Antibodies against Nitric Oxide Damaged Poly L-Tyrosine and 3-Nitrotyrosine Levels in Systemic Lupus Erythematosus

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Alterations in the amino acid structure or sequence can generate neo-epitopes from self-proteins causing autoaggressive immune attack. Reactive nitrogen species are an important factor that induces post-translational modification of proteins by cellular reduction and oxidation mechanisms: cysteinyl-nitrosylation or tyrosine nitration leading to potentially pathogenic pathways. It was thought of interest to investigate the immunogenicity of nitrated poly L-tyrosine vis-à-vis its possible role in the induction of antibodies in systemic lupus erythematosus (SLE). Commerically available poly L-tyrosine was exposed to nitrating species and the damage was monitored by UV spectroscopy and alkaline gel electrophoresis. The results indicated the formation of 3-nitrotyrosine. Nitrated poly L-tyrosine induced higher titre antibodies as compared to the native form. Nitrated poly L-tyrosine was recognized by the autoantibodies present in the sera of patients suffering from SLE by enzyme immunoassays and band shift assay. The possible role of nitrated self-proteins has been discussed in the production of circulating anti-DNA antibodies in SLE.

Keywords: Nitric oxide, 3-Nitrotyrosine, Poly L-tyrosine, SLE autoantibodies

Introduction

Nitric oxide (NO) is a diffusible messenger known to display a variety of physiological functions, including vasodilatation, bronchodilation, inhibition of platelet aggregation, and neurotransmission. Additionally, it appears to be involved in the macrophage-dependent killing of intracellular parasites and possibly cancer cells, indicating the potential of this free radical to mediate cytotoxic and pathological effects. When produced in excess, NO can have a multitude of potentially toxic effects, which are highly dependent on its concentration and the particular microenvironment in which it is produced (Brito et al., 1999). NO has been reported to inhibit mitochondrial respiration (Cai and Radi, 1996; Brown et al., 1997) and ribonucleotide reductase (Lepoivre et al., 1991) and to damage DNA (Nguyen et al., 1992) and bring about protein modification (Oates et al., 1999). Nitric oxide damage in DNA exposes base residues in the backbone and the minor regions of single-stranded DNA (ssDNA), rendering it highly immunogenic (Dixit and Ali, 2004). The free radical damage to proteins results mainly in the modification of amino acid residues, side chain crosslinking and fragmentation. The aromatic amino acids and cysteine are more susceptible to modification affecting the general properties of the protein such as change in charge, hydrophobicity and conformation (Davies, 1987; Davies et al., 1987; Davies and Delagnier, 1987). L-tyrosine and protein bound L-tyrosine are attacked by various reactive nitrogen intermediates (RNI) including peroxynitrite to form 3-nitrotyrosine (3-NT) and protein associated 3-nitrotyrosine (Tsikas and Caidahl, 2005). Tyrosine nitration is a post-translational modification in proteins that occurs under conditions of oxidative stress and plays a role in the pathogenesis of diseases such as asthma (Xiao et al., 2005), Alzheimer’s disease (Reynolds et al., 2005), atherosclerosis, endotoxic shock, human acute lung injury (Brito et al., 1999), systemic lupus erythematosus (Oates et al., 1999) and rheumatoid arthritis (Kaur and Halliwell, 1994).

There is increasing evidence that NO may be involved in the pathogenesis of SLE (Wanchu et al., 1998; Lopez-Nevot et al., 2003) which is a multisystem autoimmune disease involving both humoral and cellular aspects of the innate and acquired immune system (Robert and Kimberly, 2001). The disease is characterized by the circulating autoantibodies against a variety of cell antigens (Scofield et al., 2005). Murine models of SLE demonstrate abnormally high levels of NO compared with normal mice, while systemic blockade of NO production reduces disease severity. Two potential
sources of excessive NO are activated endothelial cells and keratinocytes via up regulated nitric oxide synthase (Belmont et al., 1997). Serum nitrite/nitrate level, which is an index of nitric oxide production, was found to correlate with SLE disease activity and with the levels of antibodies to dsDNA. Subjects with active lupus nephritis had higher levels of serum nitrotyrosine than those without renal disease, suggesting that overproduction of NO may play a pathogenic role in SLE and lupus nephritis (Gilkeson et al., 1999; Oates et al., 1999).

In the present study, commercially available poly L-tyrosine (PLT) was nitrated with nitric oxide generated by sodium nitrite. Antibodies were induced in rabbits against the nitrated form and were characterized with respect to antigen binding specificities. The crossreactivity of naturally occurring autoantibodies in SLE with nitrated-PLT (NO-PLT) has also been examined. The binding characteristics of SLE autoantibodies with native DNA, native and nitrated poly L-tyrosine and other nitrated nucleic acids have been investigated and the role of nitrated self-proteins has been discussed in the etiopathogenesis of SLE.

