Discussion
Autoimmune diseases have been associated with a myriad of immunological abnormalities ranging from increased proinflammatory cytokines, decreased anti-inflammatory cytokines, increased expression of major histocompatibility complex molecules, skewing of T-cell receptor repertoire, increased numbers of B cells, and autoantibody production. It has been widely suspected that underlying all these immunological changes, there is a defect in the ability to eliminate self-reactive T cells or B cells. Although much knowledge has accrued regarding various mechanisms that affect autoimmune disease, the exact causes and mechanisms of malfunction and tissue damage remain unclear. This failure is probably due to complex processes involved in immune recognition and regulation. Though a host of components that could lead to autoantibody production have been discovered, it appears that it will be the unraveling of the exact way in which these species interact that may actually lead to a complete understanding of autoimmune processes. The identification of the inciting antigen has been masked by three areas of ambiguity. First, it is uncertain whether identified autoantibodies and corresponding antigens have anything to do with the primary cause or pathogenesis of a given disease. Second, the immune diversity of autoantibodies observed in some diseases causes considerable difficulty in the identification of the
inciting antigen. Third, there is uncertainty as to whether the initial trigger has any relation to the antigen recognized by the autoantibodies (Plotz, 1992; Mongey and Hess, 1993; Levinson, 1994; Mountz et al., 1994; Radic and Weigert, 1994 and Theofilopoulos, 1995).

The presence of antinuclear antibodies in the sera of patients suffering from various autoimmune disorders is of considerable importance. 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; Pisetsky et al., 1990). Amongst these antibodies those specific for DNA (anti-DNA antibodies) have received much attention as they are highly characteristic for SLE. Efforts to induce anti-DNA antibodies by immunization with native DNA have been unsuccessful indicating thereby that native DNA per se is not the immunogen for these antibodies.

Despite considerable work that has been done to understand the pathogenesis of SLE, it is still unknown whether a single factor or more than one factor participate to initiate SLE (Frank et al., 1990). The polyspecific binding of SLE autoantibodies to a whole gamut of modified nucleic acid conformers (Ali et al., 1991; Alam and Ali, 1992; Alam et al., 1992., 1993; Arjumand and Ali, 1994;
Klinman et al., 1994, and Moinuddin and Ali, 1994) has frustrated the efforts of lupus researchers to understand the origin and consequences of anti-ds DNA antibodies. It has long been recognised that more active immune responses and a higher incidence of autoimmune diseases occur in females (Lahita, 1985). Sex appears to be a potent risk factor as suggested by strong preponderance of SLE in females as compared to males, thus drawing immediate attention to the possible involvement of female sex hormones (Talal and Ahmad, 1987; Folomeev et al., 1990, 1992; Lahita, 1992a, 1992b). Estrogen has also been reported to be a potent disease accelerator in SLE prone MRL lpr/lpr mice (Carlsten et al., 1990). Furthermore, elevated binding of estradiol to serum globulins was found in SLE patients and women who had taken oral contraceptives as compared to normal men and women (Counihan et al., 1991).

In this study, two aspects were probed. One, the induction of Z-conformation in native calf thymus DNA as a result of bromination in high salt and by treatment with polyamines and second, the covalent modification of native DNA (average size 200 bp) by linking it to BSA and estradiol-albumin (E<sub>2</sub>-BSA) respectively. The possible role of these modified conformers in the pathogenesis of SLE has been investigated.

The B→Z transition of synthetic polynucleotides as a
result of bromination in high salt has been well documented (Thomas et al., 1988). Bromination of double stranded DNA altered its UV absorption characteristics. The changes were more pronounced in the case of native DNA brominated in high salt compared to the polymer brominated in low salt. A large decrease in absorbance at 260 nm and increased absorbance at around 295 nm was observed in DNA brominated in high salt, while the polymer brominated under physiological conditions of saline did not show such marked changes in the UV spectrum. Moreover, the absorbance at around 300 nm in the difference spectrum was quite enhanced for native DNA brominated in high salt compared to the low salt sample. The spectra obtained in the case of DNA brominated in 4M NaCl are quite similar to the pattern exhibited by poly(dG-dC).poly(dG-dC) when brominated in high salt, indicating thereby that native calf thymus DNA has attained Z or Z-like conformation. The absorbance ratio ($A_{295}/A_{260}$) of native DNA brominated in high salt was 0.35 while for the low salt brominated form, it was 0.188. This is also a parameter to judge the formation of Z-conformation because $A_{295}/A_{260}$ value for prototype Z-DNA is 0.3 and this value has been accepted as a characteristic of Z-conformation (Thomas and Strobel, 1988). The results were also evaluated by circular dichroism studies wherein the inversion of spectrum in the case of DNA brominated in high salt provides additional
evidence for the attainment of Z-conformation.

