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 \(^{1}OH \) 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 $O_2^-$ 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 $^{1}O_2$-O$_2^-$-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 (\(^{1}\text{O}_2\)) and superoxide anion radical (\(\text{O}_2^-\)). The production was confirmed by the use of quenchers, sodium azide and SOD. Near complete inhibition in the production of \(^{1}\text{O}_2\) was observed in presence of sodium azide. No inhibition in the production of \(\text{O}_2^-\) was observed when SOD alone was used. However inhibition in the production of \(\text{O}_2^-\) 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 \(\text{O}_2^-\) but when sodium azide was also introduced in the system at a concentration at which almost complete inhibition of \(^{1}\text{O}_2\) production was observed, SOD was not inactivated and inhibition in the production of \(\text{O}_2^-\) 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 \(\lambda_{\text{max}}\) and \(\lambda_{\text{min}}\). 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 \(^{1}\text{O}_2-\text{O}_2^-\)-DNA is presence of sodium azide shows that the damage obtained in presence of quencher of \(^{1}\text{O}_2\) may be due to the presence of \(\text{O}_2^-\) only.

The fluorescence spectra of \(^{1}\text{O}_2-\text{O}_2^-\)-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 $^{1}$O$_2$-O$_2^{-}$-DNA showed a net decrease of 8°C in the Tm value for $^{1}$O$_2$-O$_2^{-}$-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 $^{1}$O$_2$-O$_2^{-}$-DNA were subjected to nuclease S1 digestion. The results, showed partial digestion of $^{1}$O$_2$-O$_2^{-}$-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; Theofilopoulos, 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 \(^{1}\text{O}_2-\text{O}_2^-\)-DNA were induced in rabbits by immunizing with \(^{1}\text{O}_2-\text{O}_2^-\)-DNA complexed with methylated bovine serum albumin. The \(^{1}\text{O}_2-\text{O}_2^-\)-DNA was a potent immunizing stimulus, inducing high titre antibodies. Specificity of purified \(^{1}\text{O}_2-\text{O}_2^-\)-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 \(\mu\)g/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-\(^{1}\text{O}_2-\text{O}_2^-\)-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-\(^{1}\text{O}_2-\text{O}_2^-\)-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-\(^{1}\text{O}_2-\text{O}_2^-\)-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-\(^{1}\text{O}_2-\text{O}_2^-\)-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 $^1$O$_2$-O$_2^-$-DNA except one sera which showed less binding to $^1$O$_2$-O$_2^-$-DNA than native DNA. No activity was found in normal human sera. When reactivity of native and $^1$O$_2$-O$_2^-$-DNA with SLE sera, was probed competition ELISA results showed preferential binding of $^1$O$_2$-O$_2^-$-DNA over native DNA. Band shift assay further substantiated the binding of native and $^1$O$_2$-O$_2^-$-DNA with SLE anti-DNA autoantibodies. The strong binding potential of anti-DNA IgG towards $^1$O$_2$-O$_2^-$-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 $^1$O$_2$-O$_2^-$-DNA. In direct binding ELISA, almost all the cancer sera tested showed higher recognition of $^1$O$_2$-O$_2^-$-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 $^1$O$_2$-O$_2^-$-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 (Oliński et al., 1992;
Okamoto et al., 1994). Further, the binding to native and $^{1}$O$_2$-O$_2$$^-$-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 $^{1}$O$_2$-O$_2$$^-$-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 $^{1}$O$_2$-O$_2$$^-$-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 $^{1}$O$_2$-O$_2$$^-$-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-$^{1}$O$_2$-O$_2$$^-$-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-$^{1}$O$_2$-O$_2$$^-$-DNA IgG appreciably and inhibited its activity to 64% and 68%. The results indicate presence of ROS induced lesions in DNA from patients with lung cancer. DNA from two patients with oral carcinoma too inhibited antibody activity appreciably. Prostrate cancer DNA exhibited 37% and 42.79% inhibition. All these results indicate effective binding of anti-$^{1}$O$_2$-O$_2$$^-$-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 $^{1}O_2-O_2^-$-DNA than nDNA.

7. $^{1}O_2-O_2^-$-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 $^{1}O_2-O_2^-$-DNA than for its native analog.

9. Oxidative lesions were detected in DNA isolated from lymphocytes of cancer patients using anti-$^{1}O_2-O_2^-$-DNA IgG as probe.