IMMUNOGENICITY OF REACTIVE OXYGEN SPECIES MODIFIED POLY (G) AND ITS IMPLICATION IN PROGRESSIVE SYSTEMIC SCLEROSIS

ABSTRACT

THESIS SUBMITTED FOR THE DEGREE OF
Doctor of Philosophy
IN
BIOCHEMISTRY

BY
DEEPAK KUMAR GARG

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE
JAWAHARLAL NEHRU MEDICAL COLLEGE
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)
1998
Reactive oxygen species (ROS) generated during various metabolic and biochemical reactions has multifarious effects that include oxidative damage to DNA leading to various human degenerative and autoimmune diseases. ROS are formed from oxygen in one electron reduction reactions and include superoxide anion, hydrogen peroxide and hydroxyl radical.

Chronic inflammation is associated with considerable tissue injury resulting from perpetual stimulation of phagocytic cells by bacterial and immunological stimuli releasing reactive oxygen species. The hydroxyl radical (OH), formed during inflammatory processes is an extremely reactive oxidizing radical with a short half life and interact indiscriminately with all components of the DNA molecule.

Systemic lupus erythematosus (SLE) and progressive systemic sclerosis (PSS) are the chronic inflammatory disorders characterized by the presence of autoantibodies targeted against various intracellular proteins and nucleic acids. SLE is considered to be the prototype of a spontaneous immune complex disease in which anti-DNA antibody-DNA deposit in the tissues causing inflammation. Anti-double stranded DNA antibodies are the serologic hallmarks of SLE. PSS is serologically associated with the emergence of autoantibodies to SCI-70 (DNA topoisomerase I, PM-SCI, U3-RNP) with different incidence and disease specificities.

The origin of these autoantibodies and their etiology is still in dilemma. The binding of SLE autoantibodies to ROS-modified DNA, DNA-psoralen adduct, brominated DNA, DNA-RNA hybrids and various single and double stranded polynucleotides have been reported.

In the present study, a ribohomopolymer, polyguanylic acid [poly(G)] was modified with hydroxyl radical, generated by irradiation of hydrogen peroxide with 254 nm UV light. The modification of poly(G) was analysed by UV and CD spectroscopic techniques. When compared with the spectrum of native poly(G), ROS-poly(G) exhibited hypochromicity at \( \lambda_{\text{max}} \), 253 nm. This could be due to structural alterations of guanine base in ROS-poly(G). The result was substantiated by UV difference spectra, exhibiting an undefined peak, indicating the marked hypochromicity of modified poly(G). Poly(G) interacts with poly(C) to form an extremely stable poly(G).poly(C) complex. The change in absorbance ratio (\( A_{260}/A_{280} \)) from 1.82 in case of native poly(G) to 1.7 for ROS-poly(G) and 2.21 for poly(G).poly(C) to 1.78 for ROS-poly(G).poly(C) complex suggests structural and conformational changes in hydroxyl modified poly(G). CD spectrum showed no conformational transition but a remarkable loss in ellipticity of modified poly(G).
The strand breaks in modified poly(G) was detected by Sephadex G 200 gel chromatography, densitometric scanning, agarose gel electrophoresis and gel diffusion. Sephadex G 200 gel chromatography showed 52.3% modification of poly(G) after hydroxyl treatment. Densitometric scanning of ROS-poly(G) showed low intensity and high mobility. This is due to the formation of strand breaks in modified poly(G). The enhanced mobility of ROS-poly(G) on agarose gel substantiated the formation of strand breaks, resulting in the formation of low molecular weight species. Gel diffusion experiments reiterate the fragmentation of poly(G) after hydroxyl modification.

Hydroxyl radicals have been known to induce guanine base modifications in DNA. UV spectra of acid hydrolysed poly(G) showed a distinct peak at 273 nm, indicating the release of guanine base from the sugar-phosphate backbone. The broadening of absorption maxima of acid hydrolysed ROS-poly(G) at 273 nm, in contrast to sharp peak [of native poly(G)] indicated the structural perturbations of guanine base in modified poly(G). The ion exchange profile of acid hydrolysed ROS-poly(G) on DEAE Sephadex A 25 column showed 75.8% modification of guanine base.

Native and ROS-poly(G) were used to induce antibodies in rabbits. Both were found to be a potent immunizing stimulus, inducing high titre antibodies. The antigenic specificity of the induced antibodies was studied by competition ELISA. The immunogen showed a high degree of specificity for the induced antibodies. Protein A-Sepharose purified immune IgG showed higher specificity as compared to serum. The antibody diversity was checked by using various nucleic acid inhibitors.

Calf thymus DNA, buffalo thymus RNA, poly(I), poly(C), guanine base and their ROS-modified forms were used as inhibitors in inhibition ELISA. It was observed that anti-native poly(G) antibodies are more specific towards the native form of conformers than their ROS-modified forms. Anti-ROS-poly(G) antibody showed preferential recognition of ROS-modified part of inhibitor than their unmodified counterparts. ROS-guanine was inhibitory to an extent of 56%, whereas, native guanine showed only 21% inhibition. The data indicates that anti-ROS-poly(G) antibodies are more specific to ROS-modified epitopes on guanine. The polyspecific behavior of anti-ROS-poly(G) antibodies resembles the binding characteristics of SLE anti-DNA
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The frequency of single stranded anti-RNA antibodies, particularly the anti-poly(G) autoantibodies were investigated in SLE and PSS serum samples. All PSS sera showed the formation of precipitating immune complexes with native and ROS-poly(G) by counterimmunoelectrophoresis, whereas, eight out of twelve SLE sera (67% frequency)
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Dated: .....................
Approved: ..................

Prof. Rashid Ali (Supervisor)

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JAWAHARLAL NEHRU MEDICAL COLLEGE
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

1998
CERTIFICATE

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

(Rashid Ali)
Professor
Department of Biochemistry
Faculty of Medicine
J.N. Medical College
Aligarh Muslim University
Aligarh-202 002
INDIA
With love
to my parents
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ABBREVIATIONS

$A_{260}$ : Absorbance at 260 nm

$A_{280}$ : Absorbance at 280 nm

BSA : Bovine serum albumin

CD : Circular dichroism

DEAE : Diethylaminoethyl

DNA : Deoxyribonucleic acid

dsDNA : Double stranded DNA

EDTA : Ethylene diamine tetraacetic acid

ELISA : Enzyme linked immunosorbent assay

$H_2O_2$ : Hydrogen peroxide

IgG : Immunoglobulin G

MBSA : Methylated bovine serum albumin

ng : Nanogram

$O_2$ : Molecular oxygen

$O_2^-$ : Superoxide anion radical

'OH : Hydroxyl radical

8-OH-G/8-oxo-G : 8-Hydroxyguanine/8-oxoguanine

8-oxo-dG : 8-Hydroxydeoxyguanosine

PBS : Phosphate buffered saline
Poly(G) : Polyguanylic acid
PSS : Progressive systemic sclerosis
RNA : Ribonucleic acid
ROS : Reactive oxygen species
ssDNA : Single stranded DNA
SDS-PAGE : Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SLE : Systemic lupus erythematosus
Tris : Tris(hydroxymethyl)aminomethane
TEMED : N,N,N',N'-tetramethylethylenediamine
UV : Ultraviolet
μL : Microlitre
μg : Microgram
λ<sub>max</sub> : Maximum wavelength
Introduction
The structure of nucleic acids has been a research target for decades, ever since Watson and Crick (1953) discovered the double-stranded helical structure of DNA. More information has been obtained about the structure of DNA molecules in crystals and fibres through X-ray diffraction (Langridge et al., 1960; Fuller and Wilkins, 1965; Arnott and Hukins, 1972; Shakked and Kennard, 1983). The solution structure of nucleic acids which may not be directly predictable from their crystal or fibre structures, depend on the variation of environmental conditions, such as solvent, pH, temperature and ionic strength (Clore and Gronenborn, 1985; Wilson and Yang, 1986; Patel et al., 1987; Antao et al., 1988; Flemming et al., 1988; Nerdal et al., 1988; Fukudome et al., 1990; Zhong and Johnson, 1990). However, the structure of nucleic acids in solution is more difficult to obtain, although a number of spectroscopic techniques have been used to investigate aspects of nucleic acid structure in various solutions (Clore and Gronenborn, 1985; Wilson and Yang, 1986; Flemming et al., 1988; Zhong and Johnson, 1990). It is well known that the right-handed double-helical DNA can exist in A, B, C and D conformations, whereas the left handed polynucleotide can adopt the Z-structure (Saenger, 1984). Though RNA prefers the A type of conformation (Woo et al., 1980), the formation of the left handed Z-RNA has also been reported (Uesugi et al., 1984; Nakamura et al., 1985; Dayis et al., 1986; Krzyzaniak et al., 1994). It has been demonstrated that the deoxyguanosine moieties play an important role in producing the left handed Z-structure in poly(dG-dC) (Wang et al., 1979), while the deoxyguanosine residues are responsible for the poly(dA).poly(dT) conformational transitions (Uesugi et al., 1984; Nakamura et al., 1985). Triple stranded nucleic acids have been found to exist in DNA using a variety of hydrogen bonding arrangements in the major groove of B-DNA (Camerini-Otero and Hseih, 1993). Another unique structure is the quadruple-stranded nucleic acids (Rich, 1958). The X-ray diffraction pattern of polyguanylic acid and polyinosinic acid indicate a four-stranded structure (Rich, 1958; Zimmerman et al., 1975; Simard and Savoie, 1994). The different conformations adopted by DNA and RNA reflects the enormous versatility that is inherent in the molecule.

Antigenicity of DNA

DNA is a complex macromolecule with immunologic properties that have been both misconstrued and underappreciated. Although anti-DNA antibodies are considered
diagnostic of systemic lupus erythematosus (SLE), they are expressed in association with multiple antinuclear antibody specificities, suggesting a role for both generalized as well as antigen specific immune abnormalities in their etiology (Pisetsky, 1992; 1997). The notion that DNA is immunologically inactive derives primarily from efforts to replicate lupus by immunization of normal animals with DNA, have been unsuccessful (Messina et al., 1993). When presented with the protein carrier methylated BSA and administered in adjuvant, mammalian DNA elicits poor responses to single-stranded DNA and fails to induce antibodies to double stranded DNA, the serologic hallmark of SLE (Madaio et al., 1984). However, DNA complexes with a synthetic immunogenic peptide Fus-1 induces an anti-double stranded DNA response in mice (Desai et al., 1993). This led to the conclusion that antigen drive in lupus involves either a substance other than DNA or DNA in a form (e.g., nucleosomes) that is not readily mimicked by artificial complexes (Burlingame et al., 1993; Mohan et al., 1993). Since the antibody reactivity to both single and double stranded forms appears independent of DNA species origin, these finding suggests recognition of conserved conformational determinants (e.g., helical backbone of B-DNA) and a lack of DNA sequence microheterogeneity on antigenicity (Stollar, 1975; Pisetsky, 1993).

In contrast to mammalian DNA which is poorly immunogenic (Isenberg et al., 1994), bacterial DNA can induce strong antibody response (Pisetsky, 1997). Contrary to prevailing dogma that anti-DNA antibodies occur only in SLE, the normal human sera showed significant binding to DNA from two bacterial species, Micrococcus lysodeikticus and Staphylococcus epidermidis, and from BK polyoma-virus as well (Fredriksen et al., 1994; Rekvig et al., 1995). Thus during either infection or colonization, bacterial, or viral DNA could drive the production of antibodies to sequence regions absent from host DNA. The immune response to foreign DNA is analogous to that of foreign proteins in that, it does not require T-cell recognition. The antibodies are of IgG2 isotype, where as lupus anti-DNA are predominantly IgG1 and IgG3. (Zouali et al., 1984; Kay et al., 1988; Pisetsky, 1997; Pisetsky and Drayton, 1997; Tsujimura et al., 1997). This pattern of isotype expression suggests that the responses to foreign DNA are distinct from the lupus anti-DNA response, which appears T cell dependent (Robertson et al., 1992). The antibodies elicited by
immunization of bacterial DNA in normal mice, bind both single stranded and double stranded DNA of the immunogen. The autoimmune NZB/NZW mice, immunized with either bacterial DNA or BK viral DNA, produce cross reactive antibodies to mammalian double stranded DNA (Fredriksen et al., 1994; Gilkeson et al., 1995). The antigenic specificities of these induced antibodies are comparable to the SLE anti-DNA autoantibodies (Gilkeson et al., 1993). Thus the presence of bacterial DNA sequences in immune complexes in lupus sera indicate the possibility of foreign DNA driven immune response. Low molecular weight DNA was found in the DNA-anti-DNA complexes of lupus patients and is enriched in CG content (Sano et al., 1983). The CG rich DNA regions has the tendency to form Z-DNA, which is highly immunogenic.

**Free Radical Biochemistry**

For many years, free radicals were dismissed as either non-existent in biological systems or simply an unimportant curiosity. Now free radical presence is undisputed and their role in health and disease has become widely accepted into the biochemical and medical orthodoxy.

A free radical is a chemical species, possessing a very short half life that contains one or more unpaired electrons. As such, free radicals can be formed by homolytic fission of a covalent bond, or by the loss/addition of a single electron from/to a normal molecule. It is now well established that free radicals and other reactive oxygen species (ROS) are continously produced in vivo, and can damage most cellular components (Martinez-Cayuela, 1995). Many toxic agents also generate intracellular oxygen radicals. In consequence, several antioxidant defense systems limit their damaging effects and also repair systems prevent the accumulation of oxidatively-damaged molecules (Fridovich, 1989; Halliwell and Gutteridge, 1989; Sies, 1985; 1991). Oxidative damage refers to the damages formed by ROS that accumulate with time in the DNA of animal cells (Ames et al., 1993).

The term reactive oxygen species includes not only oxygen-centered radicals as superoxide (\(O_2^-\)) and hydroxyl ('OH), but also some non-radical derivatives of oxygen, such as hydrogen peroxide (\(H_2O_2\)), singlet oxygen ('\(O_2\)), hypochlorous acid (HOCI) and
ozone ($O_3$), that are involved in oxygen radical production. There are numerous mechanisms for the generation of free radicals in vivo (Simic et al., 1988; Emerit et al., 1990). Components of the electron carriers that bring reducing equivalents to cytochrome oxidase are subject to oxidation by oxygen to produce the superoxide free radical anion ($O_2^-$). (Ames et al., 1993).

Hydrogen peroxide that escapes destruction can act as an oxidizing agent, but is not especially reactive. Its main significance lies in it being a source of hydroxyl radicals in the presence of reduced transition metal ions via Fenton reaction (Fenton, 1894; Halliwell and Gutteridge, 1990).

$$H_2O_2 + Fe^{2+/Cu^+} \rightarrow \cdotOH + OH^- + Fe^{3+/Cu^2+}$$

In a biosystem, where reducing conditions prevail, $Fe^{2+/Cu^+}$ can be regenerated easily by an electron donor,

$$Fe^{3+/Cu^2+} + e^- \rightarrow Fe^{2+/Cu^+}$$

The non-catalysed Haber-Weiss reaction is the reaction of superoxide directly with hydrogen peroxide (Haber and Weiss, 1934).

$$O_2^- + H_2O_2 \rightarrow \cdotOH + OH^- + O_2$$

The hydroxyl radical so formed in vivo, is an extremely reactive oxidizing radical that will react with most biomolecules at diffusion-controlled rates. It, therefore, does not diffuse a significant distance within a cell before reacting and has an extremely short half-life, but is capable of causing great damage within a limited radius at its site of production.

Beauchamp and Fridovich (1970) proposed that toxicity of superoxide radical and hydrogen peroxide could involve their conversion into a much more reactive hydroxyl radical. Nitric Oxide, another reactive oxygen species interact with superoxide radical to generate peroxynitrite which in turn forms hydroxyl radicals (Saran et al., 1990; Inoue and Kawanishi, 1995).
Free Radical Production in Cells

Free radicals are generally produced in cells by electron transfer reactions mediated by the action of enzymes or through the redox chemistry of transition metal ions. Their production in animal cells can either be accidental or deliberate. Some enzymes utilize a free radical at their active site in the process of catalysis, for example, ribonucleotide reductase (Reichard and Ehrenberg, 1983; Stubbe, 1990). Activated phagocytes also deliberately generate superoxide as part of their bactericidal role (Babior, 1978). The major source of free radicals in cells is electron 'leakage' from electron transport chains, such as those in mitochondria and in the endoplasmic reticulum, to molecular oxygen, generating superoxide. Flavin oxidases located in the peroxisomes also produce superoxide or hydrogen peroxide. Autooxidation of certain compounds including ascorbic acid (Vitamin C), thiols (e.g., glutathione, cysteine), adrenaline and flavin co-enzymes are also good sources of superoxide. This accidental production of free radicals is kept to a minimum, by the high efficiency of enzyme-mediated electron transfer and by keeping metal ions tightly sequestered, the fundamental means of preventive antioxidant defence. Such precautions are not completely efficient and leads to the production of free radicals during normal metabolic activity. Several toxic foreign compounds enhance the free radical production in cells. The generation of reactive free radicals overwhelms the antioxidant defences in the liver and results in the oxidative destruction of cellular membranes and serious tissue damage. In many cases, the free radical production may be secondary to the initial toxic mechanism, a consequence rather than the cause of cell damage.

