FINE ANTIGENIC SPECIFICITY OF HUMAN ANTI-DNA AUTOANTIBODIES AGAINST DNA OF VARYING SIZE AND CONFORMATION

ABSTRACT

Thesis Submitted for the Degree of Doctor of Philosophy in BIOCHEMISTRY

BY KHURSHID ALAM

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ALIGARH (INDIA)
1992
Antibodies reactive with cellular and nuclear components occur spontaneously in patients with systemic lupus erythematosus. The basis for spontaneous production of anti-native DNA antibodies in systemic lupus erythematosus remains a puzzling question especially in view of the difficulty in their induction on immunization with DNA in B-conformation. The anti-dsDNA antibodies seem to be closely related to the disease pathogenesis through the process of immune complex formation, and their levels are frequently indicative of the intensity of disease activity. For reasons that are not best understood, anti-DNA antibodies have always exhibited polyreactivity with DNA molecules of varying source, size and conformation. Analysis of plasma DNA from SLE patients have revealed that these molecules are 100-1000 base pair long and rich in G+C sequences. Such DNA sequences have the unique potential to undergo B- to Z- or Z-like transitions under constraint conditions.

In the present study purified native calf thymus DNA was digested with micrococcal nuclease and fractionated on Sepharose 4B. The approximate fragment size was determined by standard DNA marker. DNA-(polyllysyl-Sepharose 4B) affinity matrix was chosen for the isolation of homogeneous anti-DNA antibodies from SLE serum. The affinity purified human anti-dsDNA antibody was found to possess polyreactivity with native and modified DNA antigens.

Native DNA covalently modified with lysine under UV-A light (320-400 nm) was found to contain one lysine residue per helical turn. By the photoaddition of one lysine molecule per helical turn of DNA, the native B-conformation was considerably altered which resulted in the appearance of neoepitopes leaving much less epitope typical of B-conformation. Affinity purified anti-dsDNA IgG showed maximum affinity towards DNA-lysine photoconjugate followed closely by native DNA. The retention of crossreactivity of affinity purified anti-dsDNA IgG with DNA-lysine photoadduct strengthen the idea of an alternate antigen for the induction of antibodies crossreactive with native DNA. Lysine rich histone
HI in nucleosome thus on modification by physical, chemical or environmental agents might form DNA-histone adduct making it immunogenic and thus generating antibodies crossreactive with native B-conformation.

Hydroxyl radical generated by hydrogen peroxide and ferrous ions caused single strand breaks in native DNA, decrease in melting temperature (Tm) by 6.5 °C and modification of purine and pyrimidine bases. Experimentally induced antibodies against ROS modified DNA exhibited polyreactivity, a property commonly associated with SLE anti-DNA antibodies. Native DNA, RNA and synthetic polynucleotides in B-conformation were found to be effective inhibitor of induced antibody-immunogen interaction. The SLE antibody binding with ROS-DNA was better than native DNA. Similar results were observed in competition-inhibition assay. Because of the diminished superoxide dismutase activity in SLE patients, the excessively generated free radicals could interact with DNA resulting in its modification. Once the native structure of DNA is modified it may become immunogenic.

The binding specificity of SLE anti-DNA IgG with varying DNA conformation was analyzed in polyacrylamide or agarose gel. The altered electrophoretic mobility in gel retardation assay of A- and B-conformation complexed with anti-DNA IgG reiterates the antibody binding to these conformers. The above conformers showed high binding with SLE antibodies in competition-inhibition experiments.

The B- to Z- transition of poly(dG-dC).poly(dG-dC) in 4.0 M NaCl was evaluated in terms of "absorption ratio", the average of absorbance at 294 and 296 nm divided by absorbance at 260 nm. This ratio was observed to be 0.322. The high salt induced Z-form of poly(dG-dC). poly(dG-dC) was stabilized by bromination which exhibited Z-DNA characteristics when brought in physiological buffer. The low salt brominated poly(dG-dC). poly(dG-dC) exhibited the characteristics of B-DNA. Native DNA under low or high salt concentration showed "absorbance ratio" greater than 0.3. Under identical conditions the absorbance ratio for small DNA fragments varied between 0.2 to 0.38. The data indicate that small DNA fragments have different levels of potentiality to undergo B- to Z- or Z-like conformational transitions as the fragments will have variation
in the G+C and A+T contents. Native DNA or small DNA fragments (300 bp) brominated under low or high sodium chloride concentrations indicated regions of interspersed sequences which has the potential to undergo B- to Z- or Z-like transitions. The Z- or Z-like epitopes on brominated native DNA and small DNA fragments was confirmed by binding and inhibition data of monoclonal and polyclonal anti-Z-DNA antibodies.

The effect of varying concentrations of D- and L-polylysine or DNA showed that in native calf thymus DNA there are certain DNA sequences which are sensitive to elevated levels of polyamines and can undergo B- to Z- or Z-like transitions. Since the eukaryotic DNA is heavily associated with proteins rich in lysine, such conformational transitions might serve as molecular signal for genetic events inside the cell. That the bromination has changed the typical B-characteristics of DNA was also proved by decreased percentage inhibition of SLE anti-dsDNA IgG activity by the brominated forms of DNA compared to high percent inhibition of SLE anti-dsDNA IgG activity when the same polymers were in a typical B-conformation. This again shows that SLE antibodies are polyspecific with respect to binding towards varying DNA conformation.

In conclusion, native and small DNA fragments have interspersed base pair sequences which have the potential to undergo B- to Z- or Z-like transitions. Irrespective of salt concentrations, covalent modification (e.g. bromination) induces Z- or Z-like helix in native and small DNA fragments. That the epitopes were presenting the features of Z-DNA was confirmed by anti-Z-DNA antibodies binding data. A detailed study of SLE anti-DNA antibody interaction with modified DNA antigens (DNA-lysine photoconjugate and hydroxyl radical modified DNA) have been carried out to explore the possibility of participation of these neoantigens in the etiopathogenesis of SLE. It appears that the polyspecificity of anti-DNA antibodies is much greater than what is anticipated.
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Approved:

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Prof. RASHID ALI (Supervisor)

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1992
CERTIFICATE

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

(RASHID ALI)
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ALIGARH 202 002
INDIA
Facing my window, across the road, stands an old and elegant banyan tree. It soaks rain, filters sun, absorbs chill but provide shelter and comfort to every passerby. Through these five years be it a moment of crisis or despair I have received same comfort, guidance and protection from my supervisor Professor Rashid Ali. I thank him for giving me an opportunity to be benefitted from the sunshine of his knowledge and academic brilliance.

My sincere thanks are also due to Professor A. Salahuddin, Chairman of the department for the provision of necessary facilities.

I owe heart felt thanks to Drs. W.A. Naqvi, M.A. Qasim, A. Ali and M.U. Siddiqui for their encouragement and suggestions. Dr. Saad Tayyab of the Biotechnology unit is also thanked for his constructive criticism.

Words cannot express my deep sense of gratitude to my teachers and well wishers Professors A.M. Siddiqui, M. Saleemuddin, S.M. Hadi, A.H. Siddiqui, Dr. Sheela Shahab and Mr. Shamshad Alam for their patient listening to my genuine demands.

I am privileged to thank my research colleagues Ms. Sadia Arjumand, Mr. Moinuddin, Ms. Zarina Arif and Ms. Jahan Ara for their unmatchable cooperation and day to day scientific discussion.

Thanks are also due to my senior colleagues Dr. Rashid Hasan, Dr. Najmul Islam, Dr. Amita Gupta, Mr. Naushad Ali and Mrs. Vandana
Very sincerely I owe heartfelt thanks to my friends Abeeb Alam Khan, Abul Faiz Faizy, Mohd. Aleem, Arshad Rahman, Imranuddin, Zubair, Dilshad, Fabeha, Tabassum, Tauseef, Gaity and Lubna for their love, help and encouragement.

I will be failing in my duty if I do not acknowledge Drs. A.S. Khan and Shahzad F. Haq of the Nephrology division for providing the sera of SLE patients.

I am highly thankful to nonteaching staff of the department for their constant help. Cooperation of Lab. Assistants M/s Sabu Khan, Ashfaq Ali and Rizwan Ahmad is highly appreciated.

It takes many hands to give the final shape to Ph.D. thesis. I am thankful to Mr. Mazahir Hussain and Mr. Syed Vigar Husain for typing and Mr. Nadeemuddin for graphic work. I appreciate the help given by the staff of central photography section in preparing the gel photographs.

Lastly, the financial assistance provided by the University Grants Commission, New Delhi is gratefully acknowledged.

(KHURSHID ALAM)
<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>[i]</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>[iv]</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>[v]</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>[ix]</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>[1]</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>[30]</td>
</tr>
<tr>
<td>RESULTS</td>
<td>[51]</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>[116]</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>[126]</td>
</tr>
</tbody>
</table>
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ABBREVIATIONS

\[ A_{260} = \text{Absorbance at 260 nm} \]

BSA = Bovine serum albumin

dsDNA = Double stranded DNA

DEAE = Diethylaminoethyl

EDTA = Ethylenediaminetetra-acetic acid (disodium salt)

High salt = 4 M sodium chloride

IgG = Immunoglobulin G

Low salt = 0.15 M sodium chloride

nDNA = Native DNA

PUVA = Psoralen plus ultraviolet A light

ROS = Reactive oxygen species

RT = Room temperature

ssDNA = Single stranded DNA

SLE = Systemic lupus erythematosus

TEMED = N,N,N',N'-tetraethylenediamine

Tm = Helix-coil transition temperature

UV = Ultraviolet

ul = Microliter
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Isolation of IgG from an SLE serum by DEAE Sephacel Chromatography</td>
<td>36</td>
</tr>
<tr>
<td>2.</td>
<td>Evaluation of titer and immunocross-reactivity of antibodies against human IgG</td>
<td>52</td>
</tr>
<tr>
<td>3.</td>
<td>Direct binding ELISA of antibodies against human IgG</td>
<td>53</td>
</tr>
<tr>
<td>4.</td>
<td>Melting profile of native DNA</td>
<td>54</td>
</tr>
<tr>
<td>5.</td>
<td>Fractionation of SLE IgG on Sephadex G 200</td>
<td>57</td>
</tr>
<tr>
<td>6.</td>
<td>Elution profile of DNA digested with micrococcal nuclease on Sepharose 4B column</td>
<td>60</td>
</tr>
<tr>
<td>7.</td>
<td>Thermal denaturation curves of DNA fragments</td>
<td>61</td>
</tr>
<tr>
<td>8.</td>
<td>Immunoaffinity purification of anti-dsDNA IgG on DNA-[polylysyl-Sepharose 4B] column</td>
<td>63</td>
</tr>
<tr>
<td>9.</td>
<td>Binding of human SLE antibodies to native DNA and DNA-lysine photoconjugate</td>
<td>66</td>
</tr>
<tr>
<td>10.</td>
<td>Assay of anti-DNA antibodies by ELISA</td>
<td>69</td>
</tr>
<tr>
<td>11.</td>
<td>Inhibition of SLE antibody binding by native DNA, heat denatured DNA and RNA</td>
<td>70</td>
</tr>
<tr>
<td>12.</td>
<td>Inhibition of SLE anti-DNA antibody binding by Br4 DNA, poly(dG-me\textsuperscript{5}dC) and poly(rG), poly(dC)</td>
<td>71</td>
</tr>
<tr>
<td>13.</td>
<td>Competition ELISA of SLE anti-DNA antibodies. The competitors were poly(dA),poly (dT), poly(dG),poly(dC), poly(dA-dG),poly (dC-dT), double stranded: poly(dG-dC), poly (dI-dC) and poly(dA-dU)</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>14.</td>
<td>Binding of SLE anti-DNA IgG to A- and B-conformation analyzed by gel retardation assay</td>
<td>74</td>
</tr>
<tr>
<td>15.</td>
<td>Direct binding assay of SLE auto antibodies. The coating antigens were native DNA, poly(dA-dT)-8-MOP, poly(dG-dC)-psoralen, DNA-8-MOP and DNA-psoralen</td>
<td>76</td>
</tr>
<tr>
<td>16.</td>
<td>Circular dichroism spectra of poly(dG-dC). poly(dG-dC) in 0.15 M and 4 M sodium chloride</td>
<td>78</td>
</tr>
<tr>
<td>17.</td>
<td>Ultraviolet spectra of poly(dG-dC), poly(dG-dC) and DNA fragments (300 bp) in 0.15 M and 4 M sodium chloride</td>
<td>80</td>
</tr>
<tr>
<td>18.</td>
<td>Ultraviolet difference spectra of poly(dG-dC), poly(dG-dC) and DNA fragments in 0.15 M and 4 M sodium chloride</td>
<td>82</td>
</tr>
<tr>
<td>19.</td>
<td>Ultraviolet difference spectra of poly(dG-dC) brominated in 4 M sodium chloride</td>
<td>83</td>
</tr>
<tr>
<td>20.</td>
<td>Ultraviolet absorption and difference spectra of native and brominated DNA fragments</td>
<td>84</td>
</tr>
<tr>
<td>21.</td>
<td>Ultraviolet absorption spectra of native DNA in presence of D- and L-polylysine</td>
<td>87</td>
</tr>
<tr>
<td>22.</td>
<td>Ultraviolet absorption spectra of native DNA in presence of D- and L-polylysine</td>
<td>88</td>
</tr>
<tr>
<td>23.</td>
<td>Ultraviolet difference spectra of polylysine DNA complex</td>
<td>91</td>
</tr>
<tr>
<td>24.</td>
<td>Ultraviolet difference spectra of polylysine-DNA complex</td>
<td>92</td>
</tr>
<tr>
<td>25.</td>
<td>Agarose gel electrophoresis of plasmid DNA in presence of hydrogen peroxide and ferrous ions</td>
<td>95</td>
</tr>
</tbody>
</table>
26. Ultraviolet absorption spectra of native and hydroxyl radical modified DNA ... 96
27. Hydroxyapatite column chromatography of native and hydroxyl radical modified DNA ... 97
28. Alkaline sucrose density gradient centrifugation of native and hydroxyl radical modified DNA ... 98
29. Melting profile of native and ROS-modified DNA ... 99
30. Binding of anti-ROS-DNA IgG with native and ROS-modified DNA ... 103
31. Inhibition of immunogen-antibody interaction by ROS-DNA. The competitors were native DNA, poly(dA-dG).poly(dC-dT), double stranded : poly(dl-dC) and poly(dA-dU) ... 105
32. Determination of antibody affinity by Langmuir plot ... 106
34. Direct binding and competition ELISA of human lupus antibodies binding to native DNA and ROS-DNA ... 107
35. Inhibition ELISA of anti-Z-DNA antibodies (Z 22) by poly(dG-dC) and poly(dG-dC) brominated in 0.15 M and 4 M sodium chloride ... 109
36. Inhibition of Z 22 binding to Z-DNA by poly(dA-dG).poly(dC-dT) and poly(dA-dG).poly(dC-dT) brominated in 0.15 M and 4 M sodium chloride ... 110
37. Inhibition of SLE anti-DNA IgG binding to native DNA. The competitors were poly(dA-dG).poly(dC-dT), poly(dA-dG).poly(dC-dT) brominated in 0.15 M and 4 M sodium chloride ... 111

38. Inhibition of SLE anti-DNA IgG binding to native DNA. The competitors were native DNA, native DNA brominated in 0.15 M and 4 M sodium chloride ... 114
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Autoantibodies associated with clinical findings</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td>Direct binding ELISA of SLE anti-DNA antibodies</td>
<td>56</td>
</tr>
<tr>
<td>3.</td>
<td>Ultraviolet characteristics of immunoglobulin G</td>
<td>59</td>
</tr>
<tr>
<td>4.</td>
<td>Melting profile of DNA fragments</td>
<td>62</td>
</tr>
<tr>
<td>5.</td>
<td>Lupus autoantibody binding with native and modified DNA</td>
<td>65</td>
</tr>
<tr>
<td>6.</td>
<td>Binding profile of SLE anti-DNA IgG</td>
<td>67</td>
</tr>
<tr>
<td>7.</td>
<td>Binding inhibition of anti-DNA autoantibodies by polynucleotides</td>
<td>72</td>
</tr>
<tr>
<td>8.</td>
<td>Binding of SLE anti-DNA IgG by DNA-Furocoumarin photoadducts</td>
<td>77</td>
</tr>
<tr>
<td>9.</td>
<td>Ultraviolet absorbance characteristics of DNA polymers under low and high salt</td>
<td>81</td>
</tr>
<tr>
<td>10.</td>
<td>Ultraviolet absorbance characteristics of DNA polymers brominated under low and high salt</td>
<td>86</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of polylysine on ultraviolet characteristics of native DNA</td>
<td>90</td>
</tr>
<tr>
<td>12.</td>
<td>Binding of SLE anti-DNA IgG by amines</td>
<td>93</td>
</tr>
<tr>
<td>13.</td>
<td>Ultraviolet and thermal denaturation characteristics of native and hydroxyl radical modified DNA</td>
<td>100</td>
</tr>
<tr>
<td>14.</td>
<td>Percent base modification of DNA by hydroxyl radical</td>
<td>101</td>
</tr>
</tbody>
</table>
15. Inhibition of the binding of Z 22 and G 10 to Z-DNA and NBr4 ... 112

16. Binding of SLE anti-DNA IgG by small DNA fragments brominated under low and high salt ... 115
The DNA has been the subject of biochemical investigations almost from the time it was first isolated from pus cell nuclei (Miescher, 1879). The information buried in DNA in the form of four-unit language gets amplified to 20 unit language of protein by simple permutation and combination of nucleotides. These events of language translation produce various types of polypeptides whose function is dictated by the type of cell where the event is taking place. The utmost importance of DNA lies in its unique property to adopt multiple conformations depending on its bioenvironment. The environment could mean ions, small molecules or even proteins. These alternate conformations are likely to play crucial role in gene expression by increased or decreased synthesis of regulatory proteins which are utilized by the cell for its betterment.

Structure and Antigenicity of DNA

James D. Watson and Francis Crick proposed their double helical model for DNA in 1953 on the basis of X-ray diffraction studies. Analysis of diffraction pattern showed that DNA can exist in two forms: as right-handed B-DNA, which is stable at a relative humidity of about 92 percent and as A-DNA which appears when the relative humidity falls to about 75 percent. There are two kinds of double-ring purine bases, adenine (A) and guanine (G), and two kinds of single-ring pyrimidine bases, cytosine (C) and thymine (T). The base pairs are held together by hydrogen bonds. Adenine normally is paired with thymine by two hydrogen bonds and guanine is paired with cytosine by three bonds. The two points of attachment of a base pair to its sugar ring do not lie directly opposite each other across the pair, and this is important for the geometry of a DNA double-helix. The edge of the base pairs along which the angle between attachment is less than 180 degrees is called the minor-groove edge, and the one forming an angle larger than 180 degrees is the major-groove edge. B-DNA has an average of
10/10.5 base pairs per turn of the helix, with a spacing of 3.4 Å along
the helix axis from one base pair to the next. The A-helix has closer
to 11 base pairs per turn, and because of the way the tilted base pairs
are stacked, the distance along the axis is only 2.9 Å per base pair.
The right handed B-DNA is about 2 mm thick.

Since the discovery of B-DNA reactive antibodies in human SLE
and its murine equivalent (Robbins et al., 1957) and its clinical significance
in the disease, the biochemical and immunochemical approach of the
investigators working with this molecule have always faced problem
whenever this macromolecule was used as immunogen. Exogenous native
B-DNA has not been immunogenic in experimental animal. Even MRL
mice, which are genetically programmed to produce anti-B-DNA antibodies
"spontaneously", do not respond to exogenous DNA and it did not result
in increased anti-native DNA antibody production above the level that
resulted from injection of adjuvant alone (which does cause some increase)
(Madaio et al., 1984). Later normal animals immunized with DNA, including
DNA complexed with adjuvant, show only limited antibody production
which is almost exclusively directed to single stranded DNA (ssDNA)
(Plescia et al., 1964). The first experimental anti-DNA antibodies appeared
only in 1960, in the form of antibodies to denatured (single stranded)
DNA of the T-even bacteriophages (Levine et al., 1960). By contrast,
double stranded RNA, RNA-DNA hybrid, triple helical RNA and DNA
analogues, and double helical polydeoxyribonucleotides that differ from
average B-DNA structure, such as poly (dG). poly (dC) have been found
to be immunogenic (Stollar 1973, 1975, 1986; Anderson et al., 1988).
Injection of histone-DNA complexes yielded some anti-histone but not
anti-DNA antibodies (Stollar and Ward, 1970); nor did immunization
with isolated nucleosomes induce anti-DNA antibodies (Einck et al.,
1982). A monoclonal antibody from animal immunized with a duplex
DNA, poly (dT-dC). poly (dG-dA) gave a weak cross reaction with native
DNA (Lee et al., 1985). Recently, injection of bacterial native DNA
into normal mice induced antisera that bound native bacterial but not
mammalian DNA (Gilkeson et al., 1989b). However, the sera reacted
with denatured DNA from both sources. Injection of BK virus into rabbits induced antibody to both native DNA and histone (Flaegstad, 1988). Antibodies induced by 5-methylcytosine also include some populations that react with a native circular XP 12 phage DNA that is rich in 5-methylcytosine content (Adouard et al., 1985). The modified portion of this base would be accessible to antibody in the major groove and there is evidence to show that a portion of antibody's combining site can enter the major groove and interact with accessible surfaces of bases (Stollar, 1986). Using a highly sensitive avidin-biotin micro ELISA, the base specific binding of adenosine antibodies to double stranded DNA has been reported (Vaishnav and Antony, 1990).

To explain the unexpected presence of anti-DNA antibodies in the sera of normal subjects, it has been proposed that antibodies that are highly specific for bacterial DNA arise as part of the overall host response to infections by these organisms. In this setting, bacterial DNA would be recognized as foreign because of the presence of sequence arrays, structural features, or higher order conformations that are not usually present in human DNA. Further studies have shown that bacterial DNA differs from mammalian DNA in its content of pyrimidine clusters, patterns of base methylation, and undoubtedly, in many of its coding sequences (Wells, 1988; Szybalski et al., 1966; Wells et al., 1977; Cheng et al., 1985).

**Types of DNA within B-Family**

The B-DNA structure *per se* is a poor immunogen. There are certain right handed double stranded polydeoxyribonucleotides which differ slightly from the average B-DNA helix and are immunogenic beyond the adjuvant induced autoantibody expansion (Madaio et al., 1984). The immunogenicity itself is an indication that they differ in structure from the average B-DNA. Further, nearly all of the antibodies generated recognize those differences almost uniquely; they react with immunogen, but very weakly or not at all with DNA. This again indicates the existence of structural differences in the helices. The B-form of synthetic polydeoxyribonucleotides have been found to be immunogenic and recognize
the difference between them and native B-DNA. These polymers include poly(dG), poly(dC) (Stollar, 1970); poly (dT-dC), poly (dG-dA) (Lee et al., 1985); poly (dA), poly (dT) (Diekmann and Zarling, 1987; Arnott et al., 1983); poly (dG-dC) (Zarling et al., 1987). The other right handed structure in natural DNAs are A- and C-DNA which are found on changing the humidity and salt conditions (Arnott and Hukin, 1972; Arnott and Selsing, 1975). Double stranded RNA and RNA-DNA hybrid take on the A- or A-like form (Spencer et al., 1962; Milman et al., 1967). The sequence (dC-dA)ₙ.(dG-dT)ₙ is the most abundant purine-pyrimidine dinucleotide repeat in eukaryotic genome (Weber, 1990). Relatively short (approximately 50 bp) stretches of this sequence occur, on average, at a frequency of once 30 to 100 kb in all eukaryotic genomes thus far examined, from yeast to human (Hamada et al., 1982). Studies on the chromatin structure of the potential Z-forming sequence (dT-dG)ₙ.(dC-dA)ₙ has revealed evidence for an "alternating B" conformation on the nucleosomal surface (Gross et al., 1985). It is possible that the alternative helical conformation of these sequences yields a distinctive structure in the nucleus that is recognized by proteins functioning in recombination and replication events. Because these sequences exhibit length polymorphism, they represent a new rich source of informative genetic markers (Weber and May, 1989). An "alternating B" structure has been proposed for poly (dA-dT),poly(dA-dT) in solution based on DNase I cleavage pattern, proton and 31P nuclear magnetic resonance (NMR) spectroscopy, and X-ray fiber diffraction (Klug et al., 1979). The synthetic deoxyoctanucleotide d(G-G-G-G-T-C-C-C) crystallizes as an A-type DNA double helix containing two adjacent G-T base pair mismatches (Kneale et al., 1985). Also, the potentially Z-DNA forming sequence d(GTGTACAC) crystallizes as A-DNA (Jain et al., 1987). Poly(dG).poly(dC) sequences, under torsional stress, induce an altered DNA conformation upon neighboring DNA sequences and such altered DNA structures may play an important functional role in chromatin (Shigematsu and Kohwi, 1985).

Conformation Variant of B-DNA : Z-DNA

There have been many attempts to propose alternative models for DNA other than right-handed double helical model. In fact, Crick...
and Watson (1954) ruled out the possibility of a left handed structure for DNA because they could not build a left handed double helical model for DNA. In 1965, Fuller et al., had indeed built a left handed B-DNA, but could not build a left handed model for A-DNA. The possibility of a left handed structure for DNA was ruled out since the transition $B \leftarrow A$ could not be possible in fiber if a left handed model for B-DNA and a right handed model for A-DNA were assumed.

The discovery of left handed Z-conformation of DNA has led to a renaissance of interest in DNA structure and polymorphism (Pohl and Jovin, 1972; Wang et al., 1979). The extensive research efforts aimed at understanding the structural details and possible biological significance of the altered conformation has so far resulted in furnishing only a limited information (Jovin et al., 1983; Rich et al., 1984; Sehgal and Ali, 1990; Hasan et al., 1991; Alam and Ali, 1992). The DNA sequences shown to promote the formation of left handed Z-DNA in vivo and in vitro have been implicated in increased frequencies of genetic recombination in bacteria (Murphy and Stinger, 1986), yeast (Treco and Arnheim, 1986) and mammalian systems (Bullock et al., 1986). Segments of DNA with alternate d(GpC) sequences are most prone to form Z-DNA, the next most effective sequence being d(GpT). Other sequences in which the perfect alternate is not preserved can also adopt the Z-conformation (Klysik et al., 1981; Nordheim et al., 1982; Haniford and Pulleyblank, 1983; Nordheim and Rich, 1983). Nevertheless, a strictly alternating purine-pyrimidine is neither necessary nor sufficient (McLean et al., 1986; Singleton et al., 1983; Kilpatrick et al., 1984; Ellison et al., 1985). It has also been shown that stretches of poly(dG-dC) sequences do not contribute significantly to the presence of Z-DNA in fixed polytene chromosomes of Drosophila melanogaster (Nordheim et al., 1986). Membrane hybridization experiments and screening of data banks have shown that d(GpT) long sequences (30 > n > 1) are generally dispersed in many if not all genomes. Long d(GpC) sequences are seen less frequently (Hamada and Kakamaga, 1982; Hamada et al., 1984; Rich et al., 1984).
Z-DNA, first described at the atomic level for the sequence (dC-dG)$_3$ by Rich and his colleagues (Wang et al., 1979), is a prototype left-handed double helix whose structural properties differ significantly from those of classic B-form DNA. Z-DNA contrast with B-DNA not only in its left-handed helical sense, but in twist (12 versus 10.5 base pairs per turn), helix diameter (18 Å versus 20 Å), number of helical grooves (1 versus 2) and structural repeat unit (dinucleotide versus mononucleotide) (Wang et al., 1979; Drew et al., 1980). In addition, the purine residues of Z-DNA are rotated 180° about the glycosidic bond from the anti- to the syn-conformation. In vitro analysis have demonstrated that linear duplex (dG-dC)$_n$ sequences can adopt the left-handed Z-conformation when subjected to elevated ionic strength (Pohl and Jovin, 1972; Patel et al., 1979; Klisky et al., 1981), or modification of either guanine or cytosine residues (Sage and Leng, 1980; Lafer et al., 1981; Behe and Felsenfeld, 1981; Klisky et al., 1983). Other DNA sequences with alternating purine-pyrimidine residues, including poly(dA-S$_4$dT) and poly(dT-dG).poly(dC-dA), exhibit fiber structures that are compatible with left-handed Z-form as well (Arnott et al., 1980). In addition, the B- to Z-transition can be induced when either (dG-dC)$_n$ or (dT-dG)$_n$. (dC-dA)$_n$ sequences are embedded in a B-DNA background in recombinant plasmid or eukaryotic genome (Singleton et al., 1982; Nordheim and Rich, 1983; Haniford and Pulleyblank, 1983; Gross et al., 1985). The above sequence and certain others that contain alternating purine-pyrimidine residues have been shown to adopt the left-handed Z-DNA conformation in vitro when subjected to negative torsional stress or elevated ionic strengths (Gross et al., 1985).

In 1984, Mura and Stollar showed the ability of different lysine rich histones to cause varying conformational changes in the condensation of chromatin in DNA regions of highly biased base sequence. Recently, it has been shown that complexation of poly(dA-dC).poly(dG-dT) with spermidine and spermine could significantly alter the immunogenicity of this polynucleotide and produce antibodies that reacted with Z-DNA
form of brominated poly(dG-dC).poly(dG-dC) in addition to the immunizing antigen. That the immunizing antigen has presented the features of Z-DNA was shown by its binding to monoclonal anti-Z-DNA antibodies (Z 22) (Gunnia et al., 1991).

The results so far have failed to detect Z-conformation in calf thymus DNA (Ct-DNA). But in 1990, Hasan and Ali reported that when Ct-DNA was brominated under high salt (4M NaCl) conditions it presented the features of Z-DNA. The Z-like conformation was retained even after the removal of salt. The Ct-DNA is heterogeneous in sequence and microheterogeneity exist in B-structure (Dutta et al., 1984; Sanford et al., 1988). The B-form of poly(dG-dC).poly(dG-dC) lacks most of this heterogeneity (Drew et al., 1981). The biochemical properties of Z-DNA, cruciforms, bent DNA, anisomorphic DNA, oligopurine-oligopyrimidine structures, etc., have been the subject of numerous in vitro investigations (Wells and Harvey, 1987; Wells, 1988; Rich et al., 1984). Z-DNA cannot be incorporated within core particles, except at their termini, and that a transition from B- to Z-form in vivo might result in a significantly altered local placement of nucleosomes (Garner and Felsenfeld, 1987).

Immunochemical studies have shown that Z-DNA is a powerful immunogen eliciting a highly specific antibody response (Lafer et al., 1981). The Z-conformation of DNA was determined in interbands of polytene chromosomes (Nordheim et al., 1981) and then in banded regions (Arndt-Jovin, et al., 1983), in nuclei of protozoa (Lipps et al., 1983), cells of rat tissue (Morgenegg et al., 1983) and in metaphase chromosomes (Viegas-Pequignot et al., 1983). Both polyclonal and monoclonal antibodies specific for Z-DNA have served as sensitive reagents for the detection of Z-epitopes by relatively simple antigen-antibody binding assay (Lafer et al., 1981; Malfoy et al., 1982; Zarling et al., 1984; Pohl et al., 1982; Hanau et al., 1984). Although, the precise amount of Z-DNA present in living cells has not been established, these experiments have demonstrated the potential for Z-DNA formation in chromatin and have helped to identify the conditions under which the transition can occur. Alongwith
studies of isolated DNA, these experiments have also demonstrated the role of torsional stress of supercoiling in determining the transition from B- to Z-DNA (DiCapua et al., 1983; Hill et al., 1984; Robert-Nicoud et al., 1984; Peck et al., 1982; Haniford and Pulleyblank, 1983; Singleton et al., 1982). With monoclonal antibodies that show selectivity for particular base sequences in Z-DNA form (Zarling et al., 1984; Moller et al., 1982; Lee et al., 1984), it becomes possible to explore further what kind of sequences may or may not be responsible for Z-DNA formation in the chromosomes.

The qualitative impression from the experiments is that the prokaryotic DNA seems to form Z-DNA more readily than the eukaryotic DNA. This may be a consequence of the markedly reduced frequency of C-G sequences in eukaryotic DNA compared to prokaryotic. The reduction may be related to the methylation of C-G sequences at 5-position of cytosine found in eukaryotes but not in prokaryotes. The spontaneous deamination of methylated cytosine residues leads to the thymine which would cause considerable biological confusion. The reduction of C-G sequences in eukaryotic DNA down to approximately 10% of what is observed in prokaryotic DNA may lead to Z-DNA being found less frequently in eukaryotic DNA. A notable difference between these two systems is the presence of the middle repetitive DNA sequences \( \text{d(CA/GT)}_n \), \( n \geq 50 \), which occur frequently in eukaryotic systems and are entirely absent in prokaryotes.

**Possible Biological Role of Z-DNA**

Conformational diversity of the DNA helix appears to be used quite extensively by living organisms with regard to protein-DNA recognition and thus function (Traverse, 1989). In several cases DNA recognition by proteins seems to rely heavily-if not entirely on DNA conformations and flexibility as opposed to base sequence per se (Otwinowski et al., 1988; Goodman and Nash, 1989). Transcription by RNA polymerase is terminated by left handed \( \text{(dC-dG)}_n \) tracts (Peck and Wang, 1985).
Further work shows that transcription is associated with Z-DNA formation with a substantial increase in the binding of anti-Z-DNA antibodies in metabolically active permeabilized mammalian cell nuclei (Wittig et al., 1991). The question that naturally arises is what physiological processes act in vivo to generate the negative supercoiling (which facilitates Z-DNA formation) that is continuously being removed by the activity of topoisomerase I (Zhang et al., 1988). Wittig et al. (1991) have suggested that transcription is actually associated with a marked increase in the level of anti-Z-DNA antibody binding. This may be one of the major physiological processes that generate negative supercoiling and thus stabilizes Z-DNA formation in the intact cell. Liu and Wang (1987) have suggested that RNA polymerase movement generates negative supercoiling upstream from the transcription site.

**Reactive Oxygen Species and Genetic Apparatus**

Free radical reactions are irreversible (Pryor, 1966; Nohebel and Walton, 1974) and ubiquitous in living organisms (Harman, 1981, 1986). Enzymatic reactions serving as sources of free radical reactions (Pryor, 1966; Nohebel and Walton, 1974) include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in cytochrome P-450 system (Harman, 1988). Free radicals also arise in the non-enzymatic reaction of oxygen with organic compounds as well as those initiated by ionizing radiation (Ara et al., 1992). Major sources of endogenous oxygen radicals are hydrogen peroxide (Chance et al., 1979) and superoxide (Fridovich, 1982) generated as side products of metabolism, and the oxygen radical burst from phagocytosis after viral or bacterial infection or the inflammatory reaction (Halliwell, 1982). Observation of unique DNA-oxygen free radical reaction products formed in vivo (Floyd et al., 1986; Frenkel et al., 1986; Kasai et al., 1986) appear to be important, not only to demonstrate direct proof of their presence in vivo, but of greater significance, the lesions formed are important aspect reflecting the mechanism of
oxygen free radical mediated DNA damage. Oxidative damage is common and DNA, proteins and lipids are all at risk and therefore oxygen free radicals are thought to be linked to many diseases (Pryor, 1987). Normal oxidative damage to mitochondrial and nuclear DNA has been observed to be caused by excited oxygen species, which are produced by radiation or are by-products of aerobic metabolism. Normal cellular metabolism has been known to generate oxygen species as hydrogen peroxide, hydroxyl radical, singlet oxygen, and hydroperoxy radical (Cerruti, 1985). Moreover, superoxide radical and hydrogen peroxide are produced by activated phagocytes which are essential for the killing of many bacterial strains (Babior, 1988). Exposure of cells to visible light may enhance normal cell concentrations of hydrogen peroxide (1 x 10^{-8} M), hyperbaric oxygen and metabolism of certain dietary carcinogens lead to hydrogen peroxide acceleration.

The monitoring of macromolecular damage in tissues from humans exposed to chemical substances that interfere with DNA is being actually pursued to explain the origin of antibodies in autoimmune disorders. It is most probable that the etiological basis for oxygen free radicals as contributing factors in the development of several pathological conditions resides in their action on the genetic apparatus (Schneider et al., 1989). Oxygen free radicals have been known to cause damage to nucleic acids (Wallace, 1987; Stoewe and Prutz, 1987) and hydroxyl radicals have been implicated in the etiology of many human diseases (Halliwell and Gutteridge, 1989). The excessive concentration of the -OH overcome normal defense mechanisms and leave the DNA at risk from oxidative modification (Cotgreave et al., 1988) of deoxyribose, pyrimidines and purines (Von Sonntag, 1987). The oxidized nucleic acid base 8-hydroxyguanosine is present at a level of 130,000 bases in nuclear DNA and per 8,000 bases in mitochondrial DNA (Richter et al., 1988). The unique thing about 8-hydroxyguanosine is that it does not stop replication: but it might be relatively benign, although it does cause misreading at 8-hydroxy-2'-deoxyguanosine residue itself and at the neighboring
base (Kuchio et al., 1987). Formation of 8-hydroxy-2-deoxyguanosine either in DNA or from the free nucleoside deoxyguanosine (dG) has been shown to be mediated by hydroxyl free radicals (Kasai and Nishimura, 1984a; Floyd et al., 1986, 1988) as well as by certain other reducing substances including hydrazines (Kasai and Nishimura, 1984a), polyphenols (Kasai and Nishimura, 1984b), and intermediates in carcinogen metabolism. Recent studies have demonstrated the existence of 2-hydroxyadenine (isoguanine) in isolated human chromatin after its exposure to assay mixture of Nickel (II) and Cobalt (II) and hydrogen peroxide (Nackerdien et al., 1991). 8-hydroxyadenine have been isolated from the DNA in cancer of the female breast (Malins and Haimanot, 1991). The origin of the \( \cdot OH \) that potentially initiates the base modification is unclear, although one possibility is that this radical arises from hydrogen peroxide generated through the cytochrome P-450-mediated oxidation of estrogen (Deodutta et al., 1991). Hydroxyl radicals produced by copper (II) plus hydrogen peroxide has been shown to cause site specific DNA damage at polyguanosines resulting in single and double strand breaks and alteration in the structure of guanosines (Sagripanti and Kraemer, 1989). Guanine has previously been shown to be the target for aflatoxin (Muench et al., 1983), nitrogen mustards (Mattes et al., 1986), antitumor agents (bleomycin, neocarcinostatin, cis-platin) (D'Andres and Haseltine, 1978), and antibiotic (tetracyclin) action (Piette et al., 1986).

DNA damage involving \( \cdot OH \) would be relatively favored in diseases where copper concentration is elevated such as Menke's syndrome (Sone et al., 1987), Wilson's disease or certain neoplasms (Jendryczko et al., 1986). In normal cells Cu (I) would be oxidized mainly by oxygen with little \( \cdot OH \) formation and DNA damage. In tumorr cells, the oxidation of Cu(I) by hydrogen peroxide would be favored resulting in DNA damage (Sagripanti and Kraemer, 1989). Trace metals such as copper and iron which are present in biological system may interact with active oxygen species to damage DNA (Sagripanti et al., 1987; Imlay et al., 1988). In the presence of trace amounts of transition metal (M) ions (Fe\(^{2+}\), Cu\(^{1+}\)) found in biological system can participate readily in Fenton-like
reactions resulting in the production of \( \cdot \text{OH} \) which are short lived but extremely reactive with their bioenvironment,

\[
M^{n+} + \text{H}_2\text{O}_2 \rightarrow M^{(n+1)+} + \text{OH} + \cdot \text{OH}
\]

Under normal circumstances the concentration of reduced metal ions in chromatin would be expected to be low. Consequently, the production of a high local density of lesions in DNA by \( \cdot \text{OH} \) radicals derived from the Fenton reaction will depend on a large proportion of the potentially reactive metal ions being in the reduced state or capable of being reduced by an intrinsic mechanism or by reductants introduced from outside the cell (Jonas et al., 1989). Studies have shown that bacterial killing by hydrogen peroxides is enhanced when bacteria (Staphylococcus aureus) are grown for several days in an iron-rich environment in order to raise intrinsic iron concentrations (Repine et al., 1981). Measurement of DNA damage in the presence of an excess of DNA and 1,10-phenanthroline has been employed as a sensitive assay to measure the availability of copper ions in human body fluids (Gutteridge, 1984; Gutteridge et al., 1985; Evans et al., 1989; Dizdaroglu et al, 1990). Overall, the DNA damage seems to be produced by a Fenton-type mechanism in which transition metal ions are cycled by first being reduced by superoxide and then oxidized by hydrogen peroxide. The DNA damage in \text{vivo} may be due to a site-specific generation of this \( \cdot \text{OH} \) upon the DNA itself. This means that either the DNA has transition metal ions bound to it \text{in vitro}, or that the oxidative stress liberates such metal ions that rapidly bind to the DNA (Dizdaroglu et al., 1987). Chromosomal proteins may act as radical scavengers in reactions induced by ionizing radiation (Roti Roti et al., 1974). Moreover, the iron binding proteins transferrin (present in plasma) and lactoferrin (secreted by neutrophils) are far from saturated with iron \text{in vivo} (except during iron overload), and can bind metal ions liberated from other proteins, thus helping to diminish damaging radical reactions (Halliwell and Gutteridge, 1986). Despite
all the protective measures taken care by biological system, the free radicals generation seems to continue because in nature every reaction is liable to be altered by changes in normal physiological processes. Recently, Jiang & Chen (1992) have shown decreased activity of superoxide dismutase (SOD) in SLE patients which resulted in elevated levels of free radicals. The excessive level of free radicals in SLE than those in normal persons, probably contributing to the production of autoantibodies. These free radicals may play an important role in the pathogenesis of SLE possibly due to diminished SOD activity.

Polyamines-DNA Nexus

Polyamines are ubiquitous polycationic metabolites of prokaryotic and eukaryotic cells. These polycations are known to play critical roles in the regulation of major functions in cell growth, cell differentiation and biosynthetic pathways (Tabor and Tabor, 1984; Pegg, 1986, 1988; Schuber, 1989). Polyamines are found to be associated with ribosomes and membranes. The precise function played by these molecules inside the cell is not fully understood yet they exert an antimutagenic effect, they also prevent dissociation of 70S ribosomes to 30S and 50S components, and they increase the resistance of protoplasts to osmotic lysis. Polyamines stimulate translation (initiation and elongation) of the peptide chain and are implicated in maintaining translational fidelity, ribosomes structure and stability (Marton and Morris, 1987) thus markedly influencing protein synthesis in both prokaryotes and eukaryotes. In various growing tissues polyamine synthesis accompanies or precedes nucleic acid synthesis (Heby et al., 1976; McCann et al., 1972). The increased synthesis of RNA also depends on the stimulating effect of polyamines on RNA polymerase. The concentration of polyamines is elevated in the biological fluids and tissues in a number of disease states such as cancer, sickle cell anemia, cystic fibrosis, psoriasis and systemic lupus erythematosus (Puri et al., 1978; Gunnia et al., 1991). Similarly, the polyamine levels in the spleen cells of MRL-lpr/lpr mice are about three fold higher than that in spleen cells of normal mice (Bowlin et al., 1986; Thomas and Messner, 1989).
Spermidine and spermine are known to be distributed widely in nature. Spermine is the most abundant polyamine found in animal cells (Tabor and Tabor, 1984). Spermidine or spermine is essential for the aerobic growth of Saccharomyces cerevisiae (Balasundaram et al., 1991). The amount of spermidine varies inversely with the amount of Mg\(^{2+}\) in ribosomes, and 30S and 50S ribosomal subunits remain associated in the absence of Mg\(^{2+}\) if spermine is present.

