Discussion
It has long been hypothesized that the development of the immune system is triadic in nature. Useless cells are discarded, useful cells are retained while dangerous ones are destroyed or inactivated. Elimination or inactivation of emerging B cells reactive with membrane-bound self-antigens or soluble self-antigens respectively, has also been documented (Goodnow, 1992). Similarly, T cells with receptors of dangerously high affinity/avidity are eliminated or inactivated by negative selection (Nossal, 1994; von Boehmer, 1994). Thus, through these editing processes, a state of self-tolerance is achieved. However, despite the recent acquisition of extensive information relating to the mechanisms of self-tolerance, our understanding of the mechanisms leading to pathogenic autoimmunity is still fragmentary and incomplete. Nevertheless, recent advances in this field have begun to assemble the missing pieces of the puzzle. A wide spectrum of human and animal diseases are wholly or partially attributable to autoimmunity (Theofilopoulos, 1993). This list continually expands as autoimmune mechanisms are implicated in widely diverse disorders such as urticaria, AIDS and even neuropsychological disorders (Theofilopoulos, 1995).

Systemic lupus erythematosus is a multi-system, inflammatory disorder characterized by the production of autoantibodies of multiple specificities, especially those reactive with intracellular proteins and nucleic acids. Circulating autoantibodies to dsDNA appear to correlate most uniquely with periods of clinical activity, especially renal complications (Aitcheson and Tan, 1982). The active disease is characterized by the over production of pathogenic autoantibodies whereas the pre-clinical state is characterized by multiclonal B- and T-cell activation, which releases cytokines and that creates an immunostimulatory milieu that facilitates the subsequent anti-self responses (Klinman
and Steinberg, 1995). There is also evidence that DNA and anti-DNA antibodies comprise part of the circulating antigen-antibody complexes that precipitate in the renal glomeruli. The marked heterogeneity of SLE autoantibodies has been one of the impediments in understanding the disease. The immune abnormalities contributing to the development of systemic autoimmune disease in mice and humans are now being defined at the single-cell and molecular levels. The specific defects appear to be determined in different individuals by different genetic and environmental factors. Thus 'lupus' is not a disease but a syndrome with multiple different etiologies (Klinman and Steinberg, 1995) and expectations for a single etiological explanation may be unrealistic. There have been many theories proposed to explain the origin of autoimmune responses, for example a genetic predisposition, exaggerated random B-cell activity, cross-reactivity between host and foreign antigens or modification of host proteins as a consequence of infection, inflammation, drug administration etc. (Radic and Weigert, 1994; Theofilopoulos, 1995). It has been suggested that while neither exogenous polyclonal B- or T-cell activators nor immunoregulatory disturbances appear to be satisfactory explanations, it is clear that development of overt autoimmune disease is highly dependent on a permissive genetic constitution (Theofilopoulos, 1995).

An interesting and intriguing aspect of SLE is that while antibodies reactive with native double stranded DNA are characteristic of this chronic inflammatory disease, native DNA is a poor immunogen and hence the mechanism of anti-DNA antibody production is incompletely understood. On the other hand some synthetic homopolynucleotides, chemically modified DNA and denatured DNA are immunogenic. Also, double stranded RNA, RNA-DNA hybrid, left handed Z-DNA, triple helical RNA and DNA analogues that differ
from B-DNA have been found to be effective immunogens (Anderson et al., 1988; Stollar, 1989). This conformational variance from B-form appears to be a prerequisite for the induction of an antibody response, although the reason for this is not clear (Braun and Lee, 1988).

The damaging effects of reactive oxygen species (ROS), like superoxide anion radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($^*OH$) have gained considerable interest over the last few years (Fridovich, 1986; Halliwell, 1987; Ballmer et al., 1994). Cellular DNA is damaged by oxygen free radicals generated during cellular respiration, cell injury, phagocytosis and exposure to environmental oxidants (Floyd et al., 1986; Fridovich, 1986; Kasai et al., 1986; Klebanoff, 1988; Breimer, 1990). It has been proposed that modification of DNA increases its immunogenicity and in inflammatory disease states such as SLE, reactive oxygen species (ROS) produced from phagocytic cells have been shown to modify DNA. Different procedures of ROS generation have been found to cause distinct and characteristic changes to DNA. A chemical modification of DNA using hydrogen peroxide, which reacts in the presence of a reducing agent and metal ions bound to DNA to produce the highly reactive hydroxyl radical, can cause DNA strand breaks, base damage (Massie et al., 1972) and conformational change (Allan et al., 1988). It has been observed that denaturation of double stranded DNA by ROS results in an increased binding of human anti-DNA autoantibodies (Blount et al., 1990). There seems to be an enormous binding diversity of lupus autoantibodies to a whole spectrum of modified nucleic acid conformers (Alam and Ali, 1992; Alam et al., 1992; 1993; Arjumand and Ali, 1994; Moinuddin and Ali, 1994; Arjumand et al., 1995). Efforts to understand the origin and consequence of anti-ds-DNA antibodies are still in progress. It has also been reported earlier that DNA upon exposure to
254 nm light in the presence of hydrogen peroxide became highly immunogenic inducing high titre antibodies (Ara et al., 1992) which exhibited polyspecificity recognizing the modified polymer, native B-conformation, A and allied conformations (Ara and Ali, 1993).

In the present study native DNA was modified by UV radiation (254 nm) in the presence of hydrogen peroxide. Also individual bases adenine, thymine and their respective homopolymers poly(dA), poly(dT) were similarly modified with ROS to understand the possible role of the modified conformers in the pathogenesis of SLE. The absorption of DNA decreased at 260 nm after treatment with H₂O₂ and UV light. This observed hypochromicity could be attributed to the modification of the nitrogenous bases. However, treatment of DNA with hydrogen peroxide or UV light alone did not cause any significant change in the UV absorption profile. In the case of the bases adenine and thymine, modification with ROS caused a drastic change in their absorption spectra, with a loss of the characteristic peak at 260 nm. On the other hand, exposure of the polymers poly(dA) and poly(dT) to hydroxyl radical resulted in substantial decrease in UV absorbance at 260 nm.

The thermal denaturation profile of native and ROS-DNA showed a net decrease of 13.5°C in the Tm value for the modified DNA as compared to its unmodified native conformer. This is primarily due to a structural alteration in DNA which occurs due to the simultaneous scission of the phosphodiester backbone and the generation of single strand breaks.

The presence of single strand regions in DNA following treatment with ROS was further ascertained by alkaline sucrose gradient centrifugation. The sedimentation profile of ROS-DNA was distinct from that of unmodified native
DNA, which banded as a sharp symmetrical peak, while the modified DNA gave a broader peak indicating the generation of single strand breaks which causes changes in the molecular weight and hence the diffused pattern. These data are compatible with earlier reports implicating hydroxyl radicals in the strand breakage (Meneghini and Hoffmann, 1980; Hoffmann et al., 1984).

Hydroxyl radical appeared to generate sufficient distortions in DNA to act as substrates for nuclease S1 (Slor and Lev, 1973; Yamasaki et al., 1977) and the present results with single strand specific nuclease S1 are consistent with earlier findings. When native DNA complexed with hydrogen peroxide and exposed to UV light was subjected to nuclease S1 digestion, the single strand regions in double stranded DNA were selectively removed as seen by the decreased intensity of the band.

The hydroxyl radical was also found to produce DNA base modifications as revealed by ion-exchange chromatography. Thymine was found to be modified to the greatest extent followed by guanine which is consistent with earlier reports showing thymine to be the most susceptible to the modifying effects of hydroxyl radical (Hutchinson, 1985). In order to determine the participation of hydroxyl radical in causing alterations in thymine, its UV spectrum was compared to that of thymine dimer and was found to be clearly distinct from that of thymine dimer thereby suggesting that hydroxyl radical and not the UV radiation was responsible for the damage.

Monoclonal antibodies generated against 400 bp modified DNA (Ashok, 1996) were characterized for their fine antigenic specificity. These were found to be of IgG1 isotype. The specificity of the purified anti-ROS-DNA antibodies for antigenic determinants on modified DNA was determined by direct binding
and inhibition ELISA. Direct binding ELISA demonstrated strong binding for ROS-DNA which was further confirmed by inhibition ELISA. A maximum of 76.5% inhibition with the immunogen was observed and 4.4 μg/ml was required for 50% inhibition of antibody binding to antigen. The antibodies also showed an inhibition of 84% and 59.7% with native ROS-DNA and 200 bp ROS-DNA respectively, while the concentration required to obtain 50% inhibition was 3.0 and 10.0 μg/ml respectively. When the individual bases were used as inhibitors of anti-ROS-DNA antibody binding to the antigen, it was found that ROS-modified thymine was the most effective inhibitor followed by ROS-guanine while their unmodified forms were very poor inhibitors. On the other hand, the other two bases adenine and cytosine as well as their modified forms were not inhibitory. Also ROS-thymidine was inhibitory to the extent of 57.2% contrary to unmodified form. It was also observed that the ROS-modified homopolymer poly(dT) was an effective inhibitor of antibody binding to the antigen while its unmodified form was not. Poly(dA) and its modified form were non-inhibitory. These results clearly point out the preferential recognition of ROS-modified epitopes by the monoclonal antibodies having preference for thymine followed by guanine. Some degree of inhibition seen with native DNA and some synthetic polymers could be due to the sharing of antigenic determinants amongst ROS-modified antigens and native DNA or synthetic polynucleotides. The logical candidate for this shared epitope is a sugar-phosphate backbone that occurs in all polynucleotides (Lafer et al., 1981; Rauch et al., 1985).

The study of structure-function relationship of different amino acid residues of proteins has been successfully achieved by chemical modification of various amino acids (Cohen, 1970). Change in biological activity after chemical modification may be taken as an evidence of direct involvement of the modified
residue in catalytic function (Kaiser et al., 1985). The biological role of lysine residues in several enzymes has been demonstrated by chemical modification techniques. The acetylation of anti-ROS-DNA IgG was carried out to assess the role of lysine residues in the antigen binding characteristics of the protein. It was found that with an increase in acetylated lysine residues, there was a concomitant decrease in antibody binding, implying that such residues might be involved in the antigen binding site of the immunoglobulin molecule.

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease of unknown etiology. 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 double stranded DNA serve as an immunochemical marker for the diagnosis of SLE. However, native DNA is a poor immunogen and rarely induces antibodies in experimental animals. It has been reported that DNA after exposure to ROS presents a more discriminating antigen for the binding of SLE autoantibodies (Blount et al., 1989; 1990). 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 SLE sera were tested for their binding with various nucleic acids. It was found that appreciable binding was seen with ROS-DNA in eight different SLE sera and the concentration required to obtain 50% inhibition was found to be more for native DNA compared to ROS-modified DNA in six out of eight cases. Furthermore, in order to investigate the epitope(s) involved in anti-DNA autoantibody production in SLE, the individual base thymine modified with ROS was used as an inhibitor as ROS-thymine exhibited maximum binding with the monoclonal antibodies out of all the four bases tested. Interestingly, a high to moderate level of inhibition
was observed with almost all of the SLE sera tested with ROS-thymine as inhibitor. The homopolymer poly(dT) modified with ROS also exhibited around 50% or more inhibition in five out of the eight sera. The binding was further confirmed by a band shift and dot blot assay.

These results point out similar binding characteristics with the induced antibodies and naturally occurring SLE autoantibodies showing preference for ROS-modified epitopes. The likelihood of thymine modification as a result of ROS attack on DNA by phagocytic cells in SLE might be the trigger for the induction of circulating antibodies generated against modified epitope but cross-reacting with native DNA. The unusual DNA conformations are mostly immunogenic as they exist only transiently in cells and are therefore not subject to tolerance. Therefore, when administered to animals in a stabilized form, they can stimulate antibody production. Inhibition studies with unmodified thymine/adenine and ROS-modified adenine failed to show appreciable binding to SLE autoantibodies. It appears that pathogenic anti-DNA autoantibodies are generated by some altered conformational epitopes on nucleic acids.

The role of oxidative damage has been suggested in autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus (Loft and Poulsen, 1996). Lymphocytes isolated from patients suffering from either disease contain increased levels of 8-oxo-dG in the DNA. ROS has been found to induce the formation of 8-oxo-dG and GC to AT transitions (Epe, 1991; Emmerit et al., 1995) and 8-oxo-dG mispairs mainly with adenine (Shibutani et al., 1991; Kamiya et al., 1995b). Therefore, oxidatively modified DNA having altered base(s) and conformational difference from B-form trigger immune response and the antibodies thus generated represent circulating anti-DNA antibodies in SLE patients. Accordingly, SLE patients suffer from an increased
rate of oxidative DNA damage and are also deficient in repair process. This may contribute to the pathogenesis of SLE as well as increased chances of malignant diseases in these patients (Lunec et al., 1994). In this study the presence of oxidative damaged lesions in SLE was detected using anti-ROS-DNA monoclonal antibody as a probe. Five out of the eight DNA samples of SLE patients tested showed a high inhibition of monoclonal antibody binding to ROS-DNA, while no appreciable binding was seen with DNA isolated from normal, healthy individuals. The data indicates the presence of oxidative lesions in the DNA of these patients that are being effectively recognized by the monoclonal antibody.

ROS-DNA induced high titre antibodies in rats. Kidney sections of these rats were used to detect the deposition of immune complexes in the glomerulus. Immunofluorescence revealed the accumulation of immune complexes in the glomeruli of the kidney as evident by an apple green fluorescence. This could be correlated to the finding that lupus nephritis is a prototype of immune complex mediated disease (Tsai et al., 1992). Deposition of the complexes between DNA and antibodies to DNA in kidneys has been considered to elucidate the inflammatory reaction in lupus nephritis.

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

1. DNA modified with UV light (254 nm) in the presence of hydrogen peroxide resulted in the formation of single strand breaks and modification of DNA bases.

2. Thymine was the major base modified with ROS, followed by guanine.

3. The monoclonal antibodies against ROS-modified DNA were found to be of IgG1 subtype.
4. The monoclonal antibodies were found to be specific towards ROS-modified epitopes with some degree of binding seen with native DNA suggesting the presence of certain common epitopes on native and modified polymer.

5. Lysine residues of monoclonal antibodies were found to be involved in antigen recognition.

6. SLE anti-DNA autoantibodies showed a preferential binding for ROS-DNA compared to its native form.

7. ROS-thymine and ROS-poly(dT) exhibited high degree of binding to SLE autoantibodies.

8. Almost identical antigenic specificity of the induced anti-ROS-DNA antibodies and SLE autoantibodies as well as high binding of these antibodies with ROS-thymine suggests that ROS-thymine could be the major modified epitope, against which antibodies are produced in SLE which are cross-reacting with native DNA.

9. Monoclonal antibody used as a probe showed effective recognition of oxidative damage to DNA in SLE patients.

10. Immune complex deposition in the glomeruli of kidney of ROS-modified DNA immunized rat was observed.