Free Radical Induced DNA Damage

Oxidative DNA damage from active oxygen species has been hypothesized to play a critical role in several diverse biological processes including mutagenesis, aging, carcinogenesis, radiation induced damage, and cancer chemotherapy (Ames, 1983; Cerutti, 1985; von Sonntag, 1987; Ames and Gold, 1991; Retel et al., 1993). These pathological conditions are believed to develop due to reactive oxygen species (ROS), namely the superoxide radicals, singlet oxygen and the hydroxyl radical arising through normal cellular oxidative process, inflammatory events, ischemia and xenobiotic
metabolism (Klebanoff, 1980; Fridovich, 1983). It is now widely held that the mutagenic
capacity of oxygen is due to the direct interaction of hydroxyl radicals with DNA.
Hydroxyl radicals have been detected with electron paramagnetic resonance spectroscopy
under conditions of active oxygen-induced DNA damage (Inoue and Kawanishi, 1987).
Much evidence indicates that DNA damaging hydroxyl radicals are produced through the
interaction of hydrogen peroxide and superoxide with transition metals. For example,
hydrogen peroxide and superoxide do not directly interact with DNA to produce
oxidative lesions in vitro (Breimer, 1990). Additionally, metal chelators that block the
elaboration of hydroxyl radicals can inhibit DNA damage, mutations, and malignant
transformation induced by active oxygen species in cell-free and cellular systems, as can
agents that detoxify hydroxyl radicals or its precursors (Lesko et al., 1980; Brawn and
Fridovich, 1981; Aruoma et al., 1989; Nassi-Calo et al., 1989). The carcinogenic iron
complex nitrilotriacetic acid facilitates hydroxyl radical production from oxidants and can
enhance oxidative DNA damage (Hartwig and Schlepegrell, 1995). Iron and copper ions
can also promote DNA damage by active oxygen species in vitro (Aruoma et al.,
1991). An unstable radical like the hydroxyl radical will interact indiscriminately with all
components of the DNA molecule, producing a broad spectrum of DNA damage that
includes DNA modification of all bases as well as the production of base-free sites,
deletions, frame shifts, strand breaks, DNA-protein cross links, and chromosomal
rearrangements (Halliwell and Aruoma, 1991). In particular, endonucleases activated
through an oxidant-stimulated rise in intracellular calcium can stimulate the formation of
strand breaks and degradation products of the DNA molecule (Nicotera et al., 1990).

**Hydroxylation of Guanine Bases**

The apparently most abundant and certainly the most studied oxidative modification
of DNA bases involves the C-8 hydroxylation of guanine, frequently estimated as the
oxidize deoxynucleoside, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-G) (Floyd et al.,
1986; Shigenaga et al., 1989; Floyd, 1990; Dizdaroglu, 1991; Inoue and Kawanishi,
1995). The pulse radiolysis of 2'-deoxyguanosine (dG) and its 5'-monophosphate
dGMP) in the presence of oxidizing and reducing agents, showed the hydroxyl radical
addition to C-4, 5, and 8 of guanine, giving the radicals G4'OH, G5'OH, and G8'OH
(Fig. 1). The hydroxyl radical adducts of guanine undergo redox ambivalence. The
Fig. 1. Products formed by the action of hydroxyl radical on guanine.
reduction of G8'-OH yields ring opened product 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPy) compound (Dizdaroglu, 1991). Later studies found that FAPy is formed in DNA (Fuciarelli et al., 1990) and chromatin (Gajewski et al., 1990) in human cells (Nackerdien et al., 1992), and in mice livers after whole body irradiation (Mori et al., 1993). The oxidation of G8'-OH, rearomatized the imidazole ring giving 8-hydroxyguanine (8-OH-G) or 7,8-dihydro-8-oxo-guanine (8-oxo-G) isolated from irradiated DNA (Kasai et al., 1984; Dizdaroglu, 1985; Fuciarelli et al., 1990), chromatin (Gajewski et al., 1990), human cells (Nackerdien et al., 1992; Kasai et al., 1986) and mice liver (Mori et al., 1993). In particular, NMR studies of the tautomerism of 8-OH-G indicate that the oxygen at the C-8 position is most likely to adopt the keto form at physiological pH. Further, in contrast its normal counterpart, the oxidized base assumes the syn conformation about the glycosyl bond in DNA (Cho et al., 1990). Both these structural considerations impact the base-pairing properties of the oxidized base. NMR structural analysis have demonstrated the 8-oxo-G (syn) will form a stable base pair with A (anti) in the interior of the DNA helix without perturbation of flanking base pairs (Kouchakdjian et al., 1991). Figure 2 shows the structure and base pairing of guanine, the formation of its oxidized product, 8-oxo-G, and the abnormal base pair 8-oxo-G form in DNA. The mutation resulting from this 8-oxo-G-A mispairing will lead to G—>T transversion (Shibutani et al., 1991; Cheng et al., 1992). 8-OH-G is considered to be an excellent marker of oxidative DNA damage (Wood et al., 1990; Shibutani et al., 1991; Lunec et al., 1994; Tritscher et al., 1996) due to its highly mutagenic potential (Kuchino et al., 1987; Ames and Gold, 1991; Kasai and Nishimura, 1991; Guyton and Kensler, 1993; Moriya and Grollman, 1993; Musarrat and Wani, 1994).

Other abundant oxidatively modified purines and pyrimidines include, 8-oxo-adenine, 2-hydroxy adenine, FAPy adenine, 5-hydroxy methyl uracil, 5-hydroxy cytosine, cytosine glycol and thymine glycol (Tg). In addition, a large number of other modifications of bases and sugars have been identified (Dizdaroglu, 1991; 1994). The yield of the individual DNA modifications is highly dependent on which reactive oxygen species are involved. Thus, whereas singlet oxygen induces preferentially 8-oxo-G (Epe, 1991), superoxide has low reactivity (Fischer-Nielsen et al., 1994), and hydroxyl radical can cause almost any modification (Dizdaroglu, 1991).
Fig. 2. Oxidation at C-8 causes structural and conformational alterations in G that results in mispairing of 8-oxo-G with A.
Repair of Oxidative DNA Damage

DNA repair systems are the key lines of defense that prevent or limit the biological effects of DNA damage. It comprises of a variety of enzymes that excises the modified bases and deoxyribose sugar and the strand breaks are annealed (Ramotar and Demple, 1993; Demple and Harrison, 1994). The repair products are excreted into the urine. DNA glycosylases excise bases and subsequently endonucleases incise the phosphodiester bonds on each site of abasic sites allowing insertion of an intact nucleotide. Endonuclease III possesses both glycosylase and endonuclease activities for repair of oxidized pyrimidines. The formamidopyrimidine-DNA glycosylase enzyme (Fpg; mutM) in *E. coli* repairs 8-oxo-dG and 2,6-diamino-4-hydroxy-5-formamidopyrimidine and to a lesser extent the corresponding adenine derivatives by base excision (Boiteux et al., 1992). Exonucleases incise the apurinic site for insertion of a new nucleotide. In humans, similar two step pathways yielding 8-oxo-G have been identified in addition to repair of 8-oxo-dG by single step excision of the complete nucleotide (Bessho et al., 1993). In *E. coli* the mutM and mutT repair enzymes cooperate functionally with mutY, an enzyme which removes adenine misinserted opposite 8-oxo-G in DNA (Tajiri et al., 1995). Lack of DNA repair mechanisms would lead to have increased mutation frequencies in the early steps of carcinogenesis (Freidberg, 1985).

Antioxidants

Antioxidants are another key line of defense capable of scavenging free radicals by preventing radical formation, intercepting radicals from further activity (Cotgreave et al., 1988), or participating in repair of damage caused by free radicals (Sies, 1993). Enzymatic defense systems include superoxide dismutases (SOD), which catalyzes the superoxide dismutation to produce \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \) at \( 10^4 \) times higher rate than spontaneous dismutation at physiological pH. Catalase (CAT) mediates the detoxification of \( \text{H}_2\text{O}_2 \) from the cell when it is present in high concentrations.

\[
\text{SOD} \quad 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]
CAT

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

Selenium dependent glutathione peroxidase (GSHpx) catalyzes the reduction of \( \text{H}_2\text{O}_2 \) and organic free hydroperoxides requiring glutathione as substrate (Ernster, 1987).

\[ \text{GSHpx} \]

\[ \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \]

\[ \text{GSHpx} \]

\[ \text{ROOH} + 2\text{GSH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O} \]

Glutathione reductase reduces oxidized glutathione utilizing NADPH generated by various systems

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \]

Other enzymatic proteins such as DT-diaphorase or epoxide hydrolase are also considered to be primary antioxidant defenses (Ernster, 1987; Lind et al., 1990).

Transferrin or lactoferrin and ferritin reduce the free iron ion concentration, preventing hydroxyl radical generation through the Fenton-type reactions (Halliwell and Gutteridge, 1984; 1985; Thurnham, 1990). Albumin and ceruloplasmin transport copper in plasma, but the later does not prevent interaction between this metal and superoxide or hydrogen peroxide to generate hydroxyl radical (Thurnham, 1990; Meucci et al., 1991). Glutathione (GSH), vitamin C, uric acid, taurine and hypotaurine are some of the small molecules widely distributed in biological systems, scavenge oxygen free radicals non-enzymatically. Vitamin E (\( \alpha \)-tocopherol), the major lipid-soluble antioxidant protects against lipid peroxidation by donating a hydrogen ion to oxygen free radical. The resultant tocopheryl radical may be reduced by the ascorbic acid-GSH redox couple (Cadenas, 1989; Empey et al., 1992). \( \beta \)-carotene, the most efficient scavenger of singlet oxygen, has a synergistic action with vitamin E (Machlin and Bendich, 1987; Bendich and Olson, 1989; Di Mascio et al., 1991) Finally, bilirubin breaks the chain of damage propagation by reacting with the oxygen free radicals which cause this damage (Cotgreave et al., 1988; Stocker et al., 1990).
Autoimmunity and Autoimmune Diseases

Immune mechanisms are not always protective or beneficial to the host. Under certain conditions, immunological disbalance results in discriminating between 'self' and 'non-self' leading to antibody production against various cellular components and tissue injury, resulting in clinical disease. Thus, autoimmune diseases are those which result from immunologic reactions, humoral and/or cellular, directed against the individual's own tissue components, leading to clinical disease (Deodhar, 1992). An autoimmune disease may be organ/tissue specific, for example, Hashimoto's syndrome, thyrotoxicosis, pernicious anaemia or non-organ/tissue specific (systemic) such as progressive systemic sclerosis (PSS) or systemic lupus erythematosus (SLE). There is no single theory or mechanism that can adequately explain all features of autoimmune diseases. However, three models have been proposed for the induction of a productive autoimmune process. The first is the autoimmune response to a cross-reacting antigen, i.e., crossreactivity between host and foreign antigens or modification of host proteins due to infection, drug administration, inflammation etc. (Theofilopoulos and Dixon, 1985; Huang et al., 1988). 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. Perhaps the best approach, as suggested by Shoenfeld and Isenberg (1989), is to consider the wide spectrum of autoimmune diseases as the "mosaic" of autoimmunity with its many pieces, genetic, hormonal, immunological, and environmental, leading to diverse diseases.

Autoimmune diseases show a highly significant familial predisposition (Hochberg, 1987; Arnett, 1992). The involvement of genetic factors has been linked to the human lymphocyte antigen (HLA) system, particularly the HLA-DR sublocus. The HLA genes functions as secondary genes to allow expression of specific autoantibody or the respective disease state (Bias et al., 1986). The initiation of an autoimmune response requires that the self-reactive T cells interact with self-antigen and HLA class II antigen complex with sufficient avidity for the development of autoantibody in subsequent autoimmune diseases. The association between certain autoimmune diseases and HLA antigens (Braun and Zachary, 1988) such as SLE DR2 and DR3, Sjogren's syndrome and DR2 and for DR3, insulin-dependent diabetes mellitus or diabetes Type I DR4 and
DR3, Grave’s disease and DR3, myasthenia gravis and DR3, rheumatoid arthritis and DR4 are well documented.

In a wide variety of autoimmune diseases, the regulatory failure results in significant decrease in T-suppressor cell numbers and activity, thereby imbalancing the T-helper/T-suppressor cell ratio. The increased T-helper/T-suppressor cell ratio has been noted in a wide variety of autoimmune diseases, such as, SLE, Sjogren’s syndrome, PSS or scleroderma, rheumatoid arthritis, pernicious anemia, multiple sclerosis, immune complex-mediated renal diseases, immunologic skin diseases and many others. It is well established that the human immunodeficiency virus (HIV) associated immune dysregulation results in autoimmune phenomena or inversely some type of autoimmune mechanism may contribute to the immunopathogenesis of the HIV infection itself (Calabrese, 1988). The autoantibodies associated with HIV infection include antinuclear antibodies, rheumatoid factor, anti-cardiolipin antibodies, anti-platelet, anti-red cell and anti-lymphocyte antibodies. Other viral agents involved in initiating autoimmune phenomena include hepatitis B infection associated with chronic active hepatitis and polyarthritis nodosa, EB virus in polymyositis, HTLV-1 virus in multiple sclerosis, and related neurologic diseases (Weetman and Borysiewicz, 1990). Hormonal factors such as sex hormones, thymic hormones and corticosteroids play a significant role so that autoimmune disease occur predominantly in females than males (Talal, 1989; Counihan et al., 1991; Lahita, 1992; Moinuddin and Ali, 1994). It has been reported that testosterone and thymic hormones enhance CD8+ T-cell receptor function (Lahita and Kunkel, 1984), whereas estrogen may suppress this function (Talal and Ahmad, 1987).

Immunological crossreactivity and molecular mimicry has been an important phenomena in autoimmune diseases. It has been suggested that the degree of sequence conservation between host and a given infectious agent, heat shock proteins, because of molecular mimicry, may provide the link between infection and subsequent autoimmunity. In responding to the virus, the immune system may become activated to attack the self molecule, since only a few residues are needed to stimulate the immune response. For example, adenovirus type II has sequences very much like those of myelin basic protein (Steinman, 1993). The reason that some persons may mount an immune response against
self and others do not may be because HLA molecules from different individuals display different portions of the antigen to T cells. Thus, one person's HLA molecule may bind a self-mimicking portion while another's may not. Antibodies to cardiolipin have been shown to cross-react with DNA, thus suggesting the possibility that in SLE, native DNA may not be the initiating factor for autoimmune reactivity, rather, the pathogenic anti-DNA antibody may be an example of immunologic crossreactivity (Nakamura and Binder, 1988; Arif et al., 1994).

Polyclonal B cell activation has been proposed as one possible mechanism that may be responsible for the over-activation of B cells and production of autoantibodies in certain autoimmune diseases, particularly SLE (Dziarski, 1988; Klinman et al., 1990; Steinberg, 1992). In autoimmune prone individuals, B cells are hyper-responsive to polyclonal activators and undergo initial activation, followed by expansion of autoreactive clones, under the influence of exogeneous or endogeneous polyclonal activators. Various polyclonal B-cell activators such as Epstein Barr Virus (EBV) and its components, and endotoxin or lipopolysaccharide (LPS), certain bacterial agents and drugs may function by bypassing T cell regulatory mechanisms and activating B cells directly.

The 'modified self' hypothesis suggests that autoimmunity may arise as a result of an immune response against modified self determinants (neo-self determinants) such as new determinants created on somatically mutated antibodies during the maturation of the immune response. Although natural anti-idiotypic autoantibodies have been detected, it is thought that such idiotypic determinants generally do not reach an adequate level to induce pathogenic immune responses. Rheumatoid factors (RFs), which are anti-IgG Fc autoantibodies induced transiently during an immune response, presumably because neo-antigenic determinants are exposed on antigen-complexed IgG. Glycosylation defects in IgGs have also been proposed as playing a role in RF induction, although the specificity of this phenomenon is uncertain (Tsuchiya et al., 1993). “Nephritic factor” referring to an autoantibody against neoantigenic determinants reveals the complement protein C3 during complement activation (Spitzer et al., 1992). Other possibilities for creation of neo-self determinants occur through binding of drugs or other haptenic groups to self-molecules, as well as through molecular modifications introduced, for example, by gene mutations. A long list of drugs has been compiled, all of which may be correlated with
SLE-like symptoms, but the mechanisms for these adverse effects remain unknown. Some investigations suggest binding to MHC with subsequent induction of alloreactivity, whereas others suggest interference with gene methylation (Quddus et al., 1993). Further investigation of the mechanisms by which environmental or iatrogenic chemical compound induced autoimmunity is highly warranted.

