Results
Ultraviolet absorption spectral studies of native and MG-Lys-Cu\(^{2+}\) glycated human DNA

Pilot experiments were undertaken to work out the time of incubation and optimum concentration of MG, lysine and Cu\(^{2+}\) needed to modify the DNA. Human DNA (37.8 \(\mu\)M) was incubated with MG, lysine and Cu\(^{2+}\) along with respective controls for different time intervals (3, 6, 12 and 24 hr) at 37 °C. Maximum hyperchromicity at 260 nm was obtained at 24 hr incubation mixture and further incubation did not result in any change in the hyperchromicity. Therefore, for further characterization, human DNA was incubated for 24 hr with 40 mM each of methylglyoxal and lysine in presence and absence of copper sulphate (Cu\(^{2+}\). 300 \(\mu\)M) in phosphate buffer saline was used. Native and modified DNA samples were subjected to UV- spectroscopical analysis on UV-1700 spectrophotometer. A characteristic peak at 260 nm was observed with native DNA (Fig. 3). However, upon modification with methylglyoxal + lysine (in absence of Cu\(^{2+}\) ions). 68% hyperchromicity was observed in comparison to native human DNA. While DNA modified with methylglyoxal + lysine (in presence of Cu\(^{2+}\)) exhibited further increase in hyperchromicity, which was 76% above that of native DNA. Furthermore, a new peak, more of a shoulder, appeared at 330 nm in the modified samples. This might be due to glycated nitrogenous bases of DNA. The controls, Cu\(^{2+}\), lysine, lysine + Cu\(^{2+}\), MG, and MG + Cu\(^{2+}\) treated DNA did not show appreciable hyperchromicity under similar conditions.

Agarose gel electrophoresis of native and MG-Lys-Cu\(^{2+}\) glycated human DNA

Electrophoresis of native and modified DNA was performed on 0.8% agarose gel. The gel pattern (Fig. 4) shows an increase in the mobility of glycated DNA with increasing incubation time (lanes 2- 5). Maximum mobility was observed at 24 hr incubation, and further incubation did not have any consequential effect on DNA migration pattern. The increase in mobility may be due to the generation of single stranded breaks by glycation induced intermediates which may cause the formation of small size DNA having faster mobility compared to native DNA of lane 1.
Fig. 3 Ultraviolet absorption spectra of native human DNA (—); human DNA modified with 300 μM Cu²⁺ (—); 40 mM lysine ( ); 40 mM lysine + 300 μM Cu²⁺ ( ); 40 mM MG (—); 40 mM MG + 300 μM Cu²⁺ (—); 40 mM MG + 40 mM lysine (—) and 40 mM MG + 40 mM lysine + 300 μM Cu²⁺ (—).
Fig. 4  Agarose gel electrophoresis of native and modified human DNA. DNA samples from lane 2-5 are treated with MG-lysine (40 mM each) and Cu$^{2+}$ (300 μM).

Lane 1: Native human DNA.
Lane 2: Modified human DNA with 3 hr. incubation.
Lane 3: Modified human DNA with 6 hr. incubation.
Lane 4: Modified human DNA with 12 hr. incubation.
Lane 5: Modified human DNA with 24 hr. incubation.
Fluorescence studies of native and MG-Lys-Cu$^{2+}$ glycated human DNA

Generation of fluorogenic AGEs in glycated-DNA samples was measured using excitation wavelength of 370 nm ($\lambda_{ex}$) and emission wavelength of 450 nm ($\lambda_{em}$) (Table 4). This is a characteristic excitation and emission wavelength of AGEs fluorophor. Under identical conditions, native human DNA alone does not give any fluorescence. Glycation of DNA by methylglyoxal and lysine in the presence copper sulphate generated fluorescent DNA-AGEs characterized by emission maxima of 450 nm. An increase of 76.2% of fluorescence intensity was observed in glycated DNA when compared to native form (Fig. 5).

Circular dichroism of native and MG-Lys-Cu$^{2+}$ glycated human DNA

Circular dichroism is used for elucidating conformational changes in biomacromolecules, which measures differential absorption of right and left circularly polarized light. The CD spectra of nucleic acids result primarily due to the spatial asymmetry of the constituent nucleotides. The CD profile of human DNA (37.8 μM), was recorded at a wavelength range of 220 – 400 nm which exhibited a negative peak at 243 nm, and a positive peak at 275 nm. (Fig. 6). Structural changes in DNA were evaluated by ellipticity measurements. The CD signal of modified analogue shifted from 275 to 278 nm in the direction of higher wavelength, which is indicative of structural changes in DNA. When native DNA was compared with the modified DNA, it showed an increase in ellipticity from 6.64 to 8.47 mdeg at 275 nm. This increase in ellipticity corresponds to 27.56 % structural loss in modified DNA. The structural loss in DNA after modification might be due to unstacking of bases as a result of helix destabilization.

Genotoxicity of advanced glycation end-products in human lymphocytes by single cell gel electrophoresis (comet assay)

The photographs of comets seen on treatment of lymphocytes with different combinations of MG (40 mM), lysine (40 mM) and Cu$^{2+}$ (300 μM) are shown in Fig. 7. A comet with a tail is indicative of DNA breakage in single cell gel electrophoresis. The results clearly establish that MG in combination with lysine is capable of DNA breakage in lymphocytes as evident from the formation of distinct
TABLE 4

Fluorescence characteristics of native and modified human DNA

<table>
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<tr>
<th>Parameter</th>
<th>Native DNA</th>
<th>Modified DNA</th>
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<tr>
<td>Fluorescent intensity ($I_f$)</td>
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<td>44.14</td>
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<tr>
<td>Wavelength for max $I_f$</td>
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<td>450</td>
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<tr>
<td>Stokes shift (nm)</td>
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<td>24</td>
</tr>
<tr>
<td>Ratio of quantum efficiency</td>
<td>3.25</td>
<td>1</td>
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</table>
Fig. 5  Fluorescence emission spectra of native human DNA (—) and modified human DNA with 40 mM MG + 40 mM lysine + 300 μM Cu²⁺ (----).
Circular dichroic spectra of native human DNA (—) and modified human DNA with 40 mM MG + 40 mM lysine + 300 μM Cu$^{2+}$ (----).
Fig. 7 Single cell gel electrophoresis of human lymphocytes showing comets after treatment with MG or lysine and Cu\(^{2+}\). (A) untreated, (B) treated with 40 mM lysine, (C) with 40 mM MG, (D) with 40 mM MG + 40 mM lysine and (E) with 40 mM MG + 40 lysine + 300 µM Cu. All the lymphocytes were treated for 24 hour at 37 °C.
tail from the diffused head. However, in the presence of Cu$^{2+}$ the damage of lymphocyte DNA was found to be enhanced by 36% (Fig. 7 E). This suggests that Cu$^{2+}$ has enhanced the genotoxicity of MG+Lys system by increasing free radical (O$_2^-$ and ·OH) generation. Furthermore, MG and lysine alone did not cause a significant damage to the lymphocyte DNA.

The DNA damage parameters i.e. olive tail moment (OTM), mean percentage of tail DNA and tail length were also measured (Figs. 8-10) and found to be significantly increased in MG-Lys-Cu$^{2+}$ system as compared to MG-Lys system. A marked increase in OTM (316%) and mean percentage of tail DNA (246%) was observed in MG-Lys-Cu$^{2+}$ treated lymphocytic cells when compared to control (untreated lymphocytes). Furthermore, there was a pronounced increase in tail length, which was found to be 92% as compared to control.

**Nuclease S1 digestibility of native and MG-Lys-Cu$^{2+}$ glycated human DNA**

Native and modified DNA were digested with nuclease S1 (20 units/mg DNA) for 30 minutes and electrophoresed on 0.8% agarose to visualize the generation of single strand breaks. The controls were native and modified DNA samples, untreated with nuclease S1. Modified DNA showed decrease in fluorescence intensity following nuclease S1 digestion. On the other hand nuclease S1 treated and untreated native DNA showed almost identical electrophoretic migration pattern and fluorescence intensity (Fig. 11). These observations clearly demonstrate that sufficient distortions (formation of single strand breaks) are caused in the helical structure of DNA by MG-Lys-Cu$^{2+}$ treatment, rendering it susceptible to digestion by single strand specific nuclease S1.

**Thermal denaturation of native and MG-Lys-Cu$^{2+}$ glycated human DNA**

Thermally induced transitions were measured spectrophotometrically at 260 nm by heating nucleic acid samples at a rate of 1 °C/ minute. Melting profile of modified and unmodified human DNA were analysed in the temperature range of 30 °C to 95 °C. Increase in absorbance at 260 nm was taken as a measure of helix denaturation. The process was characterized by determining the percent DNA in
Fig. 8 Effect of lysine (40 mM), MG (40 mM), MG + lysine (40 mM each), and MG + lysine (40 mM each) + Cu^{2+} (300 µM) on olive tail moment in lymphocytic DNA.
Fig. 9 DNA damage (% DNA in tail) in the comet assay in peripheral lymphocytes after treatment with lysine (40 mM), MG (40 mM), MG + lysine (40 mM each) and MG + lysine (40 mM each) + Cu²⁺ (300 μM).
DNA damage (Tail length, μm) in the comet assay in peripheral lymphocytes after treatment with 40 mM of lysine; 40 mM of MG; MG + lysine (40 mM each) and MG + lysine (40 mM each) + Cu²⁺ (300 μM).
Fig. 11  Nuclease S1 digestibility of native and modified DNA. Lane 1 contains native DNA, while Lane 2 contains native DNA treated with nuclease S1 for 30 min; Lane 3 contains modified DNA and Lane 4 contains modified DNA treated with nuclease S1 for 30 min. Electrophoresis was carried out on 0.8% agarose gel for 2 hr at 30 mA.
denatured state as a function of temperature. The melting temperature, \( T_m \), of native DNA was found to be 86 °C, while in case of modified DNA it was recorded at 76 °C, which shows a significant decrease in the \( T_m \) value of modified DNA as compared to its unmodified native form (Fig. 12). A decrease of 10 °C in the \( T_m \) value in case of modified human DNA point towards alterations in the DNA molecule as a result of modification that may be unstabilizing the helix. Generation of strand breaks and base modifications cause altered hydrogen bonding between base pairs, resulting in thermal susceptibility of the DNA molecule. Moreover, the melting curve for modified DNA indicated a progressive and heterogeneous local melting with the increase in temperature as compared to more homogenous global melting in the control. Early onset of melting in the case of modified DNA is a definite indication of structural instability consequent to modification. The thermal denaturation characteristics of native and modified DNA are listed in Table 5.

**Quenching studies**

The modification of human DNA in the present study is a result of production of \('\text{OH} \) and \( \text{O}_2'^- \). Its *in vitro* generation was confirmed by the use of different quenchers of various radicals. Quenchers like SOD, mannitol, EDTA and catalase were exploited to study their quenching effect on MG-Lys-Cu\(^{2+}\) modified human DNA. As evident from the figure 13, the hydroxyl radical ('\(\text{OH}\) trapping agent (mannitol) and superoxide radical (\( \text{O}_2'^- \)) trapping agent (SOD) strongly inhibited (82.6% and 78.3%, respectively) the modification by MG-Lys-Cu\(^{2+}\) system, thereby indicating the involvement of these radicals. Whereas EDTA showed marked inhibition of 69%. Moreover, uric acid, an antioxidant inhibited DNA modification to the extent of 46%.

**Quantitation of hydroxyl radical in MG-Lys-Cu\(^{2+}\) system**

The generation of hydroxyl radicals in the MG-Lys-Cu\(^{2+}\) system was measured with thiobarbituric acid-reactive substance (TBARS). The incubation of 2-deoxy-D-ribose with lysine and MG produced 13 nmol TBARS ml\(^{-1}\) (Fig. 14). However, reaction of MG with lysine in the presence of Cu\(^{2+}\) enhanced it to 24 nmol TBARS ml\(^{-1}\). Radical scavengers like mannitol, catalase and a metal ion chelator, desferrioxamine significantly inhibited the production of TBARS (Fig. 15). TBARS
Fig. 12  Thermal melting profile of native human DNA (□) and modified human DNA (■).
**TABLE 5**

Ultraviolet and thermal denaturation characteristics of native and modified human DNA

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Modified DNA</th>
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<tbody>
<tr>
<td>Absorbance ratio (A_{260}/A_{280})</td>
<td>1.76</td>
<td>1.4</td>
</tr>
<tr>
<td>Hyperchromicity at 95 °C (%)</td>
<td>36.5</td>
<td>24</td>
</tr>
<tr>
<td>Melting temperature (Tm), °C</td>
<td>86</td>
<td>76</td>
</tr>
<tr>
<td>Onset of duplex melting, °C</td>
<td>72</td>
<td>36</td>
</tr>
</tbody>
</table>
Fig. 13  Effect of various scavengers of free radical (mannitol, SOD), metal ion chelator (EDTA) and antioxidant (uric acid) on human DNA modification induced by MG-Lys-Cu$^{2+}$ system.
Fig. 14 Generation of hydroxyl radicals during the glycation reaction of lysine with MG in the presence of Cu$^{2+}$. The reaction mixtures contained 100 mM 2-deoxy-D-ribose in 10 mM phosphate buffer at pH 7.4 and the following: 40mM MG, 40mM lysine, 300μM Cu$^{2+}$ with their different controls as depicted in diagram. Data represent the means ± S.D. (n=4).
The reaction mixtures contained 100 mM 2-deoxy-D-ribose along with 40 mM each of MG and lysine and 300μM Cu²⁺ in the presence of radical scavenger, mannitol (50 mM), catalase (CAT) (2mg/ml) and a metal ion chelator, Desferrioxamine (DFXA) (10mM). Data represent the means ± S.D. (n=4).
generation was inhibited up to 71.7% when mannitol was used as scavenger. However, both catalase and DFXA inhibited TBARS equally, which was found to be almost 67%. The result suggests that the MG-mediated hydroxyl radical generation may be caused by traces of transition metals. Furthermore, it is also being suggested that the redox reactions of copper may facilitate the generation of hydroxyl radical by reaction of MG and lysine.

**Quantitation of superoxide anion in MG-Lys system**

Superoxide generation was quantitated by cytochrome c reduction experiment. During incubation of MG with lysine, the formation of superoxide anion was gradually increased in time dependent manner. The incubation of MG (40 mM) with lysine (40 mM) produced 28.2 nmol \( O_2^- \) ml\(^{-1}\) h\(^{-1}\) compared to 2.46 nmol \( O_2^- \) ml\(^{-1}\) h\(^{-1}\) with MG (40 mM) alone and 1.96 nmol \( O_2^- \) ml\(^{-1}\) h\(^{-1}\) with lysine (40 mM) alone (Fig. 16). Superoxide dismutase (SOD) inhibited the superoxide radical generation. As evident from figure 17, on increasing concentration of SOD, the superoxide generation was gradually decreased.

**Anti-glycation study**

Anti-glycating agent D-penicillamine (1 mM) and pyridoxal phosphate (PLP) (10 mM) showed remarkable inhibition of 55% and 73% respectively in DNA modification as analyzed at 260 nm. However, at 330 nm D-penicillamine and PLP showed still higher inhibition of 69% and 85% respectively (Fig. 18). The enhanced inhibition at 330 nm is due to the formation of AGEs at this wavelength range (330-360 nm) (Schmitt et al., 2005). This clearly indicates the formation of AGEs in the MG-Lys-Cu\(^{2+}\) modified human DNA.

**Synthesis and characterization of \( N^2\)-(1-Carboxyethyl)-2-deoxyguanosine (C\( \bar{E} \)dG)**

Synthesis of the standard, C\( \bar{E} \)dG was performed as described by (Seidel and Pischetsrieder, 1998) with slight modifications. After final preparation, C\( \bar{E} \)dG was isolated by preparative HPLC using 50 mM ammonium acetate buffer solution and methanol as eluents. The elution of C\( \bar{E} \)dG was obtained at a retention time of 14.399
Fig. 16  Generation of the superoxide anion in the glycation reaction of lysine with MG. Reduction of cytochrome c was measured by increasing concentrations of reaction products. The reaction mixture contained 10 μM cytochrome c in 10 mM phosphate buffer at pH 7.4 and the following: 40 mM MG (■); 40 mM lysine (■); 40 mM MG and lysine (▲). The absorbance changes were monitored at 550 nm for 10 min at room temperature.
Fig. 17 Superoxide formation during the glycation reaction of lysine by MG. MG (40 mM) and lysine (40 mM) was reacted in presence of various concentrations of superoxide dismutase, 50 units (1), 100 units (2) and 500 units (3).
Fig. 18  Effect of antiglycating agent D-Penicillamine (1 mM) and pyridoxal phosphate (10 mM) on the modification of DNA induced by MG-Lys-Cu²⁺ system. Percent DNA modification was calculated at 260 nm and 330 nm after 24 hr incubation.
min when UV detector was used for the experiment. However, deoxyguanosine (dG) gave elution at a retention time of 9.1 min (Fig. 19).

**Nuclear magnetic resonance of CEdG**

For structural assignment of the compound, the CEdG peak was isolated by HPLC and then subjected to $^1$H NMR analysis. Resonance signals can be identified and characterized in $^1$H NMR spectrum of CEdG (Fig. 20), recorded in DMSO-d6. $^1$H NMR (400MHz, DMSO-d6, 20 °C) assignment for CEdG: $\delta$ 10.6 (s, 1H, N1-H), $\delta$ 7.93 (s, 1H, C8-H), $\delta$ 6.76 (d, 1H, C2-NH), $\delta$ 6.12 (dd, 1H, C1'-H), $\delta$ 5.3 (d, 1H, C3'-OH), $\delta$ 4.89 (s, 1H, C5'-OH), $\delta$ 4.36 (m, 1H, C2-NH-CH), $\delta$ 4.18 (q, 1H, C2-NH-CH), $\delta$ 3.81 (m, 1H, C3'-H), $\delta$ 3.5 (ddd, 2H, C5'-H$_2$), $\delta$ 2.64 (ddd, 1H, C2'-H), $\delta$ 1.39 (d, 3H, C2-NH-CH-CH$_3$).

The $^1$H NMR analysis indicated a $-CHX-CH_3$ group bound to the purine (guanine) ring. The chemical shifts ($\delta$), 4.18 ppm (q, 1H, C2-NH-CH) and 1.39 ppm (d, 3H, C2-NH-CH-CH$_3$) respectively, is attributed to the carboxyethyl group of the standard carboxyethyl-deoxyguanosine (CEdG).

**Characterization of native and MG-Lys-Cu$^{2+}$ glycated human DNA by HPLC**

Figures 21 and 22 show the representative HPLC chromatograms of acid hydrolysed samples of native and modified human DNA respectively. Well defined peaks at retention time 4.467 min, 7.332 min, 8.727 min were observed in native DNA. However, in the case of modified DNA these peaks shifted to 6.748 min, 8.455 min and 12.399 min respectively, suggesting considerable change in the DNA bases. The extra peak at a retention time of 14.249 min in the acid hydrolysate of modified DNA is characteristic of N$^2$-(1-Carboxyethyl)-2-deoxyguanosine (CEdG) adduct. This is in conformity with the standard CEdG results wherein also when deoxyguanosine was exposed to dihydroxyacetone, a distinct peak at retention time of 14.399 min was observed (Fig. 19). The CEdG adduct is a marker of glucose and MG induced DNA glycation.
Fig. 19 Representative HPLC chromatogram of the reaction of 2'-deoxyguanosine with dihydroxyacetone.
Fig. 20 $^1$H NMR spectra of the standard CEdG.
Fig. 21 Representative HPLC chromatogram of acid hydrolysate of native human DNA.
Fig. 22  Representative HPLC chromatogram of acid hydrolysate of modified human DNA.
Detection of $\text{N}^2$-(1-Carboxyethyl)-2-deoxyguanosine ($\text{CEdG}$) formed in modified human DNA by LC-MS

The UV spectrum of the adduct showed the maxima at 254 nm same as that for unmodified deoxy-guanosine, indicating that it is the modification of same nucleoside. The standard (CEdG) and modified DNA samples were then analyzed by mass spectrometer for full scan in negative mode, giving evidence for the adduct formed $[\text{M- H}]^-$ at $m/z$ values of 338 (Figs. 23-25). In addition, the observed masses corresponded with the calculated masses for single charged ions $[\text{M- 1}]^-$ of the nucleoside plus 1 equivalent of MG ($m/z$ 338 for dGuo). Whereas, we did not find peak at $m/z$ value of 338 for native DNA.

Characterization of DNA-AGEs by electrospray ionization mass spectrometry (ESI-MS)

In an attempt to confirm the formation of Schiff base and amadori product in glycated DNA, mass spectrometry was used to analyze the hydrolysed glycated human DNA. Figures 26-27 show the respective mass-spectral profiles of hydrolyzed native and MG-Lys-Cu$^{2+}$ glycated human DNA. The ion at $m/z$ 341 is consistent with a $[\text{Schiff base+H}]^+$ molecule resulting from the condensation reaction of dG (Mr 285.26) with methylglyoxal (Mr 70.06) in a dehydration reaction involving the loss of a water molecule. The ion at $m/z$ 679 is consistent with the formation of a $[\text{Schiff base+H}]^+$ dimer product. The ion at 268 is speculated to result from the loss of a hydroxyl group from dG. The ion with $m/z$ 385 is assumed to be fragment formed by the degradation of methylglyoxal reacting with Schiff base product, or its enaminol or Amadori intermediates.

Immunogenicity of MG-Lys-Cu$^{2+}$ modified human DNA

The antigenicity of MG-Lys-Cu$^{2+}$ modified human DNA was evaluated by inducing antibodies in female rabbits. Antigenic specificity of the induced antibodies was assayed by direct binding and competition ELISA. The binding of these antibodies to the immunogen and native human DNA was further checked by gel retardation assay.
Fig. 23 Full scan LC-MS spectral analysis of synthesized CEdG standard.
Fig. 24 Full scan LC-MS spectral analysis of hydrolyzed native human DNA (1mg/ml).
Fig. 25 Full scan LC-MS spectral analysis of hydrolyzed modified human DNA (1mg/ml).
Fig. 26 Full scan ESI-MS spectral analysis of native human DNA (1mg/ml) of HPLC resolved products.
Fig. 27 Full scan ESI-MS spectral analysis of modified human DNA (1mg/ml) of HPLC resolved glycated products.
Antibodies against MG-Lys-Cu$^{2+}$ modified human DNA

Antiserum obtained from immunized animal was subjected to direct binding immunoassay on immunogen coated polystyrene microtitre plates. The antiserum showed a high titre (> 1: 12800) in direct binding ELISA while preimmune serum, used as control, showed feeble binding on immunogen coated plates (Fig. 28). The specificity of induced anti-MG-Lys-Cu$^{2+}$ modified human DNA antibodies was evaluated by competitive inhibition ELISA. A maximum of 62.2% inhibition in antibody binding was observed at 20 μg/ml of the immunogen (Fig. 29). The concentration for 50% inhibition was 10.1 μg/ml. While MG-Lys-Cu$^{2+}$ modification of human DNA rendered it highly immunogenic, the native counterpart did not produce any appreciable immune response in experimental animals (Fig. 30 & 31 respectively).

Purification and characterization of serum IgG

Immunoglobulin G was isolated from preimmune and immune rabbit serum by affinity chromatography on Protein A-agarose column (Fig. 32). The purity of IgG was ascertained by SDS-polyacrylamide gel electrophoresis in absence of a reducing agent. Appearance of a single band in SDS-PAGE under non reducing condition (Fig.32 inset) establishes the purity of IgG. Direct binding ELISA of the purified anti-MG-Lys-Cu$^{2+}$-DNA IgG showed strong binding towards its immunogen (Fig. 33). However, the preimmune IgG showed negligible binding. The specificity of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG was evaluated by competition ELISA, wherein 88.5% inhibition in the antibody binding was achieved by the MG-Lys-Cu$^{2+}$ modified human DNA, while native DNA could cause only 35.2% inhibition in antibody binding (Fig. 34). Fifty percent inhibition for MG-Lys-Cu$^{2+}$ modified human DNA binding was observed at an inhibitor concentration of 2.8 μg/ml.

Band shift assay

Antigen-antibody interaction was further evaluated by band shift assay. Constant amount of human DNA or its MG-Lys-Cu$^{2+}$ modified form was incubated with varying amount of anti-MG-Lys-Cu$^{2+}$-DNA IgG for 2 hr at 37 °C and overnight at 4 °C. The resulting immune complex was then electrophoresed on 0.8% agarose
Fig. 28  Level of induced antibodies against MG-Lys-Cu$^{2+}$ modified human DNA. Direct binding ELISA with preimmune serum (□) and immune serum (■). The microtitre wells were coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 29 Inhibition ELISA of serum antibodies against MG-Lys-Cu$^{2+}$ modified human DNA (- ■ -) and preimmune serum (- □ -). MG-Lys-Cu$^{2+}$ modified human DNA was used as a coating antigen (2.5 μg/ml) as well as an inhibitor.
Fig. 30 Level of induced antibodies against native human DNA. Direct binding ELISA with preimmune serum (∙) and immune serum (■). The microtitre wells were coated with native human DNA (2.5 µg/m
Fig. 31 Inhibition ELISA of anti-native human DNA antibodies (-■-) and preimmune (-○-) sera. Native human DNA used as a coating antigen (2.5 μg/ml) as well as an inhibitor.
Fig. 32 Elution profile of anti-MG-Lys-Cu²⁺ modified human DNA IgG on Protein-A agarose affinity column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel
Fig. 33 Binding of affinity purified anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG (●) and preimmune IgG (□) to MG-Lys-Cu$^{2+}$ modified human DNA. Microtitre plates were coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 34 Inhibition of anti-MG-Lys-Cu\textsuperscript{2+} modified human DNA IgG binding by native human DNA (○ ○) and modified human DNA (■ ■) sera. The microtitre plate was coated with MG-Lys-Cu\textsuperscript{2+} modified human DNA (2.5 μg/ml).
for 2 hr at 30 mA. Figure 35 (a) shows the binding of IgG to MG-Lys-Cu\(^{2+}\) modified human DNA. With the increase in the amount of IgG, there was an increase in the formation of high molecular weight immune complexes, which resulted in retarded mobility and increased fluorescence intensity near the well. However, under similar experimental conditions the unmodified human DNA failed to show the shift in its mobility when incubated with anti-MG-Lys-Cu\(^{2+}\)-DNA IgG [Fig. 35 (b)]. These results clearly indicate that epitopes on native human DNA and its modified counterpart have quite distinct recognition.

**Immuno-crossreactivity of anti-MG-Lys-Cu\(^{2+}\) modified human DNA antibodies**

Antigenic specificity of the induced anti-MG-Lys-Cu\(^{2+}\) modified human DNA antibodies was investigated by competitive inhibition ELISA. The induced antibodies exhibited a wide range of heterogeneity in recognizing varied inhibitors that include nucleic acid polymers and bases. A maximum of 89.5% inhibition of the anti-MG-Lys-Cu\(^{2+}\)-DNA IgG was observed with the immunogen as an inhibitor (Fig. 34). The induced antibodies were highly specific for MG-Lys-Cu\(^{2+}\) modified human DNA as only 2.8 \(\mu\)g/ml inhibitor was required to cause 50% inhibition. While, native human DNA caused considerably reduced inhibition (35.1 %) in antibody activity (Fig. 34).

Native, ROS and MG-Lys-Cu\(^{2+}\) modified calf thymus DNA inhibited the induced antibodies by 33.3%, 48.6% and 64.2% respectively (Fig. 36). Whereas ROS modified human DNA showed maximum inhibition of 57.1% at 20 \(\mu\)g/ml. To achieve 50% inhibition, 13.2 \(\mu\)g/ml MG-Lys-Cu\(^{2+}\) modified calf thymus DNA was required. However, for ROS modified human DNA, 50% inhibition was attained at an inhibitor concentration of 15.1 \(\mu\)g/ml.

Plasmid DNA, its ROS and MG-Lys-Cu\(^{2+}\) modified forms caused 32.1%, 43.9% and 54.2% inhibition respectively in the binding of the immune IgG to the modified DNA (Fig. 37). The amount of MG-Lys-Cu\(^{2+}\) modified plasmid required for 50% inhibition was 19.3 \(\mu\)g/ml.

Lymphocyte DNA of human origin modified by MG-Lys-Cu\(^{2+}\) and ROS showed the maximum inhibition of 72.5% and 59% respectively (Fig. 38), while
Fig. 35 Band shift assay of anti-MG-Lys-Cu$^{2+}$ modified human DNA-IgG binding to (a) MG-Lys-Cu$^{2+}$ modified human DNA and (b) native human DNA. Electrophoresis was carried out on 0.8% agarose gel for 2 hr at 30mA.

(a) MG-Lys-Cu$^{2+}$ modified human DNA (0.5 $\mu$g, Lane 1) was incubated with 10, 20, 40 and 60 $\mu$g of anti-MG-Lys-Cu$^{2+}$ modified human DNA-IgG (lanes 2-5) for 2 hr at 37°C and overnight at 4°C.

(b) Native human DNA (0.5 $\mu$g, Lane 1) was incubated with 10, 20, 40 and 60 $\mu$g of anti-MG-Lys-Cu$^{2+}$ modified human DNA-IgG (lanes 2-5) under identical conditions.
Fig. 36  Inhibition of anti-MG-Lys-Cu$_{2+}$ modified human DNA IgG binding by calf thymus DNA (- Δ -), ROS modified calf thymus DNA (- ▲ -), ROS modified human DNA (- ○ -) and MG-Lys-Cu$_{2+}$ modified calf thymus DNA (- ■ -). The microtiter plate was coated with MG-Lys-Cu$_{2+}$ modified human DNA (2.5 µg/ml).
Fig. 37 Inhibition of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG binding by plasmid DNA (- ▲ -), ROS modified plasmid DNA (- □ -), and MG-Lys-Cu$^{2+}$ modified plasmid DNA (- ■ -). The microtiter plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
Fig. 38  Inhibition of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG binding by lymphocyte DNA (- ▲ -), ROS modified lymphocyte DNA (- □ -), and MG-Lys-Cu$^{2+}$ modified lymphocyte DNA (- ■ -). The microtiter plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
native lymphocyte DNA inhibited 34.5% binding of immune IgG to immunogen. Fifty percent inhibition of antibody activity was achieved with 8.8 μg/ml of MG-Lys-Cu$^{2+}$ modified lymphocyte DNA and 17.8 μg/ml of ROS modified DNA.

The DNA bases guanine, cytosine, adenine, thymine and their ROS (·OH) modified and MG-Lys-Cu$^{2+}$ modified forms were used as inhibitors of antibody binding activity. Guanine showed a moderate inhibition of 38 percent. In contrast, ROS and MG-Lys-Cu$^{2+}$ modified guanine were potent inhibitors showing maximum inhibition of 59% and 78% respectively (Fig. 39). The amount of ROS-guanine and MG-Lys-Cu$^{2+}$ modified guanine required for 50% inhibition in antibody activity was 16.6 μg/ml and 8.9 μg/ml respectively. Adenine caused inhibition 29% inhibition, whereas its ROS modified form was a moderate inhibitor, showing a maximum of 54% inhibition in antibody activity. In contrast, MG-Lys-Cu$^{2+}$ modified form was found to be a potent inhibitor, showing a maximum inhibition of 65% (Fig. 40). The amount of MG-Lys-Cu$^{2+}$ modified adenine for 50% inhibition was 14.2 μg/ml, whereas 19.4 μg/ml was required in the case of ROS-modified adenine to get 50% inhibition in the antibody activity. Thymine and its ROS modified form produced 26% and 32% inhibition respectively (Fig. 41), whereas MG-Lys-Cu$^{2+}$-thymine showed a moderate inhibition of 43%. In contrast, cytosine and its ROS and MG-Lys-Cu$^{2+}$ modified forms showed a maximum of 27%, 43% and 47% inhibition respectively (Fig. 42). Binding characteristics of the induced antibodies against MG-Lys-Cu$^{2+}$-DNA have been summarized in Table 6.

**Binding of autoantibodies against native and MG-Lys-Cu$^{2+}$ modified human DNA in diabetes patients**

In this clinical study, we have randomly selected total 85 cases of both diabetes type I (40 samples) and type II (45 samples). The sera were obtained from patients attending J.N. Medical College and hospital, A.M.U., Aligarh after the informed consent. Sera from age and sex-matched normal healthy individuals served as control. All sera were diluted to 1:100 in TBS-T and subjected to direct binding ELISA on solid phase separately coated with equal amounts of human DNA and MG-Lys-Cu$^{2+}$ modified human DNA. Out of total 40 type I diabetes sera, 27 samples
Fig. 39  Inhibition of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG binding by guanine (- ▲ -), ROS modified guanine (- □ -), and MG-Lys-Cu$^{2+}$ modified guanine (- ■ -). The microtiter plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
In Fig. 40, the inhibition of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG binding by adenine (- △ -), ROS modified adenine (- □ -), and MG-Lys-Cu$^{2+}$ modified adenine (- ■ -) is shown. The microtiter plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
Fig. 41  Inhibition of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG binding by thymine (- ▲ -), ROS modified thymine (- □ -), and MG-Lys-Cu$^{2+}$ modified thymine (- ■ -). The microtiter plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml)
Fig. 42 Inhibition of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG binding by cytosine (- ▲ -), ROS modified cytosine (- □ -), and MG-Lys-Cu$^{2+}$ modified cytosine (- ■ -). The microtiter plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
TABLE 6
Cross-reaction of anti-MG-Lys-Cu$^{2+}$ modified human DNA IgG

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Maximum% inhibition at 20 µg/ml</th>
<th>Concentration for 50% inhibition (µg/ml)</th>
<th>Percent relative affinity</th>
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<tr>
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<tr>
<td>ROS calf thymus DNA</td>
<td>48.6</td>
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<td>-</td>
</tr>
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<td>ROS human DNA</td>
<td>57.1</td>
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</tr>
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<td>MG-Lys-Cu$^{2+}$-plasmid</td>
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<td>ROS thymine</td>
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<td>MG-Lys-Cu$^{2+}$-thymine</td>
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<td>ROS cytosine</td>
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<tr>
<td>MG-Lys-Cu$^{2+}$-cytosine</td>
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</table>

Microtitre plates were coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5µg/ml).
67.5%), (Figs. 43-46) showed higher binding with MG-Lys-Cu\(^{2+}\) modified human DNA, while in type II diabetes only 16 sera (35.5%) out of 45 samples tested, showed enhanced binding with modified human DNA as compared to the native form.

The serum samples which showed enhanced binding were considered for further studies, while samples whose absorbance was less than or equal to control were not included.

**Immuno-crossreactivity of autoantibodies from diabetes type I patients**

Competition ELISA was carried out to analyze the specific binding of circulating autoantibodies in type I diabetic patients sera for native and MG-Lys-Cu\(^{2+}\) modified human DNA.

In the 27 sera chosen from type I diabetes patients, which showed enhanced binding, the observed maximum inhibition with MG-Lys-Cu\(^{2+}\) modified human DNA was in the range of 46.9 to 63.1% while with native human DNA it ranged from 22 to 33% (Figs. 47-55). Mean inhibition for the entire sample tested with native human DNA was 27±3.8%, while for MG-Lys-Cu\(^{2+}\) modified human DNA, it was 54.95±5.4%. The above results have been summarized in Table 7.

**Purification of IgG from the sera of type I diabetes patient**

IgG was isolated on a protein A-agarose column from selected high binding sera of type I diabetes patients. The purified IgG eluted as a symmetrical single peak on the affinity column (Fig. 56). IgG purity was confirmed by a single homogeneous band on SDS-PAGE under non-reducing conditions (Fig. 56 inset).

**Binding of IgG from different diabetes type I patients to native and MG-Lys-Cu\(^{2+}\) modified human DNA**

Purified IgG from type I diabetes patients, were subjected to direct binding ELISA, on a microtitre plate coated with native human DNA and MG-Lys-Cu\(^{2+}\) modified human DNA to evaluate the amount required for antigen saturation. The saturation for modified human DNA was obtained at 50 μg/ml of IgG, while for native human antigenic saturation could not be ascertained because of its negligible
Fig. 43  Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 1-10) to native (■) and MG-Lys-Cu$^{2+}$ modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml)
Fig. 44 Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 11-20) to native (■) and MG-Lys-Cu$^{2+}$ modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 45  Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 21-30) to native (●) and MG-Lys-Cu$^{2+}$ modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
Fig. 46  Direct binding ELISA of serum autoantibodies from type I diabetes patients (no. 31-40) to native (■) and MG-Lys-Cu\(^{2+}\) modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu\(^{2+}\) modified human DNA (2.5 μg/ml).
Fig. 47 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). Sera 1, 3 and 4 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 48 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (○, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). Sera 7, 8 and 9 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 49 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (○, △, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). Sera 10, 11 and 14 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 50  Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (○, △, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). Sera 15, 17 and 18 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 51 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (♦, △, ■). Sera 20, 21 and 22 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 52 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, △, □) and MG-Lys-Cu\textsuperscript{2+} modified human DNA (●, ▲, ■). Sera 23, 24 and 25 were analyzed on the microtitre plate coated with MG-Lys-Cu\textsuperscript{2+} modified human DNA (2.5 µg/ml).
Fig. 53  Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (○, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). Sera 28, 29 and 31 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml)
Fig. 54 Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu²⁺ modified human DNA (●, ▲, ■). Sera 32, 34 and 36 were analyzed on the microtitre plate coated with MG-Lys-Cu²⁺ modified human DNA (2.5 μg/ml).
Fig. 55  Competitive inhibition of serum autoantibodies in type I diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). Sera 37, 38 and 39 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
TABLE 7

Competitive inhibition data of serum autoantibodies in type I diabetes patients

<table>
<thead>
<tr>
<th>Sera no</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
</tr>
</thead>
<tbody>
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<td></td>
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<tr>
<td>38</td>
<td>20.2</td>
</tr>
<tr>
<td>39</td>
<td>31.7</td>
</tr>
</tbody>
</table>

| Mean ± SD | 26.98±3.8% | 54.8±5.4% |

The microtitre plates were coated with MG-Lys-Cu\(^{2+}\) modified human DNA (2.5μg/ml).
Fig. 56 Elution profile of IgG from the serum sample of diabetes type I patients on Protein A-agarose affinity column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel. Similar pattern was obtained in all sera.
binding. Therefore, for type I diabetes, IgG concentration was kept constant (50 μg/ml) in all further experiments unless indicated.

The binding specificity of the isolated IgG, towards native and MG-Lys-Cu^{2+} modified human DNA, was evaluated by inhibition ELISA. The IgG was mixed with varying amounts of native or MG-Lys-Cu^{2+} modified human DNA (0-20 μg/ml) and incubated for 2 hr at 37 °C and overnight at 4 °C. The inhibition results of type I diabetic sera IgG have been presented in Figs. 57-61. The observed antibody (IgG) inhibition ranged from 59-69% when modified human DNA was employed as inhibitor, while with the native human DNA it varied from 27-36%; maximum inhibitor concentration being 20 μg/ml in both the cases. The mean of inhibitions for various sample tested with the MG-Lys-Cu^{2+} modified human DNA was 66.2±7.1%, while with native human DNA, it was 31.3±3.2%. Table 8 summarizes the inhibition data of isolated IgG of diabetes type I group.

**Band shift assay**

Band shift assay was performed for the visual detection of interaction of native and MG-Lys-Cu^{2+} modified human DNA with purified IgG from type I diabetes patients IgG. Constant amount of antigens were incubated with increasing concentration of IgG for 2 hr at 37 °C and overnight at 4 °C, resulted in a proportional increase in the formation of high molecular weight immune complexes as visualized by retarded mobility and gradually increased band intensity near the wells in agarose gel electrophoresis. Normal human IgG incubated under identical conditions did not show immune complex formation (Fig. 62 a & b).

**Detection of antibodies against native and MG-Lys-Cu^{2+} modified human DNA in diabetes type II patients**

Forty five samples from diabetes type II patients were analysed for native and MG-Lys-Cu^{2+} modified human DNA by direct binding ELISA. Sera from normal healthy individuals served as controls. Out of 45 serum sample only 16 sera (35.5%), exhibited enhanced binding with MG-Lys-Cu^{2+} modified human DNA as compared to the native DNA (Figs. 63-66). However, the binding in diabetes type II patients was found appreciably lower than in type I patients. The specific binding of serum
Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). The microtitre plates were coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml). The above plot shows the inhibition in IgG isolated from serum samples 3, 7 and 8.
Fig. 58 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). The microtitre plates were coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml). The above plot shows the inhibition in IgG isolated from serum samples 14, 15 and 17.
Fig. 59 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (○, △, □) and MG-Lys-Cu\textsuperscript{2+} modified human DNA (●, ▲, ■). The microtitre plates were coated with MG-Lys-Cu\textsuperscript{2+} modified human DNA (2.5 μg/ml). The above plot shows the inhibition in IgG isolated from serum samples 18, 21 and 24.
Fig. 60 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■). The microtitre plates were coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml). The above plot shows the inhibition in IgG isolated from serum samples 25, 31 and 32.
Fig. 61 Inhibition of IgG, isolated from the sera of type I diabetes patients, by native (o, Δ, □, ◊) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■, ♦). The microtitre plates were coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml). The above plot shows the inhibition in IgG isolated from serum samples 34, 37, 38 and 39.
### TABLE 8

**Competitive inhibition data of IgG isolated from type I diabetes patients**

<table>
<thead>
<tr>
<th>Sera no</th>
<th>Maximum percent inhibition at 20 μg/ml</th>
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<td>Native human DNA</td>
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</table>

**Mean ± SD**

- Native human DNA: 31.46±3.2%
- MG-Lys-Cu\(^{2+}\) modified human DNA: 65.56±7.1%

The microtitre plates were coated with MG-Lys-Cu\(^{2+}\) modified human DNA (2.5μg/ml).
Fig. 62  Band shift of IgG from diabetic patient using (a) native and (b) MG-Lys-Cu$^{2+}$ modified human DNA. Varying concentrations of IgG were incubated with a constant amount of DNA (0.5 µg) for 2 hr at 37 °C and overnight at 4 °C. Electrophoresis was carried out on 0.8% agarose gel for 2 hr at 30mA. Lane 1 contains native (or modified) human DNA while lanes 2-5 contain native or MG-Lys-Cu$^{2+}$ modified human DNA with 20, 40, 60 and 80 µg of IgG from type I diabetes patient.
Fig. 63 Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.1-11) to native (■) and MG-Lys-Cu\(^{2+}\) modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu\(^{2+}\) modified human DNA (2.5 µg/ml).
Fig. 64  Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.12-22) to native (■) and MG-Lys-Cu$^{2+}$ modified human DNA (●). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 65  Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.23-33) to native (■) and MG-Lys-Cu\textsuperscript{2+} modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu\textsuperscript{2+} modified human DNA (2.5 μg/ml).
Fig. 66  Direct binding ELISA of serum autoantibodies from type II diabetes patients (no.34-45) to native (■) and MG-Lys-Cu$^{2+}$ modified human DNA (■). Normal human sera (NHS) served as negative control. The plate was coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
antibodies to native and MG-Lys-Cu$^{2+}$ modified human DNA was studied by competition inhibition solid phase assay. An inhibition in the range of 18% to 30.1% and 34.4% to 53% (Figs. 67-71) was recorded with native and MG-Lys-Cu$^{2+}$ modified human DNA respectively. Mean inhibition for the entire sample tested with native human DNA was 23.9±3.0%, while for MG-Lys-Cu$^{2+}$ modified human DNA, it was 44.6±5.3%. The inhibition studies results have been summarized in Table 9.

Purification of IgG from the sera of type II diabetes patient

IgG was isolated from the selected high binding serum samples of type II diabetes patients on a protein A-agarose column. IgG purified by affinity chromatography eluted as a symmetrical single peak (Fig. 72) and migrated as a single homogeneous band on SDS-PAGE under non-reducing conditions (Fig. 72 inset).

Binding of IgG from diabetes type II patients to native and MG-Lys-Cu$^{2+}$ modified human DNA

Purified IgG from type II diabetes patients, were subjected to direct binding ELISA, on a microtitre plate coated with native human DNA and MG-Lys-Cu$^{2+}$ modified human DNA to evaluate the amount required for antigen saturation. The saturation for modified human DNA was obtained at 80 µg/ml of IgG, while for native human antigenic saturation could not be ascertained because of its negligible binding. Therefore, for type II diabetes, IgG concentration was kept constant (80 µg/ml) in all further experiments.

The specific binding of the IgG isolated from type II diabetes patients was ascertained in competitive inhibition ELISA wherein an inhibition in the range of 24% to 36% and 49% to 57% was recorded with native and MG-Lys-Cu$^{2+}$ modified human DNA respectively (Figs. 73-75). Mean inhibition for the entire sample tested with native human DNA was computed to be 29.3±3.6%, while for MG-Lys-Cu$^{2+}$ modified human DNA, it was 53±7.3%. Table 10 summarizes the inhibition data of isolated IgG of diabetes type II group.
Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (●, ▲, ■). Sera 2, 7 and 8 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).
Fig. 68  Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu^{2+} modified human DNA (●, ▲, ■). Sera 19, 12 and 15 were analyzed on the microtitre plate coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).
Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (○, △, □) and MG-Lys-Cu$^{2+}$ modified human DNA (○, △, ■). Sera 19, 20 and 24 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
Fig. 70 Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (o, Δ, □) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ▼). Sera 26, 29 and 31 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 µg/ml).
Fig. 71 Competitive inhibition of serum autoantibodies in type II diabetes patients by native human DNA (○, △, □, ◇) and MG-Lys-Cu$^{2+}$ modified human DNA (●, ▲, ■, ♦). Sera 32, 37, 38 and 45 were analyzed on the microtitre plate coated with MG-Lys-Cu$^{2+}$ modified human DNA (2.5 μg/ml).
### TABLE 9

**Competitive inhibition data of serum autoantibodies in type II diabetes patients**

<table>
<thead>
<tr>
<th>Sera no</th>
<th>Native human DNA</th>
<th>MG-Lys-Cu(^{2+}) modified human DNA</th>
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<td>50.0</td>
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<tr>
<td>45</td>
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<td>34.4</td>
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</table>

| Mean ± SD | 23.9±3.0% | 44.62±5.3% |

The microtitre plates were coated with MG-Lys-Cu\(^{2+}\) modified human DNA (2.5\(\mu\)g/ml).
Fig. 72  Elution profile of IgG from the serum of diabetes type II patients on Protein A-agarose affinity column. Inset: SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.
Inhibitor Concentration, \( \mu \text{g/ml} \)

Percent Inhibition

Fig. 73  Inhibition of IgG isolated from type II diabetes patients (samples 2, 7 and 8) by native (o, Δ, □) and MG-Lys-Cu\(^{2+}\) modified human DNA (•, ▲, ■). The microtitre plates were coated with MG-Lys-Cu\(^{2+}\) modified human DNA (2.5 \( \mu \text{g/ml} \)).
Fig. 74 Inhibition of IgG isolated from type II diabetes patients (samples 10, 19 and 29) by native (○, △, □) and MG-Lys-Cu²⁺ modified human DNA (●, ▲, ■). The microtitre plates were coated with MG-Lys-Cu²⁺ modified human DNA (2.5 μg/ml).
Fig. 75  Inhibition of IgG isolated from type II diabetes patients (samples 31 and 38) by native (o, Δ) and MG-Lysine-Cu^{2+} modified human DNA (●, ▲). The microtitre plates were coated with MG-Lys-Cu^{2+} modified human DNA (2.5 μg/ml).
TABLE 10

Competitive inhibition data of IgG isolated from type II diabetes patients

<table>
<thead>
<tr>
<th>Sera no</th>
<th>Native human DNA</th>
<th>MG-Lys-Cu²⁺ modified human DNA</th>
</tr>
</thead>
<tbody>
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<tr>
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<td>49.0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>29.3±3.6%</td>
<td>53±7.3%</td>
</tr>
</tbody>
</table>

The microtitre plates were coated with MG-Lys-Cu²⁺ modified human DNA (2.5µg/ml).