Carbamylated-BSA is immunologically active and autoantibodies in sera of diabetic nephropathy patients show strong binding towards it

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Abstract
Isocyanates (ICN) have drawn considerable attention in the recent past as they can react with macromolecules (polypeptides, nucleic acids etc.) and produce toxic insults. ICN react with side chain nitrogen of amino acid residues of polypeptides. Pathophysiological implications resulting from occupational and accidental exposures of ICN are yet too elusive. It may be produced in high concentrations in conditions of chronic renal failure and chronic inflammatory diseases (diabetes mellitus, rheumatoid arthritis etc.). In this study, bovine serum albumin (BSA) has been carbamylated and characterized by UV and AGE. Antibodies against carbamylated-BSA were raised in animals and characterized by direct binding and inhibition ELISA. Presence of anti-carbamylated-BSA autoantibodies in sera of diabetic nephropathy patients was elucidated by ELISA. Carbamylated-BSA exhibited hyperchromicity at 280 nm compared to native BSA. Carbamylated-BSA was found to be highly immunogenic as compared to native BSA. Experimental induction of antibodies against carbamylated-BSA and presence of autoantibodies against carbamylated-BSA in diabetic nephropathic patients’ sera points towards the role for carbamylated-BSA in diabetic nephropathy.

Keywords: Isocyanates; Carbamylated-BSA; Autoantibodies; Diabetic nephropathy patients.

Introduction
Immune response to toxic exposures of industrial and environmental chemicals has been largely documented in the past two decades. Reports from different cohort studies suggest that immune system is a possible target of such toxic exposures\textsuperscript{[1]}. Even though our immunity, one way or other, is influenced by the environment, reduction in the number of immunocompetent cells or alterations in function, selection, and differentiation of lymphocytes following occupational or accidental exposures might have detrimental effects\textsuperscript{[2]}. Immunotoxins may interfere with processes at genomic level. Records of preliminary studies suggest that isocyanates (ICN) and their derivatives may have deleterious health effects but molecular mechanisms underlying such an effect have never been addressed\textsuperscript{[3,4]}. Methyl isocyanate (MIC), a reactive industrial byproduct is one of the most toxic ICN and is known to exert
detrimental effects on numerous organ systems and immunity. In vitro studies carried out on MIC and its reaction products have shown evidence of mutagenicity evoking probable clastogenic activity. MIC also reacts with side chain nitrogen of tryptophan and tyrosine residues of polypeptides to produce carbamylated products, producing polypeptide cross-links in turn contributing to cytotoxicity. These data implicate that occupational and accidental exposure to MIC might possibly increase individuals’ susceptibility by eliciting hypersensitivity reactions, causing autoimmunity or immune suppression.

The objectives of present study are: To modify BSA with Urea and study the changes in urea-modified BSA by physicochemical techniques like UV spectrophotometry and agarose gel electrophoresis; to evaluate the immunogenicity of urea-modified BSA in experimental animals; to use the urea-modified BSA as an antigen for detection of autoantibodies against urea-modified BSA in sera of diabetic nephropathy patients and to propose a potential role for urea-modified BSA in T2DM nephropathy.

**Materials and Methods**

**Materials**

Bovine serum albumin (BSA), urea, Freund’s adjuvants (complete and incomplete), methylated BSA, Anti-human and anti-rabbit IgG alkaline phosphatase conjugate and dialysis tubings were purchased from Sigma-Aldrich, USA. Disodium hydrogen phosphates, sodium dihydrogen phosphate, sodium chloride, ethylenediamine tetraacetic acid (EDTA) were purchased from Qualigens/Thermo-Fischer, India. Para-nitrophenyl phosphate (PNPP) was purchased from SRL, India. Dimethyl sulfoxide (DMSO) was obtained from J.T.Baker Chemical Company, USA. All other reagents and chemicals used were of the highest analytical grade available.

**Collection and processing of animal blood samples**

Use of experimental animals was approved by the Institutional Animal Ethics Committee, Katihar Medical College, Al-Karim University, Katihar. Blood (4 ml) was withdrawn from marginal ear veins of rabbits before and after completion of immunization, in plain vials. The blood was allowed to clot and sera were separated. All sera were heat decomplemented at 56°C for 30 min and stored in aliquotes at -20°C.

**Collection and processing of human blood samples**

All patients (or their legal guardians) and healthy subjects consented for blood samples. A total of 30 patients of diabetic nephropathy (17 males & 13 females) and equal number of healthy subjects (16 males & 14 females) were included in the study. All patients of diabetic nephropathy fulfilled the revised criteria of the American Diabetes Association (2013). Sera were heated at 56°C for 30 min and stored at -20°C. The study protocol was approved by the Institutional Ethics Committee, Katihar Medical College, Al-Karim University, Katihar.

**Modification of BSA by urea**

BSA (25 µg/ml) was mixed with urea (0.19 and 0.58 mM) in 10 mM PBS (pH 7.4) and kept at 25°C for 2h in sterile capped glass tubes. At the end of incubation, the samples were extensively dialyzed against PBS to remove excess urea. Solutions of urea and BSA dissolved separately in the same buffer and kept under identical conditions served as control.

**Absorbance spectroscopy**

UV profiles of native and urea-modified BSA were recorded on spectrophotometer using quartz cuvette of 1 cm path length and hyperchromicity was calculated from the following formula:

\[
\% \text{ hyperchromicity at } 280 \text{ nm} = \left( \frac{\text{OD modified sample} - \text{OD native sample}}{\text{OD native sample}} \right) \times 100
\]
Enzyme-Linked Immunosorbent Assay (ELISA)

**Direct binding ELISA**

Direct binding ELISA was performed on flat bottom maxisorp plates as described earlier[10]. Briefly, the wells were filled with 100 μl of antigen (native or urea-modified BSA) prepared in antigen coating buffer and incubated at 37°C for 2h followed by overnight incubation at 4°C. Each sample was coated in duplicate and half of the plate, devoid of antigen, served as control. Following the overnight incubation, the plates were washed thrice with TBS-T (20 mM Tris, 2.68 mM KCl, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20) and unoccupied sites were blocked by 150 μl of 1.5% fat-free skimmed milk in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4-6h at 37°C followed by 3-4 washing with TBS-T. Thereafter, 100 μl of sera (serially diluted in TBS) was added to each well and plates were placed for 2h at 37°C and overnight at 4°C. After that, the plates were washed 4 times with TBS-T and bound antibodies were assayed with anti-rabbit alkaline phosphatase conjugate (diluted in TBS). This was followed by 2h incubation at 37°C and 3-4 washing with TBS-T. Finally the plates were washed with distilled water. Para-nitrophenyl phosphate was added and after 45 min of incubation at 37°C color was read at 405 nm on an automatic microplate reader. Results were expressed as mean of A_test - A_control.

**Inhibition ELISA**

Inhibition ELISA[11] was performed for specificity of antibodies. The plates were coated with 100 μl of antigen (native or urea-modified DNA, 2.5 μg/ml) for 2h at 37°C and overnight at 4°C. Varying amounts of inhibitors (0–20 μg/ml) were mixed with a constant amount of antiserum or affinity purified IgG. The mixtures were incubated at 37°C for 2h and overnight at 4°C. The immune complex was coated in the wells instead of serum. Rest of the steps were same as mentioned above in direct binding ELISA. Percent inhibition was calculated using the following equation:

\[
\text{Percent Inhibition} = 1 - \left( \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \right) \times 100
\]

**Statistical Analysis**

Data are expressed as mean ± standard deviation (±SD). Statistical significance of the results was determined by Student’s t-test and a p value of <0.05 was considered as significant.

**Results**

**UV–visible analysis of native and urea-modified DNA**

BSA (25 μg/ml) treated with varying concentrations of NSNM showed hyperchromicity at 280 nm compared to native BSA (Fig. 1, Table 1). Significant increase in absorbance was observed between 260 and 280 nm in all the modified samples compared to native BSA.

**Immunogenicity of native and Urea-modified DNA**

Carbamylation-induced structural changes in BSA were further probed by its potential to induce antibodies in experimental animals. Antisera were subjected to direct binding ELISA on maxisorp microtitre modules coated with respective immunogens. Antigen binding specificity of the induced antibodies was ascertained by inhibition ELISA.

**Antibody induction against native and urea-modified BSA**

Rabbits immunized with native and Urea-modified BSA induced low to moderate antibody response with a titre of <1:400 to >1:3200 as revealed by direct binding ELISA (Fig. 5). Preimmune sera included as control, showed negligible binding with the immunogens. A maximum inhibition of 14.7 %, 62.2 % and 61.1 % (Table 3) was observed at 20 μg/ml of the immunogens; n BSA, 0.19 mM Urea-BSA and 0.58 mM Urea-DNA, respectively).
Detection of serum autoantibodies against NSNM-modified DNA in diabetic nephropathy

The study was extended to find out the clinical relevance of NSNM-modified DNA in diabetic nephropathy. This study included 30 patients of diabetic nephropathy (DN) and 30 healthy human subjects (HHS). These HHS served as control and were age- and sex-matched. Sera from DN patients and HHS were diluted (1:100) and direct binding ELISA (Fig. 6a and 6b) was performed on microtitre plates coated with native and NSNM-modified DNA. Autoantibodies in diabetic nephropathy sera showed enhanced binding with NSNM-modified DNA as compared to native DNA.