**Systemic Lupus Erythematosus**

Systemic lupus erythematosus is a multisystem, inflammatory disorder characterized by the production of autoantibodies of multiple specificities, especially antibodies reactive with nuclear ligands, including native DNA (Andrews et al., 1978; Smith and Steinberg, 1983; Tan, 1985; Steinberg et al., 1990; 1991; Steinberg, 1992; Harada et al., 1994). One of the impediments to understanding human systemic lupus has been its marked heterogeneity (Steinberg et al., 1991; Steinberg, 1992). Indeed, a comparable heterogeneity is observed among the several strains of mice which spontaneously develop SLE-like syndromes. Taken together, they demonstrate the genetic and pathogenetic heterogeneity of lupus (Ebling and Hahn, 1980; Theofilopoulous and Dixon, 1980; Steinberg et al., 1984).

From the last 40 years, anti-DNA antibodies were identified and linked to patients with SLE. The clinical manifestations include fever, an erythematous 'butterfly' rash across the face, lesions of discoid lupus or a vasculitic rash, polyarthralgia and arthritis, polyserositis (especially pleurisy and pericarditis), anemia, thrombocytopenia, renal, neurologic, and cardiac abnormalities. SLE, a common disease with a strong female preponderance with female/male ratio of 9:1. Although the cause of SLE remains obscure, current concepts suggest that, this is a multifactorial disorder in which there is profound disturbance of immune regulation. A defect of suppressor T lymphocytes is associated with polyclonal B cell activation and the uncontrolled production of autoantibodies and circulating immune complexes.

The immunological abnormalities found in SLE are given in Table 1. Antinuclear antibodies can be detected by indirect immunofluorescence in the serum of more than 90% of patients. Radioimmunoassays are more specific for antibodies to undenatured
### TABLE 1

Immunological Abnormalities Detectable in the Blood of Patients with SLE

<table>
<thead>
<tr>
<th>Anti-nuclear antibodies</th>
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<tbody>
<tr>
<td>Anti-DNA-histone (and LE cells)</td>
</tr>
<tr>
<td>Anti-DNA (single and double stranded)</td>
</tr>
<tr>
<td>Anti-RNA</td>
</tr>
<tr>
<td>Anti-Sm</td>
</tr>
<tr>
<td>Anti-U1 RNP</td>
</tr>
<tr>
<td>Anti-Ro/SS-A</td>
</tr>
<tr>
<td>Anti-La/SS-B</td>
</tr>
<tr>
<td>Anti-MA</td>
</tr>
<tr>
<td>Anti-PCNA</td>
</tr>
</tbody>
</table>

Depression of CH$_{50}$ and C3 and C4

Antibodies against erythrocytes, leucocytes and platelets

Antineuronal antibodies

Biological false positive tests for syphilis

Circulating anticoagulants (anti-cardiolipin antibodies)

Anti-thyroid (and other organ specific autoantibodies)

Rheumatoid factors

Cryoglobulins

Circulating immune complexes

Sm, Ro, La, MA - antibodies identified by initials of patients in whom they were discovered

CH$_{50}$ - Total haemolytic complement activity

C3, C4 - Complement components
double stranded DNA (Takeuchi et al., 1997). High levels of anti-DNA antibodies coupled with depressed total haemolytic complements (CH_{50}) activity (Nishiya and Hashimoto, 1997) and low C3 and C4 complement component suggest that there is activation of the classical complement pathway in active immune complex disease. Further evidence for circulating immune complexes may be obtained by finding a cryoprecipitate or by using one of a number of tests for C1q binding. Tissue evidence for immune complex deposition comes from detecting complement components and immunoglobulins by immunofluorescence at the dermo-epidermal junction skin (lupus ‘band’ test) or in organ biopsies.

Lupus anticoagulant activity is detected by prolongation of the partial thromboplastin time (PTTK, APTT) which is not correctable by the addition of normal plasma or by a prolongation of the dilute prothrombin time. Anticardiolipin antibodies are detected by ELISA (Verthelyi and Ansar, 1997). High titers are associated with thrombocytopenia, thrombotic manifestations, pulmonary hypertension and neurologic problems such as chorea, strokes or TIAs and in some cases recurrent abortion (the anti-phospholipid syndrome).

Historically, lupus is considered to be the prototype of a spontaneous immune complex disease in which anti-DNA antibody-DNA deposit in the tissues and induce inflammation. Although anti-double stranded DNA antibodies are considered to be the hallmarks of human lupus nephritis (van Bruggen et al., 1996), most of the anti-DNA antibodies in the murine renal lesions also bind to single stranded DNA (Theofilopoulos and Dixon, 1985; Gavalchin and Dutta, 1987). Recently, the interaction of nucleosome/antibody complexes bound to heparan sulphate in the kidney, for the initiation of nephritis have been reported (Naparstek and Madaio, 1997). A novel subset of murine monoclonal anti-DNA antibodies that deposit in cells and cross both cellular and nuclear membranes to deposit in nuclei in vivo have been described. (Foster et al., 1994; Kieber-Emmons et al., 1994; Yanase et al., 1994). Another monoclonal anti-DNA antibody, termed 3E10, have been reported to penetrate murine renal tubular cells and localizes in the nucleus.
Several genetic and environmental factors play important roles in assessing the role of anti-DNA antibodies (Isenberg, 1997; Schwartz, 1997). Polyoma T antigen binds with high avidity to mammalian DNA and the complex can be an immunogen in individuals with a genetic susceptibility to SLE (Rekvig et al., 1995). The complexes consisting of DNA and a variety of microbial DNA binding peptides inducing anti-DNA antibodies have been suggested (Schwartz, 1997). Undigested DNA, DNA-protein or DNA-peptide complexes might accumulate in lupus due to a failure to phagocytose the detritus of apoptotic cells and provide a source of lupus provoking immunogens (Schwartz, 1997). Pathogenic anti-DNA antibodies might be originated from bacterial antigens and DNA (Kalden and Marion, 1997). Nevertheless, a randomly encountered environmental stimulus is a tantalising explanation for the observation of pathogenic anti-DNA antibodies in one identical twin with SLE, and autoantibodies but no disease in the other twin.

Autoantibodies and B cells have been in the lupus limelight for decades, but now T cells have been studied extensively in SLE (Spronk et al., 1996). T cells have been cloned from lupus-prone mice, and stimulate the production of anti-DNA antibodies and renal lesions when injected in vivo. In mice and humans with lupus, the histone/DNA-binding T cells are activated, express the CD40 ligand, and have the potential to trigger B cells to produce antibodies. A finding that T cells from MRL-lpr/lpr mice recognise DNA-binding peptide from a parasite underlines the relevance of Rekvig's report implicating a viral DNA-binding protein (the polyoma T antigen) in human SLE. Perhaps many different DNA-binding peptides of microbial origin can be immunological carriers of nucleic acid or polynucleotide haptens. It is possible that the lupus patients collective experience with microbial DNA-binding proteins builds up a repertoire of carrier-specific memory T cells, members of which participate in triggering, enhancing, or modifying the activation of B cells with the capacity to produce anti-DNA antibodies.

**ROS in SLE**

DNA damage by ROS has been found in the development of autoimmune diseases, such as SLE (Blount et al., 1991). The process may involve the release of ROS intermediates from activated phagocytic cells, their passage through cell membranes, and
finally reaction with nuclear DNA (Bashir et al., 1993) producing altered DNA, stimulating DNA-antibody production. The development of autoantibodies in SLE has been supported by the enhanced reactivity of SLE anti-DNA antibodies to ROS-modified DNA and polynucleotides (Blount et al., 1989; 1990; Alam et al., 1993; Ara and Ali, 1993; Ahmad et al., 1997; Cooke et al., 1997; ). ROS modification exposes base residues in the DNA backbone and minor regions of single stranded DNA (Blount et al., 1994), rendering DNA immunogenic. The detection of 8-oxo-dG in the immune complex derived DNA of SLE (Lunec et al., 1994) reinforces the evidence that ROS may be involved in SLE. ROS-modified DNA may therefore, play a significant role in the generation of immune complexes which are of recognised importance in the pathogenesis of SLE (Lisitsyna et al., 1996; Cooke et al., 1997).

Progressive Systemic Sclerosis

Progressive systemic sclerosis (PSS), also known as scleroderma is a generalized disorder of connective tissue characterized by inflammatory, fibrotic, and degenerative changes, accompanied by vascular lesions, in the skin (scleroderma), synovium, and certain internal organs, notably the esophagus, intestinal tract (Ebert et al., 1997), heart, lung and kidney (Medsger, 1985). The disease is highly variable in its presentation. At one end of the spectrum are patients with limited scleroderma, in whom skin thickening is relatively restricted to the fingers, hands, and/or face, and in whom internal organs compromise is delayed, often for several decades or more. Patients with these symptoms occur predominantly in the CREST variant of PSS i.e., a combination of subcutaneous calcinosis, Raynaud’s phenomenon (RP), esophageal dysmotility, sclerodactyly, and telangiectasia (Winterbauer, 1964; Rodnan et al., 1979; Drosos et al., 1991). At the other end, are patients with diffuse scleroderma (Steen et al., 1980) in whom skin changes are wide-spread and often rapidly progressive, involving distal and proximal extremities and the trunk, and in whom visceral complications lead to cardiac failure, fulminant hypertensive renal disease, pulmonary complications, or intestinal malabsorption and cachexia (D'Angelo et al., 1969; Rodnan, 1972)

The origin of PSS is obscure. PSS has been linked with rheumatoid arthritis, systemic lupus erythematosus, and certain other rheumatic diseases because these
conditions have a number of clinical features in common and because they are all characterized anatomically by generalized alterations of (or in) the connective tissue (Tsankov et al., 1990). A number of patients with scleroderma present certain clinical and serologic features highly suggestive of SLE (mixed connective tissue disease or overlap syndrome), including fever, discoid rash, pleurisy, pericarditis, lymphadenopathy, anemia, leukopenia, marked hypergammaglobulinemia, and positive LE cell reactions (Dubois et al., 1971; Sharp et al., 1972; Schneider, 1993). Like rheumatoid arthritis and systemic lupus erythematosus, PSS has been associated in a number of instances with Sjogren’s syndrome and less often with Hashimoto’s thyroiditis (Yazawa et al., 1997). The serologic abnormalities has given rise to suspicion that aberrant immunity may play an important role in pathogenesis (Rodnan, 1972). Inflammatory manifestations, e.g., arthritis and myositis as well as the expression of anti-nuclear antibodies that mark particular disease patterns suggest a role for autoimmunity in PSS (Yuan and Chen, 1991). Thus autoantibodies to an entirely different set of cellular antigens are observed in scleroderma (Table 2). Antibodies to SCI-70 occur in patients with the diffuse pattern of disease and bind to the enzyme DNA topoisomerase I (Nakamura and Tan, 1978; Shero et al., 1986; Gruschwitz et al., 1993; Vazquez-Abad et al., 1997). This enzyme is a 100 kd protein in its native form and can relax supercoiled DNA; the 70 kd antigen routinely detected is a degradation product. In the CREST variant of PSS, antibodies to centromere antigens (kinetochore) are the serologic hallmark (Moroi et al., 1980; Vazquez-Abad et al., 1997). These antigens have been identified as 17kd, 80kd and 140kd proteins. Anti-centromere antibodies occurring frequently in CREST patients, do not usually coexist with antibodies to SCI-70 (Earnshaw et al., 1986; Shero et al., 1986; Earnshaw et al., 1987; Weiner et al., 1988; Fritzler, 1997).

Some patients of scleroderma produce autoantibodies which target intranucleolar antigens. These include RNA polymerase I, a complex of subunit proteins ranging from 210 to 11 kd. Microinjection of IgG autoantibody into the frog oocyte effectively inhibited synthesis of 28 and 18 S ribosomal RNA (Reimer et al., 1987). A second “nucleolar” antigen is PM-SCI (Polymyositis-scleroderma overlap), a complex of 11 polypeptides ranging from 110 to 20 kd (Reimer et al., 1986; Yuan and Chen, 1991). The third nucleolar antigen is fibrillarin, a 34 kd protein rich in dimethyl arginine and
## TABLE 2

Nature of Autoantigens and Prevalence of Autoantibodies in Progressive Systemic Sclerosis

<table>
<thead>
<tr>
<th>Designation of autoantigen</th>
<th>Prevalence(%) of autoantibody</th>
<th>Molecular characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCI-70*</td>
<td>70% in diffuse scleroderma</td>
<td>Protein 70kd-degradation product of 100 kd DNA topoisomerase</td>
</tr>
<tr>
<td>Centromere kinetochore*</td>
<td>80% in CREST</td>
<td>Proteins 17, 80, and 140 kd of kinetochore</td>
</tr>
<tr>
<td>RNA polymerase I*</td>
<td>4</td>
<td>Proteins 210 to 11 kd - subunits of RNA pol I</td>
</tr>
<tr>
<td>PM-ScI</td>
<td>3</td>
<td>Proteins 110 to 20 kd</td>
</tr>
<tr>
<td>Fibrillarin*</td>
<td>8</td>
<td>Proteins 34 kd complexed with U3RNA</td>
</tr>
<tr>
<td>To</td>
<td>Rare</td>
<td>Protein complexed with 7-2 and 8-2 RNA</td>
</tr>
<tr>
<td>Alu-RNA-protein</td>
<td>Rare</td>
<td>Protein 68 kd complexed with Alu RNA</td>
</tr>
<tr>
<td>Nuclear lamins</td>
<td>Rare</td>
<td>Proteins 70 and 60 kd</td>
</tr>
</tbody>
</table>

*Antibodies highly specific for systemic sclerosis group including diffuse scleroderma and CREST
glycine. Other autoantibodies have been detected infrequently and the antigenic targets are 7-2 and 8-2 RNAs, a 68 kd protein complexed with Alu RNA and nuclear lamin proteins (McKeon et al., 1983; Reddy et al., 1983; Kole et al., 1985).

Autoantibodies of one specificity or another of those described in Table 2 are detected in 90% of all patients with scleroderma and this places the disease squarely among lupus, MCTD, and Sjogren’s syndrome where autoantibodies have been detected in nearly all patients. However, other features commonly associated with immune hyperresponsiveness such as hypergammaglobulinemia, complement activation, and abnormal T and B cell functions have not been consistently observed. Although the immune response in scleroderma is fairly diverse, it appears that patients do not produce autoantibodies to several antigens simultaneously as they do in lupus.

Structure of Polyguanylic Acid

The synthetic polynucleotides are of considerable interest because of their close relation to the naturally occurring nucleic acids. Both the natural and synthetic polymers are often capable of assuming more than one configuration, and a great deal of attention has been focussed on these structural changes. All these molecules can be found in an amorphous, random coil form in which there is no regular regulation between the orientation of successive nucleotides among the polymer chain. In a suitable environment, many polynucleotides are also capable of forming elongated, helical molecules in successive groups have a fixed position relative to each other.

A number of homopolyribonucleotide structures have been investigated by the techniques of X-ray fibre diffraction. The results established the close structural similarity between poly(I) and poly(G) and provides one of the principal arguments that their structure is four-stranded (Zimmerman et al., 1975; Simard and Savoie, 1994). High stability of ordered poly(G) has been observed by many investigators using a variety of physical methods (Fresco and Massoulie, 1963; Lipsett, 1964; Miles and Frazier, 1964; Pochon and Michelson, 1965; Englander et al., 1972). The I.R. spectroscopy shows the existence of metastable and stable ordered, multistranded forms of poly(G) (Howard et al., 1977). One form is inverted spontaneously and irreversibly to the other, but can be regenerated by freezing and thawing of solution. The high charge density of
four-stranded poly(G) makes it especially susceptible to electrostatic destabilization by the use of tetaethylammonium counterions, which screen electrostatic repulsion of multiple strands less effectively than alkali metal ions and significantly lower the transition temperature of the helix. Zimmerman et al. (1975) proposed a structure of poly(G) in which four guanine residues are mutually hydrogen bonded N(1)H to (6)O and N(2)H to N(7) in a closed planar array. The plane of the four bases is perpendicular to the axis of the four stranded helix, and the ribose residues are in the C(3')-endo conformation. A slightly different structure was proposed by Arnott et al. (1974) in which the plane of the bases is tipped by 8.9° relative to the helix axis, and the ribose conformation is C(2')-endo. Evidence for G-G hydrogen bonding and stacking comes from the IR spectra and from NMR data (Pinnavaia et al., 1975; Saenger, 1984) on the similar helical self structure formed by 5'-GMP in neutral or slightly basic solution. High stability of poly(G) is favoured in the tetrameric bonding scheme by formation of two hydrogen bonds per base (Zimmerman et al., 1975). A search for other possible modes of G-G association in neutral solution suggested that none was likely to be as stable as the tetrameric arrangement (Hattori et al., 1975a). Guanine is unique in the naturally occurring bases in having a pair of hydrogen bond donors and a pair of hydrogen bond acceptors arranged approximately at right angles to each other. This permits the formation of a planar square array with two hydrogen bonds per base, compared with 1.5 bonds per base, in the G.C pair of DNA. This additional hydrogen bond significantly stabilize the ordered polynucleotide helices (Howard et al., 1966a and b; Ikeda et al., 1970) and enhance the thermal stability of poly(G) besides involving other factors as hydrophobic bonding and the van der Waal's interactions. Evidence on the stabilizing effect of hydrogen bonds has been provided in a variety of synthetic polynucleotides (Howard et al., 1966a; Ikeda et al., 1970; Hattori et al., 1975a and b; Howard et al., 1976). Dimethylsulphoxide is an effective denaturant for nucleic acids (Strauss et al., 1968; Englander et al., 1972), and 90% dimethylsulphoxide is evidently capable of completely destabilizing the ordered form of poly(G) if sodium ion concentration is quite low. In 50% dimethylsulphoxide, poly(G) exists in an ordered form at ambient temperature but can be melted by heating.
Antibodies to RNA

Reactions of SLE sera with ribosomes (Sturgill and Carpenter, 1965; Schur et al., 1967) and ribonucleoprotein (Koffler et al., 1971; Mattioli and Reichlin, 1971) suggest that RNA may also serve as an antigen in this disease. Various reports showing positive results in several kind of assays have been obtained with SLE sera and double stranded polynucleotides, including poly(A).poly(U), poly(I).poly(C), or naturally occurring viral double stranded RNA (Schur and Monroe, 1969; Epstein et al., 1971; Schur et al., 1971; Talal et al., 1971). Koffler et al. (1971) found positive passive agglutination reactions to occur more frequently with double stranded polynucleotides than with native DNA in diseases other than SLE. Considerable heterogeneity in antigen binding characteristics was observed when several polynucleotides were used as antigens. Schur and Monroe (1969) found that several of these sera also precipitated with the homopolymers poly(I), poly(A), and poly(G). In general SLE sera reacted better with naturally occurring double stranded RNA of viral origin than with synthetic copolymers (Schur et al., 1971; Talal et al., 1971). It is noteworthy that the poly(I).poly(C) accelerated production of both anti-double stranded RNA and anti-DNA antibodies, even though these are distinct antibody populations (Steinberg et al., 1969; Carpenter et al., 1970; Talal et al., 1971).

