DISCUSSION
Humans are exposed to a whole range of nitrogen containing compounds and nitrosating agents, such as nitrates, nitrites, peroxynitrite, nitrogen oxides, that can react in vivo to form potentially carcinogenic N-nitroso compounds, as well as several carcinogenic C-nitro or reactive diazo compounds. Nitrosating agents can also be synthesized endogenously by bacteria and activated macrophages via the nitric oxide synthase (NOS) pathway. Endogenous nitrosation can thus occur at many locations in the body, including sites of chronic inflammation or infection (Bartsch and Frank, 1996; Felley-Bosco, 1998). Nitric oxide, a multifaceted bioregulatory agent, shows an unusual divergence of action. It has been demonstrated to have a direct role in cellular signaling, vasodilation and immune response. Macrophages also use NO as a cytotoxic agent, which may lead to DNA damage and protein modifications. The effects of NO at the DNA level are complex and involve formation of N-nitrosoamines, deamination of purines and pyrimidines, or damage induced by peroxynitrite. Besides inducing DNA damage, nitric oxide may also inactivate DNA repair/replication enzymes (Delaney and Eizirik, 1996; Luperchio et al., 1996; Drew and Leeuwenburgh, 2002).

Nitric oxide is produced within cells by the action of a group of enzymes called nitric oxide synthases (NOS). Presently, there are three distinct isoforms of nitric oxide synthase: neuronal, inducible and endothelial. While NO is a relatively unreactive radical, it is able to form other reactive intermediates, which could have an effect on protein function and thus on the functions of the entire organism. These reactive intermediates can trigger nitrosative damage on biomolecules, which in turn may lead to autoimmune and age related diseases due to structural alteration of proteins, inhibition of enzyme activity, and interferences of the regulatory functions (Drew and Leeuwenburgh, 2002).

Toxicity is largely mediated via intermediates such as N2O3 and ONOO−, arising from the reaction of NO with either molecular oxygen or reactive oxygen species. In general, such reactions become significant only when high concentrations of NO are generated by the induction of NOS (Gordge, 1998; Wink et al., 1998a and b). NO is an important mediator of the inflammatory response. Over expression of NOS and overproduction of NO are parallel with the development of an autoimmune syndrome with a variety of inflammatory manifestations (Gilkeson et al., 1997). NO is involved in a number of immunopathologies, including diabetes, graft-vs-host rejection, rheumatoid arthritis, SLE and multiple sclerosis (Cook and Cattell, 1996; Parkinson et al., 1997; Kuhn et al., 1998).
Protein nitration has been suggested to be a final product of the production of highly reactive nitrogen oxide intermediates (e.g. ONOO\(^-\)) formed in reactions between NO and oxygen derived species such as superoxide (Gunther et al., 2002). The most frequently studied marker of oxidative damage to proteins is protein carbonyl groups, oxidation of tryptophan, tyrosine and cysteine residue and protein fragmentation. 3-Nitrotyrosine is thought to be a relatively specific marker of oxidative damage mediated by ONOO\(^-\). The biological significance of tyrosine nitration is a subject of great interest, because ample evidence supports the formation of 3-nitrotyrosine \textit{in vivo} in diverse pathological conditions. In addition, tyrosine nitration causes inactivation of several enzymes, influences tyrosine phosphorylation-mediated signal transduction, and induces apoptosis through impairment of microtubule formation in cells. A recent intriguing finding shows the presence of 3-nitrotyrosine denitrase activity, which repairs protein nitration in rat tissues, suggesting that a tyrosine nitration-denitration pathway participates in NO\(^-\) or ONOO\(^-\)-dependent signal transduction, similar to phosphorylation-dephosphorylation systems. These findings indicate that 3-nitrotyrosine formation has great importance not only as biomarker of nitrogen-mediated tissue injury but also as a means to gain insight into molecular mechanisms of NO-related physiological and pathophysiological phenomena (Sawa et al., 2000).

