PREPARATION AND CHARACTERIZATION OF ANTIBODIES AGAINST HYDROXYL RADICAL MODIFIED THYMIDINE MONOPHOSPHATE

ABSTRACT

THESIS
SUBMITTED FOR THE DEGREE OF
Doctor of Philosophy
IN
BIOCHEMISTRY

BY
RIZWAN AHMAD

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE
JAWAHARLAL NEHRU MEDICAL COLLEGE
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)
1998
Systemic lupus erythematosus (SLE) is a prototype autoimmune disease characterised by the production of autoantibodies, in particular antibodies to native double-stranded DNA. These naturally occurring antibodies are heterogenous, exhibiting wide antigen binding characteristics that includes a variety of nucleic acids and other biomolecules. While much has been learned in recent years of the fine specificity and cross reactivity of anti-DNA antibodies in human and murine lupus, neither their production and regulation nor the molecular genetics of their formation is known. Autoimmune phenomena manifested as antibodies to cellular components have also been described in cancer patients. These include anti-nuclear antibodies and antibodies to cytoplasmic antigens. Autoantibodies have been detected in patients with leukemias, malignant melanomas, lung, breast and hepatocellular carcinoma.

Reactive oxygen species are formed constantly in living organisms, as products of the normal metabolism, or as a result of different environmental influences. ROS have been implicated in a number of human degenerative diseases including SLE and cancer. Among the ROS, the 'OH is the most potent and can react with DNA, lipids and proteins. Of the five major DNA components, thymine and cytosine are most susceptible to 'OH damage, followed by adenine, guanine and deoxyribose moiety.

In the present thesis, thymidine monophosphate (TMP) was modified with hydroxyl radical, generated by \( \text{H}_2\text{O}_2 \) and UV light. UV absorption and difference spectra showed the characteristic loss in absorption pattern as a result of the damage caused by hydroxyl radical. Antibodies against TMP and ROS-TMP were induced in rabbits. Both the antigens were found to be non-immunogenic. TMP was conjugated to BSA as a carrier protein by
carbodiimide method to enhance its immunogenicity. TMP-BSA conjugate was characterized by UV spectra and PAGE. The results of PAGE reiterate the formation of conjugate. TMP-BSA conjugate was modified with hydroxyl radical. UV absorption and difference spectra showed hypochromicity in the modified conjugate. The formation of ROS-modified conjugate was analyzed by Sephadex G-100 column chromatography, densitometric scanning and agarose gel electrophoresis. The data conclusively demonstrates structural perturbations and formation of low molecular weight species.

Induced antibodies against TMP-BSA and ROS-TMP-BSA conjugates revealed their high immunogenicity. Antibodies thus formed were purified on Sepharose 4B column. Purified anti-TMP-BSA conjugate IgG showed 95% inhibition (at 10 µg/ml) with TMP-BSA conjugate. Almost similar inhibition of anti-ROS-TMP-BSA conjugate IgG was observed with ROS-TMP-BSA conjugate. The antigen binding characteristics of purified TMP-BSA conjugate IgG was investigated with various nucleic acid conformers. ROS-TMP-BSA conjugate showed a maximum inhibition of 42%. ROS-modified TMP, thymidine and thymine showed higher inhibitions of 69%, 56% and 82% as compared to their native forms. Similarly, ROS-adenine and ROS-guanine show higher inhibition of 57% and 61% than their native counterparts. Poly(dA-dT).poly(dA-dT) showed moderate inhibition while poly(l), ROS-poly(l) and ROS-poly(G) showed insignificant inhibition. Inhibition data showed crossreactivity of anti-ROS-TMP-BSA conjugate IgG with various conformers. The TMP-BSA conjugate gave a maximum inhibition of 89%. ROS-modified TMP, thymidine and thymine showed higher inhibitions (86%, 56% and 81%) than their unmodified counterparts (39%, 23% and 16%). Poly(dA-dU).poly(dA-dU) and poly(dl-dC).poly(dl-dC) showed inhibition of 35% each. ROS-guanine showed a
high inhibitory potential of 78% than native guanine (55%). The broad antigen binding characteristics of induced antibodies with a variety of polynucleotides might be due to recognition of the phosphodiester-backbone.

In the present study, anti-double stranded DNA autoantibodies from SLE patients were investigated for binding to native and modified conjugates. Results with purified SLE IgG showed appreciable binding with native DNA (inhibition varies from 32% to 55%). Results of direct binding ELISA show preferential binding of TMP-BSA conjugate as compared to ROS-modified conjugate. Competition ELISA of purified SLE IgG with TMP-BSA conjugate showed maximum inhibition ranging from 32% to 85% while ROS-modified form show inhibition from 10% to 52%. The data substantiated the results of direct binding ELISA.

The presence of autoantibodies in the sera of cancer patients were also studied. Direct binding ELISA of the serum samples from patients with breast, lung, liver and gall bladder cancer showed higher recognition of ROS-TMP-BSA conjugate as compared to its native form. Tonsil and oral cancer sera recognizes both native and ROS-modified conjugates. Out of the six breast cancer sera tested, two showed moderate inhibition with TMP-BSA conjugate while two gave similar binding with native and ROS-modified conjugates. Among seven serum samples from oral cancer three showed higher inhibition with TMP-BSA conjugate as compared to ROS-modified form. All three liver cancer sera had antibodies with a higher recognition to ROS-TMP-BSA conjugate as compared to its native form. Sera from lung and gall bladder cancer showed preferential recognition with ROS-TMP-BSA conjugate. Out of two sera from larynx one showed higher inhibition with TMP-BSA conjugate and the other with ROS-TMP-BSA conjugate. Purified cancer IgG was investigated for antibodies against
TMP-BSA and ROS-TMP-BSA conjugates. IgG isolated from breast cancer sera showed higher recognition of ROS-modified conjugate over that of native conjugate. One of them showed a maximum inhibition of 62% with ROS-TMP-BSA conjugate. Two IgG isolated each from liver and gall bladder cancer sera showed higher recognition with ROS-TMP-BSA conjugate. Out of the five IgGs isolated from oral cancer sera, four showed higher binding with TMP-BSA conjugate than towards ROS-modified conjugate.

In conclusion, the conjugation of TMP with BSA renders it immunogenic. The antibodies raised against TMP-BSA and ROS-TMP-BSA conjugates were found to be highly immunogenic and are precipitating in nature. The induced antibodies though highly specific for the immunogen, exhibited cross reactivity with various nucleic acid polymers, resembling the binding characteristics of SLE anti-DNA antibodies. TMP-BSA conjugate provides higher inhibitory potential to SLE anti-DNA autoantibodies which might serve as a diagnostic marker for SLE. IgG isolated from sera of breast, liver and gall bladder cancer recognize and bind to ROS-modified TMP-BSA conjugate.
PREPARATION AND CHARACTERIZATION OF ANTIBODIES AGAINST HYDROXYL RADICAL MODIFIED THYMIDINE MONOPHOSPHATE

THESIS
SUBMITTED FOR THE DEGREE OF
Doctor of Philosophy
IN
BIOCHEMISTRY

BY
RIZWAN AHMAD

Dated : ......................
Approved : ......................
....................................
....................................
Prof. Rashid Ali (Supervisor)

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE
JAWAHARLAL NEHRU MEDICAL COLLEGE
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)
1998
AS A TOKEN OF LOVE AND DEEPEST AFFECTION TO MY PARENTS
ACKNOWLEDGEMENTS

I bow in reverence to ALLAH, the Almighty, whose benign benediction gave me the required zeal and confidence to complete my work.

I fail to find words for expressing my deep and profound gratitude to my supervisor, Professor Rashid Ali under whose able guidance the present work was undertaken and completed. In fact, his sound professional approach and encouragement have infused in me the enthusiasm to proceed with this study for which I am thankful to him from the depths of my heart.

I would like to express my sincere gratitude to Dr. Asif Ali Chairman, Department of Biochemistry, Dr. Khurshid Alam, Dr. Najmul Islam and other members of the staff for their timely advice and help.

Thanks are due to Professor Shahid Siddiqui, Chairman, Department of Radiotherapy and his post graduate student Dr. M. Kafeel for providing cancer sera.

I am grateful to my seniors, Drs. B.I. Ashok, Moinuddin, Fahim H. Khan, Aftab Ahmad and Sadia Arjumand for their valuable suggestions and encouragement.

I am privileged to thank my research colleagues Waris, Deepak, Sharique, Rizwan, Aslam, Sajid, Jabeen, Humra, Subia, Kiran, Nabiha and Saba for their help, cooperation and providing me good working environment. I am also grateful to the newcomers of our lab for their help during the last phase of the completion of my thesis.

Thanks are also due to my friends Zeeshan, Abrar, Faiyaz, Arad and Omair and specially Hasseeb for all the help he has rendered to me during the course of my research work. I am highly grateful to him for his cooperation and moral support.

My special thanks are due to my wife, sons and in-laws for the patience and love they showered upon me all along. I owe heartfelt gratitude to my brother Inwan and sister for their unfailing consideration, love and encouragement. I also owe unstinted gratitude to Dr. Asif my brother-in-law for his keen interest in my work and constant affectionate encouragement.

The cooperation of the non-teaching staff of the department, particularly, Mr. Sabu Khan, Mr. Abdul Aleeem and Mr. Rizwan is no less worthy of appreciation. Thanks are also due to Mr. Mansoon and Mr. Wajid of Central photography section, J.N.M.C. Special efforts of Mr. Azad for quick and excellent page-setting is highly appreciated.

Finally, financial assistance from Indian Council of Medical Research, New Delhi is gratefully acknowledged.

(Rizwan Ahmad)

(RIZWAN AHMAD)
I certify that the work presented in the following pages has been carried out by Mr. Rizwan Ahmad and is suitable for the award of Ph.D. degree in Biochemistry of the Aligarh Muslim University, Aligarh.

Rashid Ali
(Rashid Ali)
Professor
Department of Biochemistry
Faculty of Medicine
J.N. Medical College
Aligarh Muslim University
Aligarh - 202002 INDIA
CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>(i)</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>(v)</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>(x)</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>(xi)</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>32</td>
</tr>
<tr>
<td>RESULTS</td>
<td>44</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>125</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>136</td>
</tr>
</tbody>
</table>
ABSTRACT
Systemic lupus erythematosus (SLE) is a prototype autoimmune disease characterised by the production of autoantibodies, in particular antibodies to native double-stranded DNA. These naturally occurring antibodies are heterogeneous, exhibiting wide antigen binding characteristics that includes a variety of nucleic acids and other biomolecules. While much has been learned in recent years of the fine specificity and cross reactivity of anti-DNA antibodies in human and murine lupus, neither their production and regulation nor the molecular genetics of their formation is known. Autoimmune phenomena manifested as antibodies to cellular components have also been described in cancer patients. These include anti-nuclear antibodies and antibodies to cytoplasmic antigens. Autoantibodies have been detected in patients with leukemias, malignant melanomas, lung, breast and hepatocellular carcinoma.

Reactive oxygen species are formed constantly in living organisms as products of the normal metabolism, or as a result of different environmental influences. ROS have been implicated in a number of human degenerative diseases including SLE and cancer. Among the ROS, the \( \cdot OH \) is the most potent and can react with DNA, lipids and proteins. Of the five major DNA components, thymine and cytosine are most susceptible to \( \cdot OH \) damage, followed by adenine, guanine and deoxyribose moiety.

In the present thesis, thymidine monophosphate (TMP) was modified with hydroxyl radical, generated by \( H_2O_2 \) and UV light. UV absorption and difference spectra showed the characteristic loss in absorption pattern as a result of the damage caused by hydroxyl radical. Antibodies against TMP and ROS-TMP were induced in rabbits. Both the antigens were found to be non-immunogenic. TMP was conjugated to BSA as a carrier protein by
carbodiimide method to enhance its immunogenicity. TMP-BSA conjugate was characterized by UV spectra and PAGE. The results of PAGE reiterate the formation of conjugate. TMP-BSA conjugate was modified with hydroxyl radical. UV absorption and difference spectra showed hypochromicity in the modified conjugate. The formation of ROS-modified conjugate was analyzed by Sephadex G-100 column chromatography, densitometric scanning and agarose gel electrophoresis. The data conclusively demonstrates structural perturbations and formation of low molecular weight species.

Induced antibodies against TMP-BSA and ROS-TMP-BSA conjugates revealed their high immunogenicity. Antibodies thus formed were purified on Sepharose 4B column. Purified anti-TMP-BSA conjugate IgG showed 95% inhibition (at 10 μg/ml) with TMP-BSA conjugate. Almost similar inhibition of anti-ROS-TMP-BSA conjugate IgG was observed with ROS-TMP-BSA conjugate. The antigen binding characteristics of purified TMP-BSA conjugate IgG was investigated with various nucleic acid conformers. ROS-TMP-BSA conjugate showed a maximum inhibition of 42%. ROS-modified TMP, thymidine and thymine showed higher inhibitions of 69%, 56% and 82% as compared to their native forms. Similarly, ROS-adenine and ROS-guanine show higher inhibition of 57% and 61% than their native counterparts. Poly(dA-dT).poly(dA-dT) showed moderate inhibition while poly(I), ROS-poly(I) and ROS-poly(G) showed insignificant inhibition. Inhibition data showed crossreactivity of anti-ROS-TMP-BSA conjugate IgG with various conformers. The TMP-BSA conjugate gave a maximum inhibition of 89%. ROS-modified TMP, thymidine and thymine showed higher inhibitions (86%, 56% and 81%) than their unmodified counterparts (39%, 23% and 16%). Poly(dA-dU).poly(dA-dU) and poly(dl-dC).poly(dl-dC) showed inhibition of 35% each. ROS-guanine showed a
high inhibitory potential of 78% than native guanine (55%). The broad antigen binding characteristics of induced antibodies with a variety of polynucleotides might be due to recognition of the phosphodiester-backbone.

In the present study, anti-double stranded DNA autoantibodies from SLE patients were investigated for binding to native and modified conjugates. Results with purified SLE IgG showed appreciable binding with native DNA (inhibition varies from 32% to 55%). Results of direct binding ELISA show preferential binding of TMP-BSA conjugate as compared to ROS-modified conjugate. Competition ELISA of purified SLE IgG with TMP-BSA conjugate showed maximum inhibition ranging from 32% to 85% while ROS-modified form show inhibition from 10% to 52%. The data substantiated the results of direct binding ELISA.

The presence of autoantibodies in the sera of cancer patients were also studied. Direct binding ELISA of the serum samples from patients with breast, lung, liver and gall bladder cancer showed higher recognition of ROS-TMP-BSA conjugate as compared to its native form. Tonsil and oral cancer sera recognizes both native and ROS-modified conjugates. Out of the six breast cancer sera tested, two showed moderate inhibition with TMP-BSA conjugate while two gave similar binding with native and ROS-modified conjugates. Among seven serum samples from oral cancer three showed higher inhibition with TMP-BSA conjugate as compared to ROS-modified form. All three liver cancer sera had antibodies with a higher recognition to ROS-TMP-BSA conjugate as compared to its native form. Sera from lung and gall bladder cancer showed preferential recognition with ROS-TMP-BSA conjugate. Out of two sera from larynx one showed higher inhibition with TMP-BSA conjugate and the other with ROS-TMP-BSA conjugate. Purified cancer IgG was investigated for antibodies against
TMP-BSA and ROS-TMP-BSA conjugates. IgG isolated from breast cancer sera showed higher recognition of ROS-modified conjugate over that of native conjugate. One of them showed a maximum inhibition of 62% with ROS-TMP-BSA conjugate. Two IgG isolated each from liver and gall bladder cancer sera showed higher recognition with ROS-TMP-BSA conjugate. Out of the five IgGs isolated from oral cancer sera, four showed higher binding with TMP-BSA conjugate than towards ROS-modified conjugate.

In conclusion, the conjugation of TMP with BSA renders it immunogenic. The antibodies raised against TMP-BSA and ROS-TMP-BSA conjugates were found to be highly immunogenic and are precipitating in nature. The induced antibodies though highly specific for the immunogen, exhibited cross reactivity with various nucleic acid polymers, resembling the binding characteristics of SLE anti-DNA antibodies. TMP-BSA conjugate provides higher inhibitory potential to SLE anti-DNA autoantibodies which might serve as a diagnostic marker for SLE. IgG isolated from sera of breast, liver and gall bladder cancer recognize and bind to ROS-modified TMP-BSA conjugate.
<table>
<thead>
<tr>
<th>Fig</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 1</td>
<td>Repair products of oxidative DNA damage in human urine.</td>
<td>15</td>
</tr>
<tr>
<td>Fig 2</td>
<td>UV absorption spectra of TMP and ROS-TMP.</td>
<td>45</td>
</tr>
<tr>
<td>Fig 3</td>
<td>UV difference spectra of TMP and ROS-TMP.</td>
<td>46</td>
</tr>
<tr>
<td>Fig 4</td>
<td>UV absorption spectra of TMP and TMP-BSA conjugate.</td>
<td>47</td>
</tr>
<tr>
<td>Fig 5</td>
<td>Polyacrylamide gel electrophoresis of TMP-BSA conjugate.</td>
<td>48</td>
</tr>
<tr>
<td>Fig 6</td>
<td>UV absorption spectra of TMP, TMP-BSA and ROS-TMP-BSA.</td>
<td>50</td>
</tr>
<tr>
<td>Fig 7</td>
<td>UV difference spectra of TMP-BSA and ROS-TMP-BSA conjugates.</td>
<td>51</td>
</tr>
<tr>
<td>Fig 8</td>
<td>UV absorption spectra of TMP-BSA conjugate at 35°C and 95°C.</td>
<td>52</td>
</tr>
<tr>
<td>Fig 9</td>
<td>UV absorption spectra of ROS-TMP-BSA conjugate at 35°C and 95°C.</td>
<td>53</td>
</tr>
<tr>
<td>Fig 10</td>
<td>Fractionation of TMP-BSA and ROS-TMP-BSA conjugates on Sephadex G-100 column.</td>
<td>55</td>
</tr>
<tr>
<td>Fig 11</td>
<td>Densitometric scanning of TMP-BSA and ROS-TMP-BSA conjugates on polyacrylamide gel.</td>
<td>56</td>
</tr>
<tr>
<td>Fig 12</td>
<td>Agarose gel electrophoretic profile of TMP-BSA and ROS-TMP-BSA conjugates.</td>
<td>57</td>
</tr>
<tr>
<td>Fig 13</td>
<td>Immunodiffusion of anti-TMP-BSA and anti-ROS-TMP-BSA conjugate antibodies.</td>
<td>59</td>
</tr>
<tr>
<td>Fig 14</td>
<td>Counterimmunoelectrophoresis of anti-TMP-BSA and anti-ROS-TMP-BSA conjugate antibodies.</td>
<td>60</td>
</tr>
</tbody>
</table>
Fig 15. Direct binding ELISA of antibodies against TMP-BSA conjugate.

Fig 16. Inhibition ELISA of immune sera with TMP-BSA conjugate.

Fig 17. Direct binding ELISA of antibodies against ROS-TMP-BSA conjugate.

Fig 18. Inhibition ELISA of immune sera with ROS-TMP-BSA conjugate.

Fig 19. Elution profile of anti-TMP-BSA conjugate IgG on Protein A-Sepharose column.

Fig 20. Elution profile of anti-ROS-TMP-BSA conjugate IgG on Protein A-Sepharose column.

Fig 21. Elution profile of pre-immune serum IgG on Protein A-Sepharose column.

Fig 22. Direct binding ELISA of anti-TMP-BSA conjugate IgG.

Fig 23. Direct binding ELISA of anti-ROS-TMP-BSA conjugate IgG.

Fig 24. Inhibition of anti-TMP-BSA and anti-ROS-TMP-BSA conjugate IgG binding to their respective immunogens.

Fig 25. Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate by ROS-TMP-BSA conjugate, native ctDNA and ROS-DNA.

Fig 26. Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate by TMP, ROS-TMP, thymidine, ROS-thymidine, thymine and ROS-thymine.

Fig 27. Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate guanine, ROS-guanine, adenine and ROS-adenine.
Fig 28. Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate by Poly(G), ROS-poly(G) and synthetic polynucleotides.

Fig 29. Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate by TMP-BSA conjugate, TMP, ROS-TMP, thymidine and ROS-thymidine.

Fig 30. Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate by thymine, ROS-thymine, guanine, ROS-guanine, adenine and ROS-adenine.

Fig 31. Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate by cytosine, ROS-cytosine, native ctDNA, poly(G) and ROS-poly(G).

Fig 32. Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate by synthetic polynucleotides.

Fig 33. Counterimmunoelectrophoresis of SLE anti-DNA autoantibodies with TMP-BSA and ROS-TMP-BSA conjugates.

Fig 34. Evaluation of anti-DNA autoantibodies in SLE patients.

Fig 35. Evaluation of anti-DNA autoantibodies in SLE patients.

Fig 36. Inhibition of anti-DNA autoantibody binding by nDNA with SLE serum.

Fig 37. Inhibition of anti-DNA autoantibody binding by nDNA with SLE serum.

Fig 38. Binding of SLE serum antibodies to TMP-BSA and ROS-TMP-BSA conjugates.

Fig 39. Elution profile of SLE IgG on Protein A-Sepharose column.

Fig 40. Direct binding ELISA of normal human and SLE IgGs.
Fig 41. Inhibition of anti-DNA autoantibody binding by nDNA with SLE serum.

Fig 42. Inhibition of anti-DNA autoantibody binding by nDNA with SLE serum.

Fig 43. Inhibition of anti-DNA antibody binding by TMP-BSA conjugate.

Fig 44. Inhibition of anti-DNA antibody binding by TMP-BSA conjugate.

Fig 45. Inhibition of SLE anti-DNA autoantibody binding by ROS-TMP-BSA conjugate.

Fig 46. Inhibition of SLE anti-DNA autoantibody binding by ROS-TMP-BSA conjugate.

Fig 47. Binding of breast cancer serum samples to TMP-BSA and ROS-TMP-BSA conjugate.

Fig 48. Binding of oral cancer serum samples to TMP-BSA and ROS-TMP-BSA conjugates.

Fig 49. Binding of tonsil cancer serum samples to TMP-BSA and ROS-TMP-BSA conjugates.

Fig 50. Binding of gall bladder cancer serum samples to TMP-BSA and ROS-TMP-BSA conjugates.

Fig 51. Binding of liver, lung and larynx cancer sera to TMP-BSA and ROS-TMP-BSA conjugates.

Fig 52. Detection of antibodies against TMP-BSA and ROS-TMP-BSA conjugates in breast cancer sera.

Fig 53. Detection of antibodies against TMP-BSA and ROS-TMP-BSA conjugates in oral cancer sera.

Fig 54. Detection of antibodies against TMP-BSA and ROS-TMP-BSA conjugates in liver cancer sera.

Fig 55. Detection of antibodies against TMP-BSA and ROS-TMP-BSA conjugates in lung cancer sera.
Fig 56. Detection of antibodies against TMP-BSA and ROS-TMP-BSA conjugates in gall bladder cancer sera.

Fig 57. Detection of antibodies against TMP-BSA and ROS-TMP-BSA conjugates in tonsil cancer sera.

Fig 58. Detection of antibodies against TMP-BSA and ROS-TMP-BSA conjugates in larynx cancer sera.

Fig 59. Inhibition of antibody binding by TMP-BSA and ROS-TMP-BSA conjugates with breast cancer IgG.

Fig 60. Inhibition of antibody binding by TMP-BSA and ROS-TMP-BSA conjugates with liver cancer IgG.

Fig 61. Inhibition of antibody binding by TMP-BSA and ROS-TMP-BSA conjugates with gall bladder cancer IgG.

Fig 62. Inhibition of antibody binding by TMP-BSA and ROS-TMP-BSA conjugates with oral cancer IgG.
## LIST OF TABLES

<p>| I. Autoimmune diseases and their respective autoantibodies. | 3 |
| II. Diseases/disorders linked to oxygen free radicals. | 13 |
| III. Major exogenous causes of oxidative stress involved in carcinogenesis. | 24 |
| IV. Competitive inhibition data of anti-TMP-BSA antibodies. | 77 |
| V. Competitive inhibition data of anti-ROS-TMP-BSA antibodies. | 82 |
| VI. Titre of SLE anti-DNA autoantibodies. | 87 |
| VII. Competitive inhibition of SLE autoantibodies. | 90 |
| VIII. Competitive inhibition of SLE IgG. | 96 |
| IX. Inhibition of anti-DNA autoantibody binding by TMP-BSA conjugate. | 100 |
| X. Inhibition of anti-DNA autoantibody binding by ROS-TMP-BSA conjugate | 103 |
| XI. Inhibition of circulating antibodies in cancer sera with TMP-BSA and ROS-TMP-BSA conjugates. | 118 |
| XII. Inhibition of circulating antibodies in purified cancer IgGs with TMP-BSA and ROS-TMP-BSA conjugates. | 124 |</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>MBSA</td>
<td>Methylated bovine serum albumin</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide anion radical</td>
</tr>
<tr>
<td>'OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>8-OH-G/8-oxo-G</td>
<td>8-Hydroxyguanine/8-oxoguanine</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>8-Hydroxydeoxyguanosine</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>TMP</td>
<td>Thymidine 5'-monophosphate</td>
</tr>
<tr>
<td>TMP-BSA</td>
<td>Thymidine 5'-monophosphate-bovine serum albumin</td>
</tr>
<tr>
<td>Tg</td>
<td>Thymine glycol</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylene diamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>λ max</td>
<td>Maximum wavelength</td>
</tr>
</tbody>
</table>
INTRODUCTION
The mammalian immune system functions through complex interactions between various cells and their products. Antigen specific responses by the immune system are effected by B and T lymphocytes: T cells mediate cellular immunity (for example, graft rejection) and B cells mediate humoral immunity (antibody production). Other cell types such as macrophages and natural killer cells act non-specifically to eliminate foreign elements. Discrimination between self and non-self is an important feature of the immune system. Normally, immunologic attack is directed against agents which are foreign such as bacteria, virusus, parasites and some internal changes, while it does not respond to self antigens. Since the early studies of Ehrlich, immunologists have demonstrated the presence of autoantibodies in the sera of patients with various diseases. A hallmark of most autoimmune diseases is the presence of autoantibodies against self antigens.

Autoimmunity and Autoimmune Disorders

Grabar (1975) considered autoimmunity as a normal physiological phenomenon, in which the immunological mechanisms are not necessarily 'a defense' but a physiological system of transport of metabolic and catabolic substances, i.e., a 'cleaning up' of the organism of residual products and it is the application and extension of this mechanism which may act as a defense against foreign substances. Autoimmune diseases are those which result from immunologic reactions, humoral and cellular, directed against the individual's own tissue components (Deodhar, 1992). An autoimmune disease may be organ/tissue specific, e.g., Hashimoto's syndrome, thyrotoxicosis, pernicious anemia or non-organ/tissue specific (systemic) such as systemic lupus erythematosus (SLE) and progressive systemic sclerosis (PSS). These may destroy, mimic or enhance the target
and can range in severity from being mild to fatal (Minard, 1989). There is no single theory that can explain all the features of autoimmune diseases. Shoenfeld and Isenberg (1989) suggested that the wide spectrum of autoimmune diseases as the 'mosaic' of autoimmunity with its many factors, like genetic, hormonal, immunological and environmental leading to diverse diseases.

Autoimmune diseases show a highly significant familial predisposition (Arnett, 1992; Hochberg, 1987). Relatives of a patient with a given autoimmune disease are known to be at a high risk for developing the same disease. Also, multiple autoimmune diseases are known to occur in the same patient. These findings suggest involvement of genetic factors which has been linked to the human leucocyte antigen (HLA) system, particularly the HLA-DR sublocus. The HLA genes function as secondary genes to allow expression of specific autoantibody (Bias et al., 1986). Polyclonal B cell activation has been proposed as a possible mechanism that may be responsible for the over activation of B cells and production of autoantibodies in certain autoimmune diseases, particularly SLE (Klinman et al., 1990; Dziarski, 1988). In autoimmune prone individuals, B cells are hyper-responsive to polyclonal activators and undergo initial activation including expression of autoreactive clones, under the influence of exogenous or endogenous polyclonal activators. Various polyclonal B cell activators such as Epstein Barr virus (EBV) and its components, endotoxin or lipopolysaccharide (LPS), certain bacterial agents and drugs may function by bypassing T cell regulatory mechanisms and activating B cells directly. The best example of human autoimmune disease in which such direct polyclonal activation may play a role is autoimmune thyroiditis (Goodman and Weigle, 1981).
**TABLE 1**

<table>
<thead>
<tr>
<th>Autoimmune Diseases and Their Respective Autoantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseases</td>
</tr>
<tr>
<td><strong>SLE and related diseases</strong></td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Scleroderma (PSS)</td>
</tr>
<tr>
<td>Sjogren’s syndrome</td>
</tr>
<tr>
<td>Mixed connective tissue disease</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Other autoimmune diseases</strong></td>
</tr>
<tr>
<td>Autoimmune thyroid diseases</td>
</tr>
<tr>
<td>Addison’s disease</td>
</tr>
<tr>
<td>Pernicious anemia</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
</tr>
<tr>
<td>Mysthenia gravis</td>
</tr>
<tr>
<td>Pemphigus vulgaris substance</td>
</tr>
<tr>
<td>Bullous pemphigoid</td>
</tr>
<tr>
<td>Autoimmune hemolytic anemia</td>
</tr>
<tr>
<td>Multiple sclerosis and relating de-myelinating diseases</td>
</tr>
</tbody>
</table>

(adapted from Deodhar, 1992)
The immune response is controlled mainly by the regulatory influence of T-lymphocyte subsets, T-helper and T-suppressor cells. In most autoimmune diseases, there is a significant decrease in T-suppressor cell numbers and activity, which accounts for the increased T-helper/T-suppressor cell ratio. For example, in SLE the prototype of autoimmune diseases, there is a significant decrease in T-suppressor cells during active phases of the disease (Deodhar, 1992). The increased T-helper/T-suppressor cell ratio has been noted in a wide variety of autoimmune diseases, such as SLE, Sjogren's syndrome, progressive systemic sclerosis, rheumatoid arthritis, pernicious anemia, multiple sclerosis, immune complex mediated renal diseases and many others.

In acquired immune deficiency syndrome (AIDS), the presence of autoantibodies in respective autoimmune diseases have been demonstrated in recent years (Calabrese, 1988). The autoantibodies associated with HIV infection have included anti-nuclear, rheumatoid factor, anti-cardiolipin, anti-platelet, anti-red blood cell and anti-lymphocyte antibodies. The mechanisms by which viruses and other infectious agents initiate autoimmune phenomena are probably complex and it may include structural similarity, increased HLA expression, polyclonal B cell activation and alteration of the antigen (Shoenfeld and Isenberg, 1989). Hormonal factors such as sex hormones, thymic hormones and corticosteroids play a significant role. 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). The female relatives of patients with certain autoimmune diseases have been reported to demonstrate a significant prevalence of autoantibodies and suppressor T cell defects (Miller and Schwartz, 1982).
Tolerance is a state of immunologic non-responsiveness to a substance that would be expected to evoke an immune response. Tolerance provides an essential mechanism for the prevention of self-injury. Tolerance is accomplished through three mechanisms: thymic education, thymic deletion and peripheral tolerance. Thymus is the major site for 'self-nonself' discrimination. It is here that the T cell repertoire is determined. Some cells are programmed to die (negative selection) while others are not (positive selection) (Adorini, 1993). Errors in central or peripheral tolerance at the T or B cell level have also been suggested as probable causes for autoimmunity. It has also been suggested that thymus is the critical time keeper with aging process with respect to immune responses (Rose, 1994). As the thymic cortex atrophies, the response to self antigen rises, generating the aging paradox.

**Antigenicity of DNA**

Native DNA has not been found an effective immunogen in normal experimental animals but antibodies that react with B-conformation (native DNA) are found in individuals with autoimmune diseases, most commonly in systemic lupus erythematosus (SLE) (Stollar, 1989; Karounos et al., 1988; Tan, 1982). Haskowa et al. (1959) immunized rabbits with DNA of thymus or Ehrlich ascites tumor cells, no antibodies were detected by complement fixation, gel diffusion or passive hemagglutination. Wilson et al. (1965) found that 36 rabbits failed to respond to calf thymus DNA. Negative results were also obtained on the injection of purified native or denatured salmon, calf thymus, phage T4 or E. coli DNA (Levine and Stollar, 1968; Barnett and Vaughan, 1966; Plescia et al., 1964; Pasternak et al., 1960).
The specificity of anti-DNA antibodies has been analysed in order to understand the nature of epitope responsible for their production (Stollar, 1986). Anti-DNA antibodies react to a variety of determinants on both single stranded (ss) and double stranded (ds) DNA (Diamond et al., 1992; Tillman, 1992; Shlomchik et al., 1990; Marion et al., 1989). Although, the precise mechanism of anti-dsDNA antibody production remains unknown, studies point to a role of DNA antigen drive (Radic et al., 1989). Anti-dsDNA antibodies display pattern of variable (V) region gene utilization that resemble conventional responses in the content of somatic mutations (Hirose et al., 1993; O'Keefe et al., 1992; Shlomchik et al., 1987). Since these mutations are associated with enhanced binding, to DNA, it is likely that DNA is the selecting antigen in vivo. Among structural features associated with dsDNA binding, a high content of arginine residues in the third complementary determining region (CDR3) of the heavy chain appears important (Shlomchik et al., 1990 &1987).

Recent studies have, however, shown that DNA complexes with a synthetic peptide, Fus-1 can induce an anti-dsDNA response in mice (Desai et al., 1993). Anti-DNA antibodies may also result by autoimmunization with chromatin, rather than native DNA (Theofilopoulos, 1995). Immunization of experimental animals with denatured DNA, synthetic polynucleotides like poly(dT), poly(dC), poly(A), poly(I), poly(G), double stranded RNA, left handed Z-DNA and chemically modified DNA can induced immune responses (Anderson et al., 1988; Stollar, 1986). The immunogenicity of certain dsDNA structures has been well documented in animal models. Z-DNA, a left handed helix, induces a significant antibody response in experimental animals (Madaio et al., 1984). The induced antibodies are highly specific for Z-DNA and do not
bind to right handed B-DNA (Bunyard and Pisetsky, 1994). Antibodies reactive with left handed Z-DNA arise spontaneously in the sera of patients with SLE and rheumatoid arthritis and in auto-immune MRL/1pr mice (Pisetsky et al., 1990).

DNA from various bacterial species elicit high titer responses in normal mice when immunized as complexes with methylated bovine serum albumin (MBSA) (Gilkeson et al., 1989a & b). Studies have shown that bacterial DNA differs from mammalian DNA in its content of pyrimidine clusters, patterns of base methylation and in many of its coding sequences (Wells, 1988; Cheng et al., 1985; Szybalski et al., 1966). Together these features could create local regions in bacterial DNA that are structurally distinct and rarely present in mammalian DNA. The DNA from two bacterial species, Micrococcus lysodeiktus and Staphylococcus epidermidis, was recognised by normal human sera. This finding was in contrast to normal dogma that anti-DNA antibodies are prevalent in SLE alone (Fredriksen, 1991). 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 (Pisetsky, 1997; Pisetsky and Drayton. 1997; Tsujimura et al., 1997; Kay et al., 1988). This pattern of isotype expression suggests that the responses to foreign DNA are distinct from the lupus anti-DNA response, which appears to be T cell dependent (Robertson et al., 1992).

**Free Radical Biochemistry**

A free radical is any species capable of independent existence that contains one or more unpaired electrons. It is now established that free radicals and other reactive oxygen species are continuously produced in
In vivo (Halliwell, 1993). In consequence, organisms have evolved not only antioxidant defense systems to protect against them, but also repair systems that prevent the accumulation of oxidatively-damaged molecules (Sies, 1991 & 1985; Fridovich, 1989; Halliwell and Gutteridge, 1989).

The term ‘reactive oxygen species’ (ROS) is a collective one that includes not only oxygen-centered radicals such as superoxide (O\textsubscript{2}\textsuperscript{-}) and hydroxyl (\textsuperscript{·}OH), but also some non-radical derivatives of oxygen (O\textsubscript{2}), such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), singlet oxygen (\textsuperscript{1}O\textsubscript{2}), hypochlorous acid (HOCl) and ozone (O\textsubscript{3}) (Halliwell, 1993). Most molecules are non-radicals. When radicals react with non-radicals, new radicals are generated. Only when two radicals meet and join their unpaired electrons, are the radicals lost. The usual major route for metabolism of molecular oxygen involves its complete reduction to H\textsubscript{2}O by accepting four electrons (Thornalley and Bannister, 1985; Fridovich, 1978; Freiden and Osaki, 1970). However, with one electron reduction, several free radicals and H\textsubscript{2}O\textsubscript{2} can be formed (Byung, 1994).

\[
\begin{align*}
O_2 + e^- &\rightarrow O_2^- \quad \text{superoxide radical} \\
O_2 + H_2O &\rightarrow HO_2 + OH^- \quad \text{hydroperoxyl radical} \\
HO_2 + e^- + H &\rightarrow H_2O_2 \quad \text{hydrogen peroxide} \\
H_2O_2 + e^- &\rightarrow \cdot OH + OH^- \quad \text{hydroxyl radical}
\end{align*}
\]

**Superoxide Radical**

The free radical, superoxide anion (O\textsubscript{2}\textsuperscript{-}) is formed by the addition of one electron to ground state dioxygen. It is unstable in aqueous solutions, due to its spontaneous reactivity with itself producing hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and molecular oxygen (O\textsubscript{2}) (dismutation reaction) (Cadenas, 1989;
Halliwell and Gutteridge, 1984; Fridovich, 1983). The superoxide radical in its protonated form is known as perhydroxyl radical (HO₂⁺), which exhibits even higher reactivity.

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

**Hydrogen Peroxide**

Hydrogen peroxide is the protonated form of peroxide ion (O₂²⁻), formed by the reduction of two electrons of molecular oxygen. Although, H₂O₂ by definition is not considered an oxygen free radical, it nevertheless remains the most extensively studied oxygen metabolite (Harris, 1992). The dismutation of O₂⁻ by superoxide dismutase is a major source of H₂O₂.

\[ \text{SOD} \]

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

H₂O₂ is very harmful to cells because it may cross biological membranes and can also lead to the formation of highly reactive hydroxyl radical (·OH) (Aruoma et al., 1991; Pryor and Church, 1991; Pryor, 1986; Mello-Filho et al., 1984; Jones et al., 1981).

**Hydroxyl Radical**

Hydroxyl radical is an extremely reactive chemical species which can react with any biological molecule. Both, its half life (fraction of microseconds) and radius of action (30°A) are very small. The O₂⁻ and H₂O₂ are less reactive oxidants than ·OH but they have a longer life time which allows them to react with molecules in locations far from the site of their production (Pryor, 1986; Halliwell and Gutteridge, 1984). The hydroxyl radical is produced in living systems by at least two mechanisms: reaction of transition metal ions with H₂O₂ and homolytic fission of water due to its background exposure to ionizing radiation (von Sonntag, 1987). First.
hydroxyl radicals are derived from the decomposition of hydrogen peroxide via the Fenton reaction (Fenton, 1894)

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-$$

and second, by the interaction of superoxide with hydrogen peroxide through the Haber-Weiss reaction (Haber and Weiss, 1934).

$$\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O} + \text{OH}$$

**Free Radical Production in Biological Systems**

Oxygen free radicals are being continuously produced in organisms and many of them are necessary to carry out certain biological reactions (Martinez-Cayuela, 1995). In addition to exogenous sources of free radicals such as ionizing radiation, tobacco smoke, pesticides and pollutants, oxygen free radicals are also produced by intracellular systems.

The autoxidation of small soluble molecules in the cellular cytoplasm may lead to the production of oxygen free radicals by concomitant $\text{O}_2^-$ reduction. Examples include catecholamines, flavins, tetrahydroproteins, quinones and thiols (Proctor and Reynolds, 1984; Fridovich, 1978). Some cytoplasmic enzymes generate oxygen free radicals as products of their catalytic cycles e.g., xanthine oxidase and aldehyde dehydrogenase (Southern and Powis, 1988; Freeman and Grapo, 1982; Rajagopalan, 1980). Hemoglobin may be oxidized producing oxygen free radicals. However, a small quantity of oxyhemoglobin is only transformed into methemoglobin due to the action of methemoglobin reductases (Valenzuela and Videla, 1989; Clark and Cowden, 1985; Halliwell and Gutteridge, 1984; Proctor and Reynolds, 1984). Reactions catalysed by lipoxygenase and cyclooxygenase in the synthetic pathway of leukotrienes.
thromboxanes and prostaglandins involve oxygen free radical production (Blake et al., 1987; Mottley et al., 1982; Mason et al., 1980). These radicals, which deactivate the cyclooxygenase enzyme, can be an important feedback control of prostaglandin synthesis. A main source of $O_2^{\cdot-}$ is the respiratory burst of phagocytic cells when they are activated. This process is due to an enzyme, reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, located on the external surface of the plasmic membrane.

Cytochrome c oxidase from the mitochondrial electron transport system catalyzes molecular oxygen reduction to $H_2O$ by acquiring four electrons and four protons. Autoxidation of ubiquinone and NADH dehydrogenase produces superoxide radicals (Beyer, 1990; Freeman and Grapo, 1982; Turrens and Boveris, 1980). Cytochromes P450 and b5 of the microsomic electron transport systems generate oxygen free radicals. During the catalytic cycle of these cytochromes, superoxide radicals may be formed (Sevanian et al., 1990; Valenzuela and Videla, 1989; Rush and Bielski, 1985). Cytochrome reductases involved in redox reactions of cytochromes P450 and b5 can also produce superoxide radicals and $H_2O_2$ when they undergo autoxidation (Sevanian et al., 1990; Freeman and Grapo, 1982).

**DNA Damage and its Repair**

The air we breathe is a double edged sword. It is of course, fundamental for life; yet, many intracellular reactions in which it participates results in the formation of oxygen-derived free radicals. These highly reactive electrophilic chemical species can not only permanently damage cells by reacting with nucleic acids, proteins and polyunsaturated lipids (lipid peroxidation) but may also lead to cell death (Knight, 1995).
The endogenous reactions that are likely to contribute to DNA damage are oxidation, methylation, depurination and deamination (Ames, 1989). The chemistry of DNA damage by several ROS has been well characterized in vitro (Box et al., 1995; Epe, 1993; Steeken, 1989; von Sonntag, 1987). Different ROS affect DNA in different ways e.g. \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) do not react with DNA bases at all (Halliwell and Aruoma, 1991), while \( \cdot\text{OH} \) generates a multiplicity of products from all four DNA bases (Halliwell and Aruoma, 1991). Of the five major DNA components, thymine and cytosine are most susceptible to \( \cdot\text{OH} \) damage, followed by adenine, guanine and deoxyribose moiety (Saul et al., 1987). 8-hydroxyguanine (8-OH-G), is considered to be an excellent marker of oxidative DNA damage (Tritscher et al., 1996; Lunec et al., 1994; Shibutani et al., 1991), due to its high mutagenic potential (Musarrat and Wani, 1994; Guyton and Kensler, 1993; Kasai and Nishimura, 1991; Ames and Gold, 1991). It has been shown that 8-hydroxydeoxyguanine causes GC—\( \rightarrow \)AT base pair substitutions (Kuchino et al., 1987), which can result in the activation of human \( C-\text{Ha-ras}-1 \) oncogene (Kamiya et al., 1992).

There is evidence to implicate oxygen free radical damage in the etiology of many chronic health problems such as emphysema, cardiovascular and inflammatory diseases, cataracts and cancer (Machlin and Bendich, 1987). Table II presents a list of diseases that involve radical reactions in mammalian systems (Knight, 1995). The data from studies on humans indicate that the important determinants of oxidative damage rate includes tobacco smoking, oxygen consumption and some inflammatory diseases, whereas diet composition, energy restriction and antioxidant supplements have a minimal influence (Brown et al., 1995; Lunec et al., 1994; Bashir et al., 1993; Tagesson, 1992).
TABLE II

**Diseases / Disorders Linked to Oxygen Free Radicals**

<table>
<thead>
<tr>
<th>No.</th>
<th>Disorder</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ageing</td>
</tr>
<tr>
<td>2.</td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td></td>
<td>a) heart disease</td>
</tr>
<tr>
<td></td>
<td>b) stroke</td>
</tr>
<tr>
<td>3.</td>
<td>Brain disorders</td>
</tr>
<tr>
<td></td>
<td>a) hyperbasic oxygen</td>
</tr>
<tr>
<td></td>
<td>b) aluminium toxicity</td>
</tr>
<tr>
<td></td>
<td>c) neuronal lipofuscinoses</td>
</tr>
<tr>
<td></td>
<td>d) neurotoxins</td>
</tr>
<tr>
<td>4.</td>
<td>Cancer</td>
</tr>
<tr>
<td>5.</td>
<td>Cardiac myopathy</td>
</tr>
<tr>
<td></td>
<td>a) Keshan disease</td>
</tr>
<tr>
<td>6.</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>7.</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>8.</td>
<td>Eye disorder</td>
</tr>
<tr>
<td></td>
<td>a) macular degeneration</td>
</tr>
<tr>
<td></td>
<td>b) cataractogenesis</td>
</tr>
<tr>
<td>9.</td>
<td>Inflammatory disorders</td>
</tr>
<tr>
<td>10.</td>
<td>Lung disorders</td>
</tr>
<tr>
<td></td>
<td>a) asbestosis</td>
</tr>
<tr>
<td></td>
<td>b) oxygen toxicity</td>
</tr>
<tr>
<td></td>
<td>c) emphysema</td>
</tr>
<tr>
<td>11.</td>
<td>Nutritional deficiencies</td>
</tr>
<tr>
<td>12.</td>
<td>Radiation injury</td>
</tr>
<tr>
<td>13.</td>
<td>Repurfusion injury</td>
</tr>
<tr>
<td>14.</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>15.</td>
<td>Skin disorders</td>
</tr>
<tr>
<td></td>
<td>a) solar radiation</td>
</tr>
<tr>
<td></td>
<td>b) burns</td>
</tr>
<tr>
<td></td>
<td>c) contact dermatitis</td>
</tr>
<tr>
<td></td>
<td>d) Bloom syndrome</td>
</tr>
<tr>
<td>16.</td>
<td>Toxic states</td>
</tr>
<tr>
<td></td>
<td>a) xenobiotics</td>
</tr>
<tr>
<td></td>
<td>b) metal ions</td>
</tr>
</tbody>
</table>

(adapted from Knight, 1995)
Oxidative damage to DNA has been proposed to be an important factor in carcinogenesis, a suggestion supported by experimental studies on animals and in vitro (Loft and Poulsen, 1996; Ames et al., 1995; Fraga et al., 1990; Lunec, 1990; Ames, 1989). The products of repair of these lesions are excreted in the urine and the rate of excretion of repair products in terms of oxidized bases reflect the average rate of oxidative DNA damage in the body (Loft and Poulsen, 1998). Among the possible repair products from oxidative DNA modification, 8-oxo-2'-deoxyguanosine (8-oxodG), 8-oxoguanine (8-oxoGua), thymine glycol (Tg), thymidine glycol (dTg) and 5-hydroxymethyl uracil (5-OHmU) have so far been identified in urine (Fig. 1) (Loft and Poulsen, 1996; Suzuki et al., 1995; Faure et al., 1993; Shigenaga et al., 1989; Cathcart et al., 1984). The levels of oxidized bases in lymphocyte DNA or other accessible cells will reflect the steady state levels i.e., the balance between damage and repair (Loft and Poulsen, 1998).

Oxidative DNA damage is repaired in vivo by a variety of enzymes. Strand breaks are annealed and modified bases are excised as such or as nucleotides (Demple and Harrison, 1994; Ramoter and Demple, 1993). DNA glycosylases excise bases and subsequently phosphodiester bonds on each side of the abasic site are incised by endonucleases, allowing insertion of an intact nucleotide (Loft and Poulsen, 1996). Some enzymes such as endonuclease III possess both glycosylase and endonuclease activities for repair of oxidized pyrimidines. The repair products of this excision, including thymidine and hydrouracil are excreted into the urine. The formamidopyrimidine DNA glycolylase enzyme (Fpg; mutM) in E. coli repairs the oxidized purines, 8-oxoguanine and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine and to a lesser extent corresponding adenine derivatives (Boiteux et al., 1992). The formamidopyrimidine-DNA
Fig. 1 Products of repair of oxidative DNA damage identified in human urine.
glycosylase protein appears to repair 8-oxoG in noncoding and actively transcribed mammalian DNA sequences with equal efficiency (Bohr et al., 1995). The Uvr ABC complex in E. coli repair some oxidative DNA lesions by excision of 11-13 nucleotides, including a damaged base such as Tg, 8-oxoguanine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine as well as abasic sites (Czeczot et al., 1991). Oxidized nucleosides and nucleotides from the cellular pools may be incorporated into DNA and lead to AT → GC base substitution mutations in case of 8-oxoGTP (Kamiya et al., 1992; Shibutani et al., 1991). In E. coli the mut M and mut T repair enzymes cooperate functionally with mut Y, an enzyme which removes adenine mis-inserted opposite 8-oxoguanine in DNA (Tajiri et al., 1995).

Double strand breaks and DNA-protein crosslinks formed by oxygen radicals are repaired either by homologous recombination or by non-homologous end joining (Henle and Linn, 1997). In homologous recombination, double strand breaks are initially processed by degrading the 5 ends to reveal 3-OH single strand overhangs (Shinohara and Ogawa, 1995). In mammalian cells, double strand breaks are predominantly repaired by non-homologous end joining and it seems that this mode of repair is mediated by V(D)J system, which rejoins blunt double strand breaks (Jackson and Jeggo, 1995).

**Biological Antioxidant Defenses**

Substances that neutralize the potential ill effects of free radicals are generally grouped in the so called antioxidant defense system (Cutler, 1984). Such a system possesses many substances which are often called as antioxidants, free radical scavengers, chain terminators or reductants (Heffner and Repine, 1989; Cutler, 1984). Aerobic organisms have potent
antioxidant defenses whose role is to neutralize and minimize the cytotoxic effects of reactive oxidants. The defenses that directly scavenge $O_2^-$, $H_2O_2$ and $^\cdot OH$ are known as primary antioxidant defences. There are also secondary antioxidant defenses and consist of repair mechanisms which act on biomolecules that have undergone oxidative damage.

a) Enzymatic antioxidant defenses

This group includes superoxide dismutase, catalase and glutathione peroxidase.

i) Superoxide dismutase - The enzyme superoxide dismutase (SOD) catalyses the dismutation of $O_2^-$ to $H_2O_2$ (Fridovich, 1989).

$$\text{SOD} \quad O_2^- + O_2^- \rightarrow H_2O_2 + O_2$$

SOD exists in virtually all $O_2$-respiring organisms, its activity varying among the tissues. The highest levels are seen in kidney, liver, adrenal gland and spleen.

ii) Catalase - Catalase is a heme protein that decomposes $H_2O_2$ to $O_2$ and $H_2O$.

$$\text{Catalase} \quad 2 \ H_2O_2 \rightarrow O_2 + 2 \ H_2O$$

It is present in the cytosol, mitochondria and other organelles but is difficult to detect in an extracellular environment.

iii) Glutathione peroxidase - The selenium based enzyme, glutathione peroxidase, reduces $H_2O_2$ by catalysing its reaction with the reduced form of glutathione (GSH).

$$H_2O_2 + 2 \ GSH \xrightarrow{\text{peroxidase}} 2 \ H_2O + \text{GSSG} \quad \text{(oxidized glutathione)}$$
Normally, most of the intracellular glutathione is in its reduced form. Increased intracellular concentrations of $H_2O_2$ results in a drop in GSH/GSSG ratio, which serves to detect intracellular oxidative stress (Tribble and Jones, 1990).

b) Non-enzymatic antioxidant mechanisms

There are no enzymatic mechanisms to directly protect against $'OH$ because of its extremely high reactivity and rapid consumption. Alternatively, cells possess non-enzymatic antioxidant mechanisms which scavenge $'OH$. Some of these scavengers are in hydrophilic phase (ascorbate, urate, glutathione) and others in the lipid phase ($\alpha$-tocopherol and $\beta$-carotene). Vitamin E ($\alpha$-tocopherol) is a key $'OH$ scavenger and a chain breaking antioxidant in biological membranes (Niki et al., 1988). It reacts with OH or most commonly with lipid peroxyl radicals (LOO⁻) to form the $\alpha$-tocopherol radical. Another antioxidant of importance in biological membranes is ubiquinol-10 (coenzyme $Q_{10}$). Ubiquinol-10 is as efficient as vitamin-E in chain-breaking antioxidant reactions. It is present in inner mitochondrial membrane where it also functions as an electron carrier in the respiratory chain.

The various defenses are complementary to each other because they metabolize or scavenge different species in different cellular compartments. Dietary or pharmacological enhancement of endogenous antioxidant defenses may be beneficial in disease or aging processes where oxygen radicals are involved.
Cancer

Cancer is the most common term for all malignant tumors. It derives from the Latin for crab, 'Cancer' presumably because a cancer adheres to any part that it seizes upon in an obstinate manner like the crab. The growth of cancers is accompanied by progressive infiltration, invasion and destruction of the surrounding tissue. Cancer cells possess an insidious property to migrate from the site where they originate and form masses at distant sites in the body (Weinberg, 1996). Cancer in all forms are causing about 12 percent of deaths throughout the world. In the developed countries, cancer is the second leading cause of death, next to cardiovascular diseases, accounting for 21 percent (2.5 million) of mortality. In developing countries, it ranks third as a cause of death and accounts for 9.5 percent (3.8 million) of all deaths. According to WHO estimates, by the year 2000 the number of cancer deaths may go up to 8 million annually (The World Health Report, 1997). Among men, the leading eight killer sites for cancer are the lungs, stomach, liver, colon-rectum, oesophagus, mouth-pharynx, prostate and lymphoma. In women, they are cancers of the breast, stomach, colon-rectum, cervix, lungs, ovary, oesophagus and liver (WHO, The World Health Report, 1998).

The changes in DNA like base modifications, rearrangement of DNA sequence, miscoding of DNA lesion, gene duplication and the activation of oncogenes may involve the initiation of various cancers (Cavenee and White, 1995). Mutation in several critical genes can lead to tumors (Vogelstein et al., 1989), for example, mutations in the tumor suppressor gene, p53 are found in about half of human tumors. Mutations can convert proto-oncogenes into carcinogenic oncogenes and studies have revealed the presence of human c-oncogenes with several being active
(Marshall, 1985). These genes were found to be mutated, rearranged or unusually active in many viral and non-viral tumors. Retroviruses lacking c-oncogenes are also oncogenic, giving rise to tumors more slowly than those with v-oncogenes (Weiss, 1986).

Cell division is a critical factor in mutagenesis, because when the cell divides a DNA lesion can give rise to a point mutation, deletion or translocation (Ames et al., 1993; Cohen et al., 1991; Ames and Gold, 1990). With increase in cell division, there is increased risk for cancer, which can be caused by such diverse agents as increased levels of particular hormones (Handerson et al., 1982), excess calories, chronic inflammation or chemicals at doses causing cell division (Cunningham et al., 1994; Columbano et al., 1990; Moalli et al., 1987).

Alcoholic beverages cause inflammation and cirrhosis of the lung and liver (IARC, 1988). Alcohol is an important cause of oral and oesophageal cancer (IARC, 1988) and possibly contributes to colorectal cancer (Giovannucci et al., 1995). Hepatitis B and C viruses are a major cause of chronic inflammation leading to liver cancer, which is one of the most common cancers in Asia and Africa (Tabor and Kobayashi, 1992; Yu et al., 1991). The mutagenic mold toxin aflatoxin, may cause chronic hepatitis infection in liver cancer development (Qian et al., 1994). Some cancer chemotherapeutic drugs, particularly the alkylating agents, cause malignancies, most commonly leukemias, lymphomas and sarcomas (Ellis and Lisher, 1993; Curtis et al., 1992). Potent immuno-suppressive agents such as cyclosporin also increase the risk of a variety of cancers (Ryffel, 1992).

Epidemiological evidence indicates that avoidance of smoking, increased consumption of fruits and vegetables and control of infections
will have a major effect in reducing the rates of cancer (Ames et al., 1995). Other factors include avoidance of intense sun exposure, increase in physical activity and reduction of alcohol consumption (Ames et al., 1995)

**Role of Oxygen Free Radicals in Cancer Development**

Oxygen free radicals are continuously generated in cells exposed to an aerobic environment. A number of endogenous and exogenous cancer risk factors generate free radicals *in vivo* (Swartz, 1972). In recent years convincing evidence has accumulated indicating that oxygen free radicals are indeed a relevant class of carcinogens (Cerutti, 1994; Feig et al., 1994; Guyton and Kensler, 1993). Cancer development is now commonly recognised as a microevolutionary process that requires the cumulative action of multiple events (Klein, 1987). These events can be described in a simplified three-stage model: 1) the induction of DNA mutation in a somatic cell (initiation), 2) the stimulation of tumorigenic expansion of the cell clone (promotion) and 3) the malignant conversion of the tumor into cancer (progression). Oxygen free radicals can stimulate cancer development at all three stages, initiation (Husain et al., 1994), promotion (Nakamura et al., 1988) and progression (Salim, 1993).

With respect to cancer, DNA is considered to be the most important target of ROS (Ames et al., 1995; Feig et al., 1994). Oxidative damage to DNA includes a range of specifically oxidised purines and pyrimidines as well as alkali labile sites and strand breaks, formed directly or by repair processes (Breen and Murphy, 1995; Dizdaroglu, 1994). Floyd et al. (1986) supplied the earliest *in vitro* evidence for a DNA molecular alteration by a free radical-generating tumor promoter. They have successfully isolated a product of oxidatively damaged DNA, 8-OHdG. To
determine whether such radical adducts are involved in carcinogenesis, Kasai et al. (1987) carried out an in vivo experiment in which superoxide-producing KBrO₃ was administered to animals. The 8-OHdG was detected in the kidney, confirming possible tumorigenesis by O₂⁻. Additional evidence on DNA damage in human fibroblasts was supplied by Kaneko and Leadon (1986) who detected the production of thymine glycols in DNA by the reaction of N-hydroxy-2-napthalamine. Elevated levels of modified bases in cancerous tissue may be due to the production of large amounts of H₂O₂, which has been found to be characteristic of human tumor cells (Szatrowski and Nathan, 1991). Further, evidence exists that tumor cells have abnormal levels and activities of antioxidant enzymes, such as superoxide dismutase or catalase, leading to accumulation of O₂⁻ and H₂O₂ that induces damage to DNA (Olinski et al., 1992).

In patients with diseases associated with increased risk of cancer, including Fanconi anemia, chronic hepatitis, cystic fibrosis and various autoimmune diseases, studies indicate an increased rate of oxidative DNA damage or in some instances deficient repair (Brown et al., 1995; Hagen et al., 1994; Shimoda et al., 1994; Takeuchi and Morimoto, 1993). Human studies support the experimentally based notion of oxidative DNA damage as an important mutagenic and carcinogenic factor (Loft and Poulsen, 1996). ROS can damage DNA and the division of cells with unrepaired or misrepaired damage leads to mutations. The majority of mutations induced by ROS appear to involve modification of guanine, causing G→T transversions (Denissenko et al., 1996; Du et al., 1994; Colapietro et al., 1993).

Oxidative stress arises either from the overproduction of ROS or from the deficiency of antioxidant defense or repair mechanisms and results in
reversible or irreversible tissue injury (Dreher and Junod, 1996). An important endogenous cause of chronic oxidative stress is the inflammatory response (Cerutti and Trump, 1991). Activated neutrophils stimulate mutagenesis in vitro (Weitzman and Gordon, 1990) and oxidative stress from chronic inflammation favors cancer development in many organs. Cancer induction by chronic inflammation is frequently observed in ulcerative colitis (Collins et al., 1987). Other examples of inflammation related carcinogenesis are the mesothelioma caused by asbestos deposits (Mossman et al., 1990) and urinary bladder cancer induced by Schistosoma haematobium (Rosin et al., 1994). Important examples of exogenous causes of oxidative stress and their carcinogenic consequences are shown in Table III.

A role of ROS in the development of cancer in humans is further supported by the abundant presence of oxidative DNA modifications in cancer tissue. Thus, the lungs from cancer patients contain 25-75 8-oxodG per 10^6 deoxyguanosine in the apparently normal tissue and two to three fold higher values in the tumor, in addition to a whole series of other oxidative DNA modifications (Olinski et al., 1992). Smoking, the cause of bronchogenic carcinoma (Doll and Peto, 1981), chronically exposes the bronchial epithelium to ROS (Stone and Pryor, 1994). The urinary excretion of 8-oxodG and related biomarkers suggests that the rate of oxidative DNA modification corresponds up to 10^4 affected bases per cell/day (Loft et al., 1992, and 1993; Shigenaga et al., 1989; Cathcart et al., 1984).

Free radical induced oxidative base damage most likely represents an event of considerable importance in the progression of breast tumors to the metastatic state and is likely an important etiologic
### TABLE III

**Major Exogenous Causes of Oxidative Stress Involved in Carcinogenesis**

<table>
<thead>
<tr>
<th>Cause of oxidative stress</th>
<th>Oxygen free radicals</th>
<th>Cancer associated with exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco smoke</td>
<td>NO , 'OH</td>
<td>Bronchogenic carcinoma</td>
</tr>
<tr>
<td>Ultraviolet light</td>
<td>'OH , organic radicals</td>
<td>melanoma, skin cancer</td>
</tr>
<tr>
<td>Fatty acids in food</td>
<td>lipid peroxides</td>
<td>colorectal and breast cancer</td>
</tr>
<tr>
<td>Iron and copper ions</td>
<td>'OH</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>Ethanol</td>
<td>lipid peroxides</td>
<td>hepatocellular and breast cancer</td>
</tr>
</tbody>
</table>

(Adapted from Dreher and Junod, 1996)
factor (Malins et al., 1993 and 1996). The attack of \textsuperscript{·}OH on the base structure of breast DNA would be expected to result in genetic instability causing the activation of nuclear oncogenes and the deregulation of tumor suppressor genes such as p53. One study found a 9-fold increase in 8-hydroxyguanine, 8-hydroxyadenine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine in DNA from breast carcinoma compared with control tissue (Malins and Heimanot, 1991).

Exposure to ROS and its cellular production are facts of life. ROS can cause oxidative DNA damage and protein modifications, damage to tumor suppressor genes and enhanced expression of protooncogenes (Cerutti, 1994; Jackson, 1994) and oxidative stress has been shown to induce malignant transformation of cells in culture (Weitzman and Gordon, 1990). However, the development of human cancer depends on many other factors, including the extent of DNA damage, antioxidant defences, repair enzymes, the efficiency of removal of oxidized nucleosides before they are incorporated into DNA and the cytotoxic effects of ROS in large amounts and their growth promoting effects in small amounts (Burdon et al., 1995).

**Systemic Lupus Erythematous**

Systemic lupus erythematous is an autoimmune disorder with an annual incidence of 50 to 70/million and a prevalence of 500/million population (Klippel, 1997). Major organ system involvement may occur in the heart, lungs, kidneys and central nervous system and is responsible for most of the mortality caused by the disease. The etiology of the disorder is incompletely understood (Steinberg et al., 1990 & 1991). It is a prototype of spontaneous immune complex disease in which anti-DNA antibody complexes deposit in tissue and induce inflammation (Naparstek
and Madaio, 1997). Both genetic and environmental factors are believed to contribute to disease (Gourley et al., 1992).

The major serological marker of SLE, antibodies to native DNA, were distinguished by four different groups as early as 1957 (Cepellini et al., 1957; Meisher and Strassie, 1957; Robbins et al., 1957; Seligmann, 1957). There are reports of serum anti-dsDNA antibody levels correlating with the severity of the renal disease in SLE (Wallace et al., 1993). These anti-DNA antibodies found in the sera of patients with SLE are of diverse antigenic specificities (Ali et al., 1991; Pollard et al., 1986), which include components of DNA, its different conformations including modified structures (Alam and Ali, 1992; Hasan et al., 1991). Antigen specific and antigen non-specific factors have been implicated in the autoantibody production in SLE (Klinman et al., 1990; Datta et al., 1987; Klinman; and Steinberg, 1987; Schlomchik et al., 1987). Various serological findings suggest that lupus nephritis results from deposition of DNA-anti-DNA complexes and subsequent complement mediated tissue damage (Nishiya and Hashimoto, 1997; Zouali, 1997; Pisetsky, 1992; Emlen et al., 1986). Pankewyez et al. (1987) demonstrated that eluted immunoglobulins from the kidney of female MLR-lpr/lpr mice with early nephritis was predominantly IgG with antibody activity against DNA. These antibodies were also found to be reactive with multiple nucleic antigens and non-nucleic antigens like cardiolipin, suggesting that polyreactivity might be a distinguishing feature of nephritogenic autoantibodies.

Sequencing of antibody and hybridoma technology have facilitated a lot in understanding the features of anti-DNA antibodies which may impart pathogenicity and/or allow DNA recognition. Anti-DNA IgG which is
cationic in nature produces tissue damage. Putterman et al. (1996) reported that charge and affinity may not predict potential for tissue damage. Recent studies by Swanson et al. (1996) indicated that an anti-ssDNA antibody may be a more potent pathogen compared to an anti-dsDNA antibody. In addition, it has been suggested, that it is not the positive charges which allow pathogenicity but the number of charged (positive and negative) residues within the V region which influences this property (Ohnishi et al., 1994).

Apoptosis is an internally programmed cell death pathway (frequently initiated by extracellular signals), that regulates both T cell and B cell development (Smith et al., 1989; Liu et al., 1989). Accumulating evidence suggests that in the normal situation, immature self reactive thymocytes undergo apoptotic death (negative selection) upon stimulation via the T cell receptors (Von Boehmer, 1986). Emlen et al. (1994) have demonstrated that the rate of apoptosis of lymphocytes derived from SLE patients was 2.4 times faster than seen in lymphocytes from normal controls. The release of intact nucleosomes in excessive amounts during the process of apoptosis may thus provide a source of extracellular nuclear antigens sufficient to drive an immune response and induce DNA antibody production. It has been shown that onset of the autoimmune response in murine models of SLE is characterized by the early emergence of antibodies that recognize conformational epitopes of the nucleosome particles (Burlingame et al., 1993).

There is increasing evidence to suggest a link between autoimmune diseases and cancer (Seda and Alarcon, 1995; Cash and Klippel, 1991; Sela and Shoenfeld, 1988). Patients with SLE have defects in their cellular and humoral immune systems. The basic defect in SLE in humans is
suggested to be a deficiency in suppressor T cell function, which leads to proliferation and hyper-reactivity of B lymphocytes (Fauci et al., 1978). Certain B lymphocytes (CD5+ B cells) have a strong tendency to undergo oligoclonal or monoclonal proliferation and malignant transformation (Hayakawa and Hardy, 1988). Various findings suggest that cancer especially lymphoma and soft tissue sarcoma, is more common in patients with SLE than in general population (Patterson et al., 1992).

The clinical manifestations of SLE are remarkably heterogenous. Patients of SLE also exhibit high lipid profiles, which are known to arise from renal involvement and a nephrotic state (Appel, 1991; Attman and Alaupovic, 1990). Neurological involvement is also common in SLE inflicted individuals (Adelman et al., 1986). More than 60% of patients have CNS manifestations and 30-40% have neuromuscular abnormalities (Omdal et al., 1989). The major causes of death are directly related to the disease and include acute vascular neurologic events, renal failure and coronary artery disease (Klippel, 1997). Medication includes non-steroidal anti inflammatory drugs for mild form of the disease while high doses of corticosteroids and immunosuppressive drugs are needed for chronic SLE patients (Blank et al., 1992).

**Role of ROS in SLE**

Hydroxyl radical, a prominent entity of reactive oxygen species, is known to modify cellular DNA and has been implicated in several human diseases. It has been proposed that in chronic inflammatory diseases such as RA and SLE, highly reactive oxygen species released from activated phagocytic cells at the site of injury may penetrate cellular membranes and react with nuclear DNA (Allan et al., 1988; Lunec et al., 1987; Stollar, 1981). ROS play an important role in the development of
autoimmune diseases particularly SLE (Ahmad et al., 1997; Alam et al., 1993; Ara & Ali, 1993; Blount et al., 1989 & 1990). Oxidative damage to DNA in SLE patients has also been reported (Lunec et al., 1994; Bashir et al., 1993) and supports the role of ROS in the etiopathogenesis of SLE. The presence of oxidative DNA lesions in SLE was immunochemically detected using monoclonal anti-ROS DNA antibody that preferentially recognizes ROS modified epitopes on nucleic acids (Ahmad et al., 1998).

Formation of the altered base 8-OHdG, has been shown to be a very sensitive marker of ROS-induced damage (Aruoma et al., 1989; Kasai and Nishimura, 1984). The presence of 8-OHdG in the urine of normal healthy individuals also affirms that this altered base is a by product of normal oxidative metabolism and likely to be the product of a cellular repair mechanisms. The very low levels of excreted 8-OHdG in the circulating immune complexes of SLE patients, suggests that there is an abnormal repair of damaged DNA in these patients (Lunec et al., 1994). Results from our laboratory suggests a higher specificity of ROS-poly(G) than poly(G) to SLE autoantibodies, which implies that ROS damage may lead to formation of modified guanine moieties in DNA and RNA (Garg and Ali, 1998).

**Antigenicity of Nucleotides**

Antibodies may also distinguish between nucleosides and nucleotides. With anti-nucleoside antibodies, the large charged phosphate group hinder binding, while for anti-nucleotide antibodies it appears to form part of determinant (Ungar-Waron, 1967; Halloran and Parker, 1966; Erlanger and Beiser, 1964). Seaman et al. (1965) show that antibodies specific for poly(A), poly(C) and poly(I) can be produced in rabbits immunized with
ribopolymer-MBSA complexes. The results of the immunologic studies indicate that nucleotide and DNA-protein conjugates induce the formation of antibodies with nucleotide-protein conjugate (Halloran and Parker, 1966). Immunization with modified nucleosides or nucleotides conjugated to proteins has led to the induction of antibodies (Müller and Rajewsky, 1980). Nucleosides and nucleotides by themselves are not capable of inducing antibodies. So nucleosides are coupled to a carrier protein such as BSA by periodate method and then used for immunization. Whereas nucleotides/deoxynucleotides can be conjugated to carrier protein by carbodiimide method (without cleaving the ribose/deoxyribose ring) utilising the phosphate group present in them. Thus, by retaining the intact ribose/deoxyribose ring the resulting antibodies to nucleotide-BSA conjugates will be specific for DNA or RNA depending on the type of ribose ring present (Chandira Kala and Antony, 1996). Antibodies have been raised against deoxy AMP-BSA, deoxy CMP-BSA and GMP-BSA. While antibodies to deoxy AMP-BSA, deoxy CMP-BSA bind only to DNA (Vaishnav and Antony, 1988, 1989 and 1990), antibodies to GMP-BSA predominantly binds to RNA (Chandira Kala and Antony, 1993).

In human and murine lupus, the most abundant autoantibody populations are those reacting with ssDNA (Eilat, 1986; Schwartz and Stollar, 1985; Munns et al., 1984; Tan, 1982; Andrezejewski et al. 1981). These antibodies can be defined in some detail by an ELISA that employs not only immobilized ss and dsDNA antigens, but nucleotide haptnes as well (Weisbart et al., 1983). Generally, such haptnes are covalently linked to carrier proteins (e.g. BSA), which by themselves are unreactive with autoantibodies. While anti-ssDNA antibodies recognize individual base moieties with nucleotides (Ballard and Voss, 1985; Munns et al., 1984; Weisbart et al., 1983) one cannot dismiss the possibility
that sequence-specific (deoxy) oligonucleotides are responsible for antibody interaction with nucleic acids (Deutscher and Keene, 1988; Munns et al., 1987; Lee et al., 1981). For example, several monoclonal anti-ssDNA antibodies have been shown to bind with certain TMP-containing polynucleotides but not others (Zouali and Stollar, 1986; Ballard and Voss, 1985; Lee et al., 1981). Munns, et al. (1987) suggested that a significant fraction of anti-ssDNA antibodies in human SLE sera were specific for GMP-enriched oligonucleotides.

Objectives of the Present Study

Systemic lupus erythematosus (SLE) is an autoimmune disorder of unknown etiology and is characterized by the presence of circulating anti-DNA antibodies. Reactive oxygen species have been implicated in a number of human degenerative diseases including cancer and SLE.

In the present study, thymidine 5-monophosphate (TMP) was modified with hydroxyl radical generated by irradiation of hydrogen peroxide at 254 nm. The ROS-TMP was characterized by UV absorption spectra and ion exchange chromatography on DEAE Sephadex A-25 column. TMP being a hapten was linked with BSA via carbodiimide method. TMP-BSA conjugate was modified with hydroxyl radical and characterized by UV spectral analysis, Sephadex G-100 gel chromatography, densitometric scanning and agarose gel electrophoresis.

The antigenicity of ROS-modified TMP-BSA was probed by inducing polyclonal antibodies in rabbits. Induced antibodies were characterized for their fine antigenic specificity with various nucleic acid polymers. Naturally occurring human anti-DNA autoantibodies and the circulating antibodies in sera of various cancer patients were also studied for their recognition to native and ROS modified TMP-BSA conjugates.
EXPERIMENTAL
MATERIALS

Thymidine monophosphate, calf thymus DNA, nuclease S1, bovine serum albumin, anti-human and anti-rabbit IgG-alkaline phosphatase conjugates, ethidium bromide, Coomassie Brilliant Blue G250 and R250, Tween 20, Freund's complete and incomplete adjuvants, polydeoxyribonucleotides and carbodiimide were from Sigma Chemical Company, U.S.A. Synthetic polynucleotides, Sepharose 4B, DEAE Sephadex A-25, Sephadex G-100, Ficoll 400 were obtained from Pharmacia Fine Chemicals, Sweden. Folin-Ciocalteu reagent, p-nitrophenyl phosphate and Blue Dextran 2000 were purchased from Centre for Biochemical Technology, New Delhi.

Polystyrene microtitre flat bottom ELISA plates having 96 wells (7mm diameter) were purchased from Nunc, Denmark. Acrylamide, bisacrylamide, ammonium persulphate, N,N,N',N'-tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratories, U.S.A. EDTA (disodium salt), hydrogen peroxide, isoamyl alcohol, chloroform were from Qualigens, India. Diphenylamine and ethanol were chemically pure. All other 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 254 nm (Vilber Lourmat, France), Avanti 30 table top high speed centrifuge (Beckman, U.S.A.), Polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.), UV trans-illuminator (Vilber
Lourmat, France), Agarose gel electrophoresis assembly GNA-100 (Pharmacia, Sweden), Beckman ultracentrifuge were the major equipments used in this study.

**Sera Specimens**

SLE sera were obtained from outdoor and indoor patients of the Department of Medicine, All India Institute of Medical Sciences, New Delhi. The SLE sera collected, showed high titre anti-DNA antibodies and fulfilled the American College of Rheumatology criteria for the diagnosis of SLE (Arnett et al., 1988). The sera of cancer patients were collected from Department of Radiotherapy, J.N. Medical College, A.M.U., Aligarh. Complement was inactivated at 56 °C for 30 minutes and stored at -80°C with 0.1% sodium azide.

**METHODS**

**Purification of DNA**

Commercially available, highly polymerized calf thymus DNA was purified free of proteins and single stranded regions (Ali et al., 1985). DNA was dissolved in 0.1 X SSC (15 mM sodium citrate and 150 mM sodium chloride), pH 7.3. Solutions of DNA (2 mg/ml) was mixed with equal volume of chloroform-isoamyl alcohol (24:1) in a stoppered cylinder and extracted gently for 1 hr. The DNA present in the aqueous layer was separated from the organic layer and re-extracted with chloroform-isoamyl alcohol. The DNA was precipitated with two volumes of cold 95% ethanol and collected on a glass rod. Traces of water was removed by rinsing the rod with ethanol and DNA dried by pressing against the wall of the container. The DNA was then dissolved in 30 mM acetate buffer, pH 5.0 containing 30 mM zinc chloride and treated with
nuclease S1 (150 units/mg DNA) at 37°C for 30 min to remove single stranded regions. The reaction was terminated by adding one-tenth volume of 200 mM EDTA, pH 8.0. The purified DNA was extracted twice with chloroform-isoamyl-alcohol and finally precipitated with 95% ethanol. The precipitate was dissolved in PBS (10 mM sodium-phosphate, containing 150 mM NaCl), pH 7.4.

Estimation of DNA by Diphenylamine

Colorimetric estimation of DNA was carried out by the method of Burton (1956) using diphenylamine reagent.

(a) Preparation of diphenylamine reagent

Recrystallized diphenylamine (750 mg) was dissolved in 50 ml of glacial acetic acid containing 0.75 ml concentrated sulphuric acid. The reagent was prepared immediately before use.

(b) Procedure

To varying amounts of DNA in 1.0 ml of 1N perchloric acid was mixed and incubated at 70°C in water bath for 15 min. One hundred microlitre of 5.43 mM acetaldehyde was added followed by 2.0 ml of diphenylamine reagent. The contents were mixed and allowed to stand at room temperature for 16-20 hrs. Absorbance was read at 600 nm and the concentration of DNA in unknown samples was determined from the standard plot of purified calf thymus DNA.

Protein Estimation by Bradford Method

Protein was estimated by the method of Bradford (1976). This assay is based on colour change that occurs when Coomassie Brilliant Blue G250, in acidic solution, binds strongly to protein hydrophobically and at
positively charged groups. In the environment of these positively charged
groups, protonation is suppressed and a blue colour is observed ($\lambda_{\text{max}}$ 595 nm).

(a) Dye preparation

Coomassie Brilliant Blue G250 (100 mg) was dissolved thoroughly in
50 ml of 95% ethanol. One hundred ml of 85% (v/v) orthophosphoric acid
was added to this solution. The resulting solution was diluted to a final
volume of 1 litre. On every use the dye solution was filtered, to remove
undissolved particles.

(b) Procedure

Solutions containing 10-100 $\mu$g protein in a volume of up to 0.1 ml
was pipetted into test tubes. The volume was adjusted to 1.0 ml with
appropriate buffer. Five ml of dye solution was added and the contents
were vortexed. The absorbance was read at 595 nm after 2 min and
before 1 hr against a reagent blank prepared from 0.1 ml of buffer and
5.0 ml of dye solution.

ROS Modification of TMP

Aqueous solution of native TMP (0.31 mM) in PBS, pH 7.4 was
irradiated under 254 nm light for 30 min at room temperature in the
presence of hydrogen peroxide (3.10 mM). Native TMP samples exposed
to hydrogen peroxide or UV light alone were used as corresponding
controls.

Spectroscopic Analysis

The ultraviolet spectra of modified and unmodified TMP conjugate
were recorded in the wavelength range of 200-400 nm on Shimadzu UV-
240 spectrophotometer.
Preparation of the Antigen

The hapten-protein conjugate was prepared by the carbodiimide conjugation procedure of Halloran and Parker (1966). BSA (19 mg) and thymidine 5'-monophosphate (50 mg) were dissolved in 5.0 ml of distilled water. The pH was adjusted to 7.5 with 0.5 M NaOH, and ethyl ((dimethylamino) propyl) carbodiimide (20 mg) was added. The reaction mixture was incubated in the dark at room temperature for 24 hrs. The solution was then dialyzed extensively against 10 mM Tris-HCl buffer. pH 7.6, followed by extensive dialysis against distilled water.

The ultraviolet spectra of native TMP, BSA and TMP-BSA conjugate were recorded in the range of 200-400 on Shimadzu UV-240 spectrophotometer.

Polyacrylamide Gel Electrophoresis

BSA and TMP-BSA conjugate were subjected to polyacrylamide slab gel electrophoresis according to the method of Laemmli (1970).

(a) Stock solutions

(i) Acrylamide-bisacrylamide (30:0.8):

Prepared by dissolving in distilled water 30.0 g of acrylamide and 0.8 g of bisacrylamide in a total volume of 100.0 ml. The solution was stored at 4°C in a brown bottle.

(ii) TEMED: Used as supplied.

(iii) Ammonium persulphate (1.5% w/w):

0.15 g of ammonium persulphate was dissolved in 10.0 ml water. The solution was made fresh just before use.
(iv) **Resolving gel buffer (3M Tris-HCl, pH 8.8):**

Prepared by dissolving 36.0g Tris in 48.0 ml of 1 N HCl. The contents were mixed thoroughly, pH brought to 8.8 and final volume made upto 100.0 ml with distilled water.

(v) **Stacking gel buffer (0.5 M Tris-HCl, pH 6.8):**

Tris (6.05g) was dissolved in 40.0 ml distilled water, titrated to pH 6.8 with 1N HCl and volume made upto 100.0 ml with distilled water.

(vi) **Reservoir buffer (25 mM Tris, 192 mM glycine):**

3.03 g Tris and 14.4 g glycine were dissolved in distilled water and final volume made upto one litre.

(b) **Recipe for 7.5% Resolving gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Resolving gel buffer</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.95 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15.0 µl</td>
</tr>
</tbody>
</table>

(c) **Recipe for 2.5% stacking gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>1.5% Ammonium persulphate</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>11.3 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15.0 µl</td>
</tr>
</tbody>
</table>

(d) **Procedure**

The resolving gel mixture was prepared by mixing the components in the appropriate volumes as listed above and poured into the space between the glass plates leaving sufficient space (about 3.5 cm) at the
top for the stacking gel. After the polymerization of separating gel, stacking gel mixture was poured and allowed to solidify. Samples containing 10% glycerol and 0.002% bromophenol blue were applied and electrophoresis was carried out at 24 mA for 2-3 hr. Staining of the gel was achieved with 0.1% Coomassie Brilliant Blue R 250 (in 25% isopropanol and 10% glacial acetic acid) and the gel was destained using a mixture of 10% glacial acetic acid and 30% methanol.

**ROS-Modification of TMP-BSA Conjugate**

Aqueous solution of TMP-BSA conjugate (6.20 mM) in PBS, pH 7.4 was irradiated under 254 nm light for 30 min. at room temperature in the presence of hydrogen peroxide (62 mM). Excess of hydrogen peroxide was removed by extensive dialysis against PBS, pH 7.4. The ultraviolet spectra of TMP and ROS-modified TMP were recorded.

**Absorption-Temperature Scan**

Thermal denaturation analysis of native and ROS-modified conjugates were accomplished in a Shimadzu UV-240 spectrophotometer coupled with a temperature programmer and controller assembly. Native and ROS-modified TMP-BSA conjugates were subjected to heat denaturation (Hasan and Ali, 1990). Samples were melted from 30°C to 95°C at a rate of 1.5°C/min after 10 min equilibration at 30°C. The change in absorbance at \( \lambda_{max} \) was recorded with increasing temperature. Simultaneously, UV spectra were also recorded at 30°C and 95°C in the same wavelength range.
Sephadex G-100 Gel Chromatography

Gel filtration was performed on Sephadex G-100 column. Five gm of gel was swollen in distilled water for 5 hr in a boiling water bath. The degassed gel slurry was poured into the column (42 cm x 1 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 each of native and ROS-TMP-BSA conjugates were applied separately onto the column. The sample was allowed to percolate through the upper surface of the gel. The column was connected to a reservoir containing equilibrating buffer. Fractions of 3.0 ml were collected and absorbance monitored at 269 nm.

Densitometric Scanning

Samples of native and ROS-modified conjugates were subjected to polyacrylamide gel electrophoresis on 7.5% native gel. After electrophoresis, the lanes each of native and ROS-modified conjugates were cut and scanned on gel scanner GSC-3A of Shimadzu UV-240 spectrophotometer. Gel was scanned at the rate of 10 mm/min and the spectra was recorded simultaneously at a fixed wavelength of 269 nm.

Agarose Gel Electrophoresis

1% agarose in 30 ml TAE, pH 7.9, was dissolved by heating in boiling water bath. The solution was allowed to cool at 50°C and poured onto the gel tray and left at room temperature for complete solidification. Samples mixed with one-tenth volume of stop mix dye (30% Ficoll. 0.025% xylene cyanole FF in gel buffer), was loaded in the wells of the
submerged gel and electrophoresed for 2 hr at 30 mA. The gels were stained with Coomassie Brilliant Blue R 250 and destained in tap water.

**Immunization Schedule**

Female rabbits (8-12 months, weight 1-1.5 kg) were immunized with native and ROS-modified TMP-BSA conjugates. The immunizing antigen (100 μg/rabbit) was emulsified with equal volume of Freund's complete adjuvant (FCA) for the first injection given subcutaneously at multiple sites. Subsequent injections were given in incomplete adjuvant (IFA) intramuscularly. Each animal received a total of 700 μg of antigen during the course of seven injections. Booster dose was administered after a fortnight from the last injection. Blood was collected by cardiac puncture and serum separated. The separated serum was decomplemented by heating at 56°C for 30 min. Preimmune sera were collected before immunization. The sera were stored in small aliquots at -80°C with 0.1% sodium azide as preservative.

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

Serum IgG was isolated by affinity chromatography on protein A-Sepharose CL-4B column. 0.5 ml serum diluted with equal volume of PBS, pH 7.4 was applied to column (15 cm x 0.9 cm) equilibrated with the same buffer. The wash through was recycled 2-3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding. 1976) and neutralized with 1 ml of 1 M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 280 nm. The concentration was determined considering 1.4 O.D_{280} =1.0 mg IgG/ml. The IgG was then dialyzed against PBS, pH 7.4 and stored at -80°C with 0.1% sodium azide.
Immunological Techniques

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

(a) Immunodiffusion

Immunodiffusion (ID) was carried out by Ouchterlony double immunodiffusion system using glass petri dishes as described by Tan et al. (1966). Six ml of 0.4% molten agarose in PBS, pH 7.4 containing 0.1% sodium azide was poured onto glass petri dishes and allowed to solidify at room temperature and then kept at 4°C for 4 hrs. Wells 5 mm in diameter separated by 8 mm in distance, were cut into hardened gel. Antigen and antibody were loaded and kept in moist chamber for 24-48 hrs. The petri dishes were washed with 5% sodium citrate to remove non-specific precipitin lines, if any. The precipitin lines were analyzed visually and photographed.

(b) Counterimmunoelectrophoresis

Counterimmunoelectrophoresis was performed by the method of Kurata and Tan (1976). Molten agarose (0.6%) in 25 mM barbital buffer, pH 8.4, containing 0.1% sodium azide was poured onto 2.5 mm thick glass slides (7.5 cm x 2.5 cm) and allowed to harden at room temperature and then at 4°C. Wells each 3 mm in diameter were cut and loaded with antigen and antibodies (in anodal and cathodal wells, respectively). The slides were then 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) Enzyme linked Immunosorbent Assay (ELISA)

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

(i) Buffers and substrate

*Tris buffered saline (TBS)*

10 mM Tris, 150 mM sodium chloride, pH 7.4.

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

20 mM Tris, 144 mM sodium chloride, 2.68 mM potassium chloride (KCl), pH 7.4, containing 500 µL Tween-20/L

*Carbonate-bicarbonate buffer*

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

*Substrate*

500 µg p-nitrophenyl phosphate/ml of carbonate-bicarbonate buffer.

(ii) Procedure

Polystyrene microtitre plates were coated with 100 µl of antigen (2.5 µg/ml in TBS, pH 7.4) for 2 hr at room temperature and overnight at 4°C. Unbound antigen was removed by washing thrice with TBS-T. Unoccupied sites were blocked with 200 µL of 1.5% BSA in TBS for 4-6 hr at room temperature. The plates were washed once with TBS-T. The antibody 1:100 diluted in TBS was coated in each well. After incubating the plates for 2 hrs. at room temperature and at 4°C overnight, the plate was extensively washed with TBS-T. The bound antibodies were then assayed by an appropriate anti-immunoglobulin alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The plates were incubated at 37°C for 1 hr and then read at 410 nm. Each sample was
coated in duplicate and the results were expressed as a mean of $A_{\text{test}} - A_{\text{control}}$.

(d) Competition ELISA

The antibody specificity was determined by competition ELISA (Hasan et al., 1991). Varying amounts of inhibitors (0-20 μg/ml) were incubated with a constant amount of antibody for 2 hrs. at room temperature and overnight at 4°C. The resulting immune complex was coated in wells instead of the serum IgG. The remaining steps were the same as in direct binding ELISA. The results were expressed as percent inhibition.

\[
\text{Percent inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \times 100
\]

$A_{\text{inhibited}}$ = Absorbance at 20 μg/ml inhibitor concentration

$A_{\text{uninhibited}}$ = Absorbance at zero inhibitor concentration.
RESULTS
Formation of ROS-TMP and its Characterization

A solution of thymidine monophosphate 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 UV absorption spectra was recorded on a Shimadzu UV-240 spectrophotometer in the wavelength range of 200 to 400 nm. Figure 2 shows the UV absorption spectra of TMP irradiated for 15 and 30 minutes, indicated a loss in the characteristic absorption pattern of TMP as a result of damage caused by hydroxyl radical. The perturbations incurred on TMP as a consequence of ROS modification were also analysed by UV difference spectroscopic scanning (Fig. 3). The spectral curve for TMP with respect to buffer (PBS, pH 7.4) showed $\lambda_{\text{max}}$ at 267 nm, characteristic of native TMP. The difference spectra of ROS-TMP, with reference to TMP exhibited a strong negative trough having a broad peak between 282 to 255 nm and a complete disappearance of positive peak at 267 nm.

Conjugation of TMP with BSA

TMP was linked with BSA through carbodiimide method of conjugation. The UV spectra shows a slight bathochromic shift in TMP-BSA conjugate when compared with native TMP (Fig. 4). Carbodiimides, which contain secondary and tertiary amine groups, react with nucleotides altering its structure which rendered it more immunogenic. The formation of TMP-BSA conjugate was ascertained by 7.5% PAGE. The gel profile indicated the formation of high molecular weight band of complex with increasing concentration of TMP-BSA conjugate (Fig. 5).
Fig. 2  UV absorption spectra of native TMP (---) irradiated for 15 min (---) and 30 min (-----) under UV light in presence of hydrogen peroxide. TMP alone irradiated under UV light (---), $\text{H}_2\text{O}_2$ alone (----) and unirradiated TMP + $\text{H}_2\text{O}_2$ samples (-----) served as controls.
Fig. 3  UV difference spectra of native TMP (—) with reference to buffer (PBS, 7.4) and ROS-TMP (----) with reference to native TMP.
Fig 4 The TMP-BSA conjugate was prepared by carbodiimide method of conjugation UV spectra of TMP-BSA conjugate (—..). TMP alone (—-), BSA alone (-----) and TMP-BSA without carbodiimide (—)
Fig. 5  Polyacrylamide gel electrophoresis (7.5%) of TMP-BSA conjugate with stacking gel (2.5%). Lane 1 (BSA). lanes 2-4 show increasing concentration of TMP-BSA conjugate (60.90 and 120 µg. respectively).
**ROS-Modification of TMP-BSA Conjugate**

TMP-BSA conjugate was modified with hydroxyl radical. The irradiated sample was dialyzed against PBS to remove excess of hydrogen peroxide. Figure 6 shows the UV absorption spectra of native and modified conjugate. TMP-BSA conjugate gave a sharp peak at $\lambda_{\text{max}}$ (269 nm), whereas ROS-modified conjugate indicated an appreciable amount of hypochromicity, with a shoulder around $\lambda_{\text{max}}$. This loss in characteristic absorption pattern is due to degradation of conjugate incurred as a result of hydroxyl radical modification. The perturbations occurring on TMP-BSA conjugate as a consequence of ROS-modification were also analysed by UV difference spectroscopy (Fig. 7). Difference spectrum of ROS-modified conjugate revealed an appreciable negative inversion absorption with respect to native conjugate.

**Thermal Transition Studies**

UV spectra of native and modified conjugates were recorded at 30°C and 95°C. The UV spectrum of TMP-BSA conjugate at 95°C did not show any appreciable deviation from the spectral curve recorded at 30°C (Fig. 8). However, distinct hyperchromicity was observed in case of ROS-modified TMP-BSA conjugate at 95°C as compared to the spectrum at 30°C (Fig. 9). This hyperchromicity could be due to some structural and conformational changes in TMP-BSA conjugate incurred after ROS-modification.

**Sephadex-G 100 Gel Filtration**

Equal concentration of both native and ROS-TMP-BSA conjugate was applied on Sephadex G-100 column. Native TMP-BSA conjugate appeared
Fig. 6 Ultraviolet absorption spectra of TMP-BSA conjugate (---), ROS-TMP-BSA conjugate (—) and TMP alone (---).
Fig. 7  UV difference spectra of TMP-BSA conjugate (——) against buffer (PBS, 7.4) as control and ROS-TMP-BSA conjugate (---) with reference to TMP-BSA conjugate.
Fig. 8  UV absorption spectra of TMP-BSA conjugate at 30°C (—) and 95°C (—).
Fig. 9  UV absorption spectra of ROS-TMP-BSA conjugate at 30°C (—) and 95°C (---).
in small peaks along with a sharp symmetrical peak at fraction no. 31, whereas ROS-TMP-BSA conjugate indicated a degraded product with a hypochromic shift corresponding to fraction no. 31 (Fig. 10). Formation of low molecular weight species from high molecular weight complex reiterates the modification of TMP-BSA conjugate by hydroxyl radical.

**Densitometric Scanning**

TMP-BSA and ROS-modified conjugate were resolved on 7.5% polyacrylamide gel and were subsequently subjected to densitometric scanning at 269 nm (Fig. 11). ROS-TMP-BSA conjugate showed a single peak of low intensity as compared to TMP-BSA conjugate. The data indicates that considerable amount of TMP-BSA conjugate has been modified by hydroxyl radical, which in turn leads to strand breaks and thus formation of low molecular weight species.

**Agarose Gel Eletrophoresis**

Both the native and ROS-modified TMP-BSA conjugates were resolved on a 1% agarose gel. As evident from figure 12, modified conjugate exhibits low intensity as compared to its native form which might be due to the formation of low molecular weight species, as a result of oxidative damage to the complex.

**Antigenicity of Native and ROS-Modified TMP**

(a) Antibodies against TMP and ROS-TMP

The antigenicity of native and ROS-TMP was probed by inducing antibodies in rabbits. The production of antibodies against TMP and ROS-TMP could not be elicited as revealed by direct binding and inhibition
Fig. 10  Fractionation of TMP-BSA conjugate (○) and ROS-TMP-BSA conjugate (△) on Sephadex G-100 gel filtration column.
Fig. 11  Densitometric scanning of TMP-BSA conjugate (—) and ROS-TMP-BSA conjugate (—) after 7.5% native polyacrylamide gel electrophoresis.
Fig. 12 Agarose gel electrophoresis of TMP-BSA conjugate and ROS-modified conjugate. Staining was performed with Coomassie Brilliant Blue R-250. TMP-BSA conjugate, lanes 1 (20µg) and 2 (40µg). ROS-TMP-BSA conjugate, lanes 3 (20µg) and 4 (40µg).
ELISA results. In order to make TMP immunogenic, it was linked to a carrier protein (BSA) and then modified with hydroxyl radical.

(b) Antibodies against TMP-BSA and ROS-TMP-BSA conjugates

The antibodies raised against TMP-BSA and ROS-TMP-BSA conjugates were found to be precipitating as detected by immunodiffusion and counterimmunoelectrophoresis (Fig. 13 and 14). The antiserum showed a titre of 1:12800 by direct binding ELISA on plates coated with TMP-BSA conjugate. Preimmune serum showed negligible binding (Fig. 15). The specificity of induced antibodies was determined by competition ELISA. Induced antibodies were found to be highly specific for its immunogen. A maximum of 95% inhibition of antibody binding was observed. Fifty percent inhibition was observed at an inhibitor concentration of 2.0 μg/ml (Fig. 16). Anti-ROS-TMP-BSA antibodies showed a titre of 1:12800 by direct binding ELISA on plates coated with ROS-TMP-BSA conjugate. Preimmune serum showed negligible binding (Fig. 17). In competition inhibition assay, a maximum of 92% inhibition was observed and 50% inhibition was achieved with 2.5 μg/ml (Fig. 18).

(c) Purification and characterization of immune IgG

Immunoglobulin G against TMP-BSA and ROS-TMP-BSA conjugate were isolated from the respective sera by affinity chromatography on Protein A-Sepharose CL-4B column (Fig. 19 and 20). IgG from preimmune sera was also purified (Fig. 21). The purity of IgG was ascertained by SDS-PAGE under non-reducing conditions (inset of Fig. 19-21). As seen, purified IgG migrated as a single band. A high magnitude of binding of the purified anti-TMP-BSA conjugate and ROS-modified conjugate with respective immunogen was exhibited by direct binding
Fig. 13 Immunodiffusion of anti-TMP-BSA conjugate antibodies with TMP-BSA conjugate (a) and anti-ROS-TMP-BSA conjugate antibodies with ROS-TMP-BSA conjugate (b).
Fig. 14  Counterimmunoelectrophoresis of anti-TMP-BSA conjugate antibodies with TMP-BSA conjugate (a) and anti-ROS-TMP-BSA conjugate (b). Electrophoresis was performed on 0.6% agarose gel for 2 hr at 140 V.
Fig. 15  Direct binding ELISA of TMP-BSA conjugate with pre-immune ( ▲ ) and immune sera ( △ ). The microtitre plates were coated with TMP-BSA conjugate (10 μg/ml).
Fig. 16  Inhibition ELISA of immune sera with TMP-BSA conjugate. The microtitre plates were coated with TMP-BSA conjugate (10 µg/ml).
Fig. 17 Direct binding ELISA of ROS-TMP-BSA conjugate with preimmune (▲) and immune sera (△). The microtitre plates were coated with TMP-BSA conjugate (10 µg/ml).
Fig. 18 Inhibition ELISA of immune sera with ROS-TMP-BSA conjugate (○). The microtitre plates were coated with TMP-BSA conjugate (10 µg/ml).
Fig. 19  Elution profile of anti-TMP-BSA conjugate IgG on Protein A-Sepharose CL-4B column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Fig. 20  Elution profile of anti-ROS-TMP-BSA conjugate IgG on Protein A-Sepharose CL-4B column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Fig. 21  Elution profile of preimmune IgG on Protein A-Sepharose CL-4B column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
ELISA (Fig. 22 and 23). No appreciable binding was observed in case of preimmune IgG. The specificity of the purified IgG was evaluated by competitive inhibition assays. A maximum of 95% (at 10 μg/ml) inhibition of anti-TMP-BSA conjugate IgG and 98% of anti ROS-TMP-BSA conjugate with respective immunogens (used as inhibitors) was observed (Fig. 24). Fifty percent inhibition was achieved at an inhibitor concentration of 0.3 μg/ml in case of TMP-BSA conjugate while 0.5 μg/ml was required for corresponding ROS-modified conjugate.

**Immuno-crossreactivity of Anti-TMP-BSA Conjugate Antibodies**

The antigenic specificity of the induced anti-TMP-BSA conjugate antibodies was characterized by competition inhibition assay using nucleic acids, polynucleotides and bases as inhibitors. TMP-BSA conjugate showed an inhibition of 42% in the antibody binding. Native calf thymus DNA and its modified conformer (ROS-DNA) showed moderate inhibitions of 48% and 33%, respectively (Fig. 25). TMP, thymidine and thymine showed lower inhibitions of 28%, 16% and 21%, respectively. Their ROS-modified forms were also used as inhibitors. ROS-TMP showed inhibition of 69% at 20 μg/ml. Fifty percent inhibition of antigen-antibody interaction was obtained with 15 μg/ml. ROS-thymine showed inhibitions of 56% (50% inhibition at 18 μg/ml) and 82% (50% inhibition at 5.5 μg/ml), respectively (Fig. 26). Similarly ROS-guanine and ROS-adenine showed higher inhibition of 61% and 59%, respectively. Native adenine and guanine showed 29% and 46% inhibitions (Fig. 27). Among homopolymers, poly(G) and its ROS-modified form showed moderate inhibitions of 45% and 40%, while poly(I) was found to be non-inhibitory (Fig. 28).
Fig. 22 Direct binding ELISA of preimmune (▲) and immune IgG (△) with TMP-BSA conjugate. The microtitre plates were coated with TMP-BSA conjugate (10 μg/ml).
Fig. 23 Direct binding ELISA of preimmune (▲) and immune IgG (▲) with ROS-TMP-BSA conjugate. The microtitre plates were coated with TMP-BSA conjugate (10 μg/ml).
Fig. 24  Inhibition of anti-TMP-BSA IgG and anti-ROS-TMP-BSA IgG binding to TMP-BSA conjugate (O) and ROS-TMP-BSA conjugate (Δ). The competitors were TMP-BSA conjugate and ROS-TMP-BSA conjugate respectively.
Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate. The competitors were ROS-TMP-BSA conjugate (O), native ctDNA (△) and ROS-DNA (▲). The microtitre plates were coated with TMP-BSA conjugate (10 μg/ml).
Fig. 26 Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate. The competitors were TMP (○). ROS-TMP (△), thymidine (▲), ROS-thymidine (●), thymine (▽) and ROS-thymine (+). The microtitre plates were coated with TMP-BSA conjugate (10 μg/ml).
Fig. 27  Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate. The competitors were guanine (○), ROS-guanine (△), adenine (▲) and ROS-adenine (●). The microtitre plates were coated with TMP-BSA conjugate (10 μg/ml).
To further study the structural determinants recognized by anti-TMP-BSA conjugate antibodies, their interaction with various synthetic polydeoxynucleotides was examined. Poly(dA-dT).poly(dA-dT) exhibited a moderate inhibition of 36%. Poly(dA-dU).poly(dA-dU) and poly(A).poly(U) showed negligible inhibitions (Fig. 28). Table IV summarizes the binding characteristics of anti-TMP-BSA conjugate antibodies.

**Immuno-crossreactivity of Anti-ROS-TMP-BSA Conjugate Antibodies**

Antigenic specificity of anti-ROS-TMP-BSA antibodies was also characterized by competition inhibition assay. When TMP-BSA conjugate was used as an inhibitor, a maximum of 89% inhibition in the antibody binding was observed (Fig. 29). The concentration of TMP-BSA conjugate required to inhibit 50% antibody activity was 0.2 μg/ml. Native TMP and thymidine showed inhibition of 39% and 23% while their ROS-modified forms showed a maximum inhibition to an extent of 86% and 56%, respectively (Fig. 29). ROS-modified forms of thymine, guanine and adenine showed negligible inhibition. Guanine showed inhibition of 55% (Fig. 30). Calf thymus DNA, cytosine and ROS-cytosine were found to be non-inhibitory (Fig. 31). Poly(G) gave a moderate inhibition of 38%, while ROS-poly(G) was found to inhibit to a maximum of 53% (Fig. 31).

The specificity of the anti-ROS-TMP-BSA antibodies was also assessed using synthetic polynucleotides. Poly(dA-dU).poly(dA-dU) and poly(dl-dC).poly(dl-dC) showed a maximum inhibition of 35% (Fig. 32). Poly(dA-dT).poly(dA-dT) and poly(A).poly(U) showed negligible inhibitions (Fig. 32). Table V gives a summary of the binding characteristics of anti-ROS-TMP-BSA conjugate antibodies.
Fig. 28 Inhibition of anti-TMP-BSA conjugate IgG binding to TMP-BSA conjugate. The competitors were poly(G) (○), ROS-poly(G) (△). poly(dA-dT).poly(dA-dT)(▲). poly(dA-dU).poly(dA-dU) (●) and poly(A).poly(U) (▽). The microtitre plates were coated with TMP-BSA conjugate (10 μg/ml).
### TABLE-IV

**Competitive Inhibition Data of Anti-TMP-BSA Antibodies**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Maximum percent inhibition (20μg/ml)</th>
<th>Concentration required for 50% inhibition</th>
<th>Percent relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. TMP-BSA conjugate</td>
<td>95a</td>
<td>0.35</td>
<td>100</td>
</tr>
<tr>
<td>2. ROS-TMP-BSA conjugate</td>
<td>42</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. TMP</td>
<td>28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4. ROS-TMP</td>
<td>69*</td>
<td>15</td>
<td>2.3</td>
</tr>
<tr>
<td>5. Thymidine</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6. ROS-thymidine</td>
<td>56</td>
<td>18</td>
<td>1.9</td>
</tr>
<tr>
<td>7. ctDNA</td>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8. ROS-ctDNA</td>
<td>33</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9. Thymine</td>
<td>21**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10. ROS-thymine</td>
<td>82</td>
<td>5.5</td>
<td>6.3</td>
</tr>
<tr>
<td>11. Guanine</td>
<td>46</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12. ROS-guanine</td>
<td>61</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>13. Cytosine</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14. ROS-cytosine</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15. Adenine</td>
<td>29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16. ROS-adenine</td>
<td>59*</td>
<td>1.5</td>
<td>23.3</td>
</tr>
<tr>
<td>17. Poly(G)</td>
<td>45*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18. ROS-poly(G)</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19. Poly(I)</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20. ROS-poly(I)</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21. mtDNA</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22. ROS-mtDNA</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23. Poly(A).poly(U)</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24. Poly(dA-dT). poly(dA-dT)</td>
<td>36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25. Poly(dA-dU). poly(dA-dU)</td>
<td>22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26. Poly(dl-dC). poly(dl-dC)</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

# Insignificant inhibition
* Inhibition at 15 μg/ml
a Inhibition at 10 μg/ml
** Inhibition at 5 μg/ml

The microtiter plates were coated with TMP-BSA Conjugate at 20 μg/ml.
Fig. 29 Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate. The competitors were TMP-BSA conjugate (○), TMP (△), ROS-TMP (▲). thymidine (●) and ROS-thymidine (●). The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml).
Fig. 30 Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate. The competitors were thymine (O), ROS-thymine (△), guanine (▲), ROS-guanine (●), adenine (▽), ROS adenine (+). The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml).
Fig. 31 Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate. The competitors were cytosine (O), ROS-cytosine (△), native ctDNA (▲), poly(G) (●) and ROS-poly(G) (▽). The microtitre plates were coated with ROS-TMP-BSA conjugate (10μg/ml).
Fig. 32 Inhibition of anti-ROS-TMP-BSA conjugate IgG binding to ROS-TMP-BSA conjugate. The competitors were poly(dA-dT).poly(dA-dT) (○), poly(dA-dU).poly(dA-dU) (△), poly(dl-dC).poly(dl-dC) (▲) and poly(A).poly(U) (●). The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml).
<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Maximum percent inhibition (20µg/ml)</th>
<th>Concentration required for 50% inhibition</th>
<th>Percent relative affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ROS-TMP-BSA conjugate</td>
<td>98</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>2. TMP-BSA conjugate</td>
<td>89</td>
<td>0.2</td>
<td>50</td>
</tr>
<tr>
<td>3. TMP</td>
<td>39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4. ROS-TMP</td>
<td>86</td>
<td>9</td>
<td>5.5</td>
</tr>
<tr>
<td>5. Thymidine</td>
<td>23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6. ROS-thymidine</td>
<td>56</td>
<td>4.5</td>
<td>11.1</td>
</tr>
<tr>
<td>7. Thymine</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8. ROS-thymine</td>
<td>81</td>
<td>4.5</td>
<td>11.1</td>
</tr>
<tr>
<td>9. Guanine</td>
<td>55</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>10. ROS-guanine</td>
<td>78</td>
<td>2.5</td>
<td>20</td>
</tr>
<tr>
<td>11. Adenine</td>
<td>29**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12. ROS-adenine</td>
<td>48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13. Cytosine</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14. ROS-cytosine</td>
<td>17*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15. ctDNA</td>
<td>27*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16. ROS-ctDNA</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17. PolyI</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18. ROS-polyI</td>
<td>#</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19. PolyG</td>
<td>38</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20. ROS-polyG</td>
<td>53</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21. Poly(A).poly(U)</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22. Poly(dA-dT).poly(dA-dT)</td>
<td>22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23. Poly(dA-dU).poly (dA-dU)</td>
<td>35**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24. Poly(dl-dC).poly(dl-dC)</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

** Inhibition at 5 µg/ml
* Inhibition at 15 µg/ml
# Insignificant inhibition

The microtiter plates were coated with ROS-TMP-BSA Conjugate (10 µg/ml).
Detection of Anti-DNA Antibodies in SLE Sera

The SLE anti-DNA autoantibodies were found to be precipitating with TMP-BSA and ROS-TMP-BSA conjugates as detected by counterimmunoelectrophoresis (Fig. 33). Various SLE serum samples were tested for the presence of anti-DNA autoantibodies by direct binding ELISA (Fig. 34 and 35) (Table VI). Normal human serum showed negligible binding. The specificity of SLE autoantibodies binding to native DNA was determined by competition ELISA. Native DNA showed maximum inhibitions ranging from 28% to 43% in eight SLE sera (Fig. 36 and 37). Table VII shows the inhibition data of anti-DNA autoantibodies binding to DNA from these serum samples. The binding of SLE anti-DNA autoantibodies to TMP-BSA conjugate and ROS-TMP-BSA conjugate was determined by direct binding ELISA. Out of eight high titre sera, six show higher absorbance values for TMP-BSA conjugate as compared to its ROS-modified form (Fig. 38).

Purification of SLE IgG

Protein-A binds IgG from most mammalian species through interactions with the Fc part of IgG molecules. SLE IgG was purified by affinity chromatography on Protein-A Sepharose CL-4B column. The purified IgG from normal and SLE sera were found to elute in a single symmetrical peak. The homogeneity of purified IgG was confirmed by the presence of a single band in SDS-PAGE, under non-denaturing conditions (Fig. 39). In order to investigate the binding activity of purified SLE IgG with native DNA, direct binding ELISA was performed. Figure 40 shows appreciable binding with native DNA, while normal human IgG showed negligible binding. Native DNA showed maximum inhibitions ranging from
Fig 3.3 Counterimmunoelectrophoresis of SLE anti-DNA autoantibodies with TMP-BSA (a) and ROS-TMP-BSA conjugate (b). Electrophoresis was performed on 0.6% agarose gel for 2 hr at 140 V.
Fig. 34 Evaluation of anti-DNA autoantibodies in SLE patients. The microtitre plates were coated with native DNA. SLE serum 1(▲), 2(△), 3(○), 4(●), 5(▽) and NHS (+).
Fig. 35 Evaluation of anti-DNA autoantibodies in SLE patients. The microtitre plates were coated with native DNA. SLE serum 6(▲), 7(△), 8(○), 9(●), 10(▼) and NHS (+).
**TABLE VI**

Titre of SLE Anti-DNA Autoantibodies

<table>
<thead>
<tr>
<th>SLE patients</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1600</td>
</tr>
<tr>
<td>2</td>
<td>1:6400</td>
</tr>
<tr>
<td>3</td>
<td>1:1600</td>
</tr>
<tr>
<td>4</td>
<td>&gt;1:6400</td>
</tr>
<tr>
<td>5</td>
<td>1:3200</td>
</tr>
<tr>
<td>6</td>
<td>1:6400</td>
</tr>
<tr>
<td>7</td>
<td>&gt;1:3200</td>
</tr>
<tr>
<td>8</td>
<td>1:3200</td>
</tr>
<tr>
<td>9</td>
<td>1:400</td>
</tr>
<tr>
<td>10</td>
<td>1:400</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with nDNA (2.5 ìg/ml)
Fig. 36 Inhibition of anti-DNA antibody binding by native DNA with SLE serum 1(△), 2(○), 3(▲) and 4(●). Microtitre plates were coated with native DNA (2.5 μg/ml).
Fig. 37 Inhibition of anti-DNA antibody binding by native DNA with SLE serum 5(○), 6(△), 7(▲) and 8(●). Microtitre plates were coated with native DNA (2.5 μg/ml).
<table>
<thead>
<tr>
<th>SLE Sera</th>
<th>Maximum percent inhibition at 20 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with nDNA (2.5 µg/ml)
Fig. 38 Binding of SLE serum antibodies (1:100 dilution) to TMP-BSA conjugate (■) and ROS-TMP-BSA conjugate (●) Microtitre plates were coated with 10 μg/ml of respective antigens.
Fig. 39  Elution profile of SLE IgG on Protein A-Sepharose CL-4B affinity column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Fig. 40  Direct binding ELISA of normal human IgG ( ▲ ) and SLE IgG ( △ ) with native DNA (2.5 μg/ml).
Fig. 41 Inhibition of anti-DNA antibody binding by native DNA with SLE IgG 1(○), 2(△), 3(▲) and 4(●). Microtitre plates were coated with native DNA (2.5 µg/ml).
Fig. 42  Inhibition of anti-DNA antibody binding by native DNA with SLE IgG 5(○), 6(△), 7(▲) and 8(●). Microtitre plates were coated with native DNA (2.5 μg/ml).
TABLE-VIII

Competitive Inhibition of SLE IgG by Native DNA

<table>
<thead>
<tr>
<th>SLE IgG</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
<th>Concentration required for 50% inhibition (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>55</td>
<td>19</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with nDNA (2.5 μg/ml).
32% to 55% in eight SLE IgGs (Fig. 41 and 42). Table VIII summarizes the maximum inhibition of SLE IgG with native DNA.

Binding of Anti-DNA Antibodies with Native and ROS-Modified Conjugates.

The specificity of each SLE IgG for native and ROS-TMP-BSA conjugates were evaluated by inhibition assays. In this case, the SLE IgG was first incubated with native and modified conjugates and then assayed for residual binding to double stranded DNA. The microtitre plates were coated with native DNA. TMP-BSA conjugate showed maximum inhibitions ranging from 32% to 85% (Fig. 43 and 44). ROS-TMP-BSA conjugate showed relatively low percent inhibition as compared to its native form (Fig. 45 and 46). Table IX and X summarizes the inhibition results of anti-DNA autoantibodies binding to native DNA by TMP-BSA and ROS-TMP-BSA conjugates.

Detection of Circulating Antibodies in Cancer Sera

For this study, 45 serum samples were collected from patients suffering from cancer of different organs/organ systems. Cancer sera were obtained after careful clinical examination of patients with proven histopathological diagnosis attending J.N. Medical College Hospital. A.M.U., Aligarh. Sera from normal, healthy individuals served as controls.

The binding of circulating antibodies in cancer sera (at 1:100 dilution) with TMP-BSA conjugate and ROS-TMP-BSA conjugate was assessed by direct binding ELISA. Out of eight breast cancer sera tested, six showed higher recognition of ROS-TMP-BSA conjugate (Fig. 47). Oral cancer sera recognizes both native and ROS-modified conjugates (Fig. 48).
Fig. 43 Inhibition of anti-DNA antibody binding by TMP-BSA conjugate. Curves (○), (△), (▲) and (●) represent 4 different SLE IgG with TMP-BSA conjugate as inhibitor. The microtitre plates were coated with native DNA (2.5 μg/ml).
Fig. 4.4 Inhibition of anti-DNA antibody binding by TMP-BSA conjugate. Curves (O), (△), (●) and (●) represent 4 different SLE IgG with TMP-BSA conjugate as inhibitor. The microtitre plates were coated with native DNA (2.5 μg/ml).
**TABLE-IX**

Inhibition of Anti-DNA Autoantibodies by TMP-BSA conjugate

<table>
<thead>
<tr>
<th>SLE IgG</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
<th>Concentration required for 50% inhibition (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>36*</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>0.7</td>
</tr>
<tr>
<td>7</td>
<td>57</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>73</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Inhibition at 5 μg/ml

The microtitre plates were coated with nDNA (2.5 μg/ml)
Fig. 45 Inhibition of anti-DNA autoantibodies binding by ROS-TMP-BSA conjugate. Curves ( O ), ( △ ), ( ▲ ) and ( ● ) represent 4 different SLgG IgG with ROS-TMP-BSA conjugate as inhibitor. The microtitre plates were coated with native DNA (2.5 μg/ml).
Inhibition of anti-DNA autoantibodies binding by ROS-TMP-BSA conjugate. Curves (O), (△), (▲) and (●) represent 4 different SLE IgG with ROS-TMP-BSA conjugate as inhibitor. The microtitre plates were coated with native DNA (2.5 µg/ml).
### TABLE- X

**Inhibition of Anti-DNA autoantibody binding by ROS-TMP-BSA conjugate**

<table>
<thead>
<tr>
<th>SLE IgG</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
<th>Concentration required for 50% inhibition (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>36</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>36</td>
<td>-</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with nDNA (2.5 μg/ml)
Fig 47 Binding of breast cancer serum samples (1:100 dilution) to TMP-BSA (▱) and ROS-TMP-BSA conjugates (❖) Normal human serum (NHS) showed negligible binding to either of antigens. Microtitre plates were coated with 10 μg/ml of respective antigens.
Fig. 48 Binding of oral cancer serum samples (1:100 dilution) to TMP-BSA conjugate (■) and ROS-TMP-BSA conjugate (▲). Normal human serum (NHS) showed negligible binding to either of antigens. Microtitre plates were coated with 10 μg/ml of respective antigens.
Direct binding results of sera from tonsil cancer patients revealed similar binding with native and ROS-modified conjugates (Fig. 49). Out of five serum samples from cancer of gall bladder, three gave higher binding with ROS-TMP-BSA conjugate, while one serum showed appreciable binding with TMP-BSA conjugate (Fig. 50). Serum samples from patients with liver cancer showed binding to both native and ROS-modified forms. Two sera showed slightly higher binding towards ROS-modified conjugate (Fig. 51). Similarly, sera from lung and larynx cancer revealed preferential binding with ROS-modified conjugate (Fig. 51).

The binding specificity of antibodies in cancer sera were analyzed by competitive binding assay. Six sera were tested from patients suffering from breast cancer (Aged 40 to 65 years). Out of these six, only two sera showed moderate inhibition with TMP-BSA conjugate. Two sera showed almost similar level of inhibition for native (31% and 27%) and ROS-modified conjugate (29% and 30%), while the other two serum samples showed reactivity to a lesser extent with native and ROS-modified TMP-BSA conjugates (Fig. 52).

Among the seven serum samples from patients with cancer of oral cavity (four males, 40 to 65 years old and three females, 30 to 55 years old), three showed moderate but higher inhibition to TMP-BSA conjugate than ROS-TMP-BSA conjugate. Two sera showed slight higher inhibition of 14% and 26% as compared to 8% and 18% with TMP-BSA conjugate (Fig. 53). Two serum samples among these seven samples showed no inhibition with either of the antigens (Table XI).

Three sera (two male and one female) were from patients with hepatocellular (liver) carcinoma. All three sera had antibodies with a
Fig 49  Binding of tonsil cancer serum samples (1:100 dilution) to TMP-BSA (■) and ROS-TMP-BSA conjugates (▲). Normal human serum (NHS) showed negligible binding to either of the antigens. Microtitre plates were coated with 10 μg/ml of respective antigens.
Fig 50  Binding of gall bladder cancer serum samples (1:100 dilution) to TMP-BSA (■) and ROS-TMP-BSA conjugates (□). Normal human serum (NHS) showed negligible binding to either of antigens. Microtitre plates were coated with 10 μg/ml of respective antigens.
Fig. 51 Binding of liver (n=1-3), lung (n=4-6) and larynx (n=7-8) cancer sera samples (1:100 dilution) to TMP-BSA (■) and ROS-TMP-BSA (■) conjugates. Normal human serum (NHS) showed negligible binding to either of antigens. Microtitre plates were coated with 10 µg/ml of respective antigens.
Fig. 52 Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the sera of patients with breast cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml). The curves (Δ), (○), (▲), (●) and (▽) represent sera from different patients.
Fig. 53 Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the sera of patients with oral cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml). The curves (△), (○), (▲), (●)
Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the sera of patients with liver cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml). The curves (Δ), (O) and (△) represent sera from different patients.
higher recognition to ROS-TMP-BSA conjugate (32%, 34% and 41%), whereas TMP-BSA conjugate was inhibited to a lesser extent (11%, 21% and 25%) (Fig. 54)

Three samples were collected from patients with lung cancer (all males, aged 30 to 55 years). All these serum samples showed higher inhibition with ROS-TMP-BSA conjugate (37%, 35% and 46%) than towards TMP-BSA conjugate (15%, 20% and 33%) (Fig. 55). The results point out conclusively to a higher reactivity of antibodies in lung cancer sera towards ROS-modified TMP-BSA conjugate over unmodified conjugate.

Four sera (all from females) were from patients suffering from cancer of the gall bladder. Antibodies in these serum samples showed preferential binding i.e., higher inhibition with ROS-modified conjugate (37%, 52%, 43% and 47%) over unmodified TMP-BSA conjugate (33%, 23%, 27% and 30%) (Fig. 56). Four sera were from patients with tonsil cancer. Two sera showed higher recognition of TMP-BSA conjugate (34% and 39%) over ROS-modified conjugate (17% and 35%). The other two sera showed almost same reactivity with native (50% and 45%) and ROS-modified conjugates (53% and 43%) (Fig. 57).

Two sera were collected from patients with carcinoma of larynx. One serum showed preferential binding with TMP-BSA conjugate (40%) over its ROS-modified form (33%). The other serum showed a higher recognition for ROS-TMP-BSA conjugate (25%) over its native form (17%) (Fig. 58). Sera from patients with carcinoma of cervix and rectum showed no inhibition with either native or its ROS-modified form. The binding data of the cancer sera are summarized in Table XI.
Fig. 55 Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the sera of patients with lung cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml). The curves ( △ ), ( ○ ) and ( ▲ ) represent sera from different patients.
Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the sera of patients with gall bladder cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 µg/ml). The curves (Δ), (○), (▲) and (●) represent sera from different patients.
Fig. 57 Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the sera of patients with tonsil cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml). The curves (△), (○), (▲) and (●) represent sera from different patients.
Fig. 58  Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the sera of patients with larynx cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 µg/ml). The curves (Δ) and (○) represent sera from different patients.
**TABLE-XI**

Inhibition of Circulating Antibodies in Cancer Sera with TMP-BSA and ROS-modified TMP-BSA Conjugates

<table>
<thead>
<tr>
<th>Type of Cancer</th>
<th>No. of sera tested</th>
<th>Maximum percent inhibition by TMP-BSA conjugate (20 µg/ml) **</th>
<th>Maximum percent inhibition by ROS-TMP-BSA conjugate (20 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>6</td>
<td>43*, 28, 31, 26 41, 27</td>
<td>27, 21, 29, 15 31, 30</td>
</tr>
<tr>
<td>Oral</td>
<td>7</td>
<td>8, 18, 31, 35 30, #, #</td>
<td>14, 26, 19, 27 26, #, #</td>
</tr>
<tr>
<td>Liver</td>
<td>3</td>
<td>11, 21, 25</td>
<td>32, 34, 41</td>
</tr>
<tr>
<td>Lung</td>
<td>3</td>
<td>15, 20, 33</td>
<td>37, 35, 46</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>4</td>
<td>33, 23, 27*, 30</td>
<td>37, 52*, 43, 47</td>
</tr>
<tr>
<td>Tonsil</td>
<td>4</td>
<td>50, 34, 45, 39</td>
<td>53, 17, 43, 35</td>
</tr>
<tr>
<td>Cervix</td>
<td>3</td>
<td>#, #, #</td>
<td>#, #, #</td>
</tr>
<tr>
<td>Larynx</td>
<td>2</td>
<td>40, 17</td>
<td>33, 25</td>
</tr>
<tr>
<td>Rectum</td>
<td>2</td>
<td>#, #</td>
<td>#, #</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with ROS-TMP-BSA conjugate (10 µg/ml)
* Inhibition at 10 µg/ml
# Insignificant inhibition
** Maximum inhibition in case of each cancer sera has been indicated.
Binding characteristics of IgG from Cancer Sera

Cancer IgG was isolated and purified by affinity chromatography on Protein A-Sepharose column. The IgG eluted in a single symmetrical peak. Its purity was checked by SDS-PAGE under non-denaturing conditions.

Purified cancer IgG was investigated for antibodies against TMP-BSA conjugate and its ROS-modified form. Immune complexes were made between native and ROS-modified conjugate and purified IgG. Microtitre plates were coated with ROS-TMP-BSA conjugate. IgG was isolated from four patients with breast cancer. All the IgGs showed higher recognition of ROS-modified conjugate over that of native conjugate. One of them showed a maximum inhibition of 62% with ROS-TMP-BSA conjugate (Fig. 59). Two IgG isolated from hepatocellular carcinoma showed higher recognition of ROS-TMP-BSA conjugate (maximum inhibition of 38% and 49%) over that of TMP-BSA-conjugate (maximum inhibition of 28% and 32%) (Fig. 60). Similar results were obtained with the IgG isolated from patients with gall bladder cancer. In this case, maximum inhibitions of 43% and 57% for ROS-modified conjugate, and only 30% and 38% was observed with TMP-BSA conjugate (Fig. 61). IgG isolated from five patients with oral cancer showed varied results. Out of these five IgGs, four showed maximum inhibitions with TMP-BSA conjugate (30%, 42%, 52% and 59%) than towards ROS-modified conjugate (28%, 23%, 44% and 27%). One IgG showed almost similar reactivity to TMP-BSA and its ROS-modified form (43% and 40%, respectively) (Fig. 62). The binding data of the purified IgG to native and ROS-modified TMP-BSA conjugate is summarized in Table XII.
Fig. 59  Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the purified IgG isolated from sera of patients with breast cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 µg/ml). The curves (△), (○), (▲) and (●) represent sera from different patients.
Fig. 60 Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the purified IgG isolated from sera of patients with liver cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml). The curves (O) and (Δ) represent sera from different patients.
Fig. 61 Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the purified IgG isolated from sera of patients with gall bladder cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 μg/ml). The curves (O) and (Δ) represent sera from different patients.
Fig. 62 Detection of antibodies against TMP-BSA (a) and ROS-TMP-BSA conjugates (b) in the purified IgG isolated from sera of patients with oral cancer. The microtitre plates were coated with ROS-TMP-BSA conjugate (10 µg/ml). The curves (△), (○), (▲) (●) and (▽) represent sera from different patients.
### TABLE-XII

**Competitive Inhibition Data of Cancer IgG with TMP-BSA and ROS-TMP-BSA Conjugates.**

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>No. of IgG tested</th>
<th>Maximum percent inhibition by TMP-BSA conjugate (20 µg/ml)</th>
<th>Maximum percent inhibition by ROS-TMP-BSA conjugate (20 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>4</td>
<td>20, 37, 33, 48</td>
<td>43, 43, 51, 62</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>28, 32</td>
<td>38, 49</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>2</td>
<td>30, 38</td>
<td>43, 57</td>
</tr>
<tr>
<td>Oral</td>
<td>5</td>
<td>30, 42, 52, 59, 43</td>
<td>28, 23, 44, 27, 40</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with ROS-TMP-BSA conjugate (10 µg/ml)
DISCUSSION
Antibodies reactive with a number of nuclear antigens have been found in the sera of patients with systemic lupus erythematosus (SLE). Their entire spectrum is unknown, but there is considerable data suggesting that a 'single antigen', such as DNA may command a broad heterogeneity of antibodies with specificity towards receptor sites that are quite variable not only from patient to patient but even in the same patient (Arana and Seligman, 1967; Stollar, 1967; Stollar and Levine, 1963; Stollar et al., 1962a & b). These naturally occurring antibodies not only provide diagnostic/prognostic parameters to clinicians but give molecular biologists valuable tools for the better understanding of cellular processes (Woodruff et al., 1986). The binding diversity of lupus autoantibodies to a whole spectrum of modified nucleic acid conformers seems to be enormous (Arif et al., 1995; Arjumand et al., 1995; Arjumand and Ali, 1994; Klinman et al., 1994; Moinuddin and Ali, 1994; Alam et al., 1992 & 1993; Alam and Ali, 1992; Ali et al., 1991). Efforts to understand the origin and consequence of anti-dsDNA antibodies are still in progress.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

**Conclusions**

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

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

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

3. TMP-BSA conjugate was modified with $H_2O_2$ in presence of UV light, for the generation of $\cdot OH$ radical, resulting in the formation of strand breaks and modification of thymine base as characterized by various physico-chemical techniques.

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

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

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

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