Inhibition ELISA of patients’ sera

The binding specificity of autoantibodies in DN sera with native and NSNM-modified DNA was assessed by inhibition ELISA. Patients’ sera (No. 8, 9, 17, 18, 19 and 20) exhibiting high binding with NSNM-modified DNA in direct binding ELISA were selected for the inhibition studies. Mean percent inhibition was found to be 26.98±6.18%, 43.25±3.28% and 45.1±3.60% with nDNA, 0.19 mM and 0.58 mM NSNM-DNA, respectively (Table 4).

**Fig.1.** UV absorption spectra of native DNA (― □ ―) modified with 0.19 mM (― ○ ―) and 0.58 mM (― ● ―) NSNM.
Fig. 2. Ethidium bromide (- △-) assisted emission profile of native DNA (-□-) modified with 0.19 mM (- ○-) and 0.58 mM (- ●-) NSNM. The samples were excited at 325 nm.

Fig. 3. FT–IR spectra of native DNA (- □-) modified with 0.19 mM (- ○-) and 0.58 mM (- ●-) NSNM.
Fig. 4. Melting profile of native DNA (- □ -) modified with 0.19 mM (-○-) and 0.58 mM (- ●-) NSNM.

Fig. 5. Direct binding ELISA of native and NSNM modified-DNA from animal sera (immune and pre-immune). Pre-immune native (- ◆), modified1 (- ▲) and modified2 (- ◊-) DNA. Immune native (- △ -), modified1 (- ■-) and modified2 (- □-).
Fig. 6a. Direct binding ELISA of 1:100 diluted diabetic nephropathy sera (sample 1 to 15) to native DNA (- □ -) and 0.58 mM NSNM-DNA (- ■ -). Normal human sera (NHS) served as negative control. The ELISA plate was coated with the respective antigens (2.5 µg/ml).

Fig. 6b. Direct binding ELISA of 1:100 diluted diabetic nephropathy sera (sample 16 to 30) to native DNA (- □ -) and 0.58 mM NSNM-DNA (- ■ -). Normal human sera (NHS) served as negative control. The ELISA plate was coated with the respective antigens (2.5 µg/ml).
### Table 1 UV characteristics of native and NSNM-modified DNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance at 260 nm</th>
<th>Percent hyperchromicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native DNA</td>
<td>0.499</td>
<td>------</td>
</tr>
<tr>
<td>0.19 mM NSNM-DNA</td>
<td>0.579</td>
<td>16.0</td>
</tr>
<tr>
<td>0.58 mM NSNM-DNA</td>
<td>0.695</td>
<td>39.3</td>
</tr>
</tbody>
</table>

### Table 2 Fluorescence characteristics of native and NSNM-modified DNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>FI at 595 nm</th>
<th>Percent gain in FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native DNA + EtBr</td>
<td>10.074</td>
<td>----</td>
</tr>
<tr>
<td>0.19 mM NSNM-DNA + EtBr</td>
<td>10.937</td>
<td>7.8</td>
</tr>
<tr>
<td>0.58 mM NSNM-DNA + EtBr</td>
<td>11.287</td>
<td>10.7</td>
</tr>
</tbody>
</table>

### Table 3 Summary of inhibition of anti-n DNA and anti-NSNM-DNA serum antibodies

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Maximum percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>n DNA</td>
<td>14.7 %</td>
</tr>
<tr>
<td>0.19 mM NSNM-DNA</td>
<td>62.2 %</td>
</tr>
<tr>
<td>0.58 mM NSNM-DNA</td>
<td>61.1 %</td>
</tr>
</tbody>
</table>

Microtitre plates were coated with respective antigens.

### Table 4 Inhibition of diabetic nephropathy serum autoantibodies binding by nDNA, 0.19 mM NSNM-DNA and 0.58 mM NSNM-DNA

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>Maximum percent inhibition at 20 µg/ml</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nDNA</td>
</tr>
<tr>
<td>8</td>
<td>21.5</td>
</tr>
<tr>
<td>9</td>
<td>33.5</td>
</tr>
<tr>
<td>17</td>
<td>24.3</td>
</tr>
<tr>
<td>18</td>
<td>22.1</td>
</tr>
<tr>
<td>19</td>
<td>24.5</td>
</tr>
<tr>
<td>20</td>
<td>36.0</td>
</tr>
</tbody>
</table>

Mean ± S.D. 26.98 ± 6.18% 43.25±3.28% 45.1±3.60%

Microtitre plates were coated with 0.58 mM NSNM-DNA.
Discussion

Isocyanates (ICN) are highly reactive compounds and may cause structural changes in biomolecules. These compounds form covalent adducts with critical macromolecules such as nucleic acids, resulting in a series of biotransformation events that initiate the generation of the reactive intermediates\(^3,12\). DNA damage leading to the cellular demise in mammalian cells upon treatment with ICN has been reported\(^3\). It has also been shown that “carbamate,” the reactive intermediate of ICN also induces the analogous upshot\(^14\).

