DISCUSSION
Antibodies reactive with a number of nuclear antigens have been found in the sera of patients with systemic lupus erythematosus (SLE). Their entire spectrum is unknown, but there is considerable data suggesting that a 'single antigen', such as DNA may command a broad heterogeneity of antibodies with specificity towards receptor sites that are quite variable not only from patient to patient but even in the same patient (Arana and Seligman, 1967; Stollar, 1967; Stollar and Levine, 1963; Stollar et al., 1962a & b). These naturally occurring antibodies not only provide diagnostic/prognostic parameters to clinicians but give molecular biologists valuable tools for the better understanding of cellular processes (Woodruff et al., 1986). The binding diversity of lupus autoantibodies to a whole spectrum of modified nucleic acid conformers seems to be enormous (Arif et al., 1995; Arjumand et al., 1995; Arjumand and Ali, 1994; Klinman et al., 1994; Moinuddin and Ali, 1994; Alam et al., 1992 & 1993; Alam and Ali, 1992; Ali et al., 1991). Efforts to understand the origin and consequence of anti-dsDNA antibodies are still in progress.

Association between neoplasia and autoimmunity has been described with an increasing frequency during the last two decades. Similarly, various autoantibodies have been identified in the sera of patients with epithelial and hematologic malignancies (Vainio et al., 1983; Nelson, 1977). Elevated titres of antinuclear antibodies (ANA) upto 27% in cancer sera have been reported (Bunham, 1972; Fairley, 1972; Zeronski et al., 1972). Increased levels of circulating antibodies and autoantibodies have been reported in the serum of patients with malignancies directed against the nucleus, smooth muscle (Imai et al., 1993) and phospholipids (Becker et al., 1994).
It has been suggested that antibodies in lupus sera are primarily directed to the DNA bases (Stollar et al., 1962b). There may be however, some antibodies in this complex family which are reactive with the deoxyribosephosphate backbone and nucleotide but not with the corresponding nucleoside, suggesting that the antigenic determinant might be shared by the base and the sugar-phosphate moiety. While, in some sera reaction occurred with the nucleoside but not with the corresponding nucleotide, probably indicating interference of the negative charge of phosphate with the antigen-antibody reaction (Alarcon-Segovia et al., 1970). Guanine and thymine are prominent as components of nucleotides and oligonucleotides that react with certain antibodies (Lee et al., 1981; Eilat et al., 1980).

Reactive oxygen species (ROS), generated during various metabolic and biochemical reactions have multifarious effects that include oxidative DNA damage leading to various human degenerative diseases (Halliwell and Cross, 1994; Halliwell, 1989). Among the ROS, hydroxyl radical (OH) the highly reactive oxygen species can interact with the chromatin and result in a wide variety of sugar and base derived products, DNA-protein crosslinks, single and double strand breaks (Gajewski et al., 1990; Dizdaroglu and Bergtold, 1986), showing the mutagenic and carcinogenic potential of this radical (Jaruga et al., 1994). ROS modified-DNA has been found to be a better antigen for anti-DNA autoantibodies present in SLE sera (Alam et al., 1993; Ara and Ali, 1992). Experimentally induced antibodies against ROS-modified DNA exhibit polyspecificity (Ara and Ali, 1993) recognizing B, A and allied conformations of DNA (Ara and Ali, 1995). DNA isolated from SLE patients showed high recognition by Mab having preferential binding with ROS-modified epitopes which indicates
increase oxidative stress in these patients leading to DNA damage (Ahmad et al., 1998). Thymine base is highly susceptible to 'OH modification to form products such as thymineglycol (Tg) and 5-hydroxymethyluracil. These modified bases have also been detected in human urine together with 8-hydroxydeoxyguanosine, indicating that their formation is due to oxidative stress to DNA (Halliwell and Aruoma, 1991). 8-hydroxyguanosine is a major mutagenic lesion associated with an increased risk of cancer, whereas there is no such distinct association with ROS-modified thymine products. For these reasons, it was thought desirable to investigate the immunogenicity of ROS-modified thymidine monophosphate and its possible role in SLE and development of cancer.

In the present study, a deoxyribonucleotide, thymidine monophosphate (TMP) was modified by hydroxyl radical, generated by irradiation of hydrogen peroxide with 254 nm UV light. The characteristic absorption pattern was lost as a result of the damage caused by the hydroxyl radical. UV difference spectra of ROS-modified TMP with reference to native TMP shows a negative undefined trough, clearly indicating the marked change in the modified nucleotide. This could be due to the structural alterations and modifications of thymine base.