Material and Methods

Chemical and reagents. Poly L-tyrosine, 3-nitrotyrosine, carboxy-PTIO, uric acid, ascorbic acid, desferrioxamine, BSA, calf thymus DNA, anti-human/anti-rabbit IgG- alkaline phosphatase conjugates were purchased from Sigma Chem. Co. Poly-tyrosine microtine flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from NUNC. Protein A-Sepharose CL-4B was from Genet, India. All other chemicals were of the highest analytical grade.

Nitration of poly L-tyrosine. Poly L-tyrosine was nitrated according to Ohshima et al. (1990). The pH of 5 mg/ml poly L-tyrosine solution was adjusted to 3.5 with acetic acid. Sodium nitrite (200 mM) was added to the final concentration of 1 mM and the solution was incubated at 37°C for 24 h. Subsequently the solution was dialyzed overnight to PBS (10 mM sodium phosphate, 150 mM NaCl, pH 9). 365 nm at acidic pH (pH < 3, colourless) to 420 nm at basic pH (pH > 9, yellow). The absorbance maximum ranges from 350 to 450 nm, shifting from 365 nm at acidic pH (pH < 3, colourless) to 420 nm at basic pH (pH > 9, yellow). The absorbance spectrum of a series of known concentrations of 3-nitrotyrosine at basic pH was used as reference to determine the concentration of 3-nitrotyrosine in nitrated protein samples. The modifications incurred on poly L-tyrosine were also analysed by UV difference spectroscopy.

Quantification of protein nitrination. The extent of nitrination of poly L-tyrosine was quantitated by two methods. A calibration curve was drawn using different concentrations of 3-nitrotyrosine (Sigma). The absorbance of both 3-nitrotyrosine and nitrated poly L-tyrosine was measured at 420 nm. The extent of nitrination in poly L-tyrosine was calculated from the calibration curve.

Nitration of poly L-tyrosine was also quantitated by competition ELISA (Khan et al., 1999). Standard nitrated BSA procured from Sigma, was coated onto the ELISA plate (100 µl from 1 mg/ml) in triplicate and incubated at 37°C for 2 h and overnight at 4°C. The plate was then washed twice with PBS-T (+1% Tween 20) and blocked with 2.5% ovalbumin to prevent non-specific binding. Immune complex was formed by incubating standard nitrated BSA dissolved in PBS (0.02-0.5 mg/ml) and nitrated poly L-tyrosine (upto 1:10 dilution) with constant concentration of polyclonal anti-nitrotyrosine rabbit IgG (1:30,000) purchased from Sigma, USA, at 37°C for 2 h and overnight at 4°C. Immune complex (100 µl) was coated to the ELISA plate. After incubation at 37°C for 2 h and overnight at 4°C, the plate was washed 3 times with PBS-T. The plate was then coated with goat anti-rabbit IgG alkaline phosphatase conjugate (1:5000) for 2 h at 37°C. The plate was washed extensively with PBS-T and the colour development was initiated by the addition of 100 µl of 2 mM p-nitrophenyl phosphate substrate in carbonate-bicarbonate buffer, pH 9.6. The plate was allowed to develop for up to 30 min at room temperature and terminated by the addition of 100 µl of 4 M sulphuric acid. The absorbance was read at 410 nm and a standard curve was drawn between absorbance and standard nitrated BSA concentration. The concentration of nitrated poly L-tyrosine was estimated from the standard curve.

Effect of quenchers on modification by nitric oxide. The effect of nitric oxide trapping agent (carboxy-PTIO at 10 mM), antioxidants (ascorbic acid at 10 mM, uric acid at 0.2 mM) on poly L-tyrosine nitration were studied by incubating the quencher with the nitrating reaction mixture and measuring hypochromicity at 280 nm (Yoshiie and Ohshima, 1997).

Alkaline agarose gel electrophoresis. Alkaline agarose gel was cast in buffer containing 33 mM NaOH and 2 mM EDTA. The NaOH and EDTA were added after agarose dissolution. The samples were prepared for loading by adding sample dye (10% glycerol and 0.002% bromophenol blue). Electrophoresis was carried out at 30 V for 4 h in alkaline electrophoresis buffer (30 mM NaOH, 1 mM EDTA).

Serum samples. SLE sera were collected from Indian patients in the year 2004, from outdoor and indoor patients of Department of Medicine, All India Institute of Medical Sciences, New Delhi. The SLE sera satisfied the American College of Rheumatology criteria for its diagnosis (Arnett et al., 1988) showing high titer anti-DNA antibodies (>1:12,800). No patient had an active infection, known malignancy, tuberculosis, pregnancy, or cirrhosis. Normal human sera were obtained from healthy individuals and pooled. All serum samples were decomplexed by heating at 56°C for 30 min and stored at −20°C.

Immunization schedule. Native and NO treated poly L-tyrosine solution (30 mg/ml) were emulsified with complete Freund’s adjuvant and injected intramuscularly in female rabbits. Subsequent injections were given in incomplete Freund’s adjuvant. Each animal received a total of 450 mg of antigen in the course of 9 injections. Blood was collected from marginal vein of the ear. Serum was separated and decomplexed by heating at 56°C for
Nitric Oxide, Poly L-Tyrