost equal percent inhibition (32% and 31%) with native and $^1O_2$-$O_2^{\cdot-}$-DNA (Fig. 47).

One serum sample each from Hodgkin's lymphoma and CML showed higher recognition for $^1O_2$-$O_2^{\cdot-}$-DNA with maximum inhibition of 65% and 45%. Two serum samples from carcinoma of vulva showed higher recognition for $^1O_2$-$O_2^{\cdot-}$-DNA with maximum inhibition of 68% and 54% as compared to native DNA 45% and 35% (Fig. 48). The binding data of cancer sera are shown in Table 7.

Band shift assay

The binding of native and $^1O_2$-$O_2^{\cdot-}$-DNA to lung cancer IgG was detected by band shift assay. Constant amounts of antigen were incubated with varying amounts of cancer IgG for 2 hr at room temperature and overnight at 4°C. The immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA (Fig. 49). The gel visualized under UV light revealed formation of high molecular weight immune complexes which resulted in retarded mobility and decrease in fluorescence intensity of the nucleic acid on agarose gel.

Detection of oxidative lesions in DNA

DNA was isolated from lymphocytes of various cancer patients. The purity and concentration of the DNA preparations were ascertained by A_{260} and A_{280} measurements. DNA isolated from lymphocytes of normal healthy individuals were used as controls for this study.

Anti-$^1O_2$-$O_2^{\cdot-}$-DNA IgG was used as probe to detect oxidative lesions in the DNA from cancer patients. Immune complexes, formed by incubating a fixed amount (60 μg) of anti-$^1O_2$-$O_2^{\cdot-}$-DNA IgG with increasing concentrations (0 to 20 μg/ml) of DNA were coated on microtitre plates already coated with $^1O_2$-$O_2^{\cdot-}$-DNA (2.5 μg/ml).

Two DNA samples isolated from patients with lung cancer (Fig. 50) showed a high inhibition 64% and 68.6% of immune IgG binding to immunogen. Another two

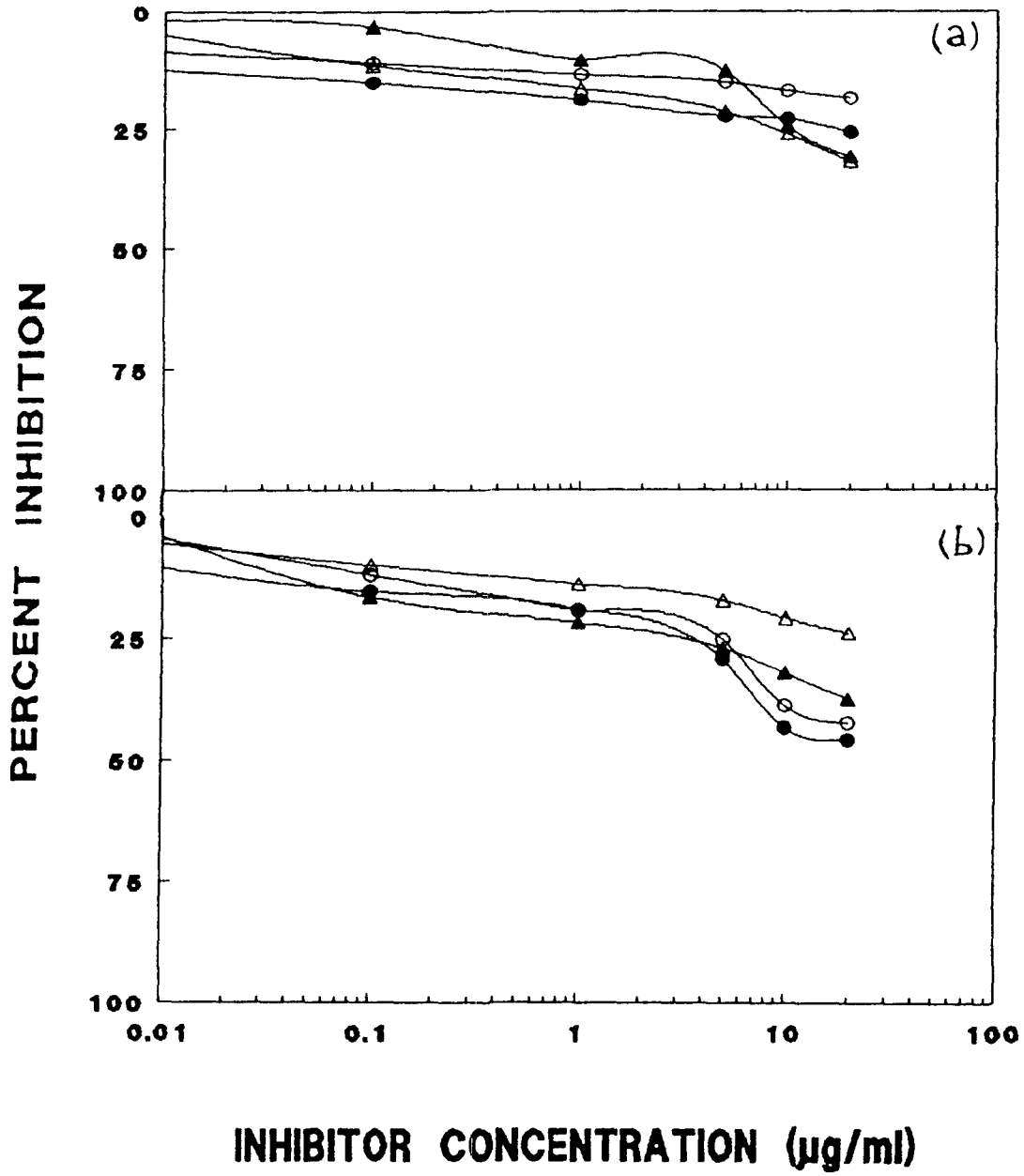

Fig. 47. Detection of autoantibodies against native and $^1O_2$-$O_2^{\cdot-}$-DNA, in the sera of patients with prostrate cancer. (a) Cancer sera 1 and 2 by native DNA (△,○), and $^1O_2$-$O_2^{\cdot-}$-DNA (▲,●). (b) Cancer sera 3 and 4 by native DNA (△,○) and $^1O_2$-$O_2^{\cdot-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\cdot-}$-DNA (2.5 μg/ml) and serum dilution was 1:100.