The barrier of interconversion of double stranded DNA between right handed and left handed helical forms was also challenged by polyamines, which are known to induce Z-conformation in synthetic polynucleotides (Thomas et al., 1990; Takeuchi et al., 1991). The importance of spermidine and spermine lies in their requirement for normal cell growth and differentiation. Their interaction with nucleic acids may be responsible, in part, for the biological function of polyamines. (Pegg, 1988). The transition of native B-form of DNA to Z-conformation in the presence of polyamines was checked by absorbance ratio and interaction with anti-Z-DNA antibodies. Taking into consideration the absorbance ratio (average 0.315) observed in case of poly(dG-dC).poly(dG-dC) and concentration of spermine required to achieve this conversion (40 µg/ml), native calf thymus DNA indicated around 15% isomerization into Z-conformation (average absorbance ratio was 0.125 at 100 µg/ml of spermine). The specific binding of anti-Z-DNA antibody to polyamine-DNA complex is another evidence of conformational isomerization of native B-epitopes into Z-conformation in the presence of increasing concentrations of spermine, spermidine and putrescine. Poly(dG-dC).poly(dG-dC) taken as a reference polymer showed similar epitope polymerization. Anti-Z-DNA antibody had no binding with
poly(dG-dC).poly(dG-dC) and native DNA when the polynucleotides were coated on the microtitre plate in the absence of polyamines. Monoclonal and polyclonal anti-Z-DNA antibodies have been used to detect the presence of left handed segments in natural DNAs (Arndt-Jovin et al., 1983; Lee et al., 1984; Nordheim et al., 1986). The solid phase enzyme immunoassay is a very sensitive technique to study the B->Z conformational transition of polynucleotides (Thomas et al., 1988).

Native calf thymus DNA (average size 200 bp), obtained as a result of controlled digestion with micrococcal nuclease was covalently modified by linking with E₂-BSA in order to see whether the female sex hormone, when conjugated to DNA, could form a possible antigenic trigger for SLE as the disease is more prevalent in women. The linked and unlinked species were separated by exclusion chromatography on Sephadex G-200 column. Two distinct peaks were obtained. However, colorimetric estimations could detect DNA only in the first peak while protein was present in both the peaks, implying that peak 1 contained E₂-BSA-DNA conjugate. The altered spectral properties of DNA signify modifications in the double helix as a result of conjugate formation.

The change in the absorbance ratio \( \frac{A_{260}}{A_{280}} \) from 2.2 in case of DNA to 1.6 for DNA-BSA conjugate and 1.45 for E₂-
BSA-DNA conjugate also suggests DNA modification as a result of conjugation.

The modified nucleic acid polymers, with the exception of brominated DNA, because in this case the helix did not melt even at 100°C, were also characterised by thermal denaturation studies. While for native DNA the observed Tm was 87°C, it was 90°C in the case of DNA-spermine complex. Moreover the DNA-spermine helix disruption started quite late (87.5°C) compared to native DNA. This means that the helix was resisting heat induced strand separation to a greater extent in case of DNA-spermine, meaning thereby that complexation of spermine has imparted stability to the DNA molecule.

The E₂-BSA-DNA and DNA-BSA conjugates were also characterised by thermal denaturation studies. The increased Tm value and decreased percent denaturation at 80°C in case of E₂-BSA-DNA as compared to native DNA fragment indicates that E₂-BSA-DNA is thermodynamically more stable than the corresponding native DNA fragments. On the contrary, the DNA-BSA conjugate was found to be less stable as judged by the decreased melting temperature and greatly increased percent denaturation at 80°C. The only difference between DNA-BSA and E₂-BSA-DNA conjugate is the presence of estradiol molecule linked to BSA in the later species. Thus inference can be drawn that estradiol is somehow imparting
stability to the helix in E₂-BSA-DNA conjugate.

Additional evidence for the structural perturbations in native DNA and 200 bp DNA fragments as a consequence of interactions with protein, polyamine and hormone was gathered by computation of thermodynamic parameters. Native DNA displayed a large negative value for Gibb's free energy of denaturation (ΔG_D) till 85°C. The results depicted in Table 7 speculate the tremendous stability exhibited by double helical native DNA. Shift in ΔG_D value from negative to positive above 85°C suggest the disruption of Watson-Crick base pairing and the transition of double helical nucleic acid from the native to denatured state.