Because of protonated amino groups (at physiological pH values), polyamines are known to bind to negatively charged cellular molecules such as nucleic acids. The interaction of polyamines with nucleic acids may be responsible in part for the biological functions of polyamines (Basu et al., 1989). Thermophilic bacteria growing under the extremes of heat are known to produce a variety of polyamines to protect their genetic material from denaturation (Oshima, 1982). The polyamines composition of the extreme thermophile depends on growth temperature (Oshima and Baba, 1981). The thermophilic cells grown at an optimum temperature (75 °C) contain two novel tetramines, thermine (Oshima, 1975) and thermospermine (Oshima, 1979). The interaction of polyamines with DNA produces profound changes in the structural organization and reactivity of DNA. These include the stabilization of double stranded DNA, collapse of DNA into toroids and spheroids (Gosule and Schellman, 1978; Thomas and Bloomfield, 1983), and the induction of left handed Z-DNA conformation of alternating purine-pyrimidine sequences and plasmids containing blocks of these sequences (Behe and Felsenfeld, 1981; Thomas and Messner, 1988; Thomas et al., 1991). Spermine and spermidine promote aggregation of DNA, increases its melting temperature (Tm), and induce B- to Z-transition (Feuerstein and Marton, 1987). Spermine induce DNA conformation that binds actinomycin D less efficiently (D'Orazi et al., 1979). On the contrary, actinomycin D is a very powerful antibiotic that forms a complex with native poly(dG-dC).poly(dG-dC) sequences of DNA (Jain and Sobell, 1972; Sobell and Jain, 1972). Behe and Felsenfeld (1981) and Thomas et al. (1985) demonstrated that polyamines and their acetyl derivatives were capable of provoking B- to Z-transition of poly(dG-
m^dC).poly(dG-m^dC). Blocks of (dA-dC)\_n(dG-dT)\_n which are known to occur widely in nature as a part of transcriptional enhancer elements of many genes undergo B- to Z- to Y- conformational transitions in the presence of polyamines, thus obviating the extreme conditions (3M NaCl; 19% ethanol or high concentrations of NiCl\_2) otherwise used to attain these conformations in vitro (Thomas and Messner, 1986). Extremely sensitive enzyme immunoassay technique have demonstrated that in the absence of polyamines, a polynucleotide sequence poly (dG-m^dC). poly(dG-m^dC) does not bind to monoclonal anti-Z-DNA antibody (Z 22) but rapid interaction occurs when polyamines are added to the system (Thomas and Messner, 1988). These immunochemical studies, along with UV spectral and circular dichroism studies corroborate the above mentioned evidence on the binding of polyamines to the polynucleotides.

In 1982, Klevan and Schumaker noticed that addition of poly-L-arginine but not poly-L-lysine to poly (dG-dC).poly(dG-dC) in high salt solution allows the Z-conformation to be retained upon dialysis to lower salt concentration. This observation suggests that the stabilization of the Z-DNA form is not due to simple electrostatic factors but may also involve some specificity in the interaction of arginine side chains with Z-helix. Analysis of interactions of H1 and H5 histones with polynucleotides of B- and Z-conformations revealed two things. Firstly, at low protein: DNA ratios, both H1 and H5 bound more Z-DNA than B-DNA. Secondly, the binding of Z-DNA was less sensitive to interfere by an increase in ionic strength (to 600 mM NaCl). Moreover, H5 histone caused more profound C.D. spectral changes than did H1 (Mura and Stollar, 1984). These findings demonstrate the ability of different lysine-rich histones to cause varying conformational changes in the condensation of chromatin in DNA regions of highly biased base sequence. The occurrence of H5 is characteristic of cells that are inactive in both transcription and replication (Billet and Hindley, 1972; Urban et al., 1980).

Recent studies further reiterates that natural polyamines are capable of altering the immunogenicity of polynucleotides by mechanisms involving the stabilization of Z-DNA conformation (Gunnia et al., 1991).
The presence of high levels of polyamines and anti-Z-DNA antibodies in some lupus patients and autoimmune mice suggest the involvement of polyamines in altering the topological state of DNA (Sehgal and Ali, 1990; Alam et al., 1992).

Autoimmunity and Causative Factors

The immune system has tremendous discrimination potential of self and nonself. The process of autoimmunity begins when the immune response is started against the self antigens. The laboratory data till date indicate that an age dependent loss of immune regulation might be a single most critical factor which is involved in the process of autoimmunity. It has not been found possible so far, to reproduce the disease by the transfer of antibody from the patient to normal individual. There are three models for the induction of a productive autoimmune process. The first is the autoimmune response to a cross reacting antigen, which is different from, but cross reactive with autologous determinants, the second is a genetic predisposition for autoimmunity through regulatory failure, and the third is the triggering with bacterial mitogen of potentially autoimmune B cells, normally held in check by suppressor cells.

Polyclonal stimulation might, in fact, favor antibodies against autologous macromolecules, since these are present in the milieu of activated cells and might provide the second stimulus, which results in the productive response by a polyclonally activated or hyperactive B cell (McHugh and Bonavida, 1978). There appears to be at least two genetic factors that are involved in autoimmune disease. A predisposition may be controlled by one gene (a controller T cell or a deviant gene for stem-cell development) and the disease may be precipitated by an external event and the reaction of this precipitating event may be under polymorphic genetic control. Since suppressor T cells appear to be important elements in the regulation of immune response, it is likely that any failure in the capacity to generate suppressor T cells may contribute to autoimmune disease. Also, genetic factors appear to affect the autoimmune disease of NZB mice and some forms of human SLE (Cleland...
et al., 1978). However, the level at which the gene controls this operation remains unknown. It is likely that the autoimmune disease of NZB/NZW F1 mice is determined by two dominant genes and that one gene of NZB strain is on chromosome 17, but not close to H-2 (Knight and Adams, 1975). There is also good evidence that systemic lupus erythematosus is under genetic controls; 5-10% of close relatives of patients with SLE have lupus (Arnett and Shulman, 1976). Furthermore, multiple autoimmune diseases, including SLE, have been associated with genetically determined products of the major histocompatibility complex (MHC) in humans, i.e. HLA (Keuning et al., 1976; McMichael et al., 1976; Klouda et al., 1979; Fye et al., 1978; Terasaki et al., 1976). There is common occurrence of two HLA-DRW types in SLE patients (HLA-DRw 2 and HLA-DRw 3), as well as high percentage of individuals with B-cell alloantigen la-715 (Decker et al., 1979). Increased frequencies of HLA-DR 3, with or without increased frequencies of DR2, have been found in several small groups of patients with SLE (Stastany, 1978; Scherak et al., 1979; Celada et al., 1980; Scherak et al., 1980). Sontheimer et al. (1981) have reported an increased frequency of DR3 in patients with subacute cutaneous lupus erythematosus, a subset of lupus erythematosus. As in the disease of NZB mice, there appears to be a familial abnormality of suppressor cell function in systemic lupus erythematosus (Miller and Schwartz, 1979; Fauci, 1980).

There is some evidence for an association of autoimmune disease with low macrophage function, as assessed by carbon blockades (Passwell et al., 1974), in vivo clearance of 125I labeled polyvinyl pyrrolidone (Morgan and Soothill, 1975) and low affinity antibody (Passwell et al., 1974). It also seems possible that the class of antibody provides the event that results in transition from autoimmunity to autoimmune disease. In New Zealand mice, the ability to clear particles was found to decline with increasing age (Morgan and Soothill, 1975; Morgan and Steward, 1976). In short, defective macrophage function may be a factor in the glomerulonephritis of NZB/W mice. It is possible that an age-dependent decline in the phagocytic function of macrophages may be directly involved
in preferential formation of low affinity antibody (Steward and Powis, 1977) but it is also possible that poor macrophage function accentuates the persistence of complex between antigen and low affinity antibody.

There are growing concern about theoretical risk of autoimmune disease in patients undergoing PUVA treatment for psoriasis, vitiligo and mycosis fungoides. Psoralens are used in the treatment of human skin diseases (Fitzpatrick et al., 1982; Parrish et al., 1982), for probing nucleic acid structure (Song and Tapley, 1979; Cimino et al., 1985; Parsons, 1980; Rinke et al., 1983; Calvet et al., 1982; Setyono and Pederson, 1984) and nucleic acid-protein interactions (Cech and Pardue, 1977; Potter et al., 1980; Cimino et al., 1985; Kittler and Lober, 1988). Alterations in DNA resulting from the photoreaction could lead to development of antibodies to DNA or could precipitate SLE (Hasan et al., 1991). There have been reports of lupus-like syndrome (erythema) following PUVA therapy (Millins et al., 1978; Eyason et al., 1979). In a recent study, cell membrane DNA has been looked as a new suitable target for psoralen photoaddition (Gasparro et al., 1990).

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by a myriad of clinical manifestations and multiple autoantibodies (Provost, 1979; Hahn, 1980). The origin of autoantibodies in SLE is an enigma poised on the verge of solution. The riddle has been a taxing one because lupus serum contains a bewildering variety of autoantibodies against nucleic acids (notably DNA), nucleoproteins, cell surface antigens and phospholipids. Such extensive diversity in the serological abnormalities of the disease is awaiting a definite mechanism of antigen activation (Harley and Scofield, 1991; Brigido and Stollar, 1991; Fronek et al., 1990).

While detection of anti-dsDNA antibodies remains the most reliable parameter to define the disease, antibodies to DNA have also been found to be associated with the nephritis of SLE and are thought to play a pathogenic role therein. Association of other autoantibodies in
many pathological conditions have been presented in Table 1. It is envisaged that certain subpopulation of DNA antibodies may be more pathogenic than others. Considerable evidence has accumulated to show that both qualitative and quantitative differences in the antibodies to DNA are important in the production of nephritis in SLE patients (Valle et al., 1985). Antibodies to dsDNA are relatively restricted to IgG1 and IgG3 subclasses whilst antibodies to ssDNA were equally distributed throughout the four subclasses i.e. IgG1, IgG2, IgG3 and IgG4.

Granholm and Cavallo (1990) have proposed that prolonged circulation of immune complexes (between DNA and anti-DNA autoantibodies) due to various altered immune functions contributes to nephritis in MRL/lpr mice. In human SLE, the early deposits of immune complexes (IC) are found in the mesangium and can be detected even before clinical or laboratory manifestations of renal involvement are evident (Cavallo et al., 1977). Theoretically altered blood cell carrier functions could attribute to nephritis by allowing large amounts of ICs to remain unbound (free) in the circulation. Such ICs are thought to localize more readily in tissues because they circulate close to the plasma-endothelial face and can be precipitated or entrapped in the microcirculation (Schifferli and Taylor, 1989). Delayed removal of ICs from the circulation is thought to favor their deposition in tissues (Schifferli and Taylor, 1989). The biologically active (pathogenic) immune complex systems that have been implicated in nephritis of autoimmune mice, i.e. DNA-anti-DNA (Dixon, 1987), gp 70-anti-gp 70 (reviewed by Cavallo et al., 1985) and RNA polymerase I-anti-RNA polymerase I (Stetler and Cavallo, 1987), could lend itself to localization in the kidney as preformed ICs, as ICs formed in situ, or both. Lupus prone mice, and many immunologically normal mice, have relatively high concentrations of a serum glycoprotein of molecular weight 70 kD (gp 70). This glycoprotein is related structurally and immunologically to the envelop glycoprotein of murine retroviruses (Yoshiki et al., 1974; Strand and August, 1976; Lerner et al., 1976; Elder et al., 1977; Hara et al., 1981). It is synthesized in liver and it behaves like an acute phase reactant (Hara et al., 1982). More
### TABLE 1

Autoantibodies Associated with Clinical Findings*

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>Clinical finding</th>
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<tbody>
<tr>
<td>Anti-dsDNA</td>
<td>Nephritis</td>
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<tr>
<td>Anti-P</td>
<td>Psychosis</td>
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<tr>
<td>Anti-Ro</td>
<td>Lymphopenia, Thrombocytopenia</td>
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<td>Interstitial pneumonitis</td>
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<td>Photosensitive skin rash</td>
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<td>Congenital heart block</td>
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<td>Neonatal lupus dermatitis</td>
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<td>Anti-La</td>
<td>Absence of nephritis</td>
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<td></td>
<td>Congenital heart block</td>
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<td>Neonatal lupus dermatitis</td>
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<td>Antiphospholipid</td>
<td>Intravascular thromboemboli</td>
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<td>Miscarriage</td>
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<td>Anti-Sm</td>
<td>Isolated central nervous system</td>
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<td>Lupus</td>
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<td>Anti-nRNP</td>
<td>Myositis</td>
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<td>Raynaud's phenomenon</td>
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<td>Interstitial pneumonitis</td>
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<td>Capillary dropout</td>
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<td>Sclerodactyly</td>
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importantly only lupus prone mice develop autoantibodies to gp 70. Part of their circulating gp 70 is complexed with IgG (Izui et al., 1979, 1981a, 1982) and the serum concentration of such complexed (heavy) gp 70 correlates with the progression of nephritis (Izui et al., 1981b, 1984a,b). A small nuclear RNA-protein complex known as Ro/SSA binds autoantibody from many patients with SLE and Sjogrens syndrome (Reichlin, 1981; Tan, 1982; Wasicek and Reichlin, 1982; Reichlin and Wasicek, 1983). The frequent coexistence of autoantibodies to a cellular DNA-binding protein complex (p70/p80) and to DNA has also been reported (Reeves, 1985).

Recent findings have indicated that development of autoimmune processes in SLE is accompanied by autoantigen driven production of autoantibodies but is preceded and predicted by polyclonal B-cell activation (Klinman, 1990). B-lymphocytes with immunoglobulin receptor for dsDNA are found in normal individuals as well as in SLE patients. The activation of these cells in SLE may be a problem of disordered immunologic regulation. In human SLE both CD5− and CD5+ B-cells are capable of anti-DNA production, although the high affinity IgG anti-DNA antibodies appear to come from CD5− subset of B-cells (Cesali et al., 1989). These findings are consistent with a model in which autoantibody production in lupus is initiated by polyclonal activation and perpetuated by autoantigen specific stimulation Klinman et al., 1988, 1990). Also, the presence of anti-receptor antibodies in SLE and related disorders (Bennett et al., 1986a,b) suggests that the DNA receptor may be an important antigen in the repertoire of autoimmune responses. Indeed, two murine monoclonal antibody have been generated with reactivity towards a 30000 molecular weight DNA binding protein found on the cell surface of human leukocytes (Bennett et al., 1988). SLE patients have decreased numbers of complement receptors on their erythrocytes as compared to normal individuals (Miyakama et al., 1981).

Polyspecific anti-DNA autoantibodies (so called natural autoantibodies) derived from normal mice showed binding to a variety of proteins including actin, myosin and spectrin. Natural antibodies against tubulin, actin,
myoglobin, thyroglobulin, fetuin, albumin and transferrin are present in normal human sera (Avrameas et al., 1981). Anti-DNA antibodies have been found to interact with cytoskeletal proteins (Andre-Schwartz et al., 1984); fibronectin (Lake et al., 1985; Gupta et al., 1988) and heparan sulfate (Termaat et al., 1990), cell surface proteins (Jacob et al., 1984) and different cell types (Tron et al., 1984; Faaber et al., 1985). Binding with hyaluronic acid, chondroitin sulfate and heparan sulfate which contain repeating negatively charged units (Faaber et al., 1986) have also been observed. Anti-dsDNA antibodies have been selectively removed from SLE serum by immunoadsorption with dextran sulfate (Kinoshita et al., 1989). Many different Fc-binding proteins exist in the biological fluids or on the membrane of various cells. Circulating proteins, such as rheumatoid factors (RF) (Nemazee and Sato, 1983), complement components (Clq) (Dorrington and Klein, 1983), or immunoglobulin-binding factors (Khayat et al., 1984; Pure et al., 1984) can specifically interact with Fc portion. Studies on human SLE hybridoma autoantibodies have been shown to bind major cytoskeletal filaments that included intermediate filaments, microfilaments, microtubules and centrioles (Senecal and Rauch, 1988). In the same study, the immunoblot results showed that the antigenic specificity of intermediate filament reactive SLE hybridoma antibodies was not restricted to vimentin (a phosphoprotein under hormonal regulation) (Browning and Sanders, 1981) but included cytokeratins and desmin.

Monoclonal IgM anti-DNA autoantibodies, produced by hybridomas prepared from lymphocytes of SLE patients have been found to bind to several different antigens including ssDNA (Shoenfeld et al., 1982, 1983), synthetic polynucleotides (Shoenfeld et al., 1982), vimentin (Andre-Schwartz et al., 1984), platelets (Asano et al., 1985), acidic glycolipids in platelets (Asano et al., 1986) and sulfated complex carbohydrates (Asano et al., 1986). These reactivities can be most simply interpreted as the interaction of an anti-DNA with a conformational determinant or charge array in space that can be represented not only by DNA, but by other molecules as well. Besides, DNA could also interact in
unanticipated ways with other polycationic structures in the body to impair cell function or homeostasis. As a polyanion, DNA could display heparin-like activity and disrupt clotting mechanisms.

Anti-DNA antibodies in normal control groups have been reported from time to time. To explain the unexpected presence of anti-DNA antibodies in normal subjects, it has been proposed to arise as part of the overall host response to infections by pathogenic bacteria. In this setting, bacterial DNA would be recognized as foreign because of the presence of sequence arrays, structural features, or higher order conformations that are not usually present in human DNA. Studies have clearly shown that bacterial DNA differs from mammalian DNA in its content of pyrimidine clusters, patterns of base methylation, and undoubtedly in many of its coding sequences (Wells, 1988; Szybalski et al., 1966; Wells et al., 1977; Cheng et al., 1985). Together, these features could create local regions in bacterial DNA that are structurally distinct and rarely present in mammalian DNA. A role of anti-DNA as protoantibodies in an initial defense against foreign antigenic challenge has been reported (Schwartz and Stollar, 1985; Pisestky et al., 1990). Anti-DNA antibodies serve an important role in normal immunity not because they bind DNA, but because they are polyspecific and can bind non-DNA antigens, especially bacterial products. The broad reactivities represent a useful initial defense against multiple antigens. Furthermore, these antibodies can be mutated into high affinity antibodies that bind more exclusively to foreign antigenic determinants (Naprastek et al., 1986). It seems clear that many anti-DNA are in the repertoire of normal individuals and have characteristics of non-autoantibodies. Thus, the binding to a self antigen, especially under certain assay conditions, may not constitute a sufficient criterion to indicate that an antibody has pathogenic potential, or is a product of aberrant regulation or abnormal idiotype expression.

In a recent study, the effect of polyamines on the immunogenicity of polynucleotides have been investigated (Gunnia et al., 1991). The elicitation of anti-Z-DNA antibodies by polyamine-polynucleotide complex suggests a possible involvement of polyamines in the production of anti-
Z-DNA antibodies in human SLE. The antigenic DNA may be genomic DNA in the serum, which has been shown to have an abnormally high guanine-cytosine (G-C) content (Sano and Morimoto, 1982) and an abnormally high occurrence of alternating purine-pyrimidine segments (Van Helden, 1985). This phenomenon may be significant, since it has been shown that segments of alternating pyrimidine-purine residues, particularly poly(dG-dC) can undergo a transition from B- to Z-conformation (Pohl and Jovin, 1972; Behe and Felsenfeld, 1981; Zacharias et al., 1982; Moller et al., 1984) and since it has also been shown that the Z-form of DNA is highly immunogenic (Lafer et al., 1983). If, therefore, Z-DNA segments occur naturally in SLE patients we may expect a greater production of anti-Z-DNA antibodies than anti-B-DNA antibodies (Van Helden, 1985). That such anti-Z-DNA antibodies do occur in SLE sera has been shown (Lafer et al., 1983; Sibley et al., 1984; Gunnia et al., 1991; Sehgal and Ali, 1990). There are several reports that serum DNase I levels are decreased in patients with SLE (Chitrabamrung et al., 1981; Frost and Lachman, 1968). At the same time it has been suggested that most of immunogenic duplex nucleic acids are nuclease resistant (Braun and Lee, 1988).

Antibodies to dsDNA play a key role in the pathogenesis of SLE and therefore considerable research effort over the past 25 years has focused on both the quantitative determination and the qualitative characterization of anti-dsDNA antibodies that are found in the circulation of patients with SLE (Zouali and Stollar, 1986; Lau et al., 1991; Rauch et al., 1985; Cairns et al., 1989; Alam and Ali, 1992; Alam et al., 1992). Analysis has revealed that pathogenic anti-DNA antibodies of both human and murine SLE are of IgG isotype and cationic in nature (Ebling & Hahn, 1980; Datta et al., 1987). Anti-DNA antibodies induced by immunization of experimental animals with nucleic acids are highly specific for the immunogen (Stollar, 1980; Lafer et al., 1983), whereas the spontaneously produced anti-DNA autoantibodies in SLE tend to crossreact with a variety of ligands (Andrzejewski et al., 1981; Pisetsky and Caster, 1982; Koike et al., 1982; Shoenfeld et al., 1983; Pisetsky et al., 1990).
There is increasing evidence for the microheterogeneity of DNA and the existence along the DNA molecule of diverse structures that differ in dimensions and topology from B-DNA. It has been debated, whether DNA from various sources differ markedly in their immunogenic potential. It has been shown that in mice certain bacterial single stranded DNA (ssDNA) and dsDNA can induce significant anti-DNA responses, including antibodies to dsDNA (Gilkeson et al., 1989a,b). In fact immunization with E. coli DNA has induced antibodies (Gilkeson et al., 1989b) that resemble spontaneous anti-DNA antibodies of lupus mice in terms of their recognition of defined antigens (Pisetsky et al., 1990). In the year 1990, Gilkeson et al., showed that mice immunized with calf thymus DNA (Ct-DNA) failed to generate an antibody response to either Ct or E. coli (Ec) DNA. The antibodies to Ec dsDNA did not crossreact with Ct dsDNA. However, immunization with Ec dsDNA did produce antibodies that were reactive with both Ec and Ct ssDNA, possibly reflecting the in vivo breakdown of some of the dsDNA antigen into ssDNA, and the induction of an antibody response specific for this form of DNA. In these experiments the crossreactive antibodies to ssDNA resembled those found in lupus: in contrast, the antibodies to dsDNA bound only the immunizing antigen and lacked activity to common dsDNA determinants that are characteristic of lupus autoantibodies. Fournie (1988) has postulated that not DNA, but DNA bound to nuclear proteins could also play a role in the pathogenesis of SLE.

Monoclonal autoantibodies have been prepared that discriminate nearly completely between synthetic poly(dG-dC) and poly(dA-dT) and protects certain restriction nuclease cleavage sites preferentially (Stollar et al., 1986). Arnott et al. (1983) have described variation in helical geometry as presentation of a "wrinkled" backbone surface, which is different in poly(dA-dT) and poly(dG-dC), both of which differ in precise dimensions from average B-DNA. Murine monoclonal autoantibody, H241 binds well to native DNA but also displays several crossreactions that include denatured DNA, and both the B-DNA and Z-DNA forms of poly(dG-dC) (Stollar et al, 1986). On the other hand, H241 showed no reactivity in either direct binding or competitive binding assay, with
several single stranded homopolymers, [poly(A), poly(C), or poly(U)]; double stranded RNA [poly(A),poly(U) or poly(I),poly(C)]; DNA-RNA hybrid [poly(A). poly(dT)]; or triple stranded helices [poly(A),poly(I),poly(I) or poly(A). poly(U),poly(U)]. Monoclonal autoantibody H241 has a striking ability to recognize helical structural features of poly(dA-dT) and poly(dG-dC) that depend on base sequence. In poly(dA-dT) the adjacent ApT and TpA segments are not identical, as revealed by two distinct peaks in the $^31$P-NMR spectrum (Patel et al., 1981); the repeating unit in this polymer is a dinucleotide rather than a mononucleotide (Klug et al., 1979; Patel et al., 1981). H241 also reacts with cardiolipin, which contains phosphate groups spaced by 3 carbon atoms but does not have purines or pyrimidines. This crossreaction of the antibody (H241) indicates that some of the binding site also interacts with the phosphate backbone. To determine whether both heavy (H) and light (L) chains of different anti-nDNA autoantibodies are uniformly involved in binding to DNA, monoclonal mouse IgG autoantibodies, H241 and 2C10 were mixed with radiolabeled oligonucleotide and UV (254 nm) irradiated for 10 min which led to specific covalent crosslinking of oligonucleotide to both the H and L chains of H241 but only to H chain of 2C10 (Jang and Stollar, 1990). The dependence of oligonucleotide-protein crosslinking on specific immune complex formation was demonstrated by the complete absence of crosslinking to non-immune IgG even after 2 h of irradiation; by which time the IgG molecules themselves had become aggregated.

Therapeutic Approach to SLE Treatment

Therapeutic approach to the treatment of SLE have included the use of corticosteroids, various cytotoxic agents, and total body irradiation (Elad et al., 1986). Because of the profound adverse effect of corticosteroids on normal functioning of the immune system due to the fundamental drawbacks of not distinguishing between physiological and pathological immune response, the recent treatment involves use of normal immunoglobulin G (IgG) (intravenous immunoglobulins) (Dietrich and Kazatehkine, 1990; Schwartz, 1990) and plasmapheresis, a new approach to interrupting the pathogenetic events in systemic lupus erythematosus (Lewis et al., 1992). In missile therapy
of SLE (Sasaki et al., 1985), anti-idiotypic antibodies suppressed multiplication of EBV transformal lymphocytes of SLE patient has been tried. It may be reiterated that despite tremendous development in understanding the possible mechanism involved in precipitating autoimmune diseases, most of SLE patients are still receiving corticosteroids.

Biological Implications of Anti-Nucleic Acid Antibodies

Anti-nucleic acid antibodies may serve as non-destructive models and a precise yardstick for examining protein-nucleic acid interactions. They may be used to follow structural changes in given nucleic acids or nucleoproteins. Anti-nucleic acid antibodies may also be utilized as reagents to detect and quantitate DNA in serum and other body fluids (released during pathological conditions). The anti-DNA antibody can be used for sensitive detection of DNA-containing structures such as chromatin in different tissues of man, rat, mouse, chicken, amphibia and insects. Cell nuclei of various cell lines such as XLK E-A6 (Xenopus), RVF-SMC (rat), PTK-2 (rat-Kangaroo) and HeLa (human) show positive reactions. They can distinguish almost quantitatively among members of different helical families (Lacour et al., 1973; Stollar, 1973, 1975, 1989) and qualitatively among different members of helical class (Lacour, et al., 1973; Guignes and Leng, 1976; Johnston and Stollar, 1978; Nahon-Merlin et al., 1980). As their specificities are defined these antibodies become useful analytical probes, serving as sensitive and specific reagents for detecting particular structure in complex biological material including mammalian genome. Experimentally induced antibodies to left handed Z-DNA (Nordheim et al., 1981; Lemeunier et al., 1982; Arndt-Jovin et al., 1983; Kmiec and Holloman, 1984), double stranded RNA (Stollar and Stollar, 1970; Miller et al., 1975) or DNA-RNA hybrids (Rudkin and Stollar, 1977; Busen et al., 1982; Alcover et al., 1982) have been able to detect corresponding structures without interference from a large excess of other nucleic acids. Monoclonal autoantibodies have been shown to protect certain restriction nuclease cleavage sites on DNA preferentially (Stollar et al., 1986). Also monoclonal anti-nucleic
acid antibodies are useful immunochemical tracer of a specific DNA conformation of utmost biological importance (Anderson et al., 1988).

Recent look at the risk factors which are involved in the pathogenesis of SLE. Genetic, environmental, immune, and hormonal factors acting either directly or indirectly lead to the production of mediator substances like autoantibodies, immune complexes, and complement activation products which cause tissue destruction and disease expression.

**Factors**

- Genetic
- Hormonal

**Mediators**

- Autoantibodies
- Immune complexes
- Complement activation

Environmental

**Objective of the Present Study**

The non-immunogenic nature of native DNA (B-form) and polyspecificity exhibited by SLE anti-DNA antibodies, with respect to multiple antigen binding, still poses the puzzling questions regarding their possible origin. Despite this, elevated levels of anti-nDNA antibodies in active SLE continue to be diagnostic marker of the disease. As the horizon of polyspecificity of SLE antibody is expanding, the scientific views on possible origin of these antibodies is also increasing and the results so far reported have been unequivocally accepted. Hence, the study of antigen binding characteristics of anti-DNA autoantibody is relevant to specify distinctly its polyreactivity with native and modified DNA
antigens of varying size and conformation. Well defined and characterized anti-DNA antibody could be utilized for monitoring changing DNA conformations during genetic activities. In this study purified calf thymus DNA and its fragments of varying size were modified by bromination in low and high salt concentrations and were evaluated as antigen for binding with anti-DNA antibodies. Effect of varying concentrations of D- and L-isomer of polylysine on B-conformation was also evaluated. The change in DNA conformation was monitored by UV spectroscopy and binding with anti-Z-DNA antibodies. The DNA was also modified with lysine, furocoumarin and hydroxyl radical. Each of the modified antigen was systematically characterized and investigated for binding to anti-DNA IgG.
Chemicals

Anti-goat, anti-human, anti-mouse and anti-rabbit IgG alkaline phosphatase conjugates, bovine serum albumin, calf thymus DNA (highly polymerized), Coomassie Brilliant Blue G 250 & R 250, dialysis tubing (of varying diameter), ethidium bromide, Millipore filter (0.45 um pore size), Freund's adjuvants, Hoechst dye 33258, 8-methoxypsoralen, methylated bovine serum albumin, nuclease S1, poly-L-glutamic acid, poly-D-lysine, poly-L-lysine, trinitrobenzene sulfonic acid (TNBS), Tris-(hydroxymethyl)-aminomethane, xylene cyanole FF were purchased from Sigma Chemical Company, U.S.A. Dextran Blue 2000, DEAE Sephacel, DEAE Sephadex A-50, Ficoll 400, micrococcal nuclease, poly(dG-dC).poly(dG-dC), poly(dG-me^5dC).poly(dG-me^5dC), poly(dA-dT).poly(dA-dT), poly(dA-dU).poly(dA-dU), poly(dI-dC).poly(dI-dC), poly(dA-dG).poly(dC-dT), poly(dA).poly(dT), poly(dG).poly(dC), poly(rG).poly(dC), poly(dA), poly(dG), poly(dC), poly(dT), poly U, Sephadex G 200, Sepharose 4B, protein A-Sepharose CL-4B were the product of Pharmacia Fine Chemicals, Sweden. Acrylamide, ammonium persulfate, bisacrylamide, hydroxyapatite, N,N,N',N'-tetraethyl methylenediamine, Tween-20 were obtained from Bio-Rad, U.S.A. Acetic acid, acetonitrile, EDTA (disodium salt), glycine, hydrogen peroxide, sodium lauryl sulfate, sucrose, ammonium sulfate, calcium chloride, methanol, sodium carbonate, sodium chloride from Qualigens, India. Chloroform and liquid bromine were the preparation of B.D.H. India. Isoamyl alcohol, sodium bicarbonate, zinc chloride were purchased from Merck, India. Ferrous sulfate and potassium chloride was from Sarabhai, India. Psoralen was a generous gift from C.D.R.I. Lucknow. P-nitrophenyl phosphate was obtained from C.S.I.R. Centre for Biochemicals, Delhi. Cyanogen bromide was a product of S.D. fine chemicals, India. Acetaldehyde was from Fluka, Switzerland, Bromophenol blue and lysine monohydrochloride was purchased from B.D.H. England. DEAE Cellulose was obtained from Whatman, England. Perchloric acid and sodium azide were products of Ferak-Berlin, Germany. ELISA plates were from NUNC Denmark & Dynatech, U.S.A. Diphenylamine and ethanol were chemically pure.
Equipment

Spectronic 20 (Bauch & Lomb, U.S.A.), UV-Visible 240 spectrophotometer (Shimadzu, Japan), spectrofluorometer RF-540 (Shimadzu, Japan), microplate reader MR-600 (Dynatech, U.S.A.), gel electrophoresis apparatus GNA-100 (Pharmacia, Sweden), pH meter L1-10T and conductivity bridge (ELICO, India), UV lamps having maximum emission at 254 and 365 nm (Vilber-Lourmat, France), desk top microfuge RM-12C (REMI, India) were the major equipments used in the present study.

Sera

The normal human sera was collected from healthy subjects without evidence of SLE or other autoimmune disorders and it was ensured that the subjects were not taking any drug known to induce antinuclear antibodies. The SLE sera was obtained from patients who had elevated levels of anti-native DNA antibodies and met preliminary criteria of the American Rheumatism Association (ARA) for the diagnosis of disease (Tan et al., 1982). All serum samples were heated at 56 °C for 30 min to inactivate complement which inhibits the formation of, and also dissolves, preformed antigen-antibody complexes (Miller and Nussenzweig, 1975; Schefferli et al., 1980). After decomplementation the sera was kept at -20 °C with 0.02 percent sodium azide as preservative.

Determination of Protein Concentration

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

Protein Estimation by Folins-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-Ciocalteu reagent**

The reagent was purchased from C.S.I.R. Centre for Biochemicals, Delhi. Before use the reagent was diluted 1:4 with high purity deionised water.

(b) **Alkaline copper reagent**

The constituents of alkaline copper reagent were

(i) Two percent sodium carbonate in 0.1M sodium hydroxide.
(ii) 0.5 percent copper sulphate in 1.0 percent sodium potassium tartarate.

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

(c) **Procedure**

Freshly prepared 5.0 ml of alkaline copper reagent was mixed with 1.0 ml of protein sample. The tubes were incubated at room temperature for 10 min. One ml of diluted Folin-Ciocalteu reagent was added followed by 30 min incubation at room temperature. The absorbance was monitored at 660 nm. The protein content of the unknown sample was determined from standard plot constructed by using bovine serum albumin.

**Protein Estimation by Bradford Method**

This assay is based on the color change that occurs when Coomassie Brilliant Blue G 250, in acid solution, binds strongly to protein hydrophobically and at positively charged groups. The environment of these positively charged groups, protonation is suppressed and a blue color is observed ($\lambda_{max} = 595$ nm).

(a) **Dye preparation**

Hundred milligram of Coomassie Brilliant Blue G 250 was dissolved thoroughly in 50 ml of 95% ethanol. To this solution was added 100ml of 85% (v/v) orthophosphoric acid. The resulting solution was diluted to a final volume of 1 liter. On every use the dye solution was filtered.
(b) Protein assay

Solution containing 10-100 ug protein in a volume of upto 0.1ml was pipetted into test tubes. The volume was adjusted to 1.0 ml with appropriate buffer. Five ml of dye solution was added and the contents were mixed by vortexing. The absorbance was taken at 595 nm after two min and before one hr against a reagent blank prepared from 0.1 ml of buffer and 5.0 ml of dye solution.

Purification of DNA

Highly polymerized calf thymus DNA was purified free of proteins, RNA and single stranded regions (Ali et al., 1985). DNA solution (2mg/ml) in 0.1 x SSC buffer (0.015 M sodium-citrate, pH 7.3 containing 0.15 M sodium chloride) was mixed with equal volume of chloroform-isooamyl alcohol (24:1 ratio) in a stoppered glass cylinder. The cylinder was sealed and the contents were mixed end-to-end gently for one hour. The DNA present in aqueous phase was separated from the organic layer by low speed centrifugation. The extraction process was repeated once and DNA was precipitated with two volumes of cold 95% ethanol and collected on a glass rod. It was dried by pressing against the wall of the container. The DNA thus obtained was dissolved in 0.1 X SSC buffer and treated with RNase A (50 ug/ml) for 30 min at 37 °C to digest RNA in case present. The digested DNA was precipitated, dried and dissolved in 30 mM acetate buffer pH 5.0 containing 30 mM zinc chloride. The digested sample was incubated with single strand specific enzyme, nuclease SI (250 units/mg DNA) at 37 °C for 30 min. The reaction was terminated by adding one-tenth volume of 0.2 M EDTA pH 8.0. The purified DNA was extracted twice with chloroform-isooamyl alcohol and reprecipitated with cold 95% ethanol. The DNA precipitate was dried and dissolved in required buffer.

Determination of DNA Concentration

DNA was estimated by diphenylamine reagent and Hoechst dye 33258.
DNA Estimation by Burton Method

Colorimetric estimation of DNA was carried out according to Burton (1956) using diphenylamine.

(a) Diphenylamine reagent

To 750 mg of recrystallized diphenylamine was added 50 ml of glacial acetic acid followed by 0.75 ml of concentrated sulphuric acid. The reagent was prepared fresh before use.

(b) Assay procedure

Varying amount of DNA in 1.0 ml was mixed with 1.0 ml of 1N perchloric acid. The tubes were incubated for 15 min in a water bath maintained at 70 °C. One hundred ul of 5.43 mM acetaldehyde was added followed by 2.0 ml of diphenylamine reagent. The tubes were mixed thoroughly and allowed to stand at room temperature for 16-20 hr for color development. Absorbance was read at 600 nm. The DNA concentration in the unknown sample was determined from the standard plot of purified calf thymus DNA.

Fluorimetric Assay of DNA

Fluorimetric estimation of DNA was carried out according to Lebarca and Paigen (1979) using Hoechst dye. The dye Hoechst 33258 [C2-[2-(4'-hydroxyphenol)-6-benzimidazolyl]-6(1-methyl-4-piperazyl)-benzimidazole] interacts with DNA with a many fold increase in fluorescence.

(a) Reagents

(i) 0.05 M sodium phosphate (pH 7.4) containing 0.002 M EDTA and 2 M sodium chloride.

(ii) 200 ug/ml of Hoechst 33258 in deionized water.

On day of use, the working dye solution is prepared by diluting (ii) 200-fold in solution (i).
(b) Method

Solution containing 0.1-1.0 ug DNA in 0.1 ml final volume was mixed with 2.4 ml of working dye solution. The mixture was incubated for 5 min at room temperature and fluorescence was read at 460 nm ($\lambda_{ex} = 355$ nm). The fluorescence of a solution containing dye plus buffer, but no DNA, was taken as control.

Isolation of IgG

The IgG was purified by ion exchange chromatography, gel filtration and affinity chromatography.

(a) Preparation of crude immunoglobulins

To 6.5 ml of fresh serum was added dropwise saturated ammonium sulphate solution allowing each drop to disperse before the next was added. When the concentration of ammonium sulphate reached about 20% of saturation, the serum had begun to turn milky. Most immunoglobulins got precipitated by 35-37 percent of saturation. The precipitation was carried out at 4 °C. The suspension was stirred for 30 min and then centrifuged at 10,000 rpm for 15 min. The pellet thus obtained was washed 2-3 times in 35 percent saturated ammonium sulphate. Repeated washing of the pellet considerably reduced contamination of non-immunoglobulins and did not lower the yield significantly. Finally the precipitate was dissolved in 0.075 M sodium phosphate buffer, pH 6.8. Ammonium sulphate was removed by overnight dialysis against 500-1000 volumes of 0.0175 M sodium phosphate buffer, pH 6.8. The dialysis fluid was changed several times at intervals of a few hours.

(b) DEAE Sephadel chromatography

Dialyzed crude immunoglobulins in 0.0175 M sodium phosphate, pH 6.8, buffer were loaded on DEAE Sephadel column (1.5 cm X 20 cm) previously equilibrated with the same buffer. The column was eluted with 0.0175 M Na-phosphate and fractions were collected until the absorbance at 280 nm reached base line (Fig. 1). First peak of the chromatogram was found to contain IgG. The homogeneity of isolated
Fig. 1. Isolation of IgG from an SLE serum by DEAE Sephacel. Thirty five percent saturated ammonium sulphate precipitated serum proteins were loaded onto the column. The peak fractions were pooled as IgG.

Inset: Polyacrylamide gel electrophoresis of isolated IgG on 7.5% gel.
IgG was confirmed by single band movement in SDS-polyacrylamide gel (Fig. 1 inset).

(c) Purification of IgG by gel filtration

The serum immunoglobulins were fractionated on Sephadex G 200 column. Immunoglobulins from serum were precipitated with 50 percent saturated ammonium sulphate and processed accordingly to method described earlier. The immunoglobulins were finally dissolved and dialysed against copious volume of 0.05 M Tris-HCl, pH 8.0 containing 0.15 M sodium chloride and loaded on a gravity packed Sephadex G 200 column (2 cm x 75 cm) previously equilibrated with the same buffer. The column was operated at a flow rate of 25 ml/hr and fractionated immunoglobulins were monitored for protein at 280 nm. The IgG peak was identified by absorbance measurements at 278 and 251 nm. A particular property of mammalian IgG is that their absorbance at 278 nm is about 2.5-3.0 higher than at 251 nm, in contrast to other serum proteins where this ratio is about 1-1.5 (in neutral buffers).

(d) Affinity isolation of IgG

Protein A-Sepharose is a popular and simple tool in the purification of IgG and its subclasses. The antigen-binding region (Fab) is preserved in its native state as the binding occurs through Fc region of the molecule.

Commercially obtained Protein A-Sepharose CL-4B (1.5 gm) was swelled in 0.01 M phosphate buffered saline (PBS), pH 7.4 at room temperature for 48-72 hr. The swelled gel (about 5.0 ml) was poured into a small column (0.9 cm x 15 cm). The column was washed once with 0.1 M sodium-citrate, pH 3.0, to elute any bound material and then equilibrated with five volumes of PBS. Serum diluted with an equal volume of PBS is passed through the column at a rate of about 20 ml/hr. The absorbance of the effluent is monitored until no protein leaves the column. The bound IgG is eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding, 1976). To prevent the effect of acidic elution buffer on IgG, the eluate was collected in 1 M Tris-HCl, pH
8.5. Fractions containing IgG was read at 280 nm and concentration was determined considering \( O.D._{280nm} = 10 \text{ mg IgG/ml} \).

Hydroxyapatite Chromatography of Single and Double Stranded DNA

Hydroxyapatite discriminates nucleic acids endowed with different secondary structures. Rigid, ordered structures having more affinity for hydroxyapatite than flexible disordered ones.

The commercial preparation of hydroxyapatite was washed with 0.01 M Na-K phosphate, pH 6.8 buffer to remove fine particles therein. Hydroxyapatite packed in glass column (1.5 cm x 5 cm) was equilibrated with 0.01 M Na-K phosphate. Native DNA dialyzed against equilibrating buffer was adsorbed to the column. The column was washed with ten volumes of equilibrating buffer, at a flow rate of 10 ml/hr, to wash any unbound DNA. The bound DNA was eluted with increasing phosphate molarity of 0.125 and 0.25 M strength. Native DNA got eluted at 0.25 M ionic strength and in the large majority of cases a complete recovery was obtained, as judged from \( A_{260} \) measurement.

DNA denatured by heating for 15 min at 100 °C, at a concentration of 50-100 ug/ml in 0.01 M Na-K phosphate (pH 6.8) buffer, showed a chromatographic behaviour quite different from that of native DNA. The stepwise elution results show that bulk of denatured DNA is eluted at 0.125 M phosphate molarity. The properties of the main fraction of denatured DNA were those of single stranded DNA. A small fraction of denatured DNA is eluted at the same phosphate molarity as native DNA.

**Micrococcal Nuclease Digestion of Native Calf Thymus DNA**

Deproteinized calf thymus DNA (2 mg/ml) in 0.006 M Tris, 0.1 M sodium chloride, 0.002 M calcium chloride, pH 8.0 was digested with micrococcal nuclease (40 units/mg) for 2.5 min at about 37 °C. The reaction was terminated by adding one-tenth volume of 0.2 M EDTA, pH 8.0. The sample, after precipitation with cold ethanol was subjected to nuclease S1 digestion (150 units/mg DNA) for 30 min at 37 °C (Ali
et al., 1985). The resulting DNA was reprecipitated, dissolved in TBS (0.01 M Tris, 0.15 M sodium chloride, pH 8.0) and passed through a Sepharose 4B column (1.5 cm x 40 cm) previously equilibrated with the same buffer. The fallthrough fractions (4.0 ml) were collected and monitored for DNA content at 260 nm, using 1.0 O.D. at 260 nm = 50 ug dsDNA/ml. Size of the DNA fragments were analyzed on polyacrylamide slab using Lambda DNA digest of EcoR I and Hind III as size marker.

Modification of DNA

Poly(dG-dC).poly(dG-dC), native DNA and small DNA fragments were treated for 1 hr with 4.0 M sodium chloride (effective concentration for B- to Z-DNA transition) respectively in citrate buffer (0.02 M trisodium citrate, pH 7.2 containing 0.001 M EDTA) at a final concentration of 100 ug/ml in 3.0 ml total volume. The same amount of polymers in PBS (0.01 M phosphate buffer, pH 7.4 containing 0.15 M NaCl) served as low salt control sample. The absorbance values were recorded at 260, 280, 294 and 296 nm to evaluate the absorbance ratio. The UV-absorbance and difference spectra of both high and low salt sample was recorded.

Nucleic acid polymers were brominated under high and low salt conditions as described by Lafer et al. (1981). Bromine saturated water was prepared by mixing 0.55 ml of liquid bromine in 49.45 ml of high purity distilled water. Bromine water (7 uL/mL DNA sample) was added to the DNA sample, with quick mixing, and kept in dark for 30 min at room temperature. After the completion of reaction, excess bromine was removed by exhaustive dialysis against TBS (0.01 M Tris, pH 7.4 containing 0.15 M NaCl). Native DNA and small DNA fragments brominated in high and low salt solution were designated as Br4 and BrN respectively. Absorbance value of brominated DNA was recorded at 260, 280, 294 and 296 nm against TBS, pH 7.4. Average of absorption at 294 and 296 nm divided by absorbance at 260 nm was referred to as "absorbance
ratio'. The ratio obtained for brominated and non-brominated poly(dG-dC).poly(dG-dC) was used as standard to which the absorbance ratio for other nucleic acids were compared. The absorption spectra of low and high salt brominated DNAs were recorded against buffer and compared against the spectra obtained with same concentration of respective DNA solution. The UV difference spectra was recorded by taking equal amount of corresponding DNA polymer as control.

DNA-Polylysine Interaction

To nDNA samples (50 ug/ml), varying amount of D- and L-polylysine (0-25 ug/ml) was added in a total assay volume of 3.0 ml containing PBS, pH 7.4. The average molecular weight of D- and L-isomer was 13,000 and 1,94,000 respectively. Control samples were prepared by taking equal amount of nDNA and polylysine separately. All assay tubes were vortexed gently and left for 30 min at room temperature. While recording the UV difference spectra of DNA-polylysine complex, nDNA in PBS (50 ug/ml) was taken as control.

Preparation of DNA-Lysine Photoconjugate

The photoconjugate was prepared by mixing calf thymus DNA with lysine (1:3 ratio, w/w) in PBS (0.01 M sodium phosphate, pH 7.4 containing 0.15 M sodium chloride), pH 7.4 followed by overnight incubation at 4 °C. The mixture was irradiated under UV-A light (320-400 nm) for 30 min in nitrogen atmosphere and dialyzed with copious volume of 0.01 M carbonate-bicarbonate buffer, pH 10.7 to remove lysine molecules bound electrostatically to phosphate of DNA backbone. The photoadduct was finally dialyzed against PBS and photobound lysine was estimated by ninhydrin reagent (Moore and Stein, 1954).

Preparation of DNA-Furocoumarin Photoadducts

DNA in TNE buffer (0.01 M Tris, 0.05 M NaCl, 0.0004 M EDTA, pH 7.6) was mixed with psoralen and 8-methoxypsoralen in the ratio of 3.2:1 (w/w, DNA:drug). The mixture kept in dark at room temperature
for 4 hr was irradiated for 30 min under 365 nm ultraviolet light. The irradiated samples were extensively dialyzed against 0.01 M phosphate buffer, pH 6.8. Finally, the photoadduct was characterized by UV absorption spectroscopy, thermal denaturation and fluorescence measurements.

**DNA Modification by Hydroxyl Radical**

The metal ions in DNA solution were chelated by pre-treatment with EDTA for 3 hr at room temperature at a final concentration of 1m mol dm$^{-3}$ in phosphate buffer, pH 7.4 (Blakely et al., 1990). Assay tube containing mixtures of DNA (1515 um bp), ferrous ions (525 um) and hydrogen peroxide (3030 uM) were incubated for 30 min at room temperature in 0.01 M phosphate buffer, pH 7.4. Deionized DNA with hydrogen peroxide and ferrous ions separately served as controls. At the end of incubation, reaction products were dialyzed exhaustively against PBS and their UV absorption characteristics were recorded on Shimadzu UV-240 spectrophotometer.

**Alkaline Sucrose Density Gradient Centrifugation**

The damage to DNA by hydroxyl radical was analyzed by alkaline sucrose gradient centrifugation. Native and modified DNA samples (0.1 ml each) were layered separately on top of a 4.8 ml linear 5-20 percent alkaline sucrose gradient containing 0.8 M sodium chloride, 0.2 M NaOH, 0.01 M EDTA and 0.015 M p-aminosalicylate at pH 12.5 (Fujiwara et al., 1977). The samples were centrifuged at 30000 rpm for 1 hr at 20 °C in an SW 50.1 rotor of a Beckman ultracentrifuge. After centrifugation, the bottom of the tubes were pierced with a needle and contents were fractionated by drop collection (0.7 ml each). The absorbance of collected fractions was monitored at 260 nm.

**Absorption-Temperature Scan**

Native and modified DNA samples were subjected to heat denaturation by an electronically controlled heating device. The cuvette temperature was raised from 30 to 95 °C at a rate of 1 °C/min after ten min
equilibration at 30 °C. The change in absorbance at 260 nm was recorded with increasing temperature. The helix-coil transition temperature (Tm) was determined from percent denaturation data which was calculated as follows:

\[
\text{Percent denaturation} = \frac{A_T - A_{30}}{A_F - A_{30}} \times 100
\]

Where,
- \(A_T\) is the sample absorbance at various temperatures.
- \(A_F\) is the final absorbance at 95 °C.
- \(A_{30}\) is the absorbance prior to heating.

The change in melting temperature (\(\Delta T_m\)) was calculated using the formula:

\[
\Delta T_m = T_m^C - T_m^S
\]

Where, \(T_m^C\) and \(T_m^S\) are the melting temperatures of native and modified DNA sample respectively.

DEAE Sephadex A-50 Column Chromatography

Native and modified DNA samples were mixed separately with perchloric acid (70%) in a stoppered tube and boiled for one hr to release the bases. The sample, after neutralization, was placed on top of a DEAE Sephadex A-50 column (1.6 cm x 30 cm) equilibrated with Tris-HCl buffer, pH 7.2. The elution was carried out with the same buffer at a flow rate of 40 ml/hr. Fractions (2.5 ml) were collected and monitored at 260 nm. Mixtures of equimolar concentration of bases (A, G, C, T) were also passed to locate the elution pattern of unmodified bases. Native and modified bases were identified on the basis of their unique absorption profile (Hasan and Ali, 1990). The extent of base modification was calculated by measuring the peak area.

Measurement of Antibody Affinity

One hundred microgram of immune IgG was incubated, in Ependorff tubes, with varying amount of DNA antigen in an assay volume of 0.2
ml. The control experiment was carried out with the same amount of antigens and preimmune IgG. The contents were vortexed and incubated for 2 hr at 37 °C and overnight at 4 °C. The soluble antigen-antibody complex was precipitated by adding cold saturated ammonium sulphate. The assay tubes were spun in microfuge at 8,000 rpm for 2.5 min and supernatant was carefully removed. The unbound antigen was estimated by Hoechst dye 33258 and diphenylamine reagent. The binding data were analyzed and antibody affinity was calculated (Langmuir, 1918).

Immunization Scheme

(a) Antibodies against normal human IgG
Female rabbits (8-12 months old, weighing 1.0 to 1.5 kg) were immunized intramuscularly, in hind limbs, with freshly prepared emulsion of purified IgG (0.5 ml of 200 ug/ml in PBS, pH 7.4) in Freund's complete adjuvant in a total volume of 1.0 ml. Blood was collected from rabbit by cardiac puncture prior to immunization. Similar injections, but with incomplete Freund's adjuvant, were given at weekly intervals for five weeks. Anti-IgG activity was evaluated in the immune serum collected after one week of the last injection.

(b) Antibodies against DNA modified by hydroxyl radical
Female rabbits were immunized with radical modified DNA as described earlier (Hasan and Ali, 1990). The immunizing antigen (100 ug) was mixed with MBSA (1:1 ratio, w/w) and emulsified with equal volume of Freund's complete adjuvant for first injection. Subsequent injections were given in incomplete adjuvant. A single animal received a total of 600 ug antigen in the course of six injections. Antibody activity was checked in the immune serum. Preimmune and immune sera were collected from the rabbit by cardiac puncture.
Immunoaffinity Purification of Antibodies

(a) CNBr activation of Sepharose 4B

Fifteen ml of supplied Sepharose 4B was suspended in distilled water, filtered and washed with 300 ml of cold distilled water on a sintered glass funnel (porosity G-2). Moist gel (about 10 g) in 10 ml of sodium carbonate was kept in ice-NaCl bath placed on magnetic stirrer. One gram cyanogen bromide in 0.8 ml acetonitrile was slowly added to the gel with gentle stirring. After 12 min, the reaction product was filtered on sintered glass funnel washed with 400 ml of cold 0.01 M sodium bicarbonate and resuspended in 10 ml of the same buffer. The unreacted CNBr (drained effluent) was reacted with ferrous sulphate to convert it into harmless ferrocyanide. All steps were carried out in a fume-hood chamber (Ali, 1984).

(b) Poly-L-lysine coupling to activated Sepharose 4B

The method described by Wilchek (1973) was followed. Immediately after CNBr activation of gel, equal volume of poly-L-lysine (100 mg in 10 ml of 0.1 M sodium bicarbonate) was added and kept at 4 °C for 12 hr with slow stirring. The buffer was drained out and the gel was washed successively with 100 ml each of cold (i) distilled water (ii) 0.1 N HCl (iii) 0.1 M sodium bicarbonate and (iv) distilled water till neutral. Finally the gel was suspended in 40 ml of 0.15 M acetate buffer, pH 4.5. The extent of poly-L-lysine depletion due to covalent coupling with activated Sepharose 4B was quantified by the treatment of drained effluent with TNBS as described by Habeeb (1966).

(c) Immunoaffinity Purification of anti-DNA antibodies

DNA -[Polylysyl-Sepharose 4B] affinity column was prepared according to method described earlier (Nicotra et al., 1982) with little modification. Twenty ml of polylysyl-Sepharose 4B was packed in a glass minicolumn (1.6 cm X 5 cm) and equilibrated with acetate buffer. Purified DNA (12.5 ml of 100 ug/ml in acetate buffer) was loaded onto the gel and unbound material was washed with 50 ml of PBS, pH 7.4.
Heat decomplemented SLE serum previously dialyzed against PBS was diluted 1:10 and passed through the column. The unbound antibodies were washed and bound proteins were eluted with linear ionic strength gradient of 0.15-3.0 M sodium chloride in 0.01 M phosphate buffer, pH 7.4. Fallthrough material was collected and monitored at 251 nm, 260 nm, 278 nm and 280 nm. The molar concentration of sodium chloride in fractions was evaluated by conductance measurements.

(d) Regeneration of affinity column
The same column was regenerated several times by washing successively with 50 ml each of the followings: (i) distilled water (ii) 0.1 N HCl (iii) 0.1 M sodium bicarbonate (iv) distilled water till neutral.

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

(i) Acrylamide-bisacrylamide (30:0.8)
Thirty gram acrylamide and 0.8 g bis-acrylamide was dissolved in a total final volume of 100 ml. The solution was mixed thoroughly, filtered and stored at 4 °C in amber color bottle.

(ii) Resolving buffer : 3 M Tris-HCl, pH 8.8
A stock solution was prepared by dissolving 36.3 g Tris base in 48.0 ml of 1 N HCl. The contents were mixed properly, pH adjusted to 8.8 and the final volume brought to 100 ml.

(iii) Stacking gel buffer : 0.5 M Tris-HCl, pH 6.8
6.05 g Tris base was dissolved in 40 ml distilled water titrated to pH 6.8 with IN HCl and the volume adjusted to 100 ml with distilled water.
(iv) Electrode buffer:

- 0.025 M Tris,
- 0.192 M glycine,
- 0.1% SDS
- pH 8.3

(v) Sample buffer

Six gm Tris was dissolved in 80 ml distilled water and pH adjusted to 6.8 with O-phosphoric acid. The volume was made to 100 ml with distilled water. To 12.5 ml of above solution was added 1 mg bromophenol blue and 12.5 ml glycerol. The contents were mixed thoroughly prior to use.

(b) PAGE procedure

The glass plates (19 cm x 16 cm) were soaked in chromic acid and thoroughly washed with tap water followed by one rinse with distilled water and ethanol. Finally the plate was cleaned with tissue paper soaked in acetone, air dried and sealed with 1% agarose. The glass plates were separated by 1.5 mm thick spacer. 7.5% separating gel was layered with 2.5% stacking gel according to recipe given below (all volumes are in ml).

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>7.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Resolving buffer</td>
<td>3.75</td>
<td>-</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.95</td>
<td>11.3</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>0.025</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The reagents were mixed and poured between glass plates. The gel was allowed to harden at room temperature. Protein samples (10-20 ug) in 0.01 M phosphate buffer, pH 7.4 were mixed with sample buffer in the ratio of 4:1 (w/w). SDS was added to a final concentration of 2% and protein sample was boiled for 5 min prior to loading. The electrophoresis was carried out for 6-8 hr at room temperature at 70
Volts. The gel was stained overnight in 0.05% Coomassie Brilliant Blue (R 250) mixed with 25% iso-propyl alcohol and 10% glacial acetic acid. Destaining was carried out in a mixture of 10% acetic acid and 10% methanol.

(c) DNA electrophoresis on polyacrylamide gel

DNA electrophoresis was carried out following the procedure of Sealey and Southern (1985). The following buffers were prepared for DNA gel electrophoresis:

(i) 40% acrylamide in distilled water
(ii) 2% bisacrylamide in distilled water
(iii) Resolving gel buffer : 0.9 M Tris-borate, pH 8.3 containing 0.025 M EDTA
(iv) Electrode buffer : 0.09 M Tris-borate, pH 8.3 containing 0.0025 M EDTA

Recipe for 7.5% DNA-PAGE (all volumes in ml)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>7.5</td>
<td>1.25</td>
</tr>
<tr>
<td>2% bisacrylamide</td>
<td>7.5</td>
<td>1.25</td>
</tr>
<tr>
<td>Gel buffer</td>
<td>4.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>21.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>50 mg</td>
<td>15 mg</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.025</td>
<td>0.010</td>
</tr>
</tbody>
</table>

The DNA sample (0.5-1.0 ug) was mixed with one-tenth volume of dye (30% Ficoll, 0.025% xylene cyanole FF in gel buffer) and electrophoresed for 6-8 hr at 70 Volts. The gel was stained with ethidium bromide (1 ug/ml) for 30-40 min & DNA bands were visualized under UV light.

(d) Agarose gel electrophoresis of DNA

Thirty ml of 1% agarose NA dissolved in electrophoresis buffer (0.04 M Tris-acetate, pH 8.0 containing 0.002 M EDTA) was poured on horizontal gel tray (sealed from both the sides) and allowed to harden for 30-60 min at room temperature. The DNA samples, prepared as
described earlier, were put into the wells and electrophoresed for 2-4 hr at 30 mA current in tank filled with the same buffer.

(e) Gel retardation assay

The antibody binding to DNA antigens was analyzed by altered electrophoretic migration. Mixtures of 0.1 ug of B-DNA fragments (average size 200 bp) and varying amounts of IgG (50-250 ug) isolated from SLE serum were incubated in a total volume of 20-30 ul of 0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M sodium chloride for 1-4 hr at 4 °C. In case of DNA-RNA hybrid, 0.5 ug polymer was incubated with varying amounts of SLE IgG (25-100 ug). At the end of incubation, 2 ul dye solution was added to each assay tube. The complex was electrophoresed on polyacrylamide or agarose gel using Tris-acetate EDTA (pH 8.0) buffer at room temperature. On completion of electrophoresis, gels were stained and photographed under UV illumination. Control assays were carried out in the presence or absence of normal human IgG and anti-DNA psoralen crosslink IgG that has been shown to be non-reactive towards native DNA (Hasan et al., 1991).

Immunological Detection of Antibodies

Immunized rabbit sera and SLE sera were tested for antibodies by immunodiffusion, counterimmunoelectrophoresis, and enzyme-linked immunosorbent assay.

(a) Immunodiffusion (ID)

ID was carried out by Ouchterlony diffusion system using glass petri dishes. Six ml of 0.4 percent molten agarose in PBS (pH 7.4) containing 0.1 percent sodium azide was poured onto glass petri dishes and allowed to solidify at room temperature. Wells, each 5 mm in diameter and separated by 8 mm distance were cut into hardened agarose gel. Appropriate concentration of antibody and antigen was placed in the wells and petri dishes were allowed to stand in moist chamber at room temperature for 48-72 hr. The gels were washed with 5% sodium-citrate for two
hr to remove non-specific precipitin lines. The result was analyzed visually and photographed.

(b) Counterimmunoelectrophoresis (CIE)

CIE was performed according to the procedure described by Kurata and Tan (1976) with partial modification. Molten agarose (0.6%) in 25 mM barbital buffer, pH 8.4 containing 0.1% sodium azide was poured onto 2.5 mm thick glass slides (2.5 cm x 7.5 cm) and allowed to harden at room temperature. The slides were stored at 4 °C and used within a week. The wells, 3 mm in diameter, were cut on the slides at a distance of 5 mm between the two opposite wells. Antigen (nucleic acid or protein in PBS, pH 7.4) was placed in cathodal well and antibodies (immune sera or IgG) in the anodal well. Electrophoresis was performed for 30 min in 50 mM barbital buffer, pH 8.4 with a current of 3-4 mA per slide. Non-specific precipitin lines were removed by treatment with 5% sodium-citrate solution.

(c) Enzyme-linked immunosorbent assay (ELISA)

(i) Buffers and substrate

Tris buffer saline (TBS) :

\[ \text{0.01 M Tris,} \]
\[ \text{0.15 M NaCl,} \]
\[ \text{pH 7.4} \]

Tris buffer saline Tween-20 :

(TBS-T)

\[ \text{0.02 M Tris} \]
\[ \text{0.144 M NaCl,} \]
\[ \text{0.00268 M KCl,} \]
\[ \text{pH 7.4 containing} \] 500 ul Tween-20

Bicarbonate buffer :

\[ \text{0.015 M sodium} \]
\[ \text{carbonate, 0.035 M Na-bicarbonate, pH 9.6} \]
\[ \text{containing 0.002 M magnesium chloride.} \]
Substrate
500 ug p-nitrophenyl phosphate per ml of bicarbonate buffer

(ii) Assay procedure
Polystyrene wells were precoated with 100 ul of polylysine (50 ug/ml in water) for 45 min at room temperature. The wells were emptied and washed thrice with TBS. Hundred ul nucleic acid antigens (2.5 ug/ml in TBS) were coated and incubated for 2 hr at room temperature followed by overnight incubation at 4 °C. The protein antigen was coated at 50 ug/ml in 0.05 M carbonate buffer (pH 9.6) without polylysine treatment of the wells. Next morning antigen coated wells were emptied and washed four times with TBS-T. Hundred ul of poly-L-glutamate were added to the antigen wells and washed after 2 hr. This was followed by 0.15 ml of BSA (1.5% in TBS) coating for 2-6 hr to saturate the binding sites. Antibody to be tested, diluted in 1% TBS-BSA was added to antigen coated and control wells for 2 hr at room temperature and overnight at 4 °C. After adding appropriate alkaline phosphatase conjugate, color was developed with p-nitrophenyl phosphate. Absorbance measurements (at 410 nm) were determined directly from microtiter plates with a Dynatech MR 600 reader.

(iii) Competition ELISA
The antigen binding specificity of antibody was defined by competition-inhibition experiments. For the assay, varying concentration of soluble competitors were incubated with antibody (serum or purified IgG). The mixture was incubated at room temperature for 2 hr and overnight at 4 °C. Inhibition of antibody binding in presence of various competitors was determined by measuring their ability to reduce the resulting absorbance relative to control assays lacking competitor. Each serum dilution or competitor was tested in duplicate. The result of inhibition were expressed as percent inhibition.
Induction in Rabbits of Antibodies Against Normal Human IgG

Rabbits immunized with normal human IgG produced precipitating antibodies. The antibodies were characterized by counterimmunoelectrophoresis (CIE), immunodiffusion (ID) and solid phase enzyme immunoassay (SPEIA). The antibody titer was found to be 1:16 by Ouchterlony double diffusion in agarose gel (Fig. 2a). The specificity of antigen-antibody reaction was ascertained by immuno-crossreactivity of induced antibodies with immunogen, preimmune rabbit IgG and serum albumin (Fig. 2b). The results showed reactivity with immunogen but nonreactivity towards preimmune IgG and serum albumin. ELISA of immune serum on polystyrene plate coated with normal human IgG showed a titer of >1:6400. The binding of preimmune serum was of very low magnitude (Fig. 3).

Double Strandedness of Native DNA

Purified calf thymus DNA was characterized for double strandedness by physico-chemical methods. Agarose gel electrophoresis of DNA (1 μg) digested with nuclease S1 (20 l.U.) showed identical mobility and fluorescence on staining with ethidium bromide in comparison to native DNA (data not given). By contrast, heat denatured DNA incubated under identical conditions was completely digested by the enzyme. The double strandedness of purified DNA was further investigated by chromatography on hydroxyapatite. The hydroxyapatite bind both single and double stranded DNA molecules which are eluted at different ionic strength. The elution of purified DNA as a single peak at 0.25 M Pi is suggestive of its double strandedness. Purified DNA was also characterized by thermal denaturation. Thermal denaturation of native DNA showed no significant change in absorbance up to 80 °C. A sharp melting profile (Tm = 87.5 °C) (Fig. 4) reiterated the double strandedness of native DNA.
Fig. 2(a). Evaluation of titer of anti-human IgG antibodies by Ouchterlony immunodiffusion. The central well (C) contained immunogen and peripheral wells 1, 2, 3, 4, 5 and 6 represents neat, 1:2, 1:4, 1:8, 1:16 and 1:32 dilution of antiserum.

(b). Immunocrossreactivity of rabbit anti-normal human IgG. The central well (C) contained anti-human IgG whereas peripheral wells 1, 2 and 3 represents immunogen, preimmune rabbit IgG and bovine serum albumin respectively.
Fig. 3. Direct binding ELISA of anti-human IgG. The wells were coated with human IgG. Immunized (---) and preimmune (---) serum.
Fig. 4. Temperature dependent melting profile of purified native DNA.
Detection of Anti-Nucleic Acid Antibodies in SLE Sera

The direct binding of SLE antibodies to native DNA was determined by ELISA. Table 2 shows the titer of serum antibodies investigated during the course of this study. Further studies were carried out on the sera which had elevated levels of anti-dsDNA antibodies, a marker antibody for the diagnosis of SLE.

Fractionation of Serum Immunoglobulins

Serum samples (control or SLE) after precipitation with fifty percent saturated ammonium sulphate were dialyzed extensively against 0.05 M Tris-HCl, pH 8.0 containing 0.15 M sodium chloride and fractionated on Sephadex G 200 column. The fractions were read at 280 nm. Two well resolved protein peaks were identified (Fig. 5). Their absorbance was monitored at 278 and 251 nm in order to identify IgG peak. Fractions from 35 - 43 were taken as IgG as their absorbance ratio (278/251) was 2.5.

Characterization of Normal Human and SLE IgG

Only antibodies of the IgG class were studied since it has been found that several cross reactions of anti-DNA antibodies are mainly associated with IgM antibodies of low avidity. Moreover, they are not the true representatives of the pathogenic anti-DNA antibodies found in SLE patients (Edberg and Taylor, 1986).

The DEAE Sephadex purified IgG from control and SLE sera were found to be nearly free from contamination by other classes of immunoglobulins as judged by their homogeneous movement in SDS-PAGE under non-reducing conditions. Purity of the isolated IgG was also characterized by ultraviolet absorption measurement at 251 and 278 nm. The absorbance ratio (278/251) was found to be constant for one class of immunoglobulins derived from rabbit, human and goat. It has been reported that mammalian IgG absorbance at 278 nm is about 2.5 - 3.0 higher than at 251 nm, in contrast to other serum proteins where this ratio is about 1 - 1.5.
TABLE 2
Direct Binding of Anti-DNA Autoantibodies to Calf Thymus Double Stranded DNA

<table>
<thead>
<tr>
<th>SLE Serum*</th>
<th>Reciprocal of Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>800</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
</tr>
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<td>26</td>
<td>1600</td>
</tr>
<tr>
<td>27</td>
<td>3200</td>
</tr>
</tbody>
</table>

* Name of SLE patients from whom the sera were derived have been changed into numbers.
Fig. 5. Fractionation of SLE serum immunoglobulins on Sephadex G 200. Fifty percent saturated ammonium sulphate precipitated serum proteins were loaded onto the column. Fractions were collected and monitored at 280 nm (— O — ) and absorbance ratio (278/251) (— — ) was evaluated. Fractions from 35-43 were taken as IgG.
Measurements of absorbance at these two wavelengths may therefore indicate the fraction(s) containing IgG. Table 3 represents the absorbance values recorded for material isolated from one of the SLE sera. Similar values were obtained for normal human IgG. Every fresh batch of IgG was tested for purity before further investigations.

Isolation of DNA Fragments

Small DNA fragments were prepared by controlled digestion of native calf thymus DNA with micrococcal nuclease. The digested samples were passed through a Sepharose 4B column. The fallthrough fractions were collected and monitored for DNA content at 260 nm (Fig. 6). Size of the DNA fragments was analyzed on non-denaturing polyacrylamide gel using Lambda DNA digest of EcoRI and Hind III. The average size of the fragments was in the range of 100 to 600 base pairs (Fig. 6 inset). The fragments behaved as double stranded molecules on thermal denaturation.

Tm Dependence on Fragment Size

The DNA fragments of varying size were melted by an electronically controlled heating device. The denaturation data were replotted to determine the helix-coil transition temperature (Tm) (Fig. 7). The Tm of the fragments was found to vary with size. The data presented in Table 4 can most simply be interpreted as inter-fragment variation of (G+C) and (A+T) residue. The fragments rich in G+C content will resist heating as compared to A+T rich fragments which denature early in the transition.

Human Anti-Native DNA Autoantibody Binding to B-DNA

B-conformation specific antibodies from human SLE serum were isolated on affinity column of DNA-[polylysyl-Sepharose 4B]. The SLE serum was diluted 1:10 in PBS and passed through the column. After application of linear sodium chloride gradient, the bound material eluted in three peaks (Fig. 8). Colorimetric estimation and ultraviolet measure-
### TABLE 3

Ultraviolet Absorption Characteristics of SLE IgG*

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Absorbance at 251 nm</th>
<th>Absorbance at 278 nm</th>
<th>Absorbance ratio 278/251</th>
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<tbody>
<tr>
<td>13</td>
<td>0.200</td>
<td>0.428</td>
<td>2.14</td>
</tr>
<tr>
<td>14</td>
<td>0.193</td>
<td>0.485</td>
<td>2.51</td>
</tr>
<tr>
<td>15</td>
<td>0.153</td>
<td>0.358</td>
<td>2.33</td>
</tr>
<tr>
<td>16</td>
<td>0.234</td>
<td>0.523</td>
<td>2.23</td>
</tr>
<tr>
<td>17</td>
<td>0.161</td>
<td>0.315</td>
<td>1.95</td>
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<tr>
<td>18</td>
<td>0.078</td>
<td>0.164</td>
<td>2.10</td>
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<tr>
<td>19</td>
<td>0.065</td>
<td>0.128</td>
<td>1.96</td>
</tr>
<tr>
<td>20</td>
<td>0.061</td>
<td>0.122</td>
<td>2.00</td>
</tr>
</tbody>
</table>

* The IgG was fractionated on DEAE Sephacel matrix.
Fig. 6. Sepharose 4B gel filtration elution profile of calf thymus DNA digested with micrococcal nuclease. Four ml fractions were collected at a flow rate of 40 ml/hr.

Inset: Alternate fractions from 24 to 42 were analyzed on 7.5% polyacrylamide slab gel along with EcoR I and Hind III digested Lambda DNA as marker.
Fig. 7. Thermal denaturation curves of DNA fragments. The temperature was raised at a rate of 1 °C/min from 30° to 95 °C. 
(a) 70 bp, (b) 90 bp, (c) 175 bp, and (d) 300 bp.
TABLE 4

Melting Temperature and Percent Hyperchromicity of DNA Fragments

<table>
<thead>
<tr>
<th>Native DNA fragments (average size)</th>
<th>Percent duplex melted at 80 °C</th>
<th>Percent hyperchromicity at 95 °C</th>
<th>Melting temperature (Tm), °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 bp</td>
<td>10</td>
<td>29.8</td>
<td>86.0</td>
</tr>
<tr>
<td>175 bp</td>
<td>12</td>
<td>40.1</td>
<td>85.0</td>
</tr>
<tr>
<td>90 bp</td>
<td>39</td>
<td>35.0</td>
<td>80.5</td>
</tr>
<tr>
<td>70 bp</td>
<td>55</td>
<td>31.0</td>
<td>79.5</td>
</tr>
</tbody>
</table>
Fig. 8. Immunoaffinity purification of anti-nDNA antibodies on DNA-[polylysyl-Sepharose 4B] column. Anti-DNA positive SLE serum was passed through the column and bound material was eluted with linear gradient (- - -) of sodium chloride (0.15 to 3 M) in 0.1 M Na-Pi buffer, pH 7.4. Inset shows the results of Ouchterlony immunodiffusion. The central well (C) contained anti-human IgG whereas peripheral wells 1, 2 and 3 represents corresponding peak.
ments revealed proteins in peak 1 and peak 2 and DNA in peak 3. The absorbance ratio of peak 1 (278/251) was found to be 2.62 typical of mammalian IgG. Ouchterlony immunodiffusion of peak fractions with anti-human IgG showed precipitin line of complete identity with peak 1 and non-reactivity with materials from peak 2 and peak 3 (Fig. 8 inset). This in turn shows that protein in peak 1 is a homogeneous population of IgG recognizing the native B-conformation of DNA.

SLE Anti-DNA Antibodies Recognize DNA-Lysine Photoadduct

Native calf thymus DNA covalently modified with lysine under UV-A light (320-400 nm) was shown to bind naturally occurring anti-DNA antibodies from human SLE sera (Alam et al., 1992). By the photoaddition of one lysine molecule per helical turn of DNA (unpublished data), the native B-conformation was considerably altered. The altered molecule differing from average B-helix geometry has been found to be immunogenic in contrast to native DNA.

Direct binding of SLE anti-DNA antibodies to chemically modified and photomodified DNA antigens has been presented in Table 5. Besides nDNA, the naturally occurring antibodies indicated appreciable binding with Z-DNA and DNA-lysine photoadduct. In another set of experiments it was found that seventy percent of the SLE sera recognized DNA-lysine photoadduct better than native DNA adsorbed directly on the polystyrene plate (Fig. 9). The binding pattern of SLE autoantibodies upto 1:3200 dilution with either of the antigens was similar to those observed at lower dilution (1:100 dilution). In competitive binding experiments anti-dsDNA IgG isolated from affinity matrix of native DNA-[polylysyl-Sepharose 4B] showed maximum affinity towards DNA-lysine photoadduct followed closely by nDNA based on the amount of competitor required for 50 percent inhibition (Table 6).

Human Anti-DNA Autoantibody Binding to A-, B-, and Z-DNA

Serum antibodies from SLE exhibited a broad spectrum of antigen recognition as demonstrated by their ability to recognize a variety of nucleic acid antigens presenting altogether divergent conformations (Alam...
TABLE 5

Binding Characteristics of Naturally Occurring Autoantibodies with Native and Modified Nucleic Acids

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Reciprocal of antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>nDNA</td>
<td>102400</td>
</tr>
<tr>
<td>DNA-lysine photoadduct</td>
<td>51200</td>
</tr>
<tr>
<td>Z-DNA</td>
<td>12800</td>
</tr>
<tr>
<td>ssDNA</td>
<td>3200</td>
</tr>
<tr>
<td>DNA complexed with cobalt hexaammine</td>
<td>1600</td>
</tr>
<tr>
<td>Poly(dG-dC)-psoralen photoadduct</td>
<td>800</td>
</tr>
<tr>
<td>DNA intercalated with ethidium bromide</td>
<td>400</td>
</tr>
</tbody>
</table>
Fig. 9. Binding of human SLE antibodies to native DNA ( ■ ) and DNA-lysine photoconjugate ( □ ). The binding of control serum with either of the antigens was negligible.
TABLE 6

Relative Binding Profile of Anti-DNA IgG

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Competitor concentration for 50% inhibition (μg/ml)</th>
<th>Maximum percent inhibition at 50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>nDNA</td>
<td>7.5</td>
<td>97</td>
</tr>
<tr>
<td>DNA-lysine photoadduct</td>
<td>5.5</td>
<td>91</td>
</tr>
<tr>
<td>ssDNA</td>
<td>38.0</td>
<td>55</td>
</tr>
<tr>
<td>nDNA UV-irradiated</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>RNA</td>
<td>-</td>
<td>24</td>
</tr>
</tbody>
</table>
and Ali, 1992). Direct binding ELISA on polystyrene plates coated with native calf thymus DNA indicated high binding (titer $>1:6400$) with different dilutions of a typical SLE serum (Fig. 10). While nDNA proved to be an effective inhibitor of DNA-anti-DNA antibody interaction, RNA (buffalo thymus) and single stranded DNA showed inhibition at higher competitor concentration (Fig. 11). Competition ELISA with Z-form of poly(dG-me$_5$ dC), poly(dG-me$_5$ dC) and high salt brominated calf thymus DNA showed appreciable inhibition in antibody activity (Fig. 12). The latter polymer has been shown to attain Z- or analogous conformation on bromination in high salt (Hasan and Ali, 1990). However, poly(dG-dC),poly(dG-dC) in low salt (B-conformation) showed inhibition similar to nDNA. With former the maximum inhibition attained was 81 percent and 50 percent inhibition obtained with only 1.1 ug of inhibitor (Table 7). Autoantibody recognition of poly(rG),poly(dC), a polymer known to be present in A- or analogous conformation, resulted in 77 percent elimination of antibody activity with 10 ug of inhibitor (Fig. 12).

**Competition Studies by Polynucleotides**

Besides native DNA, certain right handed helical double-stranded polydeoxyribonucleotides which differ slightly from the average B-DNA helix were also employed as inhibitors of nDNA-autoantibody interaction. It was found that the epitope recognition of polymers by the antibody was of varying degree (Fig. 13).

**Gel Retardation Assay**

Antibody binding to polynucleotide was studied in polyacrylamide and agarose gel. When mixtures of SLE anti-DNA IgG and DNA fragments (average size 200 bp) were subjected to slab gel electrophoresis under non-denaturing conditions, the antigen-antibody complexes showed different degree of retarded mobility (Fig. 14a). With increasing concentration of antibody the amount of immune complexes, as judged by their fluorescence intensity, was found increased whereas the amount of unbound
Fig. 10. Assay of anti-DNA antibodies by ELISA. The polystyrene plate was coated with native DNA. Normal human serum (—○—) and SLE serum (––).
Fig. 11. Inhibition ELISA of SLE anti-DNA antibodies. The competitors were native DNA (—○—), heat denatured DNA (—●—) and RNA (—×—).
Fig. 12. Inhibition ELISA of SLE anti-DNA antibodies. The competitors were Br4 DNA (---), poly(dG-m5dC).poly(dG-m5dC) (—) and poly (rG). poly(dC) (—).
TABLE 7

Inhibition of the Binding of Anti-DNA Autoantibodies by Polynucleotides

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Concentration for 50% inhibition (ug/ml)</th>
<th>Maximum Percent Inhibition at 10 ug/ml</th>
<th>% Relative Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nDNA</td>
<td>0.38</td>
<td>77</td>
<td>100</td>
</tr>
<tr>
<td>Poly(dA-dU).poly(dA-dU)</td>
<td>1.0</td>
<td>77</td>
<td>38</td>
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<tr>
<td>Poly(dG-dC).poly(dG-dC)</td>
<td>1.1</td>
<td>81</td>
<td>35</td>
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<tr>
<td>Poly(dA-dG).poly(dC-dT)</td>
<td>3.6</td>
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<td>ssDNA</td>
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<td>10</td>
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<tr>
<td>Poly(dA).poly(dT)</td>
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<td>68</td>
<td>07</td>
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<tr>
<td>Poly(rG).poly(dC)</td>
<td>3.2</td>
<td>77</td>
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<tr>
<td>Poly(dG-me\textsuperscript{5}dC).poly(dG-me\textsuperscript{5}dC)</td>
<td>0.9</td>
<td>55</td>
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<tr>
<td>Br-DNA</td>
<td>9.5</td>
<td>85\textsuperscript{*}</td>
<td>04</td>
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<tr>
<td>RNA</td>
<td>NIL</td>
<td>44</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dl-dC).poly(dl-dC)</td>
<td>NIL</td>
<td>39</td>
<td>-</td>
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<tr>
<td>Poly(dG).poly(dC)</td>
<td>NIL</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dC)</td>
<td>NIL</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dG)</td>
<td>NIL</td>
<td>NIL</td>
<td>-</td>
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<tr>
<td>Poly(U)</td>
<td>NIL</td>
<td>NIL</td>
<td>-</td>
</tr>
</tbody>
</table>

* Inhibition at 100 ug/ml.
Fig. 13. Competition ELISA with SLE anti-DNA antibodies. The coating antigen was native calf thymus DNA. The competitors were poly(dG).poly(dC) (---), poly(dA).poly(dT) (---), poly(dA-dG).poly(dC-dT) (---) and double stranded: poly (dG-dC) (---), poly(dI-dC) (---) and poly (dA-dU) (---).
Fig. 14(a). Binding of SLE IgG to B-DNA fragments as analyzed by gel retardation assay. B-DNA fragments (200 bp) (0.1 ug) were incubated with buffer (lane 1), 100 ug of normal human IgG (lane 2), 100 ug of anti-DNA-psoralen crosslink IgG (lane 3) or various concentrations of SLE antibodies (50 ug, lane 5; 100 ug, lane 6; 150 ug, lane 7; 200 ug, lane 8; 250 ug, lane 9) for 1 hr at 4 °C and electrophoresed on polyacrylamide gel (2.5% stacking on 7.5% separating). Lane 4 contained 100 ug of SLE IgG.

(b). Binding of SLE IgG to poly (rG).poly(dC). The hybride (0.5 ug) was incubated with buffer (lane 1), 100 ug of normal human IgG (lane 2) or various concentrations of SLE IgG (25 ug, lane 3; 50 ug, lane 4; 75 ug, lane 5; 100 ug, lane 6) for 1 hr at 4 °C and electrophoresed on 1% agarose.
DNA fragments indicated proportional decrease in their concentration (Lanes 5 thru 9). Normal human IgG and anti-DNA-psoralen crosslink IgG (Hasan et al., 1991) showed no immune complex formation (lanes 2 and 3) and were negative controls.

The antibody binding to poly(rG).poly(dC) was also studied by agarose gel electrophoresis (Fig. 14b). In this case too, retardation in the mobility of hybrid was observed as a consequence of immune complexes formation (lanes 3 to 6). The fluorescence was proportional to the size of immune complex. Normal human IgG incubated under identical conditions did not show immune complex formation.

**Binding of Anti-DNA Antibodies to DNA- Furocoumarin Photoadducts**

Psoralens (furocoumarins) are a class of compounds which can intercalate between base pairs of double stranded nucleic acids. Upon UV-A irradiation, the intercalated psoralens can photoreact prominently with pyrimidine bases to yield monoadducts as well as interstrand diadduct (Song and Tapley, 1979; Cimino et al., 1985; Parsons, 1980).

Furocoumarins modified native DNA preparations (after extensive dialysis) were characterized by UV, fluorescence and thermal denaturation studies. All these experiments suggested that furocoumarins were covalently bound to pyrimidine bases of DNA forming diadducts (Hasan et al., 1991). Direct binding of SLE autoantibodies to furocoumarin-DNA adducts has been shown in Fig. 15. The true recognition of PUVA modified DNA was evaluated by competition-inhibition assay. The DNA-8-MOP photoadducts were effective inhibitors of nDNA-anti-DNA IgG interactions (Table 8). The level of inhibition by DNA-psoralen and poly(dG-dC)-psoralen was 49 and 38 percent respectively.

**Characteristics of Brominated Nucleic Acid Polymers**

The sodium chloride induced B → Z transition in case of poly (dG-dC) can be measured by circular dichroism (Fig. 16 taken from Pohl and Jovin, 1972) and ultraviolet absorbance spectroscopy (Pohl, 1983). The C.D. spectra of poly(dG-dC) in high salt showed near inversion
Fig. 15. Direct binding assay of SLE antibodies. The polystyrene plate was coated with (a) native DNA (—•—), poly(dA-dT)-8-MOP (—<——) and poly(dG-dC)-psoralein (—•—) (b) native DNA (·—•·), DNA-8-MOP (—<——) and DNA-psoralein (—•—).
### TABLE 8

**Inhibition of the Binding by DNA-Furocoumarin Photoadducts of SLE Anti-DNA IgG**

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Maximum percent inhibition</th>
</tr>
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<tbody>
<tr>
<td>nDNA</td>
<td>76*</td>
</tr>
<tr>
<td>DNA-8-MOP</td>
<td>78*</td>
</tr>
<tr>
<td>DNA-psoralen</td>
<td>49*</td>
</tr>
<tr>
<td>Poly(dA-dT)-8-MOP</td>
<td>77**</td>
</tr>
<tr>
<td>Poly(dG-dC)-psoralen</td>
<td>38**</td>
</tr>
</tbody>
</table>

* at 50 ug/ml

** at 30 ug/ml
Fig. 16. Circular dichroism spectra of poly(dG-dC)•poly(dG-dC) in low (—) and high salt (---) at pH 7.2. The low and high salt represent 0.15 M and 4.0 M sodium chloride in phosphate citrate buffer. Taken from Pahl and Jovin, 1972.
with a positive band at 264 nm and a negative band at 294 nm. Fig. 17 shows the ultraviolet right to left transition of poly(dG-dC) with apparent red shift in absorption spectrum. The change in UV absorption spectrum resulted in a change of absorbance ratio \([(294+296)/260]\) from 0.119 in low salt to 0.322 in high salt (Table 9). The red shift observed for poly(dG-dC) in high salt has been explained by Pohl in 1971. He proposed that binding of cations to two adjacent base pairs can bring about the shift of absorption spectrum due to an ion-dipole interaction. The ultraviolet difference spectra of poly(dG-dC) in 4 M sodium chloride showed positive band maxima at 259 and 294 nm and minima at 240 nm (Fig. 18). Unlike poly(dG-dC), DNA fragments in high salt did not show apparent red shift but hypochromic effect was clearly seen (Fig. 17). The UV difference spectra of DNA fragments showed positive band with absorbance maximum at 257 nm and minimum at 240 nm (Fig. 18). Native DNA under high salt conditions exhibited the similar UV and difference spectra.

The high salt induced Z-DNA form of poly(dG-dC) and altered conformations of native DNA and DNA fragments of varying size was stabilized by bromination (Moller et al., 1984). The bromine atoms have been shown to add predominantly to the C8 position of the guanine imidazole ring and less frequently to the C5 position of cytosine. The UV difference spectra of brominated poly(dG-dC) form brought under low salt conditions exhibited a positive peak at 300 nm. The negative band was found to be peaking at 238 - 283 nm (Fig. 19). Fig. 19 inset (taken from Moller et al., 1984) shows the C.D. spectra of brominated polymers with partially converted (θ = 0.6) and fully converted Z-DNA (θ = 1). Although both polymers exhibit near inversion of C.D. spectra but partially converted DNA showed bromination of 22% guanine and 8% cytosine residues and fully converted Z-DNA exhibited 38% guanine and 18% cytosine modification. The UV absorption spectra of native DNA fragments after bromination in low and high salt conditions showed loss in absorbance at 260 nm with a concomitant increase at 295 nm (Fig. 20a). The λ_{min} was shifted from 230 nm to 240 nm (under low salt) and 245 nm (under high salt) respectively. The difference spectra of native DNA fragments after bromination in either low or high salt conditions (Fig. 20b) showed
Fig. 17. UV absorption spectra of double stranded poly (dG-dC) in 0.15 M (---) and 4.0 M NaCl (-----) and DNA fragments (300 bp) in 0.15 M (-----) and 4.0 M NaCl (-----) respectively. The spectra were recorded using buffer as control.
### TABLE 9

The UV Absorbance Ratio of DNA Polymers Under Low and High Salt Conditions

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Sodium Chloride concentration</th>
<th>Absorbance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>260/280</td>
</tr>
<tr>
<td>Poly(dG-dC),poly(dG-dC)</td>
<td>0.15 M</td>
<td>2.043</td>
</tr>
<tr>
<td>Poly(dA-dG),poly(dC-dT)</td>
<td>0.15 M</td>
<td>1.865</td>
</tr>
<tr>
<td>Native DNA</td>
<td>0.15 M</td>
<td>1.874</td>
</tr>
<tr>
<td>Native DNA fragments:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 bp</td>
<td>0.15 M</td>
<td>1.381</td>
</tr>
<tr>
<td>90 bp</td>
<td>0.15 M</td>
<td>1.447</td>
</tr>
<tr>
<td>120 bp</td>
<td>0.15 M</td>
<td>1.502</td>
</tr>
<tr>
<td>175 bp</td>
<td>0.15 M</td>
<td>1.401</td>
</tr>
<tr>
<td>300 bp</td>
<td>0.15 M</td>
<td>1.457</td>
</tr>
<tr>
<td>Poly(dG-dC),poly(dG-dC)</td>
<td>4 M</td>
<td>1.520</td>
</tr>
<tr>
<td>Poly(dA-dG),poly(dC-dT)</td>
<td>4 M</td>
<td>1.800</td>
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<tr>
<td>Native DNA</td>
<td>4 M</td>
<td>1.836</td>
</tr>
<tr>
<td>Native DNA fragments:</td>
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<tr>
<td>70 bp</td>
<td>4 M</td>
<td>1.352</td>
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<tr>
<td>90 bp</td>
<td>4 M</td>
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<td>1.378</td>
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<tr>
<td>300 bp</td>
<td>4 M</td>
<td>1.441</td>
</tr>
</tbody>
</table>
Fig. 18. UV difference spectra of poly(dG-dC) (---) and DNA fragments in 4.0 M NaCl (-----). Respective polymer in 0.15 M NaCl served as corresponding control.
Fig. 19. UV difference spectra of poly(dG-dC) brominated in 4.0 M sodium chloride. Unbrominated poly (dG-dC) was taken as control. The brominated polymer was brought in low salt buffer before it was subjected to UV measurements.

Inset: Circular dichroism spectra of double stranded poly(dG-dC).poly(dG-dC) in B-form (- - - -) and brominated polymers with partially converted Z-DNA, \( \theta = 0.6 \) (- - - -) and fully converted Z-DNA, \( \theta = 1.0 \) (---) in 0.01 M sodium-phosphate (pH 7.0) containing 0.15 M sodium chloride and 0.001 M EDTA. Taken from Möller et al., 1984.
Fig. 20(a). Ultraviolet absorption spectra of native and brominated DNA fragments. DNA fragments in 0.15 M sodium chloride (---), fragments brominated in 0.15 M (--•--) and 4.0 M sodium chloride (---).

(b). UV difference spectra of brominated polymer in low (---) and high salt (--•--) concentrations. Unbrominated DNA fragments served as control.
partial resemblance with difference spectra of high salt brominated poly(dG-dC). The positive band was found peaking around 306 nm and the negative band was found in between 238-288 nm (low salt brominated polymer) and 238-286 nm (high salt brominated polymer). Table 9 and 10 summarizes the absorbance ratios for unbrominated and brominated polymers under low and high salt conditions including native DNA fragments of varying size.

Effect of Polylysine on DNA Conformation

The effect of polylysine addition on the native B-conformation was monitored by UV spectroscopy. After the addition of varying amounts of polylysine to DNA, the complex was mixed thoroughly and left at room temperature for 1 hr to attain equilibrium. The UV absorbance and difference spectra of polylysine-DNA complex was recorded by taking DNA as control. Effect of both D- and L-polylysine was evaluated to see if they can cause different levels of effect on native B-conformation under identical conditions. At low concentrations of poly-L-lysine there was little increase in absorbance at 260 nm while at around 300 nm absorbance was significantly higher (Fig. 21a). Also, the \( \lambda_{	ext{min}} \) was shifted by 2 nm from 230 to 232 nm. When the concentration was raised to 10 \( \mu \)g/ml the absorption spectra showed hyperchromicity at 260 nm and a further increase in absorbance at 300 nm. The \( \lambda_{	ext{min}} \) was shifted towards higher wavelength by 4 nm. In UV absorption spectra, at 260 nm, the effect of poly-D-lysine on native DNA was similar to poly-L-lysine but the absorbance around 300 nm was higher for poly-D-lysine (Fig. 21b). At 10 \( \mu \)g/ml, the absorbance at 300 nm was 0.140 and 0.190 for poly-L-lysine and poly-D-lysine respectively. When polylysine concentration was further raised to 12.5 \( \mu \)g/ml the absorption spectra of D- and L-form were totally different (Fig. 22). At 12.5 \( \mu \)g, poly-L-lysine showed a decrease in absorbance at 260 nm and increased absorbance at 300 nm. By contrast, poly-D-lysine at the same concentration caused hyperchromicity at 260 nm. The high absorbance at 400 nm could arise as a result of turbidity in sample at high ligand concentration.

To monitor any significant topological change in native conformation,
TABLE 10
The UV Absorbance Ratios of DNA Polymers Brominated Under Low and High Salt Conditions

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Sodium Chloride concentration</th>
<th>260/280</th>
<th>(294+296)/260</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(dG-dC).poly(dG-dC)</td>
<td>0.15 M</td>
<td>1.790</td>
<td>0.212</td>
</tr>
<tr>
<td>Poly(dA-dG).poly(dC-dT)</td>
<td>0.15 M</td>
<td>1.949</td>
<td>0.203</td>
</tr>
<tr>
<td>Native DNA</td>
<td>0.15 M</td>
<td>1.532</td>
<td>0.436</td>
</tr>
<tr>
<td>Native DNA fragments:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 bp</td>
<td>0.15 M</td>
<td>2.382</td>
<td>0.221</td>
</tr>
<tr>
<td>90 bp</td>
<td></td>
<td>2.189</td>
<td>0.217</td>
</tr>
<tr>
<td>120 bp</td>
<td></td>
<td>2.337</td>
<td>0.198</td>
</tr>
<tr>
<td>175 bp</td>
<td></td>
<td>1.879</td>
<td>0.311</td>
</tr>
<tr>
<td>300 bp</td>
<td></td>
<td>2.111</td>
<td>0.244</td>
</tr>
<tr>
<td>Poly(dG-dC).poly(dG-dC)</td>
<td>4 M</td>
<td>1.160</td>
<td>0.294</td>
</tr>
<tr>
<td>Poly(dA-dG).poly(dC-dT)</td>
<td>4 M</td>
<td>1.529</td>
<td>0.441</td>
</tr>
<tr>
<td>Native DNA</td>
<td>4 M</td>
<td>1.421</td>
<td>0.321</td>
</tr>
<tr>
<td>Native DNA fragments:</td>
<td>4 M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70 bp</td>
<td>4 M</td>
<td>1.650</td>
<td>0.380</td>
</tr>
<tr>
<td>90 bp</td>
<td></td>
<td>2.216</td>
<td>0.211</td>
</tr>
<tr>
<td>120 bp</td>
<td></td>
<td>1.901</td>
<td>0.295</td>
</tr>
<tr>
<td>175 bp</td>
<td></td>
<td>1.901</td>
<td>0.290</td>
</tr>
<tr>
<td>300 bp</td>
<td></td>
<td>1.648</td>
<td>0.379</td>
</tr>
</tbody>
</table>
Fig. 21. UV absorption spectra of native DNA in presence of (a) 2.5 (-----), 5.0 (----) and 10 ug/ml (-----) of poly-L-lysine and (b) 2.5 (-----), 5.0 (-----) and 10 ug/ml (-----) of poly-D-lysine. (-----) represents the UV spectra of native DNA.
Fig. 22. UV absorption spectra of native DNA in presence of (a) 12.5 ug/ml (- - - -) poly-L-lysine and (b) 12.5 ug/ml of poly-D-lysine (-- -- --). (- - - -) represents the UV spectra of native DNA.
the absorbance ratios of 260/280 and average of \((294 + 296)/260\) is a significant parameter. Table 11 summarizes the effect of polylysine concentrations on absorbance ratio. The effect of poly-D-lysine seems to be more pronounced. Data analysis by absorbance ratios can give further insight into the changing DNA conformation as this parameter was independent of DNA concentrations.

The UV difference spectra of polylysine-DNA complex was recorded to monitor change in secondary structure. Equal amount of DNA without polylysine served as control. At low concentrations of poly-L-lysine the positive peak at 280 nm was very shallow but as the concentration was raised the sharpness of the peak became more distinct (Fig. 23a), thereby indicating that native B-conformation is being perturbed by the added ligand and the molecule is heading towards a neoconformation. On the other hand poly-D-lysine showed a positive peak which showed maximum at 295 nm and a more steep negative band at 255 nm (Fig. 23b). Further increase in polylysine concentration gave birth to a new type of spectra which cannot be solely due to aggregation, if it is there at all (Fig. 24). At 25 \(\mu\)g/ml of poly-D-lysine, the difference spectra exhibited a shallow positive peak at 300 nm and a large negative band at 248-280 nm. The UV difference of high salt brominated poly(dG-dC) showed a positive peak at 300 nm and a negative band peaking between 238-283 nm. Although the high concentration of poly-D-lysine caused some turbidity (may be due to aggregation) in the solution but turbidity alone cannot bring about a gross structural change. The binding of SLE anti-dsDNA IgG to some of the simple amines have been shown in Table 12.

Characterization of DNA Modified by Hydroxyl Radical

It is an established fact that Fenton's reagent (hydrogen peroxide + ferrous ions) results in the production of hydroxyl radical (Floyd, 1981). The radical formation was evident by its ability of causing single strand breaks in supercoiled DNA. As a result, nicked circular form showed slow electrophoretic migration compared to fast migrating supercoiled
TABLE II

Effect of Polylysine Addition on the UV Absorbance Characteristics of Native DNA

<table>
<thead>
<tr>
<th>Microgram polylysine</th>
<th>Absorbance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>260/280</td>
</tr>
<tr>
<td>L-isomer</td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>1.883</td>
</tr>
<tr>
<td>2.5</td>
<td>1.805</td>
</tr>
<tr>
<td>5.0</td>
<td>1.746</td>
</tr>
<tr>
<td>10.0</td>
<td>1.635</td>
</tr>
<tr>
<td>12.5</td>
<td>1.739</td>
</tr>
<tr>
<td>25.0</td>
<td>1.440</td>
</tr>
<tr>
<td>D-isomer</td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>1.896</td>
</tr>
<tr>
<td>2.5</td>
<td>1.834</td>
</tr>
<tr>
<td>5.0</td>
<td>1.717</td>
</tr>
<tr>
<td>10.0</td>
<td>1.557</td>
</tr>
<tr>
<td>12.5</td>
<td>1.357</td>
</tr>
<tr>
<td>25.0</td>
<td>1.089</td>
</tr>
</tbody>
</table>
Fig. 23. Ultraviolet difference spectra of polylysine-DNA complex. (a) at 2.5 (--•--•), 5.0 (--•--) and 10 μg/ml (--•--) of L-polylysine and (b) at 2.5 (--•--•), 5.0 (--•--) and 10 μg/ml (--•--) of D-polylysine. Native DNA without polylysine was taken as control.
Fig. 24. Ultraviolet difference spectra of polylysine-DNA complex at 12.5 (----) and 25 ug/ml (-----) of L-polylysine and 12.5 (-----) and 25 ug/ml (-----) of D-polylysine.
TABLE 12

Inhibition of Binding by Amines of SLE Anti-DNA IgG to Native DNA

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Percent inhibition at 50 ug/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermine</td>
<td>53</td>
</tr>
<tr>
<td>Ethionine</td>
<td>44</td>
</tr>
<tr>
<td>Agmatin Sulfate</td>
<td>32</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>26</td>
</tr>
<tr>
<td>Histamine</td>
<td>24</td>
</tr>
<tr>
<td>Tyramine</td>
<td>18</td>
</tr>
<tr>
<td>Lysine</td>
<td>NI</td>
</tr>
<tr>
<td>Poly-D-lysine</td>
<td>NI</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>NI</td>
</tr>
<tr>
<td>Histone H₂A</td>
<td>NI</td>
</tr>
<tr>
<td>Arginine</td>
<td>NI</td>
</tr>
<tr>
<td>Putrescine</td>
<td>NI</td>
</tr>
</tbody>
</table>

NI = No inhibition
form in agarose gel electrophoresis (Fig. 25).

The ultraviolet absorption scan of modified DNA showed hypochromicity at 260 nm compared to native DNA under identical conditions (Fig. 26). That the DNA modified by hydroxyl radical did not lose its secondary structure is revealed by its desorption from hydroxyapatite in the molarity region of double stranded DNA (Fig. 27). Native DNA sedimentation in alkaline sucrose gradient showed a single sharp boundary of high molecular weight species. Treatment of hydroxyl radical modified DNA under identical conditions was found to lose the sharp boundary pattern and instead it revealed a sedimentation profile quite distinct from the one observed for nDNA (Fig. 28). The altered sedimentation profile of modified DNA indicates that the single strand breaks are randomly situated which appeared as DNA fragments of varying size that sediment more slowly than the intact single strands of alkali denatured native DNA. Temperature controlled denaturation of native and modified DNA showed a significant decrease in melting temperature (Fig. 29). A 6.5 °C decrease of melting temperature could be attributed to the local helix distortion caused by base modification and partial unstacking. The physico-chemical characteristics of native and modified duplex has been presented in Table 13.

Native and modified DNA were analyzed on DEAE Sephadex A-50 matrix after acid hydrolysis to separate and quantitate the modified bases. Column chromatography of modified DNA hydrolysate indicated ROS induced base modification (Table 14). The relative susceptibility of DNA to modification by hydroxyl radical was as follows:

Thymine > Cytosine > Adenine = Guanine

The immunogenicity of modified DNA was investigated in rabbits immunized with modified DNA complexed with Freunds' complete/incomplete adjuvant. Unlike native DNA, hydroxyl radical modified calf thymus DNA induced high titer antibodies. Preimmune serum showed negligible binding with immunogen. DEAE Sephacel purified immune IgG showed
Fig. 25. Gel electrophoresis of supercoiled DNA in presence of Fentons' reagent. Lane 1, DNA; Lane 2, DNA + hydrogen peroxide; Lane 3, DNA + hydrogen peroxide + Fe^{2+} ions.
Fig. 26. Ultraviolet absorption spectra of native (----) and hydroxyl radical modified DNA (--.--.--).
Fig. 27. Hydroxyapatite column chromatography of native (---) and modified DNA (——). The elution was carried out stepwise using Na-Pi buffer (pH 6.8) of increasing ionic strength.
Fig. 28. Profile showing sedimentation of native (---) and hydroxyl radical modified DNA (-----) through alkaline sucrose density gradient. The arrow on top shows the direction of sedimentation.
Fig. 29. Effect of temperature on the melting of native (—) and ROS-modified DNA (——). The Tm values are shown on the left corner.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Native DNA</th>
<th>Modified DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance ratio (260/280)</td>
<td>2.20</td>
<td>1.95</td>
</tr>
<tr>
<td>Percent hyperchromicity at 95 °C</td>
<td>36.03</td>
<td>25.10</td>
</tr>
<tr>
<td>Melting temperature (Tm), °C</td>
<td>89.0</td>
<td>82.5</td>
</tr>
<tr>
<td>Onset of duplex melting, °C</td>
<td>80.0</td>
<td>74.0</td>
</tr>
</tbody>
</table>
TABLE 14

Modification of Bases in DNA Exposed to Hydroxyl Radicals

<table>
<thead>
<tr>
<th>Base</th>
<th>Modification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>30.05</td>
</tr>
<tr>
<td>Guanine</td>
<td>29.20</td>
</tr>
<tr>
<td>Cytosine</td>
<td>38.80</td>
</tr>
<tr>
<td>Thymine</td>
<td>49.25</td>
</tr>
</tbody>
</table>
high degree of binding with native DNA besides immunogen (Fig. 30). Preimmune IgG as control showed negligible binding to these antigens. To ascertain the antibody specificity, competition ELISA with immunogen and other double stranded nucleic acids were carried out. In competition-inhibition assay, the induced antibody binding was fifty percent inhibited by the immunogen at a concentration of 0.32 µg/ml (Fig. 31a). The cross-reactivity of induced antibodies with B-conformations like native DNA, poly(dA-dG).poly(dC-dT), poly(dA-dT).poly(dA-dU) and poly(dI-dC). poly(dI-dC) was of varying strength (Fig. 31b). The immunogen-antibody interaction was also analyzed by precipitating the complex with saturated ammonium sulphate. The unbound antigen was estimated in the supernatant by diphenylamine reagent. Fig. 32 represents that at 50 µg of antigen concentration all the antibody binding sites were saturated and further increase in antigen concentration does not improve the binding. Almost fifty percent of the added antigen was bound by the anti-ROS-DNA antibodies. The induced antibody affinity was evaluated from Langmuir plot (Fig. 33). The antibody affinity was computed to be $2.7 \times 10^{-8}$ M.

To probe the possible role of hydroxyl radical (or ROS in general) in the pathogenesis of SLE, four sera having different levels of anti-DNA autoantibodies were tested. Binding of these antibodies (at 1:100 dilution of serum) with native and modified DNA has been depicted in Fig. 34a. The binding pattern of some of these autoantibodies up to 1:6400 dilution of serum with either of the antigens was similar to those observed at lower dilution. It was found that SLE autoantibodies recognition of modified DNA was better than native DNA under identical conditions. However, one serum sample (No. 1) showed high binding with native DNA as compared to modified DNA preparation. Competition-inhibition assay of SLE autoantibodies with native and modified DNA as inhibitors have shown that ROS modified DNA is a better inhibitor of autoantibody-nDNA interaction. The amount of competitor required for 50 percent inhibition of autoantibody binding was found to be 0.03 µg/ml and 0.13 µg/ml in case of modified DNA and nDNA respectively.
Fig. 30. Binding of anti-ROS-DNA IgG with native (■) and ROS-DNA (■). The bars having absorbance less than 0.2 represent preimmune IgG binding with native DNA and immunogen.
Fig. 31(a). Inhibition of immunogen-antibody interaction by ROS-DNA. (b) Inhibition of immunogen-antibody interaction by native DNA (—•—), poly(dA-dG),poly(dC-dT) (—○—), double stranded: poly(dI-dC) (—□—) and poly (dA-dU) (—△—).
Fig. 32. Determination of antigen bound by immune (●) and preimmune IgG (○). The assay was performed by quantitative precipitin titration.
Fig. 33. Determination of antibody affinity by Langmuir plot.
Fig. 34(a). Binding of human lupus antibodies to ROS-DNA (■) and native DNA (□). (b) Inhibition of autoantibody-nDNA interaction by native DNA (○) and ROS-DNA (●).
(Fig. 34b). Similar results were obtained with other SLE serum samples.

**Antibody Detection of Z- or Analogous Conformations**

Chemical modification with different reagents has been extensively used as a tool to study the structure and function of nucleic acids in the free state or in nucleoprotein complexes. Antibodies with predefined specificity can be used as a yardstick to locate a particular conformation in a complex biological system such as mammalian genome.

The poly(dG-dC) brominated under high salt conditions was found to attain Z-conformation. The monoclonal anti-Z-DNA antibody (Z 22) binding was nearly 80 percent inhibited at a Z-DNA concentration of 10 μg/ml (Fig. 35). The fifty percent inhibition was seen at 4.25 μg/ml of Z-DNA. This demonstrates that Z 22 is a highly specific probe for Z-DNA conformation. Z 22 recognition of native poly(dG-dC) or low salt brominated poly(dG-dC) was negligible. These antibodies were also used as tracer of Z- or Z-like epitopes appeared on poly(dA-dG).poly(dC-dT) brominated under 4 M and 0.15 M sodium chloride. The Z 22 binding to Z-DNA was inhibited to the extent of 14 and 23 percent by high and low salt brominated poly(dA-dG).poly(dC-dT) (Fig. 36). These type of sequences are widely distributed in mammalian genome and have potentiality to undergo conformational transition besides formation of multistranded structure when the pH of the medium is lowered.

The anti-dsDNA IgG derived from human lupus were also tested for their binding to poly(dA-dG).poly(dC-dT). It was found that with alteration in poly(dA-dG).poly(dC-dT) native conformation (as a result of bromination), the autoantibody binding changes accordingly (Fig. 37). The anti-DNA antibody binding dropped to 51 percent in low salt and 34 percent in high salt against 80 percent when the polymer was in its native conformation. Native DNA brominated under low and high sodium chloride showed that polymers have undergone a conformational transition that deviates from native B-conformation. The Z- or Z-like epitopes on brominated polymer was detected by Z-DNA specific monoclonal (Z 22) and polyclonal (G 10) antibodies (Tal' et al. 15). The SLE anti-dsDNA
Fig. 35. Inhibition of anti-Z-DNA antibody (Z 22) binding to Z-DNA by poly(dG-dC) (---X---), poly (dG-dC) brominated in 0.15 M (---O---) and 4 M sodium chloride (---o---).
Fig. 36. Inhibition of Z 22 antibody binding to Z-DNA by poly(dA-dG), poly(dC-dT) (---), poly(dA-dG).poly(dC-dT) brominated in 0.15 M (-----) and 4 M sodium chloride (--o--).
Fig. 37. Inhibition of SLE anti-DNA IgG binding to native DNA by poly(dA-dG).poly(dC-dT) (—), poly(dA-dG).poly(dC-dT) brominated in 0.15 M (×) and 4 M sodium chloride (○).
<table>
<thead>
<tr>
<th>Antigen on plate</th>
<th>Inhibitor</th>
<th>Maximum percent inhibition with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Z22</td>
</tr>
<tr>
<td>Z-DNA</td>
<td>NBr4</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>N Br0.15</td>
<td>12</td>
</tr>
<tr>
<td>N Br4</td>
<td>N Br4</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>N Br0.15</td>
<td>27</td>
</tr>
</tbody>
</table>

* Monoclonal mouse anti-Z-DNA antibody
** Polyclonal goat anti-Z-DNA antibody
NT = Not tested

NBr4 & NBr0.15: Native DNA brominated under 4 M and 0.15 M sodium chloride respectively.
IgG showed different levels of binding with native DNA and its brominated analogues (Fig. 38). The binding of autoantibodies to brominated DNA can be explained in two ways: Firstly, the SLE antibodies are recognizing the remaining B-epitopes on brominated polymers (as not all the sequences in native DNA are capable of undergoing B → Z transition) and secondly, they are exhibiting polyspecificity (a feature common to DNA autoantibodies) and hence recognizing two different conformations. The SLE anti-dsDNA IgG binding to brominated DNA fragments have been presented in Table 16. Some of the brominated DNA fragments did not bind anti-DNA IgG at all. A possible explanation for such non-reactivity can be given if it is assumed that such DNA fragments have undergone a radical conformational transition, which is possible in small DNA fragments. The DNA fragments rich in G+C content are expected to undergo B → Z transition more readily against DNA fragment of same length rich in A+T. Besides, the DNA molecule is a dynamic structure it would be expected that this would alternate between these two states (namely B and Z) passing through many intermediate stages.
Fig. 38. Inhibition of SLE anti-DNA IgG binding to native DNA by native DNA (---) and native DNA brominated in 0.15 M (--*) and 4 M sodium chloride (---).
TABLE 16

**Inhibition of the Binding of SLE Anti-DNA IgG to DNA Fragments of Varying Size Brominated Under Low and High Salt Conditions**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Maximum percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in low salt</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Native DNA fragments: (average size)

<table>
<thead>
<tr>
<th>Size (bp)</th>
<th>Inhibition in Low Salt</th>
<th>Inhibition in High Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 bp</td>
<td>70</td>
<td>41</td>
</tr>
<tr>
<td>175 bp</td>
<td>56</td>
<td>26</td>
</tr>
<tr>
<td>120 bp</td>
<td>51</td>
<td>20</td>
</tr>
<tr>
<td>90 bp</td>
<td>74</td>
<td>NI</td>
</tr>
<tr>
<td>70 bp</td>
<td>66</td>
<td>NI</td>
</tr>
</tbody>
</table>

NI = No inhibition
Autoimmune diseases are a diverse group of conditions characterized by antibody recognition of normal components of the host. In many of these diseases, antibodies with self specificity or autoantibodies are important effectors. These antibodies mediate disease by a variety of distinct pathological mechanisms. Although anti-DNA antibodies were discovered more than twenty five years ago and despite the fact that numerous studies have suggested various aspects of autoimmune phenomena, spontaneous antibodies to nucleic acids remain puzzling in many aspects. Questions about the mechanisms leading to their formation, their specificity, their relationship with antibodies directed towards other self constituents is still unsolved (Harley and Scofield, 1991; Jacob et al., 1989; Cairns et al., 1989; Pisetsky et al., 1990; Brendel et al., 1991).

Investigations designed to assess antibody specificity are important for many reasons. First, they permit diagnostic tests to be developed to detect and monitor disease activity (Hashizume et al., 1987). Second, specificity studies will provide insight as to the nature of autoantigens that conceivably are required for eliciting spontaneously occurring anti-DNA antibodies (Brigido and Stollar, 1991). Double-stranded DNA (B-conformation) is unlikely to be involved in SLE pathogenesis in view of its poor immunogenicity (Madaio et al., 1984; Pisetsky et al., 1990). Although commonly referred to as a discrete material, DNA displays a host of structural forms and sequence patterns that can serve as antigenic determinants (Stollar, 1975; Wells et al., 1980). The structural diversity and flexibility of DNA is inherent in its role in gene expression and providing recognition sites for regulatory molecules. A number of different conformations have been characterized by physiochemical and immunological methods (Lafer et al., 1986; Sanford et al., 1988), and structurally distinct regions probably exist along such DNA molecule. This microheterogeneity of structure provides for a multideterminant antigen.

Antibodies reactive with cellular and nuclear components occur
spontaneously in SLE. The complex serology of SLE seems to indicate response to a large number of different autoantigens (Zouali et al., 1988). The binding diversity of lupus autoantibodies seems even greater when different nucleic acids and their modified preparations are used as test antigens (Ali et al., 1991; Alam and Ali, 1992; Alam et al., 1992; Ara et al., 1992; Ara and Ali, 1992). Because a mixed picture, with aspects of wide cross reactivity and aspects of selectivity, were observed in the preliminary studies, a more detailed analysis was performed to probe the basis for anti-DNA antibody binding to DNA of varying size and conformation.

The absorption ratio measurement (278/251) was found to be a quick assessment of IgG purity. IgG fractions having an absorbance ratio of less than 2.25 were further purified by exclusion chromatography on Sephadex G 200. Removal of proteins other than IgG was thought necessary in order to define the true binding specificities of anti-DNA IgG.

Native DNA and DNA fragments of varying size prepared by controlled digestion with micrococcal nuclease were indeed double stranded as revealed by sharp melting profile. The T\textsuperscript{m} dependence on fragment size was quite obvious. Moreover the samples were also digested with single strand specific nuclease S I to get rid of single stranded regions in various DNA preparations.

The immunological recognition of various DNA conformations by SLE anti-DNA antibodies is of immense importance in relation to their polyspecificity. Double stranded DNA has repetitious antigenic determinant on the deoxyribose-phosphate backbone whereas most protein antigens have multiple but different antigenic determinants. The binding specificity of anti-DNA antibodies with varying DNA conformations was analyzed by inhibition ELISA in which the ability of a competitor to block the DNA-autoantibody interaction is assessed. The binding of SLE antibodies to native B-conformation, polymers differing from average B-helix geometry, DNA-RNA hybrid, Z-form of poly(dG-me\textsuperscript{5}dC) clearly demonstrated that native DNA is not always the preferred antigen for
antibodies in SLE and that both A- and Z-DNA shares immunogenic epitopes with native DNA. The altered electrophoretic mobility in gel retardation assay of A- and B-conformation complexed with anti-DNA antibodies reiterates the antibody binding to these conformers. The gel retardation assay (also known as band-shift assay) is a sensitive technique to detect DNA-protein interactions. Both A- and Z-DNA have been reported to be highly immunogenic in experimental animals (Nakazato, 1980; Lafer et al., 1981) and the participation of these non-B conformations in the etiology of SLE cannot be ruled out. Double stranded regions of RNA (as in cruciform) and RNA-DNA hybrids adopt a right handed double helical form very similar to that of A-DNA and the occurrence of hybrid is closely linked with transcriptional activity of the cell (Busen et al., 1982). Moreover, Z-conformation is a reversible structural form of DNA and its role in genetic activities like transcription is well documented (Kmeic et al., 1983; Peck and Wang, 1985). The existence of segments of left handed helices in native DNA has also been documented (Ueda et al., 1990; Wittig et al., 1991; Jimenez-Ruiz et al., 1991).

Polyamines are known to bind negatively charged macromolecules such as nucleic acids. The binding of SLE anti-DNA antibodies to DNA-lysine photoadduct has further expanded the horizon of antibody poly-specificity. DNA molecules differing from average B-helix geometry has been found to be immunogenic (Stollar, 1989) and DNA-lysine photoadduct has induced high titer antibodies (Islam, 1991). The anti-DNA IgG purified through DNA-(polylysyl-Sepharose 4B) affinity column was homogeneous and found to be recognized equally well by native DNA and DNA-lysine photoconjugate. Moreover, the relative binding affinity of anti-DNA antibodies to DNA-lysine photoconjugate was appreciably higher than that with native DNA. By the photoaddition of one lysine molecule per helical turn of DNA, the native B-conformation was considerably altered which has resulted in the generation of high affinity neo-epitopes leaving much less epitope typical of B-conformation. The results conclusively suggest that affinity purified anti-DNA IgG is indeed recognizing the altered conformation of DNA-lysine photoconjugate. The retention
of cross reactivity of affinity purified anti-DNA IgG with DNA-lysine photoadduct further strengthen the idea of an alternate antigen for the induction of antibodies cross reactive with native DNA. The DNA-lysine photoconjugate could be placed on the existing list of alternate antigens for the induction of autoantibodies in SLE. Lysine rich histone H1 in nucleosomes thus on modification by physical, chemical or environmental agents might form DNA-histone adduct making it immunogenic and thus resulting in the production of autoantibodies cross reactive with nDNA. The findings of Mura and Stollar (1984) have suggested the ability of different lysine-rich histones to cause varying conformational changes in native DNA molecules of interspersed repetitive sequences.

There has been considerable interest on the damaging effect of reactive oxygen species (ROS), like superoxide radical (O$_2^-$) hydrogen peroxide and hydroxyl radical (Fridovich, 1986; Halliwell, 1987) on nucleic acids, proteins and lipids. These radicals modify DNA at various sites that includes base damage and cross linking between cytosine and tyrosine in nucleohistone (Dizdaroglu et al., 1991; Gajewski and Dizdaroglu, 1990). It has been shown that DNA after exposure to reactive oxygen species presents a more discriminating antigen than native DNA for the binding of SLE autoantibodies (Blount et al., 1989). Oxidative DNA damage resulting from active oxygen species has been hypothesized to play a critical role in several biological processes including aging, cancer and autoimmune diseases (Ames, 1983, 1989; Cerruti, 1985; Von Sonntag, 1987). In our experimental conditions, the yield of free radical induced base modification predominantes over that of single strand breaks in DNA as revealed by decrease in UV absorbance at 260 nm. The decrease in Tm value by 6.5 °C with simultaneous decrease in total percent hyperchromicity of modified DNA is suggestive of the fact that DNA has been modified without undergoing major alterations in its secondary structure. In case of gross changes in the secondary structure of modified DNA the decrease in Tm value would have been much more than what has been observed. The alteration in the topological state of native DNA
has rendered it immunogenic as shown by experimentally induced high
titer antibodies. The polyspecificity exhibited by immune IgG was evident
from inhibition ELISA results. Native DNA, RNA and synthetic poly-
nucleotides in B-conformations besides immunizing antigen were effective
inhibitors. In an earlier study, native DNA on ultraviolet irradiation
in presence of hydrogen peroxide induced antibodies which showed recogni-
tion for B-conformation (Ara et al., 1992). The polyspecificity exhibited
by immune IgG was comparable to well established heterogeneity of
murine and human SLE anti-DNA antibodies. Naturally occurring anti-
DNA antibodies of SLE patients showed enhanced binding with modified
DNA and binding of anti-DNA antibodies to native DNA was inhibited
by modified DNA antigen. Since little is known about the stimulus that
causes the production of anti-DNA antibodies in SLE, the potential
reactive oxygen mediated DNA damage may provide a new concept
for the production of anti-nucleic acid antibodies in autoimmune disorders.
The spontaneous production of anti-DNA antibodies in SLE might arise
as a consequence of antigenic changes in DNA due to damage caused
by hydroxyl radicals. The augmented levels of ROS in tissues is well
related to many pathological conditions (Willson, 1979). Hydrogen peroxide
on diffusion to a vulnerable part of the cell might generate hydroxyl
radicals on reaction with ferrous ion, often bound to phosphates of DNA
backbone and to various protein including those associated with chromatin
which in turn could modify DNA (Floyd 1981, 1983). The iron promoters
of hydroxyl radical formation exist in vivo based on the observation
that iron salts never exist "free" in biological system; they will always
be found to be associated with proteins, membranes, nucleic acids or
low molecular weight chelating agents.

The conformational and dynamical properties of DNA are strongly
dependent upon both its sequence and microenvironment. It has been
suggested that when a B-DNA segment is flanked by any different struc-
tural motifs (e.g. A, Z or B), there is an increase in overall flexibility
provided by bending and enhanced fluctuations at the conformational
interfaces (Reich et al., 1991). In 1972, when Pohl and Jovin reported
right handed to left handed conformational transition for poly(dG-dC) in high salt, it was presumed that DNA polymers rich in alternating purine-pyrimidine bases will readily undergo $B \rightarrow Z$ transition. Later, it was discovered that a strictly alternating purine-pyrimidine is neither necessary nor sufficient criteria for $Z$-DNA formation (McLean et al., 1986; Singleton et al., 1983; Kilpatrick et al., 1984; Ellison et al., 1985). Nordheim et al., (1986) showed that stretches of poly(dG-dC)$_n$ sequences do not contribute significantly to the presence of $Z$-DNA in fixed polytene chromosomes of *Drosophila melanogaster*. Further studies showed that the potentially $Z$-DNA forming sequence d(GTGTACAC) adopts a right handed double helical conformation belonging to the A-DNA family (Jain et al., 1987). In C.D. spectra the $Z$-form of poly(dG-dC) shows near inversion with a positive band at 264 nm and a negative band at 294 nm. Comparison of C.D. and UV result suggests that red shift in UV absorption is solely due to $B \rightarrow Z$ transition. In 0.15 M and 4 M sodium chloride concentration the average of absorbance at 294 and 296 nm divided by absorbance at 260 nm, termed as absorbance ratio, was evaluated to monitor conformational transition in synthetic and native DNA polymers. For poly(dG-dC) in $Z$-conformation this value was computed to be 0.322. This reference value for prototype $Z$-DNA was used to monitor conformational transitions in native DNA, DNA fragments of varying size and poly(dA-dG),poly(dC-dT). Neither polymers resulted in absorbance ratio of 0.322 but data in Table 9 clearly indicates that in high salt this ratio is changing which could occur as a result of conformational transition. The low and high salt conformations were stabilized by bromination (Moller et al., 1984). The bromination had profound effect on the absorbance ratio of poly(dA-dG),poly(dC-dT), native DNA and DNA fragments. Native DNA brominated under low or high salt concentration, showed absorbance ratio greater than 0.3. Under identical conditions the absorbance ratio for DNA fragments of varying size varied between 0.2 to 0.38. This inturn shows that DNA fragments of varying size have different levels of potentiality to undergo $B$- to $Z$- or $Z$-like conformations as the fragments will have variation in the G+C and A+T content. Also native DNA or its fragments might undergo $Z$- or $Z$-like conformational
changes on bromination under low or high salt concentrations. The UV difference spectra of Z-form of brominated poly(dG-dC) had resemblance with native DNA or DNA fragments brominated under low or high sodium chloride concentrations. This further reiterates that both in native DNA and DNA fragments there are regions of interspersed sequences which has the potential to undergo B- to Z- or Z-like transitions upon bromination in high or low salt conditions.

A variety of cations such as simple amines or polyamines that effect B- to Z-transition in poly(dG-me dC) are known to induce the collapse of native DNA into compact structural forms. These forms have been visualized by electron microscope as toroids, folded fiber, rods and spheroids (Gosule and Schellman, 1976, 1978; Chattoraj et al., 1978; Widom and Baldwin, 1980; Allison et al., 1981). The polyamines have been shown to stabilize DNA against thermal denaturation, alkaline denaturation, enzymatic degradation, shear induced strain and induce in vitro condensation of DNA (Vertino et al., 1987). The condensation of naked DNA takes place in a narrow range of polyamine concentration whereas the condensation of chromatin can occur in the presence of a wider range of polyamine concentration. The UV absorption spectra of D- and L-isomers of polylysine indicated their interaction with DNA on charge basis. The effect of D-isomer was appreciably higher than L-isomer. At low concentrations of these isomers the absorbance at 260 nm showed little change but the $\lambda_{\text{min}}$ was found to be red shifted to the extent of 2-4 nm. The shift could be attributed to the presence of a ligand in DNA solution. The increase of absorbance around 300 nm is considered to be an indication of conformational transition in native B-conformation. The UV difference spectra clearly shows that at low concentrations of either D- or L-isomer, the primary interacting sites are phosphate groups on DNA backbone but once the charge neutralization has been completed the B-conformation is on the verge of changing conformation with further increase in polylysine concentration. The most significant change in UV difference spectra was seen when poly-D-lysine concentration was raised to 25 ug/ml from 12.5 ug/ml. On comparision
the spectra showed the characteristic of high salt brominated poly(dG-dC) which assumes 100 percent left handed Z-conformation. The data analysis of polyllysine-DNA interaction suggests that in calf thymus DNA there are certain base pair sequences which are sensitive to changing polyllysine level and can assume Z- or Z-like conformations. Since the eukaryotic DNA is heavily associated with proteins sufficiently rich in lysine, such conformational transitions might serve as molecular signal for genetic events inside the cell. Recently, Gunnia et al. (1991) have shown that rabbits immunized with poly(dA-dC).poly(dG-dT) complexed with spermidine or spermine produced antibodies reacting with Z-DNA in addition to those binding toward poly(dA-dC).poly(dG-dT) and ssDNA. Antibodies elicited by polynucleotide-polyamine complexes had no reactivity towards polyamines. This finding suggests that polyamines are capable of altering the immunogenicity of polynucleotides by mechanism involving the stabilization of Z-DNA conformations.

The conformational transition in native calf thymus DNA, poly(dA-dG).poly(dC-dT) and DNA fragments of varying size was evaluated by monoclonal anti-Z-DNA antibody (Z22) and polyclonal goat antibody (G10). Both Z22 and G10 are immunological probes for the detection of Z- or analogous conformation. The binding of Z22 was nearly 80 percent inhibited at a Z-DNA concentration of 10 μg/ml. This demonstrate the high specificity of Z22 for Z-conformation as poly(dG-dC) (B-form) or brominated under low salt showed no binding with Z22. Competition experiments with Z22 of poly(dA-dG).poly(dC-dT) brominated in 0.15 M or 4 M sodium chloride showed that potentiality of this polymer to undergo Z-transition was limited. Furthermore, competition ELISA with SLE anti-dsDNA IgG showed that poly(dA-dG).poly(dC-dT) has undergone conformational transition because binding of anti-dsDNA IgG to brominated polymers has declined significantly as compared to binding with native poly(dA-dG).poly(dC-dT). The Z- or Z-like epitopes on native DNA brominated under low and high sodium chloride concentrations was probed by both monoclonal (Z22) and polyclonal (G10) anti-Z-DNA antibodies. The binding of Z22 and G10 was inhibited by both low and high salt brominated polymers.
This finding again reiterates that dispersed sequences of calf thymus DNA has potential to adopt Z- or Z-like conformation. This means calf thymus DNA exhibits microheterogeneity whose conformation is dictated by its microenvironment. The binding of SLE anti-DNA IgG to brominated native DNAs could be explained in two ways:

I. In native DNA not all the epitopes typical of B-conformation has undergone conformational transition and hence naturally occurring anti-dsDNA IgG is recognizing the remaining B-epitopes.

II. A population of SLE anti-dsDNA IgG is exhibiting polyreactivity with respect to varying DNA conformation and hence Z-like epitope could also interact with antibodies.

To determine, whether reactive structure could be maintained in smaller fragments of native DNA the fragments of varying size after bromination in low and high salt were used as competitors of nDNA-anti-DNA IgG interaction. Unbrominated native DNA fragments were used as true competitors of SLE IgG to which inhibition values of low and high salt were compared. The data indicates that binding of anti-DNA IgG was significantly reduced when the polymer has undergone conformational transition. The level of transition seem to be affected by two factors: namely length of the DNA fragment and percentage of G+C and A+T content.

If anti-DNA antibodies from human and murine SLE are capable of exhibiting polyspecificity, the DNA molecule is a step ahead by its potentiality to adopt multiple conformations. The conformational flexibility of DNA depends on its microenvironment and any unusual structure which is stable at physiological conditions, suggest that such a structure might be formed in vivo. The polyspecificity phenomenon of naturally occurring anti-DNA antibodies is most likely due to epitope that recur in different molecules, an economy of nature whose meaning awaits vigorous elucidation.

Based on the results of present study, the following points of conclusion have been drawn:

I. Native DNA and DNA fragments of varying size, brominated
under low and high sodium chloride concentrations is both structurally and immunologically distinct from B-form DNA. That the bromination has induced neoepitopes capable of presenting Z- or Z-like features was detected by monoclonal (Z22) and polyclonal (G10) anti-Z-DNA antibodies.

2. In native calf thymus DNA there are distinctive distribution of DNA sequence which has the potential to undergo B- to Z- or Z-like transitions upon binding with nucleoproteins rich in basic residues.

3. The modified nucleic acid antigens may participate as antigenic stimulus for the production of a population of DNA cross reactive antibodies in SLE patients. The dietary, physical, chemical, or environmental agents might act, directly or indirectly, as modifier.

4. The hydroxyl radical modified DNA may play an important role in the pathogenesis of SLE because of markedly decreased level of superoxide dismutase in active SLE patients; the excessively generated free radicals may exert its modifying effect on DNA and thus rendering it immunogenic.

5. The binding of A- or A-like conformation by SLE anti-DNA antibodies suggest that such conformations may also act as potential auto-immunogen for the induction of antibodies cross reactive with native DNA.


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