Methyl isocyanate (MIC), one of the most toxic ICN is known to exert immunological, mutagenic and genotoxic effects\(^15-17\). Previous studies conducted on ICN derivative, such as isothiocyanates, suggest that they are capable of inducing apoptosis\(^18\). Although the exact mechanism of such induction is largely unknown, majority of studies have reported the involvement of mitochondria-mediated pathway, i.e. release of cytochrome C (Cyt C) into the cytoplasm and activation of caspase 9 and caspase 3\(^19-21\).

Isocyanate derivatives (carbamates) are also known to enhance the levels of inflammatory cytokines such as IL-6, TNF-α, and IL-1 β\(^22\), whereas it is also evident that acute and chronic inflammatory status acts as a mediator in various pathological disorders although the exact mechanisms are unknown\(^23\). The capacity of inflammatory cells to generate and release a spectrum of ROS and free radicals during oxidative burst and their probable role in tumor production are well documented\(^24-27\).

In the present study, calf thymus native DNA was carbamylated with N-succinimidyl N-methylcarbamate. Modification in DNA was characterized by different physicochemical techniques (UV-visible, fluorescence and FT-IR spectroscopy, thermal melting and enzyme immunoassay).

Under our experimental conditions the carbamylated-DNA (NSNM-modified DNA) exhibited hyperchromicity at 260 nm compared to native DNA. This might be due to generation of single stranded breaks in DNA helix, inter alia unstacking of bases resulting in enhanced absorption of UV light\(^28\). The modification was further characterized by ethidium bromide-assisted fluorescence measurement. In presence of ethidium bromide the carbamylated-DNA exhibited enhancement in emission intensity compared to native DNA treated with identical dose of ethidium bromide.

FT-IR spectroscopy is a sensitive tool to study DNA damage\(^29-30\). Band fitting was performed in the spectral region of 800–1800 cm\(^{-1}\). Earlier studies have suggested that bands falling in the region 980–1149 cm\(^{-1}\) and 1151–1350 cm\(^{-1}\) correspond to symmetric and asymmetric phosphate stretching\(^31\). Furthermore, symmetric stretching of P–O–C bond and ribose-phosphate skeletal motion has been observed at 1110 cm\(^{-1}\) and 970 cm\(^{-1}\), respectively\(^32\). In this study, appearance of prominent new bands at different wave numbers suggest carbamylation-induced changes in the vibration of sugar-phosphate, base(s) etc. In other words, addition of carbamoyl group with base(s) in DNA is bound to cause stretching or bending of bonds vis-à-vis generation of a new signal at different wave number.

Carbamylation induced destabilization of native DNA was confirmed by melting of NSNM-modified DNA. The observed decrease in \(T_m\) of NSNM-modified DNA samples suggests that hydrogen bonding between bases have been changed during adduction of carbamoyl group. This may cause thermal destabilization in DNA\(^33\).

Native DNA injected into rabbits did not elicit significant immune response. But NSNM-modified DNA produced antibodies of low to moderate titre. This suggests generation of immunogenic epitopes during the course of carbamylation. Many studies have also observed induction of antibodies against modified-DNA\(^33-35\).
We have also studied the association between carbamylated-DNA and serum autoantibodies in diabetic nephropathy patients. Binding characteristics of circulating autoantibodies with native and NSNM-modified DNA was studied by direct binding and inhibition ELISA. Direct binding ELISA gave a strong clue that NSNM-modified DNA is better recognized by serum autoantibodies in DN patients. Inhibition ELISA was performed to check the specificity of circulating autoantibodies towards NSNM-modified DNA. There was high inhibition of DN serum autoantibodies with NSNM-modified DNA compared to native DNA. This suggests true binding of autoantibodies with NSNM-modified DNA. Thus, the autoantibodies exhibited preference for NSNM-modified DNA. These autoantibodies might cross react with organ tissues, causing damage to them. Therefore, regular monitoring of serum urea level in diabetes mellitus seems important to prevent kidney disease.

Conclusion
From the studies carried out, it may be concluded that NSNM is capable of causing carbamylation in DNA. Carbamylated-DNA showed hyperchromicity and increased EtBr-assisted fluorescence. Perturbations in the B-conformation of DNA were apparent as observed in FT-IR. Decreased Tm of carbamylated-DNA points towards helix destabilization. Carbamylation has conferred immunogenicity on DNA. There are autoantibodies against carbamylated-DNA in sera of diabetic nephropathy patients included in this study. Progression in the level of autoantibodies against carbamylated-DNA may serve as good indicator of start or progression of kidney dysfunction in diabetics.

Conflict of interest
All authors declare no conflict of interest.

Acknowledgements
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