In comparison to native DNA, the DNA-spermine complex displayed negative ΔG_D values beyond 85°C suggesting that DNA-spermine complex is thermodynamically more stable than native DNA. This could be attributed to the fact that polyamine (spermine) having multiple positively charged amino groups interacts electrostatically or ionically with the negatively charged phosphate backbone of DNA and during the course of interaction, it encompasses the DNA macromolecule thereby enhancing helix stability.

Apart from native DNA, the DNA fragments (average size 200 bp) were also employed in this study to probe their interaction with BSA and E₂-BSA respectively. In case of DNA fragments negative ΔG_D value was obtained only till 70°C
while for native DNA negative $\Delta G_D$ value persisted till 85°C implying thereby, that compared to native DNA, the 200 bp DNA fragments are thermodynamically less stable. The decreased stability of 200 bp fragments could be attributed to low G=C contents. In case of DNA-BSA conjugate, the negative $\Delta G_D$ values were observed only till 45°C. Thus a net difference of around 25°C was observed in the persistence of negative $\Delta G_D$ values when compared with the corresponding control (200 bp DNA). The thermodynamic results speculate that a large portion in the DNA-BSA conjugate was already in the unstacked form. Clearly, the covalent conjugation of BSA with the amino groups of nucleic acid bases appears to completely obliterate the favourable A=T and G=C pairing interactions of the double helical DNA. The instability may be attributed to the conjugation of BSA to the potential sites or substrates in DNA rendering these conjugated sites in DNA topologically less constrained than the corresponding fully paired and fully stacked Watson-Crick interactions.

However, when estradiol-BSA was covalently linked to DNA fragments (~200 bp), a totally different type of observation was recorded. In this case, surprisingly, large negative $\Delta G_D$ values were observed till 77°C of thermal supplementation. Thus, a priori to DNA-BSA, where destabilization was observed, entirely reversed results were
observed (tremendous stability) in the case of $E_2$-BSA-DNA conjugate. The thermodynamic parameters suggest that, although the glutaraldehyde aided conjugation of BSA alone resulted in helix opening, the conjugation of $E_2$-BSA has somehow stabilized the helix. The increased stability of this hormone-protein aducted DNA is suggestive of the formation of crosslinks in the 200 bp DNA macromolecule. The thermodynamic parameters observed here substantiate the mid-point melting ($T_m$) data described earlier.

The results of time dependent denaturation of native as well as modified nucleic acid were also interesting. The lag period shown by DNA-spermine complex was a bit larger as compared to native DNA indicating the induction of an additional kinetic barrier in native DNA as a consequence of ionic interaction with spermine. The increase in the $t_{1/2}$ (half life) value for the denaturation of DNA-spermine complex in comparison to native DNA further substantiates the above argument.

Native DNA fragments (average size 200 bp) exhibited a relatively shorter lag phase, thereby suggesting that the kinetic barrier for 200 bp DNA was relatively weaker than native DNA. On the contrary, DNA-BSA conjugate formed as a result of covalent conjugation by glutaraldehyde exhibited a minor transition but not the large lag period, perhaps due to the denaturation of a "segment" involving the conjugated
sites in the DNA-BSA conjugate, which consequently initiates
the rapid denaturation of nucleic acid protein conjugate.
The decrease in the half life ($t_{1/2}$) for melting of DNA-BSA
conjugate supports the above explanations. Surprisingly, 200
bp DNA covalently linked to hormone protein (E$_2$-BSA)
conjugate exhibited a small increment over the lag period of
native DNA (~200 bp). This increase was substantial when
compared to the lag period of DNA-BSA conjugate. This could
be due to the introduction of a large kinetic barrier in the
200 bp DNA as a result of E$_2$-BSA conjugation, perhaps due to
the formation of crosslinks in the helix. The increase in
$t_{1/2}$ for melting of E$_2$-BSA-DNA conjugate further
substantiates the above argument.

The E$_2$-BSA, DNA-BSA and E$_2$-BSA-DNA conjugates were
found to be potent immunogens and induced high titre
antibodies in rabbits. The induced antibodies were specific
for their respective immunogens. The binding of anti-E$_2$-BSA
antibodies to E$_2$-BSA-DNA and vice-versa clearly indicates
that antibodies are recognizing common epitopes on these two
antigens. The inhibition in the activity of anti-E$_2$-BSA and
anti-E$_2$-BSA-DNA antibodies by estradiol is due to the
presence of a small population of antibodies that is cross-
reactive with estradiol. None of the three antibodies showed
inhibition with either poly(rG).poly(dC) or native calf
thymus DNA brominated in high salt. The data indicates that
the antibodies are not recognizing either A/A-like or Z/Z-like conformations meaning thereby that E₂-BSA-DNA and DNA-BSA conjugates have attained neither A-nor Z-conformation. The induced antibodies did not recognize native DNA. However, anti-E₂-BSA-DNA antibodies were inhibited by heat denatured DNA (ssDNA) and RNA, but at a high inhibitor concentration (75 μg/ml), pointing towards the presence of a minor population of base specific antibodies.