Antibodies against TMP and ROS-modified TMP were induced in rabbits by complexing with methylated bovine serum albumin. Both native and ROS-TMP were found to be non-immunogenic as revealed by counterimmunoelectrophoresis, direct binding and inhibition ELISA results. Our results correlate with earlier findings that nucleotides by themselves are not capable of inducing antibodies. Therefore, nucleotides can be conjugated to a carrier protein by carbodiimide method (without
cleaving the ribose/deoxyribose ring) utilising the phosphate group present in them to enhance their antigenicity (Chandira Kala and Antony, 1996).

Thymidine monophosphate was linked with BSA and the conjugate characterized by ultraviolet absorption spectra. A slight bathochromic shift in $\lambda_{\text{max}}$ was observed. The results of polyacrylamide gel electrophoresis reiterate the formation of a TMP-BSA conjugate. The TMP-BSA conjugate was modified with hydroxyl radical. A substantial decrease in absorption was observed at $\lambda_{\text{max}}$. This could be attributed to the fact that hydroxyl radicals produced by UV irradiation, simultaneously cause damage to TMP-BSA conjugate. UV difference spectra of ROS-TMP-BSA conjugate with reference to TMP-BSA conjugate shows a negative peak at $\lambda_{\text{max}}$, clearly indicating the marked hypochromicity of the modified conjugate. This could be due to structural alterations in thymine base.

The spectral changes at 30°C and 95°C were also recorded. An appreciable degree of hyperchromism was displayed by UV spectral curves of ROS-modified TMP-BSA conjugate when thermally agitated to 95°C. Hydroxyl radical abstracts hydrogen atoms from deoxyribose, giving rise to sugar radical that can fragment in various ways. Photochemical changes have also been found to occur in purine and pyrimidine bases on attack of $^{1}$OH radical (Halliwell and Aruoma, 1991). The pyrimidine bases in DNA are attacked to give several products like thymine and cytosine glycols (Breen and Murphy, 1995).

The elution pattern of $^{1}$OH modified TMP-BSA conjugate was studied by gel chromatography through a Sephadex G-100 column. The broadening of major peak of ROS-TMP-BSA conjugate in contrast to the
sharp peak of TMP-BSA conjugate suggests that hydroxyl radical causes structural perturbations of the thymine residues. The presence of low molecular weight species was further ascertained by densitometric scanning of native and ROS modified conjugate following electrophoresis on 7.5% native polyacrylamide gel. The hydroxyl radical is highly electrophilic and thus preferentially attacks the site of highest electron density, that is C-5. In thymine, the methyl group reduces the amount of attack at C-5 by steric hindrance and stabilizes the C(6) OH-5-yl radical adduct slightly, thus reducing the C(5) OH:C(6)OH ratio (Pryor, 1988).

Native DNA is known to be a poor immunogen (Stollar, 1986; Madaio et al., 1984), whereas, double stranded RNA, RNA-DNA hybrid, double helical polydeoxyribonucleotides, synthetic ribohomopolymers poly(I), poly(G), poly(U) and calf thymus DNA modified with drugs, hormones, free radicals, etc., have been reported to induce antibodies (Garg and Ali, 1998; Arjumand et al., 1997; Arif and Ali, 1996; Hasan et al., 1995; Theofilopoulous, 1995; Moinuddin and Ali, 1994; Desai et al., 1993; Anderson et al., 1988; Stollar, 1973, 1975 & 1986). Antibodies to dihydrothymidine were elicited by immunizing rabbits with dihydrothymidine monophosphate conjugated by carbodiimide to BSA (Hubbard et al., 1989). The present studies demonstrate the fine immunogenicity of TMP-BSA conjugate and its hydroxyl radical modified counterpart i.e., ROS-TMP-BSA conjugate.