Systemic lupus erythematosus is a prototype autoimmune, multisystem and multifactorial disease characterized by the presence of autoantibodies to a variety of nuclear antigens such as DNA and histones, as well as protein antigens and protein-nucleic acid complexes. The marked heterogeneity of SLE autoantibodies has been one of the impediments in understanding the disease. SLE is also characterized by abnormal lymphocyte function that may be responsible for many of the clinical manifestations that are important in disease diagnosis (Ahsan et al., 2003; Dixit and Ali, 2004; Waris and Alam, 2004). The role of apoptosis in the pathogenesis of SLE has been suspected. One of the inducers of apoptosis is the Fas (receptor/ligand) system. Nitric oxide plays a vital role in immunity. Apoptosis is an important mechanism by which NO may contribute to the pathogenesis of SLE. Elevated NO production has been suggested in the pathogenesis of lupus-like syndrome in MRL-lpr/lpr mice. NO concentration in exhaled air significantly increased in patients with SLE compared to healthy controls (Rolla et al., 1997).

Since nitrates and nitrites are only indicators for enhanced NO production, protein associated nitrotyrosine might be a more suitable marker for damage induced by reactive
nitrogen intermediates. Furthermore, most proteins have a longer life in circulation than those of nitrates and nitrites. The presence of nitrotyrosine has been detected in various inflammatory processes (celiac disease, chronic renal failure, septic shock and atherosclerotic plaques) and rheumatic diseases including systemic lupus erythematosus, Sjoegren's syndrome, vasculitis, and rheumatoid arthritis.

In the present study, an aqueous solution of commercially available poly L-tyrosine was exposed to nitric oxide generated by sodium nitrite in acidic medium. The production of nitric oxide radical was confirmed by the use of quenchers i.e., ascorbate, carboxy-PTIO, uric acid, desferrioximine and mannitol. Nearly complete inhibition in the production of NO was observed in the presence of carboxy-PTIO, and above 70% in the case of ascorbate and uric acid. However, very low inhibition in the production of NO was observed when mannitol and desferrioximine were used.

The UV absorption spectra of native poly L-tyrosine showed only one peak at 280 nm, whereas there were two peaks observed in the case of nitrated poly L-tyrosine. Peak at 280 nm showed hypochromicity of 25% and a peak shift of 4 nm towards shorter wavelength. The observed change could be attributed to structural alterations as well as damage of chromophoric groups. Another peak was observed at wavelength of 420 nm, which is characteristic of 3-nitrotyrosine at pH >9. Nitration of tyrosine residues in purified proteins is relatively easy to detect by visible spectroscopy owing to the characteristic yellow colour, which was observed in this case. The UV difference spectra exhibited decrease in absorbance at 300 nm, and an increase in absorbance at around 420 nm. These are indicative of an appreciable portion of NO induced damage of the aromatic ring of tyrosine, as well as nitration of tyrosine forming 3-nitrotyrosine.

Disruption of the chromophoric group of tyrosine was confirmed by fluorescence spectra when nitrated poly L-tyrosine showed a decrease in fluorescence intensity at both excitation and emission wavelengths. This was further confirmed when tyrosine was estimated by Lowry’s method. The data showed a decrease in absorbance for nitrated poly L-tyrosine when compared to native poly L-tyrosine.

The right-handed α-helix structure observed for native poly L-tyrosine seems to be completely lost in the case of nitrated poly L-tyrosine, as observed in the circular dichroic spectra.
Formation of high molecular weight species indicating aggregation of nitrated poly L-tyrosine was seen in the alkaline agarose gel electrophoresis. Low molecular weight species were also observed in the case of nitrated poly L-tyrosine which indicated peptide bond cleavage, whereas native poly L-tyrosine showed a single band. The formation of low molecular weight species was confirmed by gel filtration chromatography. In the case of nitrated poly L-tyrosine, a number of peaks ranging from 43 kDa to 14.3 kDa were observed, whereas for native poly L-tyrosine, only one peak was seen in the range of 90 kDa to 40 kDa.

Another experiment confirmed the fact that NO had caused peptide bond cleavage. The interaction of poly L-tyrosine with denatured DNA was studied. It has been reported that the interaction between the two species is non-electrostatic in nature and highly specific in respect of DNA bases (Friedman and Ts’o, 1971). Tyrosine is found to bind more strongly with purines than with pyrimidines. The interaction is dependent on the conformation of the nucleic acid, i.e., poly L-tyrosine can form insoluble complexes with denatured DNA but not with native DNA. The interaction also requires the polymer of tyrosine to have a sufficient size, smaller the polymer, lesser will be the binding.

The thermal denaturation profile of native poly L-tyrosine showed gradual protein denaturation till 48°C after which protein aggregation occurred. While in the case of nitrated poly L-tyrosine, there was no aggregation point indicating that all the protein structure was already lost. This could be attributed to the disruption and structural alteration of the polymer on exposure to nitric oxide.

Tyrosine is a non-essential amino acid that is synthesized *in vivo* from phenylalanine. As a building block for several important brain chemicals, tyrosine is needed to synthesize epinephrine, norepinephrine, serotonin and dopamine. It also aids in the proper functioning of organs in the body responsible for making and regulating hormones including that of adrenal, thyroid and pituitary glands. Tyrosine also has anti-aging benefits as it works as a mild antioxidant. Tyrosine in itself may not be immunogenic but its homopolymeric form may be immunogenic, being a large polypeptide having a right handed α-helix structure.

Both free and protein bound 3-nitrotyrosine dramatically increased in pathologic conditions associated with the production of reactive oxygen and nitrogen species. There is mounting evidence that nitration of tyrosine residues in proteins can profoundly alter protein function, suggesting that protein nitration may be fundamentally related to, and be predictive of oxidative cell injury. At the very least, the presence of 3-nitrotyrosine in biological
samples indicate that reactive nitric oxide derived species were produced \textit{in vivo}, although the exact nature of these species remains to be determined (Crow, 1999).

3-Nitrotyrosine immunoreactivity has been reported in several human pathological conditions mentioned before. In addition, numerous other disease states using non-human models have been shown to involve 3-nitrotyrosine formation (Crow, 1999).

We raised polyclonal antibodies against native and nitrated poly L-tyrosine and both were found to be immunogenic in rabbits as revealed by the result of direct binding and inhibition ELISA. However nitratred poly L-tyrosine was found to be more immunogenic as compared to the un-nitrated form. Band shift assays and dot blot assay clearly demonstrated the absolute specificity of the purified immune IgG towards their respective immunogen. Antigenic specificity of purified IgG was confirmed by inhibition ELISA. A maximum of 56\% inhibition of anti-native poly L-tyrosine antibody with immunogen as inhibitor was observed at 20 $\mu$g/ml and 50 \% inhibition was achieved at 17.8 $\mu$g/ml. A maximum of 83\% inhibition in the binding of anti-nitrated poly L-tyrosine antibody with immunogen as inhibitor was observed at 20 $\mu$g/ml and, 50\% inhibition was achieved at 8.1 $\mu$g/ml. The data shows that although native poly L-tyrosine was found to be immunogenic in experimental animals, nitrated poly L-tyrosine was found to be more immunogenic in comparison to native polymer. These results suggest that the nitration of tyrosine results in the generation of neo-epitopes, making it a potential immunogen.

Western blot analysis of serum from experimental animals immunized with NO-poly L-tyrosine revealed anti-3-nitrotyrosine antibody binding to 3-nitrotyrosine in serum proteins, indicating the presence of 3-nitrotyrosine in immunized animals. HPLC separation and quantitation of 3-nitrotyrosine was also carried out for the immune sera. The serum from the animals immunized by native poly L-tyrosine showed negligible 3-nitrotyrosine level when compared to 3-nitrotyrosine present in the nitrated poly L-tyrosine immune serum. This could be correlated to a feature in SLE wherein nitrated free or protein bound tyrosine (neoantigen) play a major role in the production of SLE autoantibodies.

Autoantibodies targeted against intracellular proteins and nucleic acids are the serological hallmark of the systemic rheumatic diseases, such as systemic lupus erythematosus (SLE), progressive systemic sclerosis (PSS), Sjogren’s syndrome (SS), mixed connective tissue disease (MCTD) and polymyositis (PM). Each one of these diseases is identified by the unique autoantibodies. Antibodies to dsDNA serve as an immunochemical
marker for the diagnosis of SLE. It has been shown that DNA after exposure to ROS presents a more discriminating antigen for the binding of SLE autoantibodies (Blount et al., 1989; 1990; Ara and Ali, 1992; 1993; Cooke et al., 1997).

Several retrospective studies have indicated a correlation between serum nitrate/nitrite level and disease activity. It has also been found that serum 3-nitrotyrosine level is elevated among patients with SLE. In the present study, the possible role of native and nitrated poly L-tyrosine in SLE was probed. Autoantibodies in twenty-four different SLE sera were screened by direct binding and inhibition ELISA. All sera showed stronger binding to nitrated poly L-tyrosine when compared to native poly L-tyrosine and nDNA. No detectable antibody activity was found in the pooled normal human sera.

Competition ELISA results showed 39% to 65% inhibition in the SLE autoantibodies binding to native DNA, whereas 19% to 56% was observed with native poly L-tyrosine as inhibitor. Similarly 49% to 78% inhibition was observed with NO-poly L-tyrosine as inhibitor. These results indicate that the nitrated poly L-tyrosine is an effective inhibitor of nDNA-anti-nDNA antibody interaction showing substantial difference in the recognition over native poly L-tyrosine as well as nDNA. Band shift assay and inhibition ELISA using IgG isolated from SLE serum (3 and 16) further substantiated the binding of native and nitrated poly L-tyrosine with SLE autoantibodies. Results of these experiments demonstrated that nitration of tyrosine resulted in the formation of neoantigen(s) thus inducing explicit immune response as compared to native polymer. The body's immunosurveillance may prove ineffective if the generation of nitric oxide is enhanced tremendously, as seen in chronic inflammation and in injured tissues. Once nitric oxide levels increase, the damage and nitration would be inevitable and the immunoregulatory network would be activated to deal with this alien nitration and modification. The subsequent production of autoantibodies would be a natural step.

SLE sera were analyzed for the presence of 3-nitrotyrosine by Western blot revealing anti-3-nitrotyrosine antibody binding to serum proteins, suggesting that nitration of tyrosine moieties by nitric oxide affected some, if not all, tyrosine containing serum proteins. No blot was seen in the case of pooled normal human sera.

The separation and measurement of the concentration of 3-nitrotyrosine in SLE sera was carried out by HPLC. Eight serum samples showing high inhibition of DNA-anti-DNA antibody by NO-poly L-tyrosine were chosen. The concentration of 3-nitrotyrosine in these
samples was found to be 1.065 μM ± 0.556. Serum collected from healthy volunteers showed no detectable levels of 3-nitrotyrosine indicating that serum 3-nitrotyrosine level is elevated among patients with SLE. Serum 3-nitrotyrosine correlates directly with disease activity in the patients. When compared with dsDNA, and native poly L-tyrosine, 3-nitrotyrosine level correlated better with SLE subjects. This data confirms the overproduction of NO in human SLE and points out serum 3-nitrotyrosine as a new diagnostic tool for studying the role of nitric oxide in SLE and other diseases where production of NO is prevalent. Measurement of 3-nitrotyrosine may be a useful probe in demonstrating NO-mediated pathology and testing the effectiveness of therapeutic aids in preventing damage by reactive nitrogen species.

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

1. Poly L-tyrosine exposed to sodium nitrite at pH 3.5 resulted in the generation of nitric oxide, which resulted in the formation of 3-nitrotyrosine, structural alterations and peptide bond cleavage.
2. 3-Nitrotyrosine was highly immunogenic in experimental animals when compared to native poly L-tyrosine.
3. High level of 3-nitrotyrosine was detected in nitrated poly L-tyrosine immune serum when compared to native poly L-tyrosine immune serum.
4. Induced antibodies were highly specific for respective immunogens.
5. SLE autoantibodies showed preferential binding to nitrated poly L-tyrosine over native poly L-tyrosine and nDNA.
6. Elevated level of 3-nitrotyrosine was seen in the sera of SLE patients when compared to healthy subjects.
7. 3-nitrotyrosine presents a discriminating antigen for the binding of SLE autoantibodies.
8. Nitrated tyrosine presents unique epitopes, which may be one of the factors in antigen-driven autoimmune response in systemic lupus erythematosus.