The recognition of various DNA conformations by SLE anti-DNA antibodies has got far reaching significance for the polyspecificity of SLE autoantibodies. The binding specificity of anti-DNA antibodies to various modified forms of DNA was analyzed by inhibition ELISA in which the ability of a competitor to block the DNA-autoantibody interaction is assessed. Human autoantibody recognition of the Z-conformation induced either as a result of bromination in high salt or by polyamines (under physiological conditions of saline) is significant because Z-DNA can arise in vivo as a result of various molecular events and also, the formation of Z-DNA can be facilitated by protonated amines. It is worth while to mention here that significantly elevated levels of polyamines in the case of active SLE patients (Puri et al., 1978) can, in vivo, lead to the attainment of Z-conformation by native DNA. Since Z-DNA is highly immunogenic, the possibility of this polymer acting as
antigen for the production of human autoantibodies cross-reactive with native DNA could be one of the factors for the pathogenesis of SLE.

A unique finding of this study is the binding of $E_2$-BSA-DNA conjugate to naturally occurring anti-DNA antibodies derived from the sera of SLE patients.

The results indicated the recognition of altered conformation of the modified polymer. The $E_2$-BSA-DNA conjugate was an effective inhibitor in DNA-anti-DNA system. So far, the possible explanations put forth for the production of anti-DNA autoantibodies have focussed mainly on the modification of DNA either as a result of environmental factors, hydroxyl/free radicals, radiations or some other factors including genetic and viral (Steinberg, 1992; Ara and Ali, 1992; Theofilopoulous, 1995). It is quite possible that in diseased state there may be altered conformation of estradiol-DNA conjugate rendering the hormone-receptor-DNA complex as 'alien' to the immune system, which might account for the predominance of SLE in females and also for the production of antibodies that cross-react with DNA. Compared to native DNA, SLE autoantibodies exhibited enhanced binding to $E$-estradiol in ELISA experiments performed by using both colorigenic and fluorogenic substrates. It has been reported that the fluorogenic substrate increases the sensitivity of ELISA
(Ali and Ali, 1983). This provides further credence to the emerging hypothesis that native DNA is not the antigen responsible for the production of autoantibodies in SLE. This finding lends further support to the view that female sex hormone, might in one way or the other, be responsible for the production of lupus autoantibodies as the disease is more prevalent in women. It is possible that some sex hormonal disturbances that could occur as a result of contraceptive therapy, irregular menstrual cycle, child birth etc. might be contributing towards the production of autoantibodies. What appears from the studies undertaken so far and from the present work is that SLE is not a consequence of a single factor. The disease seems to be the result of various factors acting together or it is also possible that a single factor, might be leading to a chain of reactions involving other factors. The finding of Waters et al. (1992) wherein they have reported increased polyamine levels as a result of estradiol treatment to Atlantic salmon (Salmo salar), a fish, provides a direct relation between increased estradiol concentration and elevated polyamine levels in the body. If such a condition could also occur in humans than one might be able to explain and correlate the female predominance of SLE and the Z-DNA binding of anti-DNA antibodies from lupus patients, as it is well documented that high polyamine levels induce B→Z transition.
Based on this study the following points of conclusion could be drawn:

1. Induction of Z-conformation in native calf thymus DNA depends on its microenvironment. Z-DNA can be formed either by bromination in high sodium chloride concentration or as a result of polyamine interaction with DNA under physiological conditions of saline. Z-conformation in native calf thymus DNA is maintained even when the high salt brominated polymer is dialyzed in a buffer containing 0.15 M NaCl.

2. The binding of SLE anti-DNA antibodies to Z-conformer suggest that Z-DNA may be a potential putative autoantigen for the induction of antibodies cross-reactive with native DNA.

3. The modified forms, viz., DNA-spermine and E₂-BSA-DNA conjugate are thermodynamically more stable than their respective controls, i.e native DNA and DNA fragment (~200bp).

4. The binding of naturally occurring autoantibodies to E₂-BSA-DNA as well as the interaction of these antibodies with 8-estradiol clearly points towards the role of female sex-hormone in the pathogenesis of SLE.

5. It is suggested that female sex-hormone, either alone or in conjugation with DNA, might be challenging the immune system leading to autoantibody production.