Direct binding and inhibition ELISA revealed high immunogenicity of both native and ROS modified TMP-BSA conjugate. A maximum of 95% inhibition of anti TMP-BSA conjugate antibody (serum) binding to TMP-BSA conjugate was observed. Fifty percent inhibition in the antibody
activity was achieved with 2 μg/ml of TMP-BSA conjugate. Purified anti-TMP-BSA IgG was inhibited to an extent of 95% (at 10 μg/ml) and 50% inhibition was observed with only 0.35 μg/ml of TMP-BSA conjugate. The data clearly indicate the higher specificity of purified IgG as compared to serum activity towards TMP-BSA conjugate. ROS-TMP-BSA conjugate showed a maximum inhibition of 42%. ROS-modified TMP, thymidine and thymine showed higher inhibitions of 69%, 56% and 82% respectively. whereas, unmodified forms showed lower inhibitions of 27%, 16% and 18%, respectively. The data indicates the specificity of IgG towards the ROS-modified form of conformers (than their native forms), preferably recognizing the common epitope, the phosphodiester backbone. ROS-adenine and ROS-guanine shows a high inhibitory potential of 57% and 61% than their native forms. Poly (l). ROS-poly (l) and ROS-poly (G). superoxide DNA and mitochondrial DNA showed insignificant inhibition. Inhibition studies by poly(dA-dT).poly(dA-dT) shows the polyreactivity of the induced antibodies. Experimentally induced antibodies to conjugates of BSA with purines or pyrimidines are quite specific for the base used, but cross-react extensively with nucleic acids from numerous sources which contain the individual base (Alarcon-Segovia et al., 1970).

Purified anti-ROS-TMP-BSA conjugate IgG showed a maximum inhibition of 98% (92% with serum) with ROS-TMP-BSA conjugate. Fifty percent inhibition was attained with only 0.5 μg/ml of ROS-TMP-BSA conjugate (2.5 μg/ml in case of serum). This indicates the higher specificity of purified immune IgG than serum activity. Inhibition data showed cross-reactivity of IgG with various nucleic acid polymers. The TMP-BSA conjugate gave a maximum inhibition of 89%. ROS-modified TMP, thymidine and thymine showed higher inhibitions (86%, 56% and
81%) than their unmodified counterparts (39%, 23% and 16%). Double stranded polynucleotides poly(dA-dU).poly(dA-dU) and poly(dl-dC). poly(dl-dC) showed inhibition of 35% each. This broad polyspecificity of the induced antibodies with a variety of polynucleotides might be due to the recognition of the phosphodiester-backbone (Ballard and Voss, 1982). ROS-guanine showed a high inhibitory potential of 78% than native guanine (55%). This indicates that the immune IgG is more specific to the ROS-modified epitopes on guanine. Earlier reports have demonstrated guanine to be highly susceptible to OH modification leading to the formation of 8-hydroxyguanosine (Garg and Ali, 1998; Lunec et al., 1994).

The SLE autoantibodies react with dsDNA from a wide range of species (Pisetsky, 1996). It has been proposed that in chronic inflammatory diseases such as SLE, the phagocytic cells release reactive oxygen species (Allan et al., 1988) which penetrate cellular membranes and react with nuclear DNA (Bashir et al., 1993). Subsequent release of this altered DNA during apoptosis, may enable it to act as an antigen inducing autoantibodies cross-reacting with native DNA (Casciola-Rosen and Rosen, 1997; Herrman et al., 1996). It was suggested that DNA antibodies appear to be directed predominantly against other determinants that include the purine and pyrimidine bases (Munns et al., 1984).

In the present study, anti-double stranded DNA autoantibodies were screened from patients with SLE by direct binding and inhibition ELISA. Results with purified SLE IgG showed appreciable binding with native DNA and inhibition varies from 32% to 55%. When reactivity of TMP-BSA conjugate and its ROS modified form was probed, direct binding ELISA
results showed the preferential binding of TMP-BSA conjugate over ROS-TMP-BSA conjugate. Further, competition immunoassay substantiated the above results with TMP-BSA conjugate showing inhibition ranging from 32% to 85%, while ROS-modified TMP-BSA conjugate shows inhibitions in the range of 10% to 52%.

SLE antibodies that interact with the bases can also bind to nucleoside-protein conjugates (Munns et al., 1984; Alarcon-Segovia et al., 1970). Such conjugates can absorb nearly all of the anti-denatured DNA activity of certain murine lupus sera (Munns et al., 1984). Both murine and lupus sera with anti-denatured DNA antibodies react predominantly with guanosine-protein conjugates in ELISA (Zouali and Stollar, 1986). Conjugation of nucleotide was required for a reaction of measurable affinity. Similarly, free nucleosides or mononucleotides often bind only at high concentrations, if at all, to SLE serum antibodies (Casperson and Voss, 1983). When nucleosides and nucleotides are presented on macromolecule carriers, the great increase in binding suggests that the optimal determinant is larger than the base of a single nucleotide and that bivalent binding of antibody to one molecule of antigen is required for measurable affinity (Zouali and Stollar, 1986).

The presence of autoantibodies in the sera of cancer patients were also studied. The study, consisted of 45 sera from patients with various types of malignancies. Most of the sera showed positive recognition and binding to TMP-BSA conjugate and its ROS-modified form. Sera from normal healthy individuals showed little or no reactivity with TMP-BSA conjugate and ROS-TMP-BSA conjugate. Earlier studies have demonstrated increased levels of circulating antibodies and autoantibodies
in the serum of patients with malignancies (Becker et al., 1994; Imai et al., 1992). In particular, elevated titres of ANA have been correlated with the clinical course of the disease in breast cancer (Turnbull et al., 1978) and hepatocellular carcinoma (Imai et al., 1992 & 1993).

The data presented here clearly indicates the presence of circulating antibodies in cancer sera reactive to TMP-BSA and ROS-TMP-BSA conjugate. A high proportion of sera (7 out of 8) from breast cancer showed reactivity to these antigens. When competition immunoassay was performed with purified IgG isolated from 5 high titre breast cancer sera, it showed higher reactivity towards ROS-modified TMP-BSA conjugate. It has been well accepted and established that ROS damage to DNA plays a fundamental role in carcinogenesis, particularly of breast cancer (Ashok and Ali, 1998; Ashok et al., 1997; Malins et al., 1996; Jaiyesimi et al., 1992). A high degree of reactivity of autoantibodies to ROS-TMP-BSA conjugate was observed in our study substantiating these observations. Lung and hepatocellular cancers are also strongly correlated with oxidative DNA damage (Olinski et al., 1992; Mc Elrude et al., 1991). Oxidative damage to DNA is postulated to be a major event in the development of these cancers. It would be interesting to note that two sera from patients with hepatocellular carcinoma also had chronic hepatic infections which could again explain the onset of cancer as described earlier (Stein. 1991: Beasley. 1988). Inhibition ELISA results with purified cancer IgG further reiterates the preferential binding of ROS-TMP-BSA conjugate over TMP-BSA conjugate. Hepatocellular carcinoma is actively associated with DNA damage and mutations by aflatoxin (Loeb and Preston, 1986) or viral hepatitis infections leading to free radical generation (Shimoda et al.,
1994; Chisari et al., 1989). Similar results were obtained with sera from cancer of lung and gall bladder.

Recent results from our laboratory suggest that the anti-ROS-DNA monoclonal antibodies has been clearly shown to represent an alternative immunochemical probe to detect oxidative lesions in DNA from cancer patients (Ashok and Ali, 1998). Earlier studies report the use of antibodies to specific DNA base alterations, DNA-carcinogen adducts or to UV-DNA (Yin et al., 1995; Herbert et al.; 1994). Our results also demonstrate the presence of anti-ROS-TMP-BSA conjugate antibodies in cancer patients sera, which could perhaps have a prognostic significance.

Conclusions

Based on the above studies, the following conclusions can be drawn.

1. TMP per se was found to be non-immunogenic in experimental animals.

2. TMP was linked with BSA through carbodiimide resulting in the formation of TMP-BSA conjugate.

3. TMP-BSA conjugate was modified with $\text{H}_2\text{O}_2$ in presence of UV light, for the generation of $\cdot\text{OH}$ radical, resulting in the formation of strand breaks and modification of thymine base as characterized by various physico-chemical techniques.

4. Both native and ROS-modified TMP-BSA conjugates were found to be highly immunogenic, in experimental animals. The induced antibodies were precipitating in nature.
5. The induced antibodies though, highly specific for the immunogen, exhibited some cross-reactivity with various nucleic acid polymers, thus resembling the binding characteristics of SLE anti-DNA antibodies.

6. TMP-BSA conjugate as compared to ROS-TMP-BSA conjugate provides higher inhibitory potential to SLE anti-DNA autoantibodies which points out the presence of unique potential epitopes on TMP-BSA conjugate.

7. IgG isolated from sera of cancer patients recognize and bind to ROS-modified TMP-BSA conjugate.

8. It is suggested that thymidine 5-monophosphate linked with BSA might serve as a diagnostic marker for SLE.