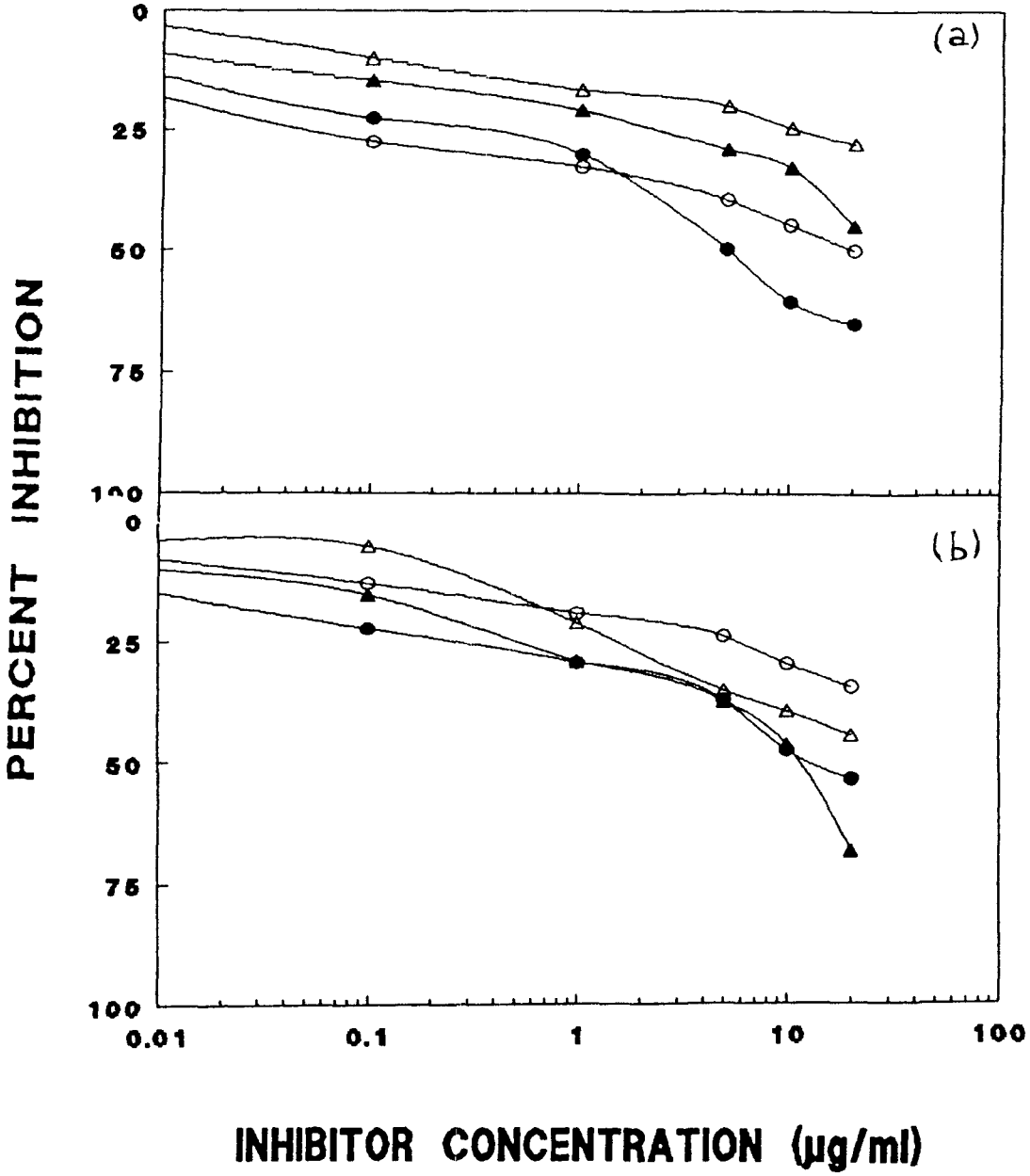

Fig. 48. Detection of autoantibodies against native and $^1O_2$-$O_2^{\bullet-}$-DNA, in the sera of patients with Hodgkin's lymphoma, CML, cancer of vulva. (a) Hodgkin's lymphoma and CML with native DNA (△,O), and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). (b) Cancer of vulva (sera 1 and 2) by native DNA (△,O) and $^1O_2$-$O_2^{\bullet-}$-DNA (▲,●). The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml) and serum dilution was 1:100.

TABLE – 7

Inhibition of the binding of antibodies in cancer sera to $^1O_2$-$O_2^{\cdot-}$-DNA by native and $^1O_2$-$O_2^{\cdot-}$-DNA

Type of cancer	No. of sera tested	Maximum percent inhibition at 20 μg/ml	
		Native DNA	$^1O_2$-$O_2^{\cdot-}$-DNA
Lung	5	32.3, 16.5, 39.3, 15, 20.4	65.3, 61.3, 70.7, 66.8, 60.8
Urinary bladder	5	38.6, 32.8, 35.6, 32.9, 45	59.3, 52.6, 55.4, 55.4, 50
Breast	4	39.7, 28.5, 24.5, 30.2	59, 46, 50.7, 53.6
Head and neck	4	39.9, 35.7, 40.7, 43	65, 67.2, 45, 48.5
Oral	4	34.4, 38.7, 46,42	69.6, 65.6, 40, 48
Gall bladder	4	20.8, 25.6, 27, 28	39.7, 42.8, 45, 41
Prostrate	4	32, 18.6, 23.7, 41.9	31, 25.8, 37, 45.4
Vulva	2	45, 35	68, 54
CML	1	28	45
Hodgkin's	1	50	65

The microtitre plates were coated with $^1O_2$-$O_2^{\cdot-}$-DNA (2.5 μg/ml) and serum dilution was 1:100.