H241, a murine monoclonal autoantibody binds well to native DNA and displays several cross reactions (Stollar et al., 1986). It reacts with higher concentrations of cardiolipin probably by recognizing repeated and appropriately spaced phosphates of the phospholipid (Lafer et al., 1981). H241 shows wide range of cross reactivity then do most induced anti-nucleic acid antibodies, as it combines with poly(I), poly(G), native DNA, denatured DNA and Z-DNA. In contrast it shows little or no reactivity with several single stranded homopolymers, poly(A), poly(U), poly(U); double stranded RNA, poly(A).poly(U), 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).

Poly(G) and poly(I) were the only polynucleotides to react with H241, suggesting that either the antibody recognized some feature common to these purines, such as 6-carbonyl and a portion of the imidazole including the 7-N, or else that the
polynucleotides of guanine and hypoxanthine had the appropriate spacing of phosphates in the backbone whereas other polyribonucleotides did not. X-ray fibre diffraction of poly(G) and poly(I) shows their structure to be four stranded (Zimmerman et al., 1975), and contain its pentoses in a C(2')-endo, a conformation more characteristic of DNA than RNA (Arnott et al., 1974). Among the double helical polydeoxyribonucleotides of varying base composition, H241 exhibits a preference for poly(dG-dC). Stollar et al. (1986) found that H241 bound portion of guanine and cytosine in the major groove and the 2-amino group of a guanine in the minor groove.

**Objective of the Present Study**

Human exposure to reactive oxygen species has been implicated in the etiology of a number of human diseases due to formation of various modified DNA bases, most abundantly the 8-hydroxyguanine. In the present study, a synthetic ribohomopolymer [poly(G)] was modified with hydroxyl radical generated by irradiation of hydrogen peroxide at 253.4 nm. The modified poly(G) was characterized by UV absorption and CD spectroscopy and thermal denaturation studies. The strand breaks in modified poly(G) was detected by Sephadex G-200 gel chromatography, densitometric scanning, agarose gel electrophoresis and gel diffusion. In addition, ion exchange chromatography on DEAE Sephadex A25 column was used to quantify the guanine base modifications in ROS-poly(G). Both the native and ROS-poly(G) induced high titer antibodies in rabbits. Induced antibodies were characterized for their fine antigenic specificity with various nucleic acid polymer.

In order to assess the possible role of ROS-modified epitopes in the etiology of inflammatory autoimmune diseases, sera from SLE and PSS patients were investigated for the presence of antibodies to native and modified poly(G). In addition, the frequency of precipitating antibodies to native and ROS-poly(G) in SLE and PSS sera were investigated by counterimmunoelectrophoresis. Furthermore, native and ROS-poly(G) was used to induce antibodies in rats which were tested for immune complex deposition in the kidney glomeruli by immunofluorescence.
Experimental
MATERIALS

Polyguanylic acid [poly(G)], and other synthetic polynucleotides, DEAE Sephadex A 25, Protein A-Sepharose CL-4B, Sephadex G 200, agarose, Ficoll 400, xylene cyanole FF were purchased from Pharmacia Fine Chemicals, Sweden. Calf thymus DNA, buffalo thymus RNA, nuclease S₁, bovine serum albumin, methylated bovine serum albumin, anti-human/-anti-rabbit- IgG-alkaline phosphatase conjugate, anti-rat-IgG-HRP and FITC conjugate, p-nitrophenyl phosphate, ethidium bromide, Coomassie Brilliant Blue G 250 and R 250, sodium dodecyl sulphate, Tween-20, orcinol, Millipore filter (0.45 µM pore size), Freund’s complete and incomplete adjuvants, were purchased from Sigma Chemical Company, U.S.A. Folin-Ciocalteau reagent and Blue Dextran 2000 were purchased from Centre for Biochemical Technology, New Delhi.

Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from Nunc, Denmark. Acrylamide, ammonium persulphate, bisacrylamide, N,N,N',N'-tetramethylethylene diamine (TEMED) were from Bio-Rad Laboratories, U.S.A. EDTA (disodium salt), hydrogen peroxide, chloroform, isoamyl alcohol, methanol, glacial acetic acid were from Qualigens, India. Diphenylamine and ethanol were chemically pure. All other reagents/chemicals were of highest analytical grade available.

Equipment

ELISA microplate reader MR-600 (Dynatech, U.S.A.), ELISA microplate washer (Denley, England), Elico pH meter model L1-120, Shimadzu UV-240 spectrophotometer equipped with thermo-programmer and controller unit, gel scanner GSC-3A, ultraviolet lamp having maximum emission at 253.4 nm and UV-transilluminator (Vilber Lourmat, France), agarose gel electrophoresis assembly (GNA-100) and gradient mixer GM-1 (Pharmacia, Sweden), Avanti 30 table top high speed centrifuge (Beckman, U.S.A.), power supply model PAC 300 and polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.), fluorescent microscope (Nikon, Japan) were the major equipments used in this study.
Serum Specimens

Normal human sera were obtained from healthy subjects. SLE and PSS sera were obtained from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi and J.N. Medical College Hospital, A.M.U., Aligarh. The SLE sera showed high titre anti-DNA antibodies and fulfilled the American College of Rheumatology (formerly American Rheumatism Association) revised criteria for the classification of SLE (Arnett et al., 1988). PSS sera fulfilled the American Rheumatism Association (ARA) criteria for the classification of definite systemic sclerosis (Masi et al., 1980). Serum samples were decomplemented at 56°C for 30 min and stored in aliquots at -20°C with 0.1% sodium azide as preservative.

METHODS

Determination of DNA Concentration

DNA concentration was estimated colorimetrically by the method of Burton (1956) using diphenylamine reagent.

(a) Crystallization of diphenylamine

Diphenylamine (2 g) was dissolved in 200 ml boiling hexane. After adding 0.5 g of activated charcoal, the hot mixture was filtered through Whatman No.1 filter paper and the filtrate was kept overnight at 4°C and dried at room temperature before use.

(b) Preparation of diphenylamine reagent

750 mg of recrystallized diphenylamine was mixed with 50 ml of glacial acetic acid and 0.75 ml concentrated sulphuric acid. The reagent was prepared fresh before use.

(c) Procedure

One ml of DNA sample was mixed with 1.0 ml of 1 N perchloric acid and incubated at 70°C for 15 min. One hundred µL of 5.43 mM acetaldehyde was added followed by 2.0 ml of freshly prepared diphenylamine reagent. The contents
were mixed and incubated at room temperature for 16-20 hr. Absorbance was read at 600 nm and the concentration of DNA in unknown samples was determined from a standard plot of calf thymus DNA purified free of RNA and proteins.

**Determination of RNA Concentration**

RNA was estimated by ferric ion catalyzed orcinol reaction (Cerriotti, 1955).

(a) **Crystallization of orcinol**

Five gm of commercial orcinol was dissolved in 100 ml boiling benzene and decolorized with 1 g of activated animal charcoal. The suspension was filtered while hot and kept at room temperature for 1 hr and at 4°C until crystallization. The crystals were separated by filtration and dried at room temperature.

(b) **Orcinol reagent**

(i) One hundred mg ferric chloride was dissolved in 100 ml concentrated HCl

(ii) Six per cent (w/v) orcinol in distilled ethanol

The reagent was prepared fresh by mixing 3.5 ml of (ii) with 100 ml of (i).

(c) **Procedure**

Two ml of RNA solution was mixed with 3 ml of orcinol reagent and placed in boiling water for 30 min. The tubes were chilled in ice water and absorbance was read at 665 nm. RNA concentrations of unknown samples were calculated from standard plot of buffalo thymus RNA.

**Determination of Protein Concentration**

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

**Protein estimation by Folin's-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-Ciocalteau reagent**

The reagent was purchased from Centre for Biochemical Technology, New Delhi and diluted 1:4 with distilled water before use.

(b) **Alkaline copper reagent**

The components of alkaline copper reagent were prepared as follows:

(i). Two per cent sodium carbonate in 100 mM sodium hydroxide

(ii). 0.5 per cent copper sulphate in 1 per cent sodium potassium tartarate

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

(c) **Procedure**

To one ml of protein sample was added 5 ml of alkaline copper reagent and incubated for 10 min at room temperature. One ml of working Folin-Ciocalteau reagent was added and the tubes were read at 660 nm after 30 min. The concentration of protein in unknown sample was determined from a standard plot of bovine serum albumin.

**Protein estimation by dye-binding method**

This assay is based on colour change when Coomassie Brilliant Blue G 250 in acidic medium, binds strongly to protein hydrophobically and at positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue colour is observed ($\lambda_{max}$ - 595 nm).

(a) **Dye preparation**

One hundred mg Coomassie Brilliant Blue G 250 was dissolved in 50 ml of 95% ethanol and 100 ml of 85% (v/v) orthophosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre and filtered through Whatman No.1 filter paper to remove undissolved particles.

(b) **Protein assay**

To one ml of solutions containing 10-100 µg protein was added 5 ml of dye solution and contents mixed by vortexing. The absorbance was read at 595 nm after 5 min against a reagent blank.
Purification of Calf Thymus DNA

Commercially obtained calf thymus DNA was purified free of proteins and single stranded regions as described by Ali et al. (1985). DNA (2 mg/ml) was dissolved in 0.1x SSC (15 mM sodium citrate and 150 mM sodium chloride), pH 7.3. and then mixed with an equal volume of chloroform-isooamyl alcohol (24:1) in a stoppered container and shaken for 1 hr. The aqueous layer containing DNA was separated from the organic layer and re-extracted with chloroform-isooamyl alcohol. The DNA was precipitated with two volumes of cold absolute ethanol and collected on a glass rod. After drying in air, the DNA was dissolved in acetate buffer (30 mM sodium acetate containing 30 mM zinc chloride), pH 5.0 and treated with nuclease S₁ (150 units/mg DNA) at 37°C for 30 min to remove single stranded regions. The reaction was stopped by adding one-tenth volume of 200 mM EDTA, pH 8.0. The nuclease S₁ treated DNA was extracted twice with chloroform-isooamyl alcohol and finally precipitated with two volumes of cold ethanol. The precipitate was dissolved in phosphate-buffered saline (PBS) (10 mM sodium phosphate containing 150 mM sodium chloride), pH 7.4.

Polyacrylamide Gel Electrophoresis

Nucleic acid samples were subjected to PAGE under non-denaturing conditions as described by Laemmli (1970). The following stock solutions were prepared:

(a) Acrylamide-bisacrylamide (30:0.8)

Thirty gm acrylamide and 0.8 gm bisacrylamide was dissolved in distilled water to a final volume of 100 ml. The solution was filtered and stored at 4°C in an amber colored bottle.

(b) Resolving gel buffer

Thirty six gm Tris was dissolved in 48 ml of 1 N HCl, pH adjusted to 8.8 and final volume made upto 100 ml with distilled water.

(c) Stacking gel buffer

Six gm Tris was dissolved in 40 ml distilled water, pH adjusted to 6.8 with 1 N HCl and final volume made upto 100 ml with distilled water.
(d) Electrode buffer
The electrophoretic buffer used was TAE (40 mM Tris, 1.14 ml glacial acetic acid and 1 mM EDTA), pH 7.9.

(e) Procedure
The PAGE assembly was set up and the glass plates separated by 1.5 mm thick spacer were sealed with 1% agarose from the sides and bottom. The non-denaturing gel was prepared and poured between the glass plates and allowed to polymerize at room temperature. Nucleic acid samples were mixed with one-tenth volume of 'stop mix' (30% Ficoll, 0.025% xylene cyanole FF and 500 mM EDTA in 10 times concentrated TAE buffer) and applied onto the gel. The gel was electrophoresed for 8 - 10 hr at room temperature at 80 Volts, stained with ethidium bromide (0.5 μg/ml) and visualized under UV light.

Recipe for 7.5 % non-denaturing PAGE

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>23.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20.0 μL</td>
</tr>
</tbody>
</table>

PAGE FOR PROTEINS

A 7.5% resolving gel was layered with 2.5% stacking gel according to the composition given below.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Stacking gel</th>
<th>Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>2.5 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>5.0 ml</td>
<td>-</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>-</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>11.3 ml</td>
<td>16.95 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.0 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15.0 μL</td>
<td>15.0 μL</td>
</tr>
</tbody>
</table>
Protein samples were mixed with one-fourth volume of sample buffer (10% glycerol and 0.002% bromophenol blue in 10 times concentrated 0.5 M Tris HCl, pH 6.8) and applied onto the gel and electrophoresed at 70 Volts for 6-8 hr using Tris-glycine buffer (25 mM Tris, 192 mM glycine containing 1% SDS), pH 8.3. The gels were stained overnight with 0.1% Coomassie Brilliant Blue R 250 (in 25% isopropanol and 10% glacial acetic acid). Destaining was carried out with a mixture of 10% methanol and 10% glacial acetic acid.

**ROS Modification of Poly(G)**

A solution of commercially obtained synthetic RNA homopolymer polyguanylic acid (0.275 mM) in PBS, pH 7.4 were irradiated under 254 nm light for 1 hr at room temperature in the presence of hydrogen peroxide (27.5 mM). Excess of hydrogen peroxide was removed by extensive dialysis against PBS, pH 7.4. The molecular weight (M) and average length of poly(G) were calculated from the sedimentation coefficient ($S_{20}$, = 7.8) using formula $\log M = 2.179 (\log S_{20} + 1.523)$ and found to be 183 kd and 504 nucleotides, respectively.

**Spectroscopic Analysis**

The ultraviolet spectra of native and ROS modified poly(G) were recorded between 200 to 400 nm on Shimadzu UV-240 spectrophotometer.

**Absorption-Temperature Scan**

Thermal denaturation analysis of nucleic acids was performed in order to ascertain the degree of modification incurred on the nucleic acids. Native and modified nucleic acid samples were subjected to heat denaturation on a Shimadzu UV-240 spectrophotometer coupled with a temperature programmer and controller assembly (Hasan and Ali, 1990). All the samples were melted from 30°C to 95°C at a rate of 1.5°C/min after 10 min equilibration at 30°C and the change in absorbance at $\lambda_{max}$ was recorded with increasing temperature. The UV spectra was recorded simultaneously at 30°C and 95°C in the wavelength range of 200-400 nm.
Circular Dichroism Measurements

Circular dichroism (CD) measurements of native and ROS-poly(G) in PBS, pH 7.4 were performed in 1 nm path length cells at a constant temperature of 25°C. The wavelengths chosen ranged from 220 nm to 350 nm. Scans were recorded at 0.2 nm intervals, and three spectra of each sample were recorded and average determined. A scan of buffer alone was recorded to adjust the base line. The concentration of the polymers was 0.275 mM per nucleotide. Molar ellipticities \([\theta]\) were calculated in terms of the nucleotide concentration according to

\[ [\theta] = \frac{\theta}{10cL} \]

where, \(\theta\) is the measured ellipticity (mdeg), \(c\) is the molar concentration of polymer per nucleotide, and \(L\) is the path length in cm.

Detection of Strand Breaks

The damage to poly(G) mediated by hydroxyl radical was characterized by the presence of strand breaks. The strand breaks in poly(G) was ascertained by Sephadex G 200 gel chromatography, densitometric scanning, agarose gel electrophoresis and gel diffusion.

(a) Sephadex G 200 Gel Chromatography

Gel filtration was performed on Sephadex G 200 column. About 5 g of gel was allowed to swell in distilled water for 5 hr in a boiling water bath. The degassed gel slurry was poured into the column (1 x 42 cm) and left overnight at room temperature to settle under gravity. The flow rate was increased gradually and adjusted to 20 ml/hr. Uniform packing of column was checked by passing 0.2% (w/v) solution of Blue Dextran 2000. The column was equilibrated with PBS, pH 7.4.

One ml of native and ROS-poly(G) was applied onto the column. The sample was allowed to percolate through the upper surface of the gel and the column was connected to a reservoir containing equilibrating buffer. Fractions of 3 ml were collected and absorbance monitored at 253 nm.
(b) **Densitometric Scanning**

Samples of native and ROS-poly(G) were mixed with stop mix without xylene cyanole FF, subjected to polyacrylamide gel electrophoresis on 7.5% native gel. The corresponding 6 cm long lanes were cut and scanned on Gel Scanner GSC-3A of Shimadzu UV-240 spectrophotometer. Gel was scanned at the rate of 20 mm/min and the spectra was recorded simultaneously at a fixed wavelength of 253 nm.

(c) **Agarose Gel Electrophoresis**

One per cent agarose in 30 ml TAE buffer (40mM Tris, 1.14 ml glacial acetic acid containing 1 mM EDTA) was dissolved by heating in boiling water bath. The solution was cooled to 50°C and poured into the gel tray and left at room temperature for complete solidification. One-tenth volume of stop mix dye was added to the sample and loaded in the wells of the submerged gel and electrophoresed for 2 hr at 30 mA. The gels were stained with ethidium bromide (0.5 μg/ml), visualized under UV light and photographed.

(d) **Gel Diffusion**

Native and ROS-poly(G) were mixed with one tenth volume of 'stop mix' without xylene cyanole FF and electrophoresed on 1% agarose gel for 2 hr at 30 mA. The gel was cut into lanes and each lane was sliced into 5 mm pieces. Each piece was gently macerated with glass rod in 0.2 ml of 10 mM Tris HCl buffer, pH 8.0 containing 150 mM NaCl (Maxam and Gilbert, 1980). After overnight incubation at 37°C, the mixture was centrifuged at 10,000 g for 10 min. The supernatant containing poly(G) was filtered through Millipore filter (0.45 μm) and read at 253 nm.

**Separation and Quantitation of Guanine Base**

The separation and quantitation of the modified guanine base in ROS-poly(G) was done according to the method described by Hasan and Ali (1990).
(a) Acid hydrolysis of poly(G)

Native and ROS-poly(G) were precipitated with two volumes of cold ethanol and dissolved in 70% perchloric acid. The samples were heated at 100°C for 1 hr to release the bases. The hydrolysate was neutralized and chromatographed on DEAE Sephadex A 25 matrix.

(b) DEAE Sephadex A 25 column chromatography

The swollen ion exchanger was suspended in the equilibrating buffer (1 mM Tris-HCl, pH 7.6) and poured in a column. The column was extensively equilibrated and 3 ml of sample was applied and eluted with a linear gradient of 1-20 mM Tris-HCl, pH 7.6 at a flow rate of 40 ml/hr. Fractions of 3 ml were collected and absorbance recorded at 260 nm. Elution profile of standard guanine base and hydrolysed native poly(G) served as controls.

Immunization Schedule

Native and ROS-poly(G) (50 μg/rabbit) was complexed with an equal amount (w/w) of methylated BSA and emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites in female rabbits (Hasan et al., 1991). Subsequent injections were given intramuscularly in incomplete adjuvant at weekly intervals. A total of 300 μg of native and ROS-poly(G) were injected during the course of six injections. Animals were bled by cardiac puncture and serum separated, and decomplemented at 56°C for 30 min. Pre-immune serum was collected prior to immunization. The sera were stored in small aliquots at -80°C with 0.1% sodium azide as preservative.

Isolation of Serum IgG by Protein A-Sepharose CL-4B

Serum IgG was isolated by affinity chromatography on Protein A-Sepharose CL-4B column. Diluted serum (0.5 ml) with equal volume of PBS, pH 7.4 was applied to column (0.9 x 15 cm) equilibrated with PBS, pH 7.4. The wash through was recycled 2-3 times and finally the column was washed extensively with PBS to remove unbound material. The bound IgG was eluted with 0.58% acetic acid in
0.85% sodium chloride (Goding, 1978) and neutralized with 1 ml of 1 M Tris-HCl, 
ph 8.5. Three ml fractions were collected and read at 280 nm. IgG concentration 
was determined considering 1.4 O.D$_{280}$ = 1.0 mg IgG/ml. The isolated IgG was 
dialedyzed against PBS, ph 7.4 and stored at -20°C with 0.1% sodium azide.

**Immunological Detection of Antibodies**

Immune sera, SLE sera and PSS sera were tested for antibodies by 
immunodiffusion, counterimmunoelectrophoresis, enzyme linked immunosorbent assay 
and gel retardation assay.

(a) **Immunodiffusion**

Immunodiffusion (ID) was carried out by Ouchterlony double diffusion system 
using glass petri dishes as described by Tan *et al.* (1966). Six ml of 0.4% agarose 
solution in PBS, ph 7.4 was brought into molten state, allowed to cool to 50-60°C 
and subsequently poured onto 5 x 1.5 cm glass petri dishes. Wells each 5 mm in 
diameter, separated by 8 mm distance were cut onto hardened gel. Appropriate 
amount of antibody and antigen was placed in the wells and the reaction was 
allowed to proceed for 48-72 hr in a moist chamber at room temperature. The gels 
were washed with 5% sodium citrate to remove non-specific precipitin lines. The 
precipitin lines were analyzed visually and photographed.

(b) **Counterimmunoelectrophoresis**

Counterimmunoelectrophoresis (CIE) was performed as described by Kurata 
and Tan (1976) with slight modifications. Three ml of 0.6% molten agarose solution 
in 25 mM barbital buffer, pH 8.4 was poured onto microscopic slides (7.5 x 2.5 
cm) and allowed to solidify at room temperature. Wells 4 mm in diameter were cut 
at a distance of 5 mm between the two opposite wells. Antigen was placed in 
cathodal well and antibodies in the anodal well, electrophoresed for 45-60 min in 
50 mM barbital buffer, pH 8.4 with a current of 3-4 mA per slide. Non-specific 
precipitin lines were removed with 5% sodium citrate.
(c) **Gel Retardation Assay**

Gel retardation assay was performed for the visual detection of antigen-antibody interaction (Sanford *et al*., 1988). Constant amounts of nucleic acid samples were incubated with varying amounts of IgG in PBS, pH 7.4 for 2 hr at 37°C and overnight at 4°C. One-tenth volume of 'stop mix' dye was added to the mixture and electrophoresed on 0.8% agarose for 2 hr at 30 mA in TAE buffer, pH 7.9. The gels were stained with ethidium bromide (0.5 μg/ml), visualized under UV light and photographed.

(d) **Enzyme Linked Immunosorbent Assay**

ELISA was performed using polystyrene (96 well) flat bottom microtitre plates as described by Aotsuka *et al*. (1979).

(i) **Buffers and reagents**

*Tris buffered saline (TBS)*

10 mM Tris, 150 mM NaCl, pH 7.4

*Tris buffered saline-Tween 20 (TBS-T)*

20 mM Tris, 144 mM NaCl, 2.68 mM KCl, pH 7.4, containing 500 μL Tween 20/L

*Carbonate-bicarbonate buffer*

15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6, containing 2 mM magnesium chloride

*Citrate-phosphate buffer*

22 mM citric acid, 50 mM Na₂HPO₄, pH 5.0

Substrates

(i) 500 μg p-nitrophenyl phosphate (p-NPP)/ml of carbonate-bicarbonate buffer
(ii) 500 µg o-phenylenediamine (OPD)/ml of citrate-phosphate buffer, containing 1 µl/ml of hydrogen peroxide

(ii) Procedure

Polystyrene microtiter plates were coated with 100 µl of nucleic acid antigen (2.5 µg/ml in TBS, pH 7.4) for 2 hr at room temperature and overnight at 4°C. The antigen coated wells were washed three times with TBS-T to remove unbound antigen and unoccupied sites in test and control wells were blocked with 150 µL of 1.5% BSA in TBS for 4-6 hr at room temperature. The plates were washed with TBS-T and antibody (100 µl/well) to be tested, diluted in TBS was added to each well. After incubating plates for 2 hr at room temperature, the bound antibodies were assayed by an appropriate anti-immunoglobulin alkaline phosphatase or horse radish peroxidase conjugate using p-nitrophenyl phosphate or the OPD/H₂O₂ substrate respectively. The plates were incubated for 1-2 hr and reaction stopped in the case of OPD/H₂O₂ system with 50 µl of 5 N H₂SO₄ and read at 490 nm. In the case of p-NPP, the absorbance was read at 410 nm. Each sample was coated in duplicate. Results were expressed as a mean of $A_{\text{test}} - A_{\text{control}}$.

Competition ELISA

The antigenic specificity of the antibodies was determined by inhibition experiments (Hasan et al., 1991). Varying amounts of inhibitors (0-20 µg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated at room temperature for 2 hr and overnight at 4°C. The immune complex thus formed was coated in the wells instead of the serum. The remaining steps were the same as in direct binding ELISA. The results were expressed as per cent inhibition.

$$\text{Per cent inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100$$
Detection of Immune Complexes Deposition in Kidney

Kidneys from immunized rats were screened for the detection of immune complexes deposition in glomeruli.

(i) Immunization protocol

Groups of four female rats (Sprague-Dawley), were immunized subcutaneously at multiple sites on the back with native and ROS-poly(G) (50 µg/rat) complexed with methylated BSA (w/w) and emulsified in Freund’s complete adjuvant. After 21 days, three booster doses (25 µg/rat) were given intraperitoneally at weekly intervals with incomplete adjuvant. A group of animals were immunized similarly, but without any antigen, whereas another group was not immunized at all. Both these groups served as controls. The sera were decomplemented at 56°C and screened for immune response by ELISA. Where necessary, another booster was given and finally the rats were sacrificed and kidneys stored at -80°C.

(ii) Immunofluorescence

Kidney were quickly frozen and sectioned in a cryostat at -30°C of thickness 4-6 µm. Sections were fixed on slides with acetone and incubated with anti-rat IgG fluorescein isothiocyanate conjugate (1:10 diluted) for 30 min. Subsequently, slides were washed three times with PBS and mounted with 50% glycerol. The deposition of immune complex was detected with a fluorescent microscope.
Results
Formation of ROS-poly(G) and its Characterization

A solution of polyguanylic acid in PBS, pH 7.4 was modified with reactive oxygen species. UV irradiation (254 nm) in the presence of hydrogen peroxide is known to generate hydroxyl radical. The irradiated samples were dialyzed against PBS to remove excess of hydrogen peroxide.

The UV absorption spectra was recorded on a Shimadzu UV-240 spectrophotometer in the wavelength range from 200 nm to 400 nm. Figure 3 shows the UV absorption spectra of poly(G) irradiated for 10, 20 and 30 min indicating the corresponding decrease in absorbance at 253 nm, the $\lambda_{\text{max}}$. Poly(G) irradiated for 1 hr showed marked hypochromicity (41% decrease in absorbance) as compared to unirradiated poly(G) (Fig. 4a). The sample was used for further studies. The UV difference spectra of ROS-poly(G) with reference to native poly(G) exhibited negative band, having absolute peak between 234 nm to 282 nm and significantly increased absorbance around 300 nm (Fig. 4b).

Native and ROS-poly(G) were mixed with poly(C) to form poly(G).poly(C) complexes. UV absorption spectra of poly(G) and poly(C) showed $\lambda_{\text{max}}$ at 253 nm and 269 nm respectively (Fig. 5). After mixing and allowing it to anneal, the $\lambda_{\text{max}}$ shifts to 261 nm. The shift in $\lambda_{\text{max}}$ shows the formation of poly(G).poly(C) double stranded complex. The UV-absorption characteristics of native and ROS-poly(G) complexed with poly(C) are depicted in Table 3. The absorbance ratio ($A_{260}/A_{280}$) was 1.82 for native poly(G) and 1.70 for ROS-poly(G). After complexing with poly(C), the ratio was 2.21 for poly(G).poly(C) and 1.78 for ROS-poly(G).poly(C) complexes. This difference in absorbance ratio could be due to some structural and conformational changes in poly(G) incurred after ROS modification.

Circular Dichroism of Native and ROS-Poly(G)

The CD spectra of the native and ROS-poly(G) was recorded in the wavelength range of 220 nm to 350 nm and showed characteristic changes in ellipticity (Fig. 6). Native poly(G) shows negative ellipticity from 222 nm to 246 nm and positive ellipticity from 246 nm to 309 nm with an unresolved
Fig. 3. UV absorption spectra of native poly(G) (——), irradiated for 10 min (---), 20 min (----) and 30 min (-----) under UV light in presence of hydrogen peroxide.
Fig. 4. (a) UV absorption spectra of native poly(G) (----) and ROS-poly(G) (--.--) irradiated for 1 hr. (b) UV difference spectra of ROS-poly(G) (---). Native poly(G) served as control.
Fig. 5. UV absorption spectra of native poly(G) (—), ROS-poly(G) (........), poly(C) (-----), poly(G).poly(C) (----) and ROS-poly(G).poly(C) (-----).
TABLE 3

UV Absorption Characteristics of Native and ROS-poly(G) and Their Complex with Poly(C)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Absorbance ratio (A&lt;sub&gt;260&lt;/sub&gt;/A&lt;sub&gt;280&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(G)</td>
<td>253</td>
<td>1.82</td>
</tr>
<tr>
<td>ROS-poly(G)</td>
<td>253</td>
<td>1.70</td>
</tr>
<tr>
<td>Poly(C)</td>
<td>269</td>
<td>1.29</td>
</tr>
<tr>
<td>Poly(G).poly(C)</td>
<td>261</td>
<td>2.21</td>
</tr>
<tr>
<td>ROS-poly(G).poly(C)</td>
<td>261</td>
<td>1.78</td>
</tr>
</tbody>
</table>
Fig. 6. Circular dichroism spectra of native poly(G) (—) and ROS-poly(G) (---) in PBS, pH 7.4.
shoulder at 285 nm. The minima and maxima of CD curve correspond to 238.8 nm and 260.2 nm, respectively (Fig. 6). ROS-poly(G) shows negative ellipticity below 247 nm and positive ellipticity from 247 nm to 307 nm. The minima and maxima correspond to 261.4 nm and 240 nm, respectively. The result showed 47% decrease in ellipticity of poly(G) after hydroxyl radical treatment.

**Thermal Melting of Poly(G)**

Thermally induced transitions of poly(G) were measured spectrophotometrically at their $\lambda_{\text{max}}$ by heating the polymer at a rate of 1.5°C/min from 30°C to 95°C. The UV spectra was recorded simultaneously at 30°C and 95°C (Fig. 7). Table 4 shows the absorbance ratio ($A_{260}/A_{280}$) at 30°C and 95°C. Native poly(G) and poly(G).poly(C) complex did not show significant change in absorbance at 30°C and 95°C (Figs. 7a and c). The absorbance ratio ($A_{260}/A_{280}$) was found to be 1.82 and 1.80 at 30°C and 95°C, respectively for native poly(G) and 2.21 and 2.19 respectively for poly(G).poly(C). These findings suggest the high stability of poly(G) due to its existence in multi-stranded form. The absorbance ratio of ROS-poly(G).poly(C) was 1.78 at 30°C and 1.39 at 95°C, while for ROS-poly(G) it is 1.70 and 1.59, respectively (Figs. 7b and d). The change in absorbance ratio of ROS-poly(G) and ROS-poly(G).poly(C) complex at 30°C and 95°C suggests structural alterations and a partial destruction of the secondary structure of poly(G) on ROS-modification.

**Strand Breaks**

**(a) Sephadex G 200 Gel Filtration**

Figure 8 shows the gel chromatography profile of native and ROS-poly(G) on Sephadex G 200 column. Native poly(G) was eluted in a single, symmetrical sharp peak, while ROS-poly(G) showed an additional peak of low molecular weight. The minor peak may be attributed to the generation of strand breaks and consequent formation of smaller fragments of poly(G). The extent of modification was determined by measuring the peak area of the ROS-modified and unmodified poly(G). The data indicates the modification of poly(G) to an extent of 52.3%.
Fig. 7. UV absorption spectra of native and ROS-poly(G) and their complex with poly(C) at 30°C and 95°C.

(a) Native poly(G) at 30°C (—) and 95°C (— —).
(b) ROS-poly(G) at 30°C (— —) and 95°C (— —).
(c) Poly(G).poly(C) at 30°C (— —) and 95°C (— —).
(d) ROS-poly(G).poly(C) at 30°C (— —) and 95°C (— —).
**TABLE 4**

**Thermal Melting Characteristics of Native and ROS-poly(G) and Their Complex with Poly(C)**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Absorbance ratio ($A_{260}/A_{280}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at 30°C</td>
</tr>
<tr>
<td>Poly(G)</td>
<td>1.82</td>
</tr>
<tr>
<td>ROS-Poly(G)</td>
<td>1.70</td>
</tr>
<tr>
<td>Poly(G).poly(C)</td>
<td>2.21</td>
</tr>
<tr>
<td>ROS-poly(G).poly(C)</td>
<td>1.78</td>
</tr>
</tbody>
</table>
Fig. 8. Fractionation of native poly(G) (○) and ROS-poly(G) (△) on Sephadex G 200 gel filtration column.
(b) Densitometric Scanning of Native and ROS-Poly(G)

Native and ROS-poly(G) were electrophoresed on 7.5% polyacrylamide gel and scanned on Gel Scanner GSC-3A of Shimadzu UV-240 spectrophotometer. Figure 9 shows the densitometric scanning of native and ROS-poly(G) at a fixed wavelength of 253 nm. ROS-poly(G) shows low intensity and high mobility than native poly(G). The data reflects the formation of low molecular weight species and strand breaks in poly(G) after hydroxyl radical modification.

(c) Agarose Gel Electrophoresis

The presence of strand breaks in hydroxyl radical modified poly(G) was ascertained by performing agarose gel electrophoresis of native and ROS-poly(G) in 1% agarose gel. As evident from Figure 10, modified poly(G) migrated with enhanced mobility as compared to native poly(G). This difference in migration may be due to the changes in the secondary structure of modified poly(G) as opposed to the native poly(G). The enhanced mobility of ROS-poly(G) may also be attributed to the formation of low molecular weight species as a result of strand breaks.

(d) Gel Diffusion of Native and ROS-Poly(G)

To further ascertain the enhanced mobility of modified poly(G) due to the presence of strand breaks, gel diffusion experiment was performed. Native and ROS-poly(G) electrophoresed agarose gel was cut into strips and sliced into 0.5 cm pieces. The individual slices were extracted by diffusion, clarified by membrane filtration and read at 253 nm. The elution profile of ROS-poly(G) shows a shift in the peak fraction relative to native poly(G) (Fig. 11). The data clearly indicate the enhanced mobility of ROS-poly(G) in comparison to native poly(G) which might be due to the formation of strand breaks after ROS-modification.

Quantitation of Modified Guanine Base

Poly(G) was subjected to acid hydrolysis in order to separate the guanine base from the sugar-phosphate backbone. UV absorption spectra of acid hydrolysed
Fig. 9. Densitometric scanning of native poly(G) (——) and ROS-poly(G) (---) on 7.5% native polyacrylamide gel. Inset: Polyacrylamide gel electrophoresis of native poly(G) (lane 1) and ROS-poly(G) (lane 2) on 7.5% native gel.
Fig. 10. Agarose gel electrophoresis of native poly(G) (lane 1) and ROS-poly(G) (lane 2). Electrophoresis was carried out on 1% agarose for 2 hr at 30 mA and stained with ethidium bromide.
Fig. 11. Elution profile of native poly(G) (○) and ROS-poly(G) (△) from agarose gel by diffusion.
native and ROS-poly(G) showed a distinct peak at 273 nm, a characteristic feature of guanine base (Fig. 12). This shift in $\lambda_{max}$ from 253 nm of poly(G) or ROS-poly(G) to 273 nm indicates the release of guanine residues from the sugar-phosphate backbone of poly(G) after acid hydrolysis. UV absorption spectra of guanine base released from ROS-poly(G) showed broadening of absorption maxima at 273 nm in contrast to a sharp peak of the base from native poly(G). The broadening of peak suggests structural alterations of guanine residues in poly(G) after ROS-modification.

The neutralized hydrolysate of native and ROS-poly(G) was applied to a DEAE Sephadex A-25 column and eluted with a linear gradient of 1-20 mM Tris HCl, pH 7.6. Figure 13a shows the elution profile of standard guanine base. The elution profile of native poly(G) hydrolysate was similar to that of standard guanine base, while ROS-poly(G) eluted in two peaks (Fig. 13b). The major and minor peak represents modified and unmodified guanine base, respectively. The modified guanine base eluted first followed by unmodified species. The extent of modification as determined by measuring the peak area shows 76% modification of guanine base.

**Antigenicity of Native and ROS-Poly(G)**

The antigenicity of native and ROS-poly(G) was probed by inducing antibodies in rabbits. Direct binding and inhibition ELISA were used to evaluate the repertoire of specificities of the induced antibodies. The binding of these antibodies to the immunogen was further assessed by band shift assay.

**(a) Antibodies Against Native Poly(G)**

The antiserum against native poly(G) in rabbits induced non-precipitating antibodies as determined by immunodiffusion and counterimmunoelectrophoresis. Direct binding ELISA on plates coated with native poly(G) indicated high binding activity with different serum dilutions showing a titre of at least 1:6400 (Fig. 14). Preimmune serum showed negligible binding. Induced antibodies were found to be highly specific for the immunogen. Inhibition ELISA showed a maximum of 67% inhibition of antibody binding to native poly(G) (Fig. 15). Fifty percent inhibition was achieved with only 1.5 $\mu$g/ml of poly(G).
Fig. 12. UV absorption spectra of native poly(G) (———), ROS-poly(G) (……….), standard guanine base (———) and acid hydrolysed native poly(G) (——) and ROS-poly(G) (———).
Fig. 13. Elution profile of (a) standard guanine base (○) and (b) acid hydrolysed native poly(G) (●) and ROS-poly(G) (△) on DEAE Sephadex A-25 column. ROS-poly(G) eluted in two peaks. major peak represents modified guanine base and minor peak represent unmodified guanine base.
Fig. 14. Direct binding ELISA of immune (△, ▲) and preimmune (○) rabbit serum antibodies to native poly(G). Microtitre plates were coated with native poly(G) (5 μg/ml).
Fig. 15. Inhibition of the binding of anti-native poly(G) antibody (serum) (○) and affinity purified IgG (△) by native poly(G). Microtitre plates were coated with native poly(G) (5 μg/ml).
(b) Antibodies Against ROS-poly(G)

ROS-poly(G) was highly immunogenic in rabbits. The antiserum showed a titre of 1:6400 by direct binding ELISA on plates coated with ROS-poly(G) (Fig. 16). The binding of preimmune serum was of low magnitude. In competition inhibition assay, a maximum of 64% inhibition was observed and 50% inhibition was achieved with 9 µg/ml of ROS-poly(G) (Fig. 17).

(c) Purification and Characterization of Immune IgG

Anti-native and ROS-poly(G) IgG was isolated from serum by affinity chromatography on Protein A-Sepharose CL-4B column (Figs. 18a and b). The purity of IgG was ascertained by SDS-PAGE under non-reducing conditions (Figs. 18a and b, inset), moving as a single homogeneous band.

Direct binding ELISA of the purified IgG against poly(G) and ROS-poly(G) exhibited a strong binding to respective immunogens (Figs. 19 and 20). Preimmune IgG as negative control showed negligible binding. The specificity of the purified IgG was evaluated by competitive inhibition assays. A maximum of 71% inhibition of anti-native poly(G) IgG and 77% of anti-ROS-poly(G) IgG binding to the solid phase bound respective immunogen was observed with the immunogens as inhibitors (Figs. 15 and 17). Fifty percent inhibition in the corresponding antibody activity was achieved with only 0.1 µg/ml of native poly(G) and 0.7 µg/ml of ROS-poly(G) respectively. The data shows the definite antigen binding characteristics of purified IgG.

Immunocrossreactivity of Anti-Native Poly(G) Antibodies

The anti-native poly(G) antibodies exhibited a broad spectrum of reactivity as observed by its binding to a variety of nucleic acid antigens. Competitive experiments with ROS-poly(G) showed 57% inhibition in the antibody activity (Fig. 21a). Fifty percent inhibition was achieved with 3.0 µg/ml of ROS-poly(G). Total RNA from buffalo thymus and calf thymus DNA showed moderate inhibitions of 45% and 41% respectively. Their ROS-modified forms showed only 38% and 30%
Fig. 16. Direct binding ELISA of immune (Δ, ▲) and preimmune (○) rabbit serum antibodies to ROS-poly(G). Microtitre plates were coated with ROS-poly(G) (5 μg/ml).
Fig. 17. Inhibition of the binding of anti-ROS-poly(G) antibody (serum) (○) and affinity purified IgG (△) by ROS-poly(G). Microtitre plates were coated with ROS-poly(G) (5 µg/ml).
Elution profile of (a) anti-native poly(G) IgG and (b) anti-ROS-poly(G) IgG on Protein A-Sepharose 4B affinity column. Insets: SDS-PAGE of purified IgG on 7.5% acrylamide gel.
Fig. 19. Binding of affinity purified anti-native poly(G) IgG ( ▲ ) and preimmune IgG ( △ ) to native poly(G). Microtitre plates were coated with native poly(G) (5 µg/ml).
Fig. 20. Binding of affinity purified anti-ROS-poly(G) IgG (▲) and preimmune IgG (△) to ROS-poly(G). Microtitre plates were coated with ROS-poly(G) (5 μg/ml).
Fig. 21. Inhibition of anti-native poly(G) IgG binding to native poly(G). Microtitre plates were coated with native poly(G) (5 μg/ml). The competitors were (a) ROS-poly(G) (○), RNA (△), DNA (▲) and (b) ROS-guanine (○), ROS-RNA (▲), ROS-DNA (△).
inhibition respectively (Fig. 21b). The nitrogenous base guanine showed 45% inhibition of antibody activity (Fig. 22a), and ROS-guanine shows 42% inhibition (Fig. 21b). Homopolymers poly(I) and poly(C) were non-inhibitory (Fig. 22a). Antibody recognition of poly(rG).poly(dC), a polymer known to attain A-/analogous conformation, resulted in 46% elimination of antibody binding (Fig. 22b).

To further study the structural determinants recognized by anti-native poly(G) antibodies, their interaction with various synthetic polydeoxyribonucleotides was studied. Poly(dA-dT).poly(dA-dT) and poly(dA-dU).poly(dA-dU) showed a maximum inhibition of 42% and 33% respectively (Fig. 22b), while poly(dI-dC).poly(dI-dC), poly(dG-dC).poly(dG-dC) and poly(dA-dG).poly(dC-dT) showed negligible inhibition (Fig. 23). Table 5 shows a summary of the data on the binding characteristics of anti-native poly(G) IgG as determined by inhibition ELISA.

Immunocrossreactivity of Anti-ROS-Poly(G) Antibodies

Competition experiments of anti-ROS-poly(G) antibodies with a variety of nucleic acid polymers showed a wide range of heterogeneity. Native poly(G) showed a maximum of 54% inhibition in the antibody activity. Fifty percent inhibition was achieved with 5.5 µg/ml of native poly(G) (Fig. 24a). Native calf thymus DNA and buffalo thymus RNA showed moderate inhibitions of 39% and 41% respectively (Table 6). Their ROS-modified forms were also used as inhibitors. ROS-DNA showed a maximum inhibition of 42%, while ROS-RNA was inhibitory to an extent of 55% (Fig. 24b). Fifty percent inhibition of antigen-antibody interaction was obtained with 14 µg/ml of ROS-RNA. Guanine showed negligible inhibition of 21%, while ROS-guanine was a potent inhibitor (Fig. 24b), showing a maximum inhibition of 56%. The amount of ROS-guanine required for 50% inhibition in the antibody activity was 17.5 µg/ml (Table 6). Among the homopolymers, poly(I) and poly(C) showed negligible inhibition of 18% and 31% respectively, while ROS-poly(I) showed moderate inhibition of 45% (Fig. 25a). Synthetic double stranded polydeoxyribonucleotides, poly(dI-dC).poly(dI-dC), poly(dA-dU).poly(dA-dU) and poly(dA-dT).poly(dA-dT) showed maximum inhibitions of 25%, 30% and 39% respectively (Fig. 25b). Table 6 summarizes the inhibition data of anti-ROS-poly(G) IgG and shows the preferential recognition of ROS-modified epitopes over their unmodified counterparts.
Fig. 22. Inhibition of anti-native poly(G) IgG binding to native poly(G). Microtitre plates were coated with native poly(G) (5 µg/ml). The competitors were (a) poly(I) ( ), poly(C) ( ), guanine base ( ) and (b) poly(rG).poly(dC) ( ), poly(dA-dU).poly(dA-dU) ( ), poly(dA-dT).poly(dA-dT) ( ).
Fig. 23. Inhibition of anti-native poly(G) IgG binding to native poly(G). The competitors were poly(dA-dG).poly(dC-dT) (○), poly(dG-dC).poly(dG-dC) (△) and poly(dl-dC).poly(dl-dC) (▲). Microtitre plates were coated with native poly(G) (5 μg/ml).
### TABLE 5

**Antigenic Specificity of Anti-Native Poly(G) Antibodies**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Maximum % inhibition at 20 μg/ml</th>
<th>Concentration for 50% inhibition (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(G)</td>
<td>71</td>
<td>0.1</td>
</tr>
<tr>
<td>ROS-poly(G)</td>
<td>57</td>
<td>3.0</td>
</tr>
<tr>
<td>RNA</td>
<td>45</td>
<td>-</td>
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<tr>
<td>ROS-RNA</td>
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<td>-</td>
</tr>
<tr>
<td>DNA</td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td>ROS-DNA</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Guanine</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>ROS-guanine</td>
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<td>-</td>
</tr>
<tr>
<td>Poly(I)</td>
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<td>-</td>
</tr>
<tr>
<td>Poly(C)</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Poly(rG).poly(dC)</td>
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<td>-</td>
</tr>
<tr>
<td>Poly(dA-dT).poly(dA-dT)</td>
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<td>-</td>
</tr>
<tr>
<td>Poly(dA-dU).poly(dA-dU)</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dI-dC).poly(dI-dC)</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dG-dC).poly(dG-dC)</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dA-dG).poly(dC-dT)</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

Plates were coated with native poly(G) (5 μg/ml)
Fig. 24. Inhibition of anti-ROS-poly(G) IgG binding to ROS-poly(G). Microtitre plates were coated with ROS-poly(G) (5 μg/ml). The competitors were (a) native poly(G) (○), RNA (△), DNA (▲) and (b) native guanine (▲), ROS-guanine (●), ROS-RNA (△), and ROS-DNA (○).
TABLE 6

Antigenic Specificity of Anti-ROS Poly(G) Antibodies

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Maximum % inhibition at 20 µg/ml</th>
<th>Concentration for 50% inhibition (µg/ml)</th>
</tr>
</thead>
<tbody>
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<td>Poly(G)</td>
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<tr>
<td>ROS-poly(G)</td>
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<tr>
<td>DNA</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td>ROS-DNA</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>RNA</td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td>ROS-RNA</td>
<td>55</td>
<td>14.0</td>
</tr>
<tr>
<td>Guanine</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td>ROS-guanine</td>
<td>56</td>
<td>17.5</td>
</tr>
<tr>
<td>Poly(I)</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>ROS-poly(I)</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Poly(C)</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dI-dC).poly(dI-dC)</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dA-dU).poly(dA-dU)</td>
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<td>-</td>
</tr>
<tr>
<td>Poly(dA-dT).poly(dA-dT)</td>
<td>39</td>
<td>-</td>
</tr>
</tbody>
</table>

Plates were coated with ROS-poly(G) (5 µg/ml).
Fig. 25. Inhibition of anti-ROS-poly(G) IgG binding to ROS-poly(G). Microtitre plates were coated with ROS-poly(G) (5 µg/ml). The competitors were (a) poly(C) ( △ ), poly(I) ( ○ ), ROS-poly(I) ( ▲ ) (b) poly(dI-dC).poly(dI-dC) ( ○ ), poly(dA-dU).poly(dA-dU) ( △ ) and poly(dA-dT).(dA-dT) ( ▲ ).
Band Shift Assay

The binding of native and ROS-poly(G) to their immune IgG was further ascertained by band shift assay. Constant amounts of native and ROS-poly(G) were incubated with varying amounts of respective immune IgG for 2 hr at room temperature and overnight at 4°C. The immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA. Figure 26 shows the formation of complexes between anti-native poly(G) IgG and native poly(G). With increasing concentration of IgG, the formation of high molecular weight immune complexes increased as judged by their fluorescence intensity, whereas, the amount of unbound poly(G) showed a proportional decrease in its intensity. The recognition of ROS-poly(G) by anti-ROS-poly(G) IgG was reiterated by the remarkable loss in fluorescence intensity of unbound ROS-poly(G) (Fig. 27).

Detection of Anti-DNA Antibodies in SLE Sera

Various SLE serum samples were tested for the presence of anti-DNA autoantibodies by ELISA. Figure 28 shows the binding of 1:100 diluted SLE serum to native DNA. Normal human serum shows negligible binding. The specificity of SLE autoantibodies binding to native DNA was determined by competition experiments. Native DNA showed maximum inhibitions ranging from 47% to 68% in eight SLE sera (Figs. 29a and b). Table 7 shows the inhibition data of anti-DNA autoantibodies binding to DNA from these serum samples.

Reactivity of Anti-DNA Antibodies with Native and ROS-Poly(G)

The binding of SLE anti-DNA autoantibodies to native and ROS-poly(G) was determined by direct binding ELISA. All the eight SLE sera showed stronger binding to native and ROS-poly(G) with preferential binding to ROS-poly(G). Figure 30 shows 10% to 35% higher absorbance values for ROS-poly(G) than native poly(G) in all serum samples. No binding was observed with the sera of normal subjects.
Fig. 26. Band shift assay of anti-native poly(G) IgG binding to native poly(G). Native poly(G) (5 μg) were incubated with buffer (lane 1) and 25, 50, 75 and 100 μg IgG through lanes 2 to 5, respectively for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose for 2 hr at 30 mA.
Fig. 27. Band shift assay of anti-ROS-poly(G) IgG binding to ROS-poly(G). ROS-poly(G) (5 μg) were incubated with buffer (lane 1) and 25, 50, 75 and 100 μg IgG through lanes 2 to 5, respectively for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose for 2 hr at 30 mA.
Fig. 28. Binding of 1:100 diluted SLE serum samples to native DNA (□). Normal human serum (NHS) showed negligible binding. Microtitre plates were coated with native DNA (2.5 μg/ml).
Fig. 29. Inhibition of SLE anti-DNA autoantibodies binding by native DNA. Microtitre plates were coated with native DNA (20 µg/ml). (a) SLE sera 1 (○), 2 (△), 3 (▲), 4 (●) and (b) SLE sera 5 (○), 6 (△), 7 (▲), 8 (●).
TABLE 7

Inhibition in the Binding of Autoantibodies from SLE Sera by Native DNA

<table>
<thead>
<tr>
<th>SLE serum</th>
<th>Maximum % inhibition at 20 µg/ml</th>
<th>Concentration for 50% inhibition (µg/ml)</th>
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<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>12</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>5</td>
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<td>6</td>
<td>68</td>
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<td>7</td>
<td>53</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>63</td>
<td>13</td>
</tr>
</tbody>
</table>

Plates were coated with native DNA (2.5 µg/ml).
Fig. 30. Binding of 1:100 diluted SLE serum samples to native poly(G) ([ ]) and ROS-poly(G) ( [ ]). Normal human sera (NHS) showed negligible binding with either of the antigen. Microtitre plates were coated with 5 μg/ml of respective antigens.
The specificity of each SLE serum for native and ROS-poly(G) was evaluated by inhibition assays (Figs. 31 and 32). In this case, the antibody was first incubated with native or ROS-poly(G) and then assayed for residual binding to double stranded DNA. The microtitre plates were coated with native DNA. Native poly(G) showed maximum inhibitions ranging from 31% to 57% in eight SLE sera (Table 8). ROS-poly(G) showed relatively high percent inhibition in six SLE sera. One of these sera was inhibitory to an extent of 73%. Rest of the two sera showed low inhibitions of 29% and 31%, respectively.

Purification of SLE IgG

SLE IgG was purified by affinity chromatography on Protein A-Sepharose CL-4B column. Protein A has been known to bind IgG from most of the mammalian species. SDS-PAGE of purified IgG under non-reducing conditions showed a single homogeneous band.

In order to investigate the binding activity of purified SLE IgG with native and ROS-poly(G), direct binding ELISA was performed. Figure 33 shows an appreciable binding of SLE IgG to native and ROS-poly(G), while normal human IgG showed negligible binding.

Band Shift Assay

Band shift assay was employed to further confirm and visualize the interaction of native and ROS-poly(G) with SLE autoantibodies. A constant amount of the antigen was incubated with increasing amounts (0-120 µg) of SLE IgG for 2 hr at 37°C and overnight at 4°C. These immune complexes were then electrophoresed on 1% agarose for 2 hr at 30 mA. Figures 34 and 35 show the binding of anti-DNA autoantibodies to native poly(G) and ROS-poly(G) respectively. With increasing concentration of IgG there is corresponding increase in the formation of high molecular weight immune complexes which resulted in retarded mobility and corresponding decrease in the fluorescence intensity of unbound antigen. Normal human IgG incubated under identical conditions did not show immune complex formation.
Fig. 31. Inhibition of SLE anti-DNA autoantibody binding by native and ROS-poly(G). Microtitre plates were coated with native DNA (2.5 μg/ml). (a) SLE sera 1 and 2 by native poly(G) (O, △) and ROS-poly(G) (△, ●) respectively. (b) SLE sera 3 and 4 by native poly(G) (O, △) and ROS-poly(G) (△, ●) respectively.
Fig. 32. Inhibition of SLE anti-DNA autoantibody binding by native and ROS-poly(G). Microtitre plates were coated with native DNA (2.5 μg/ml). (a) SLE sera 5 and 6 by native poly(G) (○, ▲) and ROS-poly(G) (△, ●) respectively. (b) SLE sera 7 and 8 by native poly(G) (○, ▲) and ROS-poly(G) (△, ●) respectively.
### TABLE 8

**Inhibition of Anti-DNA Autoantibodies Binding to Native DNA by Native and ROS-Poly(G)**

<table>
<thead>
<tr>
<th>SLE serum</th>
<th>Maximum percent inhibition at 20 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native poly(G)</td>
</tr>
<tr>
<td>1</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
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<td>40</td>
</tr>
<tr>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
</tr>
</tbody>
</table>

Plates were coated with native DNA (2.5 µg/ml).
Fig. 33. Binding of affinity purified SLE IgG to native poly(G) (▲) and ROS-poly(G) (△). Normal human IgG (○) to either of the antigen (5 μg/ml).
Fig. 34. Band shift assay of SLE IgG binding to native poly(G). Native poly(G) (5 μg) were incubated with buffer (lane 1) and 30, 60, 90 and 120 μg IgG through lanes 2 to 5, respectively for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose for 2 hr at 30 mA.
Fig. 35. Band shift assay of SLE IgG binding to ROS-poly(G). ROS-poly(G) (5 μg) were incubated with buffer (lane 1) and 30, 60, 90 and 120 μg IgG through lanes 2 to 5, respectively for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose for 2 hr at 30 mA.
Detection of Autoantibodies in PSS Sera

Direct binding ELISA results showed the presence of high titre anti-DNA antibodies (Figs. 36 and 37) and anti-poly(G) autoantibodies (Figs. 38 and 39) in sera from eight patients with PSS. In contrast, no binding was observed with the sera from normal subjects.

The specificity of the binding of native and ROS-poly(G) to PSS sera was tested using native poly(G) as an antigen in a competition assay. The results obtained are tabulated in Table 9. ROS-poly(G) effectively inhibited the PSS autoantibodies as compared to native poly(G). It was observed that from eight samples tested, native poly(G) showed inhibitions less than 50%, ranging between 33% and 46%. ROS-poly(G) showed inhibitions greater than 50% in six out of eight PSS sera i.e., 57%, 61%, 53%, 51%, 60% and one sera showed high inhibition of 73%. Two sera exhibited lower inhibitions of 46% and 41% (Figs. 40 and 41). These results suggests that oxygen free radicals under certain conditions can lead to the formation of 8-hydroxyguanine residues in DNA and RNA and thus may generate its potential immunogenic forms and induce autoantibody production in SLE and PSS patients.

PSS IgG was purified from serum by affinity chromatography an Protein A-Sepharose CL-4B column. Band shift assay of native and ROS-poly(G) with PSS IgG resulted in retarded mobility, further strengthening the direct binding and inhibition results (Fig. 42 and 43).

Detection of Precipitating Antibodies in SLE and PSS Sera

In order to detect the presence of precipitating antibodies to native and ROS-poly(G) in SLE and PSS sera, counterimmunoelectrophoresis was performed. Out of twelve, eight SLE sera showed precipitin arc with native and ROS-poly(G) (Fig. 44), whereas, all the eight PSS sera showed the presence of precipitating antibodies to native and ROS-poly(G) (Fig. 45). No precipitin line was observed with normal human sera.
Fig. 36. Direct binding ELISA of PSS serum autoantibodies to native DNA. PSS sera 1, 2, 3 and 4 (▲, △, ○, ●) and normal human sera (▽). Microtitre plates were coated with native DNA (2.5 μg/ml).
Fig. 37. Direct binding ELISA of PSS serum autoantibodies to native DNA. PSS sera 5, 6, 7 and 8 (▲, △, ○, ●) and normal human sera (▽). Microtitre plates were coated with native DNA (2.5 μg/ml).
Fig. 38. Direct binding ELISA of PSS serum autoantibodies to native poly(G). PSS sera 1, 2, 3 and 4 ( △ , △ ,○,●) and normal human sera ( ▽ ). Microtitre plates were coated with native poly(G) (5 μg/ml).
Fig. 39. Direct binding ELISA of PSS serum autoantibodies to native poly(G). PSS sera 5, 6, 7 and 8 (▲, △, ○, ●) and normal human sera (▽). Microtitre plates were coated with native poly(G) (5 μg/ml).
<table>
<thead>
<tr>
<th>PSS serum</th>
<th>Maximum percent inhibition at 20 µg/ml</th>
<th>Native poly(G)</th>
<th>ROS-poly(G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39</td>
<td>46</td>
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</tr>
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<td>8</td>
<td>46</td>
<td>73</td>
<td></td>
</tr>
</tbody>
</table>

Plates were coated with native poly(G) (5 µg/ml).
Fig. 40. Inhibition of PSS serum autoantibody binding by native and ROS-poly(G). Microtitre plates were coated with native poly(G) (5 μg/ml). (a) PSS sera 1 and 2 by native poly(G) (O, △) and ROS-poly(G) (Δ, ●) respectively. (b) PSS sera 3 and 4 by native poly(G) (O, △) and ROS-poly(G) (Δ, ●) respectively.
Fig. 41. Inhibition of PSS serum autoantibody binding by native and ROS-poly(G). Microtitre plates were coated with native poly(G) (5 μg/ml). (a) PSS sera 5 and 6 by native poly(G) (O, △) and ROS-poly(G) (△, ●) respectively. (b) PSS sera 7 and 8 by native poly(G) (O, △) and ROS-poly(G) (△, ●) respectively.
Fig. 42. Band shift assay of PSS IgG binding to native poly(G). Native poly(G) (5 μg) were incubated with buffer (lane 1) and 30, 60, 90 and 120 μg IgG through lanes 2 to 5, respectively for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose for 2 hr at 30 mA.
Fig. 43. Band shift assay of PSS IgG binding to ROS-poly(G). ROS-poly(G) (5 µg) were incubated with buffer (lane 1) and 30, 60, 90 and 120 µg IgG through lanes 2 to 5, respectively for 2 hr at 37°C and overnight at 4°C. Electrophoresis was performed on 1% agarose for 2 hr at 30 mA.
Fig. 44. Counterimmunoelectrophoresis of SLE anti-DNA autoantibodies with native and ROS-poly(G). Arrow shows the formation of precipitin line. Normal human sera and SLE sera were placed in anodal well (wells 1 and 3, respectively). (a) Native poly(G) (2 µg) and (b) ROS-poly(G) (2 µg) were placed in cathodal well (wells 2 and 4, respectively). Electrophoresis was performed on 0.6% agarose gel for 1 hr at 4 mA.
Fig. 45. Counterimmunoelectrophoresis of PSS serum autoantibodies with native and ROS-poly(G). Arrow shows the formation of precipitin line. Normal human sera and PSS sera were placed in anodal well (wells 1 and 3, respectively). (a) Native poly(G) (2 μg) and (b) ROS-poly(G) (2 μg) were placed in cathodal well (wells 2 and 4, respectively). Electrophoresis was performed on 0.6% agarose gel for 1 hr at 4 mA.
Detection of Immune Complex(es) Deposition in Kidney

Rats immunized with native and ROS-poly(G) induced high titre antibodies as screened by direct binding ELISA (Figs. 46 and 47). Preimmune sera showed negligible binding, whereas, rats immunized with adjuvant only did not show significant binding with native and ROS-poly(G). Immunofluorescence of kidney sections of native and ROS-poly(G) immunized rats shows the deposition of immune complex(es) as revealed by an apple green fluorescence (Figs. 48 and 49). Kidney sections from control rats did not show any fluorescence.
Fig. 46. Direct binding ELISA of immune ( ▲ , △ , ○ , ● ), preimmune ( □ ) and adjuvant immunized ( ▼ , ▼ ) rat serum antibodies to native poly(G). Microtitre plates were coated with native poly(G) (5 μg/ml).
Fig. 47. Direct binding ELISA of immune (▲, △, ○, ●), preimmune (□) and adjuvant immunized (▽, ▼) rat serum antibodies to ROS-poly(G). Microtitre plates were coated with ROS-poly(G) (5 μg/ml).
Fig. 48. Immunofluorescence of kidney sections from rats immunized with native poly(G). Dense granular mesangial IgG deposits with fine granular deposits of IgG involving some capillary walls were seen under fluorescent microscope, using anti-rat-FITC conjugate.
Fig. 49. Immunofluorescence of kidney sections from rats immunized with ROS-poly(G). Immune complexes deposition in the glomerulus were seen under fluorescent microscope, using anti-rat-FITC conjugate.
Discussion
Reactive oxygen species such as hydroxyl radical, superoxide radical, hydrogen peroxide and singlet oxygen are continuously produced during normal cellular metabolism. These highly reactive species are augmented after cellular exposure to ionizing radiation, carcinogens, cellular photosensitizers and certain metal catalysts among others, and have been implicated in a number of human degenerative diseases including aging, cancer (Halliwell, 1994; Knight, 1995; Loft and Poulsen, 1996) and inflammatory autoimmune diseases (Lunec et al., 1994). Amongst ROS, the hydroxyl radical is an extremely reactive oxidizing radical, formed \textit{in vivo} via ionizing radiations (Breen and Murphy, 1995) or through Haber-Weiss or Fenton reactions (Aruoma et al., 1991). These radicals have been known to interact indiscriminately with all components of the DNA molecule. A broad spectrum of DNA damage includes modification of all the four nitrogeneous bases leading to mutations, single and double strand breaks and apurinic sites (Halliwell and Aruoma, 1991).

The lethal and mutagenic effects of ionizing radiation are postulated to be due to damage induced in DNA. A lesion implicated in cell death is the DNA double strand break produced from the passage of a single radiation track (Wolf et al., 1993). The apparently most abundant and certainly the most studied hydroxyl radical attack on DNA involves the C-8 hydroxylation of guanine base, the 8-oxo-7,8-dihydro-2'-deoxyguanosine or 8-hydroxyguanine (8-OH-G) (Dizdaroglu, 1991; 1994). 8-OH-G has been well established as an indicator of oxidative damage to calf thymus DNA exposed to a variety of oxygen generating systems including UV light (Beehler et al., 1992; Rosen et al., 1996).

Human exposure to reactive oxygen species has been implicated in the etiology of a number of human autoimmune diseases, due to the formation of various modified DNA bases: the promutagenic lesion 8-OH-G, in particular, is a major product (Povey et al., 1993). The lesion induces a G to T transversion, suggesting that it is a mutation in DNA that might be a cause for carcinogenesis (Cheng et al., 1992; Musarrat and Wani, 1994; Rosen et al., 1996). Lymphocytes isolated from patients suffering from rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) also have been found to contain increased levels of 8-OH-G in DNA (Bashir et al., 1993). The level was found to be significantly high in SLE patients than in RA patients (Lunec et al., 1994).
In the present study, a ribohomopolymer, polyguanylic acid [poly(G)] was modified by hydroxyl radical, generated by irradiation of hydrogen peroxide with 254 nm UV light. The modification of poly(G) includes the generation of strand breaks and modified guanine base. A substantial decrease in the absorption of poly(G) at $\lambda_{max}$, 253 nm was observed with increasing duration of UV irradiation. This could be attributed to the fact that hydroxyl radicals are being continuously produced by UV irradiation, simultaneously causing damage to poly(G). The hypochromicity of ROS-poly(G) could be a result of structural modification of the guanine base.

UV difference spectra of ROS-poly(G) with reference to native poly(G) shows a negative undefined peak at $\lambda_{max}$, clearly indicating the marked hypochromicity of the modified polymer. This could be due to the structural alterations and modifications in guanine base. The positive peak in the negative range of UV difference spectra, shows the retention of sugar moieties of poly(G) after hydroxyl modifications.

The structure of associated poly(G) has been shown by X-ray diffraction to exist in multistranded form (Zimmerman et al., 1975). Base stacking as well as hydrogen bonding are involved in stabilizing the native structure of poly(G). Poly(G) interacts with poly(C) to form an extremely stable poly(G).poly(C) complex. The change in absorbance ratio ($A_{260}/A_{280}$) from 1.82 in case of native poly(G) to 1.7 for ROS-poly(G) and 2.21 for poly(G).poly(C) to 1.78 for ROS-poly(G).poly(C) complex suggests structural and conformational changes in hydroxyl radical modified poly(G). Circular dichroism spectra showed positive bands at 260 nm, and negative bands at 210 nm, a characteristic feature of A-form helix. There is no shift in the peaks of CD spectra indicating that no conformational transition has occurred in poly(G) after modification. The decrease in ellipticity of ROS-poly(G) indicated the reduced stacking of guanine base as a result of strand breaks.

The thermal behaviour of self associated poly(G) was evaluated from the absorption data obtained by heating the polymer from 30°C to 95°C. UV absorption spectra of native poly(G) showed no considerable change in the absorbance ratio ($A_{260}/A_{280}$) at 30°C and 95°C (1.82 and 1.80, respectively), accounting for its high thermal stability. This is due to its existence in multi-stranded forms. The stacking
of guanine base in poly(G) is the basis of its stability, because it permits to form two hydrogen bonds per base compared with 1.5 bonds per base in the G.C pair of DNA. This additional hydrogen bond significantly enhances stability of the ordered poly(G) helices. The change in absorbance ratio of ROS-poly(G) from 1.7 at 30°C to 1.59 at 95°C, point towards the destabilization of the interaction between guanine base and consequent helix disruption incurred by hydroxyl radical. An extremely high thermal stability of poly(G).poly(C) complex is due to the presence of an additional hydrogen bond between G.C pair of the double stranded homopolymer, compared to A.T pair. The change in absorbance ratio from 1.78 at 30°C to 1.39 at 95°C of ROS-poly(G).poly(C) accounts for the instability of interaction between ROS-poly(G) and poly(C). These studies suggest that hydroxyl radical is somehow imparting instability to the helix in ROS-poly(G).poly(C) complex.

Structural alterations in ROS-poly(G) are caused due to simultaneous scission, resulting in the formation of strand breaks, disruption of hydrogen bonding leading to destabilization of guanine base stacking. These results are in agreement with earlier reports where, photo-ionization of polynucleotides and DNA could lead to the formation of electron loss centres localized at the guanine moiety (Candeias et al., 1992). Strand breaks have been known to be induced in poly(G) by sulphate radical anion (Wolf et al., 1993).

The generation of strand breaks in poly(G) incurred by hydroxyl radical was studied by Sephadex G 200 gel chromatography. Unmodified poly(G) eluted in a single symmetrical peak indicating the ordered form of poly(G). In contrast, ROS-poly(G) eluted in two peaks. The first peak corresponds to the peak obtained for native poly(G) whereas, the second peak constitutes 52.3% of total ROS-poly(G) applied to the column. This additional peak may be attributed to the generation of strand breaks, disruption of hydrogen bonding between guanine bases, leading to the formation of low molecular weight species which eluted as a second peak.

The presence of strand breaks in ROS-poly(G) was further ascertained by densitometric scanning of native and ROS-poly(G) following electrophoresis on 7.5% native acrylamide gel. Low intensity of ROS-poly(G) attribute the destruction of guanine base. High mobility indicate the formation of strand breaks in poly(G).
The observed effects on the mobility of poly(G) under non-denaturing conditions suggest that a difference in the migration between native and modified poly(G) may be a consequence of hydrogen peroxide exposure. The resulting electrophoretic patterns were characterized by fast (modified) and slow (native) moving components, possibly representing strand scission in poly(G). Thus hydrogen peroxide in presence of UV light seems to affect the secondary structure of poly(G).

The agarose gel electrophoretic pattern of native and ROS-poly(G) was further analyzed by their elution profile after extraction from agarose gel. The modified poly(G) showed a shift in peak fraction, thereby implicating the enhanced mobility due to fragmentation of poly(G).

In addition to the alterations within sugar phosphate backbone, hydroxyl radicals have been known to cause extensive damage to guanine moieties of DNA producing 8-hydroxyguanine (Lunec et al., 1994). In order to determine the participation of hydroxyl radical in mediating alterations in guanine residue, the UV spectra of acid hydrolysed native and modified poly(G) were compared. A distinct peak at 273 nm clearly indicated the release of guanine base from the sugar phosphate backbone of poly(G). The broadening of absorption maxima of ROS-poly(G) at 273 nm, in contrast to the sharp peak [of native poly(G)] suggests that hydroxyl radical causes structural perturbations of the guanine residues. Elution profile of acid hydrolysed ROS-poly(G) on DEAE Sephadex A 25 column showed 75.8% modification of guanine base. These results are consistent with earlier studies which demonstrate greater susceptibility of guanine modification by hydroxyl radical leading to the formation of 8-OH-G (Rosen et al., 1996; Tritscher et al., 1996).

Native DNA per se is known to be a poor immunogen (Madio et al., 1934; Stollar, 1986), whereas, double stranded RNA, RNA-DNA hybrids, double helical polydeoxyribonucleotides, ribohomopolymers, poly(G), poly(I), poly(U), DNA modified with drugs, hormones, chromatin or DNA in complexes with DNA binding proteins induce antibodies (Stollar, 1973; 1975; 1986; Anderson et al., 1988; Desai et al., 1993; Moinuddin and Ali, 1994; Arjumand et al., 1995; Hasan et al., 1995; Theofilopoulous, 1995; Arif and Ali, 1996; Arjumand et al., 1997). Recent evidence suggests that DNA from various species differ in their immunological activity and
that bacterial DNA can induce the *in vitro* proliferation of normal murine B cells (Messina *et al.*, 1993). The mitogenic activity of DNA is dependent on sequence-specific determinants that can be presented by synthetic DNA duplexes (GC-rich region) as well as bacterial single stranded and double stranded DNA, suggesting that DNA regions enriched in GC content have enhanced immunological activity. Reactive oxygen species modified DNA have been implicated in the pathogenesis of SLE (Ara and Ali, 1992; 1993; 1995; Ahmad *et al.*, 1997; Cooke *et al.*, 1997). The present studies demonstrated the fine immunogenicity of a ribohomopolymer, polyguanylic acid [poly(G)] and its hydroxyl radical modified counterpart i.e., ROS-poly(G). The unique property of poly(G) is its existence in multistranded form, most probably the quadruple-strand. X-ray diffraction pattern of polyguanylic acid indicated a four-stranded structure (Zimmerman *et al.*, 1975; Simard and Savoie, 1994).

Both the native and ROS-poly(G) were found to be highly immunogenic in rabbits as revealed by direct binding and inhibition ELISA results. Band shift assay clearly demonstrated the absolute specificity of the purified immune IgG towards their respective immunogen. The fine antigenic specificity of purified IgG was confirmed by inhibition ELISA.

A maximum of 67% inhibition of anti-native poly(G) antibody (serum) binding to native poly(G) was observed. Fifty percent inhibition in the antibody activity was achieved with 1.5 μg/ml of poly(G). Purified anti-native poly(G) IgG was inhibited to an extent of 71% and 50% inhibition was observed with only 0.1 μg/ml of native poly(G). The data clearly indicate the higher specificity of purified IgG as compared to serum activity towards native poly(G). ROS-poly(G) showed a maximum inhibition of 57%. Buffalo thymus RNA and calf thymus DNA showed 45% and 41% inhibitions respectively, whereas, their ROS-modified forms showed lower inhibitions of 38% and 30% respectively. The data indicate the specificity of IgG towards the native form of conformers than their ROS-modified forms, preferably recognizing the phosphodiester-ribose backbone than the phosphodiester-deoxyribose backbone. Inhibition studies by a nitrogenous base guanine, double stranded polynucleotides, poly(dA-dT).poly(dA-dT), poly(dA-dU).poly(dA-dU), poly(rG).poly(dC) shows the polyreactivity of the induced antibodies. Higher
inhibitions by unmodified poly(G), DNA, RNA, and guanine as compared to their ROS-modified forms suggest that ROS-modification alters the antigenic determinants. This broad polyspecificity of the induced antibodies with a variety of polynucleotides might be due to the recognition of the phosphodiester-backbone (Ballard and Voss, 1982; Rauch et al., 1985).

Purified anti-ROS-poly(G) IgG showed a maximum inhibition of 77% (64% with serum) with ROS-poly(G). Fifty percent inhibition was attained with only 0.7 μg/ml of ROS-poly(G) (9 μg/ml in case of serum). This indicates the higher specificity of purified immune IgG than serum activity. Inhibition data showed wide crossreactivity of IgG with various nucleic acid polymers. ROS-modified DNA, RNA, poly(I) and guanine showed higher inhibitions (42%, 55%, 45% and 56% respectively) than their unmodified counterparts (39%, 41%, 18% and 21% respectively). The data shows the preferential recognition of ROS-modified part of the polymer, and cross reactivity is due the presence of common epitope, the phosphodiester backbone.

ROS-guanine showed a high inhibitory potential of 56% than native guanine (21%). This indicates that the immune IgG is more specific to the ROS-modified epitopes on guanine. Earlier reports demonstrated guanine to be highly susceptible to 'OH modification leading to the formation of 8-OH-G (Lunec et al., 1994). Modification of guanine by ROS could, therefore, generate potential epitopes against which antibodies are elicited.

Autoantibodies targeted against intracellular proteins and nucleic acids are the serologic hallmarks of the systemic rheumatic diseases, such as systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS), Sjogren's syndrome (SS), mixed connective tissue disease (MCTD), and polymyositis (PM). Each of these diseases are identified by unique autoantibodies (Fritzler, 1996). The present study was designed in view of progressive increase of autoantibodies against polyguanylic acid in systemic lupus erythematosus and progressive systemic sclerosis. Recent studies postulated that ROS-modified DNA elicits a more discriminating antigen for the diagnosis of SLE (Blount et al., 1990; 1991; Ara and Ali, 1992, 1995; Ahmad et al., 1997; Cooke et al., 1997). The detection of 8-OH-G in the immune complex derived DNA of SLE (Lunec et al., 1994) reinforces the evidence that ROS may be involved in SLE.
Two groups of patients with the inflammatory conditions of SLE and PSS were used to study the role of ROS-induced damage to a ribohomopolymer of guanine base, poly(G), in inflammation. Patients with SLE have diverse clinical manifestations including arthritis, vasculitis, skin rashes and renal complications, and the disease is notable for the wide variety of autoantibodies found in serum; anti-double stranded DNA antibodies being the most specific serologic marker in SLE. An additional abnormality of SLE is the presence of circulating immune complexes (Lunec et al., 1994).

Progressive systemic sclerosis is a generalized disorder of the connective tissue characterized by inflammatory, fibrotic, and degenerative changes accompanied by vascular lesions in skin, synovium and certain internal organs. PSS is serologically associated with the emergence of autoantibodies to SCI-70 (DNA topoisomerase I), to centromere/kinetochore protein and to nucleolar epitopes (RNA polymerase I, PM-SCI, U3-RNP) with different incidence and disease specificities (Ebert et al., 1997; Vazquez-Abad et al., 1997).

In the present study, anti-double stranded DNA autoantibodies were screened from patients with SLE by direct binding and inhibition ELISA. An appreciable binding was seen with native DNA and inhibition results show 47% to 68% inhibitions in the anti-DNA autoantibody binding to native DNA in eight different SLE sera. In order to investigate the involvement of guanine residues in the recognition of anti-DNA autoantibodies, native and modified poly(G) were used as competitors. Direct binding ELISA results demonstrate the preferential recognition of ROS-poly(G) over native poly(G) in all SLE serum samples. Native poly(G) showed maximum inhibitions ranging from 31% to 57%, whereas, ROS-poly(G) showed relatively high per cent inhibitions from 37% to 73% in six out of eight SLE sera. Band shift assay further substantiated the binding of native and modified poly(G) with anti-DNA autoantibodies.

The results are consistent with earlier studies, in recognizing the guanine moieties of DNA by anti-DNA autoantibodies (Stollar et al., 1986; Zouali and Stollar, 1986). Furthermore, the preferential recognition of hydroxyl modified poly(G) could indicate the possible presence of 8-OH-G in oxidatively damaged DNA, which may lead to mutations of GC to AT transitions (Emmerit et al.,
1995). Therefore, oxidatively modified DNA having altered base guanine might be triggering immune stimulus and the antibodies thus generated represent circulating anti-DNA autoantibodies in SLE patients, cross reacting with native DNA.

Avian scleroderma of UCD 200 chickens closely resembles human PSS in many pathomorphological, pathogenetic and serological aspects (Haynes and Gershwin, 1984; van de Water et al., 1984; van de Water and Gershwin, 1986; van de Water et al., 1989). The collagen and fibronectin deposition and the composition of the inflammatory infiltrate in the skin as well as quantitative and functional T cell deficiency in peripheral blood lymphocytes in UCD-200 have been demonstrated (Gruschwitz et al., 1991) to resemble human PSS. A progressive increase in autoantibodies to single stranded DNA, histones and less prominently to double stranded DNA have been detected. A similar pattern was also seen for antibodies to poly(G). The presence of anti-cardiolipin antibodies may be involved in the pathogenesis of PSS by causing vascular damage through inhibition of prostacyclin production in vascular endothelium (Carreras and Vermylen, 1982; Schorer et al., 1989).

Serum from PSS patients were investigated for the presence of autoantibodies to poly(G) and ROS-poly(G). Direct binding ELISA results showed the presence of high titre anti-poly(G) autoantibodies in all PSS sera. Furthermore, ROS-poly(G) inhibit the activity of PSS autoantibodies to a better extent than unmodified poly(G). Band shift assay reiterate the binding of native and ROS-poly(G) to purified PSS IgG.

PSS and SLE were further investigated to determine the frequency of single stranded RNA antibodies, particularly the anti-poly(G) autoantibodies. All PSS sera showed the formation of precipitating immune complexes with native and ROS-poly(G) by counterimmunoelectrophoresis, whereas, eight out of twelve SLE sera (67%) were positive by this technique. This could suggest the higher prevalence of anti-RNA antibodies in scleroderma which is due to ribose moiety of RNA and is quite distinct from those of anti-DNA antibodies in SLE (Alarcon-Segovia and Fishbein, 1975).
Anti-DNA autoantibodies are important contributors to glomerular immune deposit formation in individuals with systemic lupus erythematosus (Koffler et al., 1967; Hahn, 1980; Wilson, 1991). It has been suggested that both the site of immune deposit formation and the resulting pathologic and clinical abnormalities may be dependent on properties unique to subsets of anti-DNA autoantibodies (Vlahakos et al., 1992). The lupus nephritis phenotype are dependent on both the inflammatory and antigen binding properties of the pathogenic autoantibody.

Rats immunized with native and ROS-poly(G) induced high titre antibodies. Kidney sections of the immunized rats were used to detect the deposition of immune complexes in the glomerulus. Immunofluorescence revealed the deposition of immune complexes in the glomeruli of the kidney as evident by an apple green fluorescence. The immune complex deposition may cause kidney inflammation and tissue destruction. These findings could suggest that the antibody ligand interactions participate in immune deposit formation in individuals with lupus and the predominant autoantibody autoantigen interaction(s) influence both the morphologic and clinical expression of nephritis.

These results demonstrate that the guanine residue of DNA and RNA are highly susceptible to ROS-mediated damage in the chronic inflammatory disorders, SLE and PSS. Earlier reports have demonstrated the formation of 8-OH-G in oxidatively damaged DNA, in inflammatory disorders including aging and cancer (Knight, 1995; Loft and Poulsen, 1996). Therefore, it could be possible that oxidatively modified guanine base of DNA and RNA might be contributing towards the production of autoantibodies. Based on the above studies, the following conclusion can be drawn.

1. Poly(G) modified with UV light (254 nm) in the presence of hydrogen peroxide resulted in the formation of strand breaks and modification of guanine base.
2. Guanine was modified to an extent of 76%.
3. Both the native and ROS-poly(G) were highly immunogenic in experimental animals.
4. The induced antibodies recognized a variety of native and modified nucleic acid polymers.

5. Anti-native poly(G) antibodies preferentially recognize the native conformations of the polynucleotides as compared to their ROS-modified counterparts.

6. Anti-ROS-poly(G) antibodies are more specific to ROS-modified epitopes than their native forms.

7. The antigen binding behavior of anti-ROS-poly(G) antibodies resembles the binding characteristics of SLE anti-DNA autoantibodies.

8. Anti-DNA autoantibodies showed preferential binding of ROS-poly(G) than native poly(G).

9. ROS-poly(G) as compared to native DNA provides higher inhibitory potential to SLE anti-DNA autoantibodies which points out the presence of unique potential epitopes on modified poly(G), most probably due to the formation of 8-OH-G, a marker of oxidative DNA damage in SLE.

10. Serum from PSS patients preferentially recognize ROS-poly(G) than native poly(G).

11. High frequency of precipitating anti-poly(G) autoantibodies in PSS sera than SLE sera suggest the prevalence of anti-RNA antibodies in scleroderma.

12. Rats immunized with native and ROS-poly(G) induced high titre antibodies.

13. Kidney sections of native and ROS-poly(G) immunized rats showed the deposition of the immune complexes in the glomeruli, by immunofluorescence.

14. The possibility of oxidatively damaged guanine residues of DNA and RNA, acting as a stimuli in inducing lupus anti-DNA autoantibodies and anti-RNA autoantibodies in PSS could not be ruled out.
References