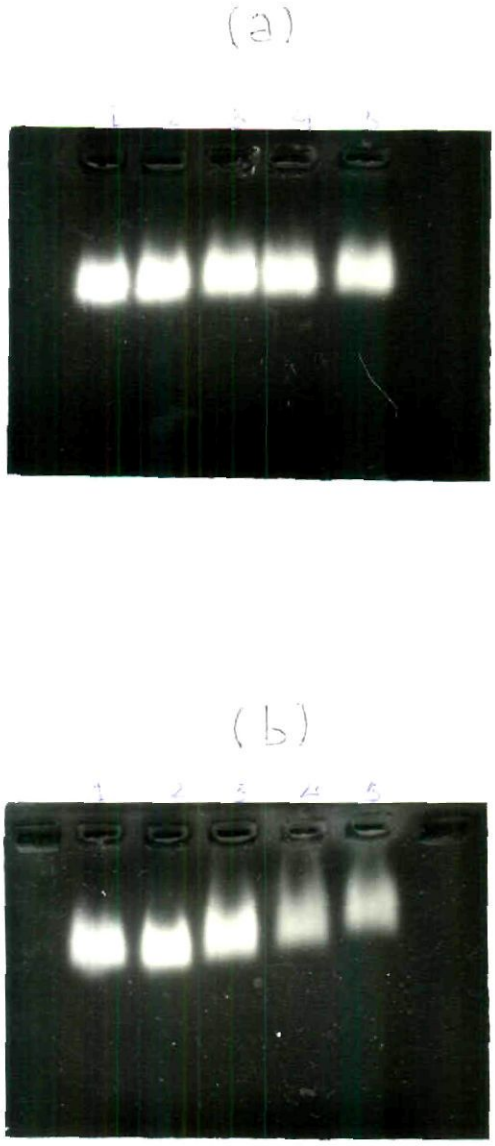

Fig. 49. Band shift assay of cancer IgG binding to (a) native DNA and (b) $^1O_2$-$\dot{O}_2^-$-DNA. Native DNA and $^1O_2$-$\dot{O}_2^-$-DNA (1μg) each were incubated with 10,20,40 and 80 μg of IgG for 2 hr at 37°C and overnight at 4°C. Electrophoresis was carried out on 1% agarose gel for 2 hr at 30 mA. Lane 1 contains native or $^1O_2$-$\dot{O}_2^-$-DNA, while lanes 2,3,4, and 5 contain native or $^1O_2$-$\dot{O}_2^-$-DNA with increasing concentrations of cancer IgG.

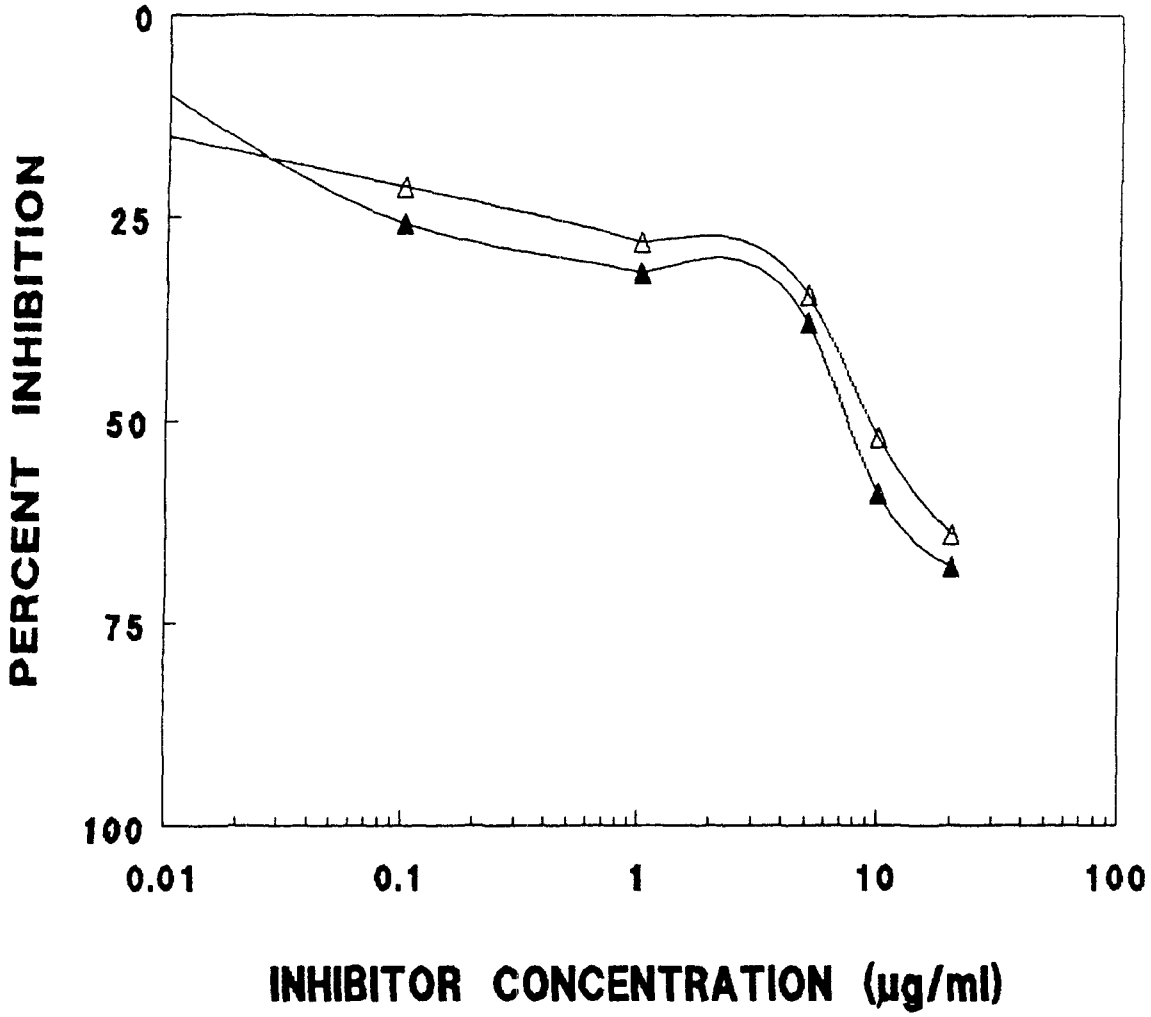

Fig. 50. Inhibition of binding of anti-$^1O_2$-$O_2^{\bullet-}$-DNA IgG by genomic DNA isolated from lymphocytes of patients with cancer of lung. The microtitre plates were coated with $^1O_2$-$O_2^{\bullet-}$-DNA (2.5 µg/ml). The curves (△) and (▲) represent DNA isolated from two different individuals with cancer of lung.

samples of DNA were from oral cancer (Fig. 51). These DNA samples showed a high inhibition of 61.1% and 60% in antibody binding. Two DNA samples isolated from cancer of prostrate showed inhibition 42.7% and 37% of antibody activity (Fig. 52). On the contrary, DNA isolated from two normal healthy individuals showed negligible inhibition of 20% and 25% with anti-$^1O_2$-$O_2^{\bullet -}$-DNA IgG binding to the immunogen (Fig. 53). These results have been summarised in Table 8.

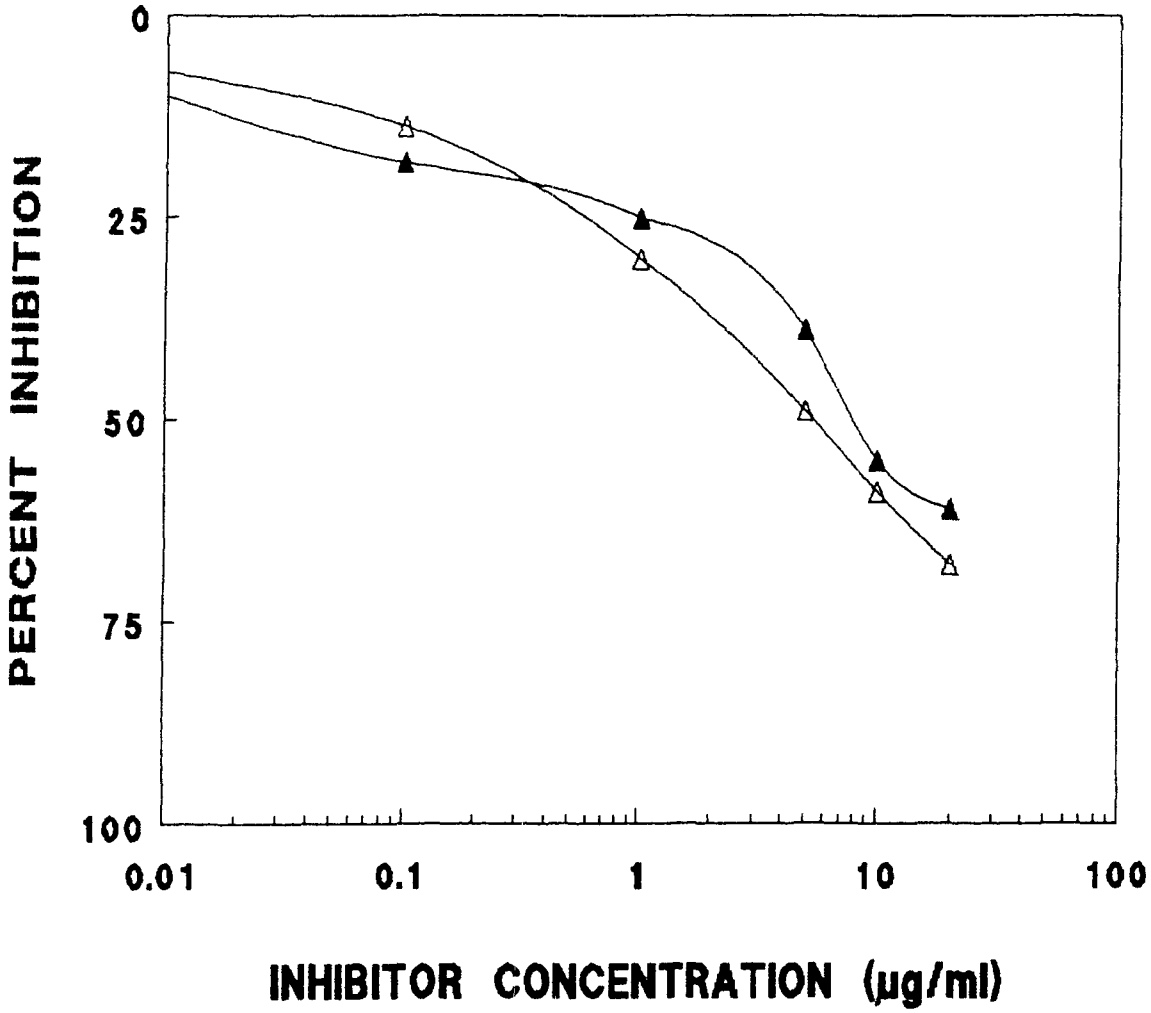

Fig. 51. Inhibition of binding of anti-$^1O_2$-$O_2^{\bullet -}$-DNA IgG by genomic DNA isolated from lymphocytes of patients with oral cancer. The microtitre plates were coated with $^1O_2$-$O_2^{\bullet -}$-DNA (2.5 µg/ml). The curves (△) and (▲) represent DNA isolated from two different individuals with oral cancer.

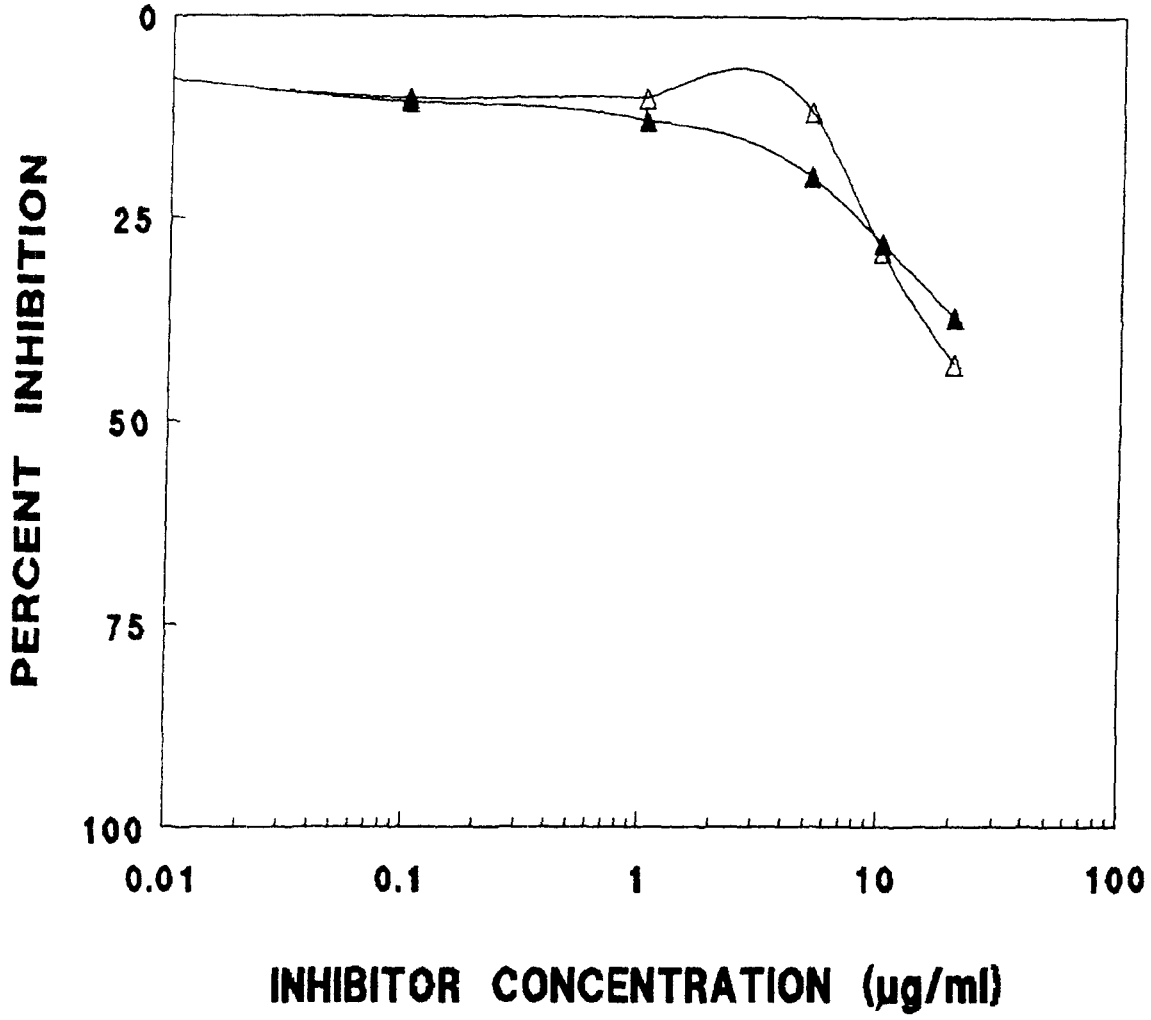

Fig. 52. Inhibition of binding of anti-$^1O_2$-$\dot{O}_2^-$-DNA IgG by genomic DNA isolated from lymphocytes of patients with prostrate cancer. The microtitre plates were coated with $^1O_2$-$\dot{O}_2^-$-DNA (2.5 µg/ml). The curves (△) and (▲) represent DNA isolated from two different individuals with prostrate cancer.

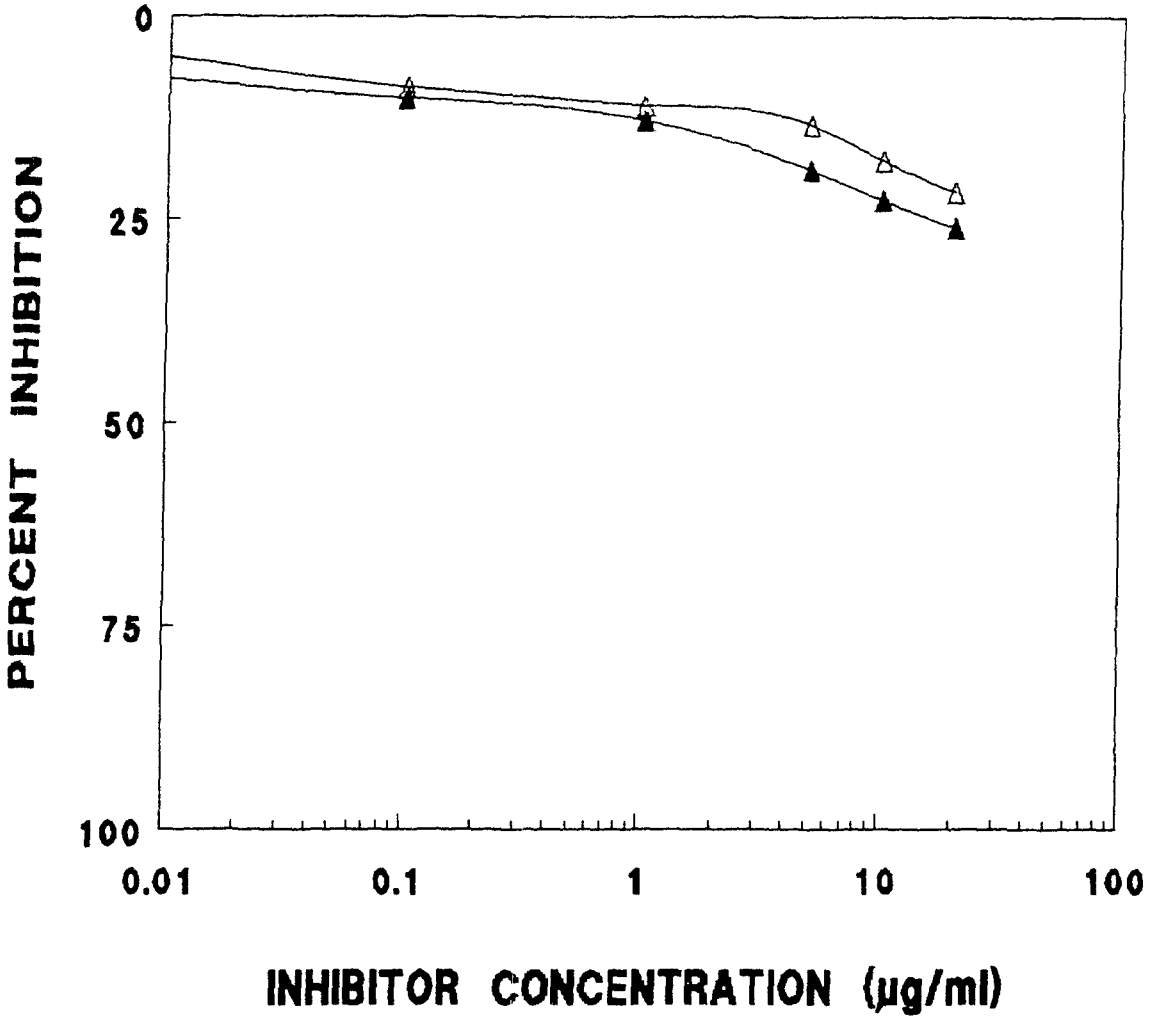

Fig. 53. Inhibition of binding of anti-$^1O_2$-$\dot{O}_2^-$-DNA IgG by genomic DNA isolated from lymphocytes of normal individuals. The microtitre plates were coated with $^1O_2$-$\dot{O}_2^-$-DNA (2.5 µg/ml). The curves (△) and (▲) represent DNA isolated from two different individuals.

TABLE - 8

Binding of anti-$^1O_2$-$O_2^{\cdot -}$-DNA IgG to DNA isolated from lymphocytes of cancer patients

Type of cancer	No. of sera tested	Maximum percent inhibition at 20 μg/ml
Lung	2	64.8 68.0
Oral	2	61.1 60.0
Prostrate	2	42.8 37.0

The microtitre plates were coated with $^1O_2$-$O_2^{\cdot -}$-DNA (2.5 μg/ml)

DISCUSSION

In the living cells, ROS are formed continuously as a consequence of both normal metabolic activity as well as external factors. The damage from these free radical has been proposed to be involved in carcinogenesis and various degenerative diseases (Halliwell and Aruoma, 1991; Ames *et al.,* 1995; Pryor, 1997). ROS modify DNA at various sites that include base damage (Dizdaroglu *et al.,* 1991) leading to mutations (Moody and Hasan, 1982; Brawn and Fridovich, 1985). Although, aerobes have developed antioxidant defence to control harmful effects of activated oxygen species (Lunec and Blake, 1990; Sandstrom and Buttke, 1993; Singh *et al* 1994) a certain fraction escapes the cellular defences. This can happen if antioxidants are depleted and/or if the formation of reactive oxygen species is increased beyond the ability of the defence to cope with them (Sies, 1991). Induction of mutation following DNA damage represents a failure of the repair system to remove DNA damage and is one of the critical events in carcinogenic transformation and other human pathologies (Henley and Linn, 1997; Moller and Wallin, 1998).

Singlet oxygen generated by various photosensitization reactions is a reactive oxygen species (ROS) involved in a variety of biological functions such as gene expression, photoaging and apoptosis (Grether-Beck *et al.,* 1996; Ryter and Tyrrell, 1998; Zhuang *et al.,* 1999; Krutmann, 2000). DNA is one of the main targets of $^1O_2$ and the abundance of oxidative DNA damage poses two biological problems: (i) blocking of DNA synthesis, which is lethal; and (ii) miscoding, which is premutagenic. It has been demonstrated that $^1O_2$ reacts preferentially with guanine residues either as free nucleosides (Cadet *et al.,* 1983) or as components of the DNA molecule (Menck *et al*, 1993), yielding a variety of DNA lesions selectively at guanine sites. These include DNA cleavage (Devasagayam *et al.,* 1991), alkali and piperidine-labile sites (including abasic sites) (Blazek *et al.,* 1989), cyanuric acid (Cadet *et al.,* 1983), 2,6-diamino-4-oxo-5-formamidopyrimidine (Fapy G, a guanine derivative with an open imidazole ring) (Boiteux *et al.,* 1992) and 7,8-dihydro 8-oxodeoxyguanine (8-oxodG) (Floyd *et al.,* 1989). DNA damage profile of $^1O_2$ is completely different from the one caused by hydroxyl radicals. As compared to $^{\bullet}OH$ radical $^1O_2$ causes fewer strand breaks (Epe *et al*, 1988).

Superoxide is the most abundant reactive species generated *in vivo* by several enzymatic and non-enzymatic pathways in mammalian tissue (Fridovich, 1986; Devasagayam *et al.*, 1991; Sies and Menck, 1992). It has been implicated in several disease states and unfavourable alteration of tissues and biomolecules (McCord, 1985; Cross, 1987; Epe *et al.*, 1988). It has been demonstrated that $O_2^{\cdot -}$ itself can exert deleterious effect in biological systems (Fridovich, 1986). It is possible that superoxide radical produced in excessive amounts might be responsible for DNA damage in autoimmune diseases.

Systemic lupus erythematosus (SLE) is a highly variegated and pleomorphic autoimmune disease (Andrzejewski *et al.*, 1980) of unknown etiology. It is characterized by the presence of anti-DNA autoantibodies of multiple specificities to numerous self-components which includes nuclear and cytoplasmic antigens (Tan, 1989; Pisetsky, 1994). The marked heterogeneity of SLE autoantibodies has been one of the impediments in understanding the disease. Both genetic and environmental factors are believed to contribute to this disease (Steinberg *et al.*, 1991). The presence of anti-DNA autoantibodies in the sera of SLE patients has long been considered as a marker of SLE as well as the pathogenic factor for its renal disease manifestations (Koffler, 1974).

The binding diversity of lupus autoantibodies to a whole spectrum of modified nucleic acid conformer (Ali *et al.*, 1991; Alam and Ali, 1992; Alam *et al.*, 1992; 1993; Arif *et al.*, 1994; Klinman *et al.*, 1994; Ahmad *et al.*, 1997; Garg and Ali, 1998) seems to be enormous. The studies to understand the origin and consequence of anti-dsDNA antibodies are still in progress. For these reasons, it was thought desirable to investigate the immunogenicity of $^1O_2$-$O_2^{\cdot -}$-DNA and its possible role in SLE and development of cancer.

Cancer, the biological consequence with the most complex etiology, has been implicated in free radical induced damage to DNA (Ames *et al.*, 1993a; 1995). Despite enzymatic repair and other defences, continuous ROS damage and division of cells with unrepaired and misrepaired lesions lead to mutations. If these relate to critical genes such as oncogenes or tumor suppressor genes initiation and/or progression of cancer can occur.

In the present study, 200 bp calf thymus DNA was modified by singlet oxygen and superoxide anion radicals generated by illumination in presence of riboflavin. This study shows that the damage observed by illumination of riboflavin system in a metal free solution is due to the production of singlet oxygen ($^1O_2$) and superoxide anion radical ($O_2^{\cdot -}$). The production was confirmed by the use of quenchers, sodium azide and SOD. Near complete inhibition in the production of $^1O_2$ was observed in presence of sodium azide. No inhibition in the production of $O_2^{\cdot -}$ was observed when SOD alone was used. However inhibition in the production of $O_2^{\cdot -}$ was observed when both SOD and sodium azide were used. This could be due to the reason that when SOD is exposed to a singlet generating system it becomes susceptible to oxidative modification and damage as indicated by the loss of activity (Kim, *et al.*, 2001) and hence SOD alone could not inhibit the production of $O_2^{\cdot -}$ but when sodium azide was also introduced in the system at a concentration at which almost complete inhibition of $^1O_2$ production was observed, SOD was not inactivated and inhibition in the production of $O_2^{\cdot -}$ was observed.

The photochemical reaction of a free radical with DNA resulted in an increase in absorption at 260 nm with a peak shift of 10 nm towards longer wavelength side in both λ_{max} and λ_{min}. This could be attributed to single strand breaks and modification of bases.

The UV difference spectral curves exhibited appreciable perturbation in native DNA as a consequence of photochemical modification. The spectral curve exhibited an appreciable negative inversion in absorption between 240 nm - 260 nm, followed by increased absorption at 270 nm. Moreover the spectral curve also exhibited a shoulder at around 290 nm. Elimination of the characteristic 260 nm peak is indicative for the loss of the double helical structure of DNA due to loss of DNA helical organization, single strand breaks and modification of bases. These changes in the curve are also indicative of the possible ring opening of the nucleic acid bases as a consequence of the modification.

UV absorption spectra of $^1O_2$-$O_2^{\cdot -}$-DNA is presence of sodium azide shows that the damage obtained in presence of quencher of $^1O_2$ may be due to the presence of $O_2^{\cdot -}$ only.

The fluorescence spectra of $^1O_2$-$O_2^{\cdot -}$-DNA showed decrease in fluorescence intensity as compared to native DNA, which indicates the generation of strand breaks as

lesser amount of ethidium bromide gets intercalated in modified DNA as compared to native DNA.

The thermal denaturation profile of native and $^1O_2$-$O_2^{\cdot-}$-DNA showed a net decrease of 8°C in the Tm value for $^1O_2$-$O_2^{\cdot-}$-DNA as compared to its native conformer. This may be due to structural alteration of DNA which occurs upon generation of single strand breaks and base modifications. Base stacking and hydrogen bonding interaction are known to stabilize the native structure of DNA, thus high temperature which disrupts these interactions favours denaturation of DNA (Casperson and Voss, 1983; Thomas, 1993). The lowered melting temperature of ROS-DNA suggests reduced base stacking, single strand breaks, disruption of hydrogen bond and a consequent helix disruption in DNA following attack by ROS.

Earlier studies demonstrate the structural alteration in DNA following damage by various agents and may be large enough to be recognized by single strand specific nucleases (Slor and Lev, 1973; Kato and Fraser, 1973; Shisido and Ando, 1974; Yamasaki *et al.,* 1977). In view of this, native and $^1O_2$-$O_2^{\cdot-}$-DNA were subjected to nuclease S1 digestion. The results, showed partial digestion of $^1O_2$-$O_2^{\cdot-}$-DNA on treatment with nuclease S1, while native DNA remained undigested. This observation clearly demonstrates that sufficient single strand breaks are generated in DNA by ROS rendering it susceptible to digestion by single strand specific nuclease S1.

Native DNA *per se* is a weak immunogen, (Madaio *et al.,* 1984; Stollar, 1986). However, its oxidatively damaged analog with altered bases and conformational variance from B-form can trigger immune response (Lafer *et al.,* 1981; Lee *et al.,* 1984; Santella *et al.,* 1985; Sundquist *et al.,* 1987; Moinuddin and Ali, 1994; Hasan *et al.,* 1995). Double-stranded RNA, RNA-DNA hybrid, left handed Z-DNA, triple helical RNA and DNA analogues and double helical polydeoxyribonucleotides, DNA modified with drugs, hormones, chromatin or DNA in complexes with binding proteins (Stollar 1973; 1975, 1986; Anderson *et al.,* 1988a; Desai *et al.,* 1993; Moinuddin and Ali, 1994; Hasan *et al.,* 1995; Theofilopoulous, 1995; Arjumand, *et al.,* 1995; 1997; Arif and Ali, 1996). Reactive oxygen species modified DNA have been implicated in the pathogenesis of SLE and cancer (Ara and Ali, 1993; Du *et al.,* 1994; Cooke *et al.,* 1997; Ahmad *et al.,* 1997).

Antibodies against $^1O_2$-$O_2^{\cdot-}$-DNA were induced in rabbits by immunizing with $^1O_2$-$O_2^{\cdot-}$-DNA complexed with methylated bovine serum albumin. The $^1O_2$-$O_2^{\cdot-}$-DNA was a potent immunizing stimulus, inducing high titre antibodies. Specificity of purified $^1O_2$-$O_2^{\cdot-}$-DNA IgG was assessed by competition ELISA. A maximum of 84.5% inhibition was observed with the immunogen. The concentration of immunogen required for 50% inhibition of IgG binding to immunogen was observed to be 1.9 μg/ml. In addition, the induced antibodies showed lesser binding to native DNA. The data indicates the higher specificity of the immune IgG towards ROS-modified epitopes. Modification of DNA by ROS might have generated potential epitopes against which the antibodies are raised. Native DNA, 200 bp DNA showed inhibitions of 47.8%, 32.3% respectively, whereas ROS-DNA showed higher inhibition of 50.25%. This indicates the increased recognition of free radical modified DNA by the induced anti-$^1O_2$-$O_2^{\cdot-}$-DNA antibodies. Chromatin and ROS-chromatin showed inhibition of 7.9% and 12.5% respectively.

To further define the structural determinant recognized by the anti-$^1O_2$-$O_2^{\cdot-}$-DNA antibodies, their interaction with various synthetic polynucleotides was studied. These compounds present a limited set of determinants than natural DNA and can be used as probe for specificity analysis. Poly(dA-dU).poly(dA-dU), poly(dI-dC).poly(dI-dC) and poly(dA-dT).poly(dA-dT) showed an inhibition of 12.7%, 38.7% and 44.4% respectively. The broad recognition of the induced antibodies with a variety of polynucleotides might be due to the recognition of the phosphodiester backbone (Ballard and Voss, 1982; Shoenfeld *et al.*, 1983; Rauch *et al.*, 1985). Besides binding to various nucleic acids and synthetic polynucleotides, the anti-$^1O_2$-$O_2^{\cdot-}$-DNA IgG also showed cross reactivity towards chondroitin sulphate and cardiolipin. It has been suggested that phosphate sugar-phosphate moiety of cardiolipin mimics the backbone of DNA, thus explaining the cross-reactivity of immune IgG (Rauch *et al.*, 1984). Analysis of the data indicates that anti-$^1O_2$-$O_2^{\cdot-}$-DNA IgG is polyspecific and the various cross-reacting antigens with which it reacts shares a common antigenic determinant or epitope.

SLE is a multisystem autoimmune disease involving both humoral and cellular aspects of the innate and acquired immune systems and is characterized by autoantibodies with a spectrum of specificities that participate in disease pathogenesis (Kimberly, 2001).

Sera of SLE patients contain a variety of autoantibodies of which a subset may be responsible for the array of chemical symptoms (Chastagner *et al.,* 1994). Antibodies to dsDNA serve as a serological marker for the diagnosis of SLE. However, native DNA *per se* is non-immunogenic. It has been reported that denaturation of dsDNA by ROS results in an increased binding of anti-DNA antibodies present in sera of SLE patients (Blount, *et al.,* 1989) and that ROS modification of DNA produces a more discriminating antigen for the diagnosis of SLE (Blount, 1990). The detection of 8-hydroxyguanosine in the immune complex derived DNA of SLE (Lunec *et al.,* 1994) reinforces the evidence that ROS may be involved in SLE. Thus, it appears that ROS-modification exposes epitopes on DNA that are recognized by circulating anti-DNA antibodies in SLE sera.

In the present study, anti-DNA antibodies from twenty four different SLE sera showed considerable binding to $^1O_2$-$O_2^{\bullet -}$-DNA except one sera which showed less binding to $^1O_2$-$O_2^{\bullet -}$-DNA than native DNA. No activity was found in normal human sera. When reactivity of native and $^1O_2$-$O_2^{\bullet -}$-DNA with SLE sera, was probed competition ELISA results showed preferential binding of $^1O_2$-$O_2^{\bullet -}$-DNA over native DNA. Band shift assay further substantiated the binding of native and $^1O_2$-$O_2^{\bullet -}$-DNA with SLE anti-DNA autoantibodies. The strong binding potential of anti-DNA IgG towards $^1O_2$-$O_2^{\bullet -}$-DNA demonstrates the possible role of modified nucleotides in SLE pathogenesis.

Increased levels of circulating antibodies and autoantibodies have been reported in sera of patients with malignancies (Anderson *et al.,* 1988b; Faiderbe *et al.,* 1992; Chagnaud *et al.,* 1992; Becker *et al.,* 1994). Elevated levels of anti-nuclear antibodies up to 27% in cancer have been reported (Zeronski *et al.,* 1972; Bunham, 1972). In the present study, serum of cancer patients were tested for the presence of autoantibodies reactive towards native and $^1O_2$-$O_2^{\bullet -}$-DNA. In direct binding ELISA, almost all the cancer sera tested showed higher recognition of $^1O_2$-$O_2^{\bullet -}$-DNA by the circulating autoantibodies. The antigenic specificity of circulating antibodies analyzed by competition binding assay.

Five sera from lung cancer and three sera from oral carcinoma showed higher recognition for $^1O_2$-$O_2^{\bullet -}$-DNA as compared to native DNA. These patients have history of smoking and support for strong oxidative stress *in vivo.* It has been reported that lung and oral carcinomas are strongly correlated with oxidative DNA damage (Olinski *et al.,* 1992;

Okamoto *et al.*, 1994). Further, the binding to native and $^1O_2$-$O_2^{\bullet-}$-DNA with circulating cancer autoantibodies were substantiated by gel retardation assay.

The results obtained with sera from cancer with urinary bladder, prostrate and Hodgkin's lymphoma have more preference for $^1O_2$-$O_2^{\bullet-}$-DNA as compared to nDNA thus suggesting the role of ROS damage to DNA in the development of these cancers.

Patients suffering from breast cancer showed a higher reactivity towards $^1O_2$-$O_2^{\bullet-}$-DNA than to its native analog. These results reiterate previous findings which suggest involvement of ROS damaged DNA in induction and progression of breast cancer (Jaiyesimi *et al.*, 1992; Malins *et al.*, 1993; 1996). Clinical significance of autoantibodies in cancer is unclear, however, the presence of anti-nuclear antibodies do indicate a worse prognosis or a more frequent recurrence of breast cancer (Turnbull *et al.*, 1978; Wasserman et al, 1975).

The results obtained from sera of patients with gall bladder, head and neck, CML and vulva showed higher reactivity with $^1O_2$-$O_2^{\bullet-}$-DNA as compared to its native analog. The data represented suggests that naturally occurring circulating autoantibodies in cancer patient have higher reactivity towards ROS modified DNA than towards native DNA. ROS appears to enhance antigenicity of native DNA, thus suggesting role of ROS damaged DNA in the production of autoantibodies in cancer patients.

In the present study, attempts have been made to detect the oxidative lesions in DNA isolated from lymphocytes of various cancer patients by using anti-$^1O_2$-$O_2^{\bullet-}$-DNA IgG as an immunochemical probe.

DNA was isolated from lymphocytes of a group of six patients suffering from cancer of either lung, oral and prostrate. DNA from two patients with lung cancer recognized anti-$^1O_2$-$O_2^{\bullet-}$-DNA IgG appreciably and inhibited its activity to 64% and 68%. The results indicate presence of ROS induced lesions in DNA from patients with lung cancer. DNA from two patients with oral carcinoma too inhibited antibody activity appreciably. Prostrate cancer DNA exhibited 37% and 42.79% inhibition. All these results indicate effective binding of anti-$^1O_2$-$O_2^{\bullet-}$-DNA IgG to DNA isolated from various cancer patients and confirms presence of ROS induced oxidative DNA lesions in cancer patients.

Based on this study, the following conclusions can be drawn:

1. Illumination of DNA in presence of riboflavin resulted in structural alterations such as single strand breaks, disruption of hydrogen bonds and a consequent helix destabilization.
2. Thermal transition studies showed that modified conformers are less stable as compared to native form.
3. ROS induced certain conformational changes in DNA, rendering it highly immunogenic in experimental animals.
4. The induced antibodies are highly specific for immunogen.
5. The induced antibodies exhibited some cross-reactivity with various nucleic acids, synthetic polynucleotides thus resembling binding characteristics of SLE anti-DNA antibodies.
6. SLE anti-DNA autoantibodies showed preferential binding for $^1O_2$-$O_2^{\bullet -}$-DNA than nDNA.
7. $^1O_2$-$O_2^{\bullet -}$-DNA presents a discriminating antigen for the binding of SLE autoantibodies.
8. Antibodies in sera of various cancer patients were found to be more specific for $^1O_2$-$O_2^{\bullet -}$-DNA than for its native analog.
9. Oxidative lesions were detected in DNA isolated from lymphocytes of cancer patients using anti-$^1O_2$-$O_2^{\bullet -}$-DNA IgG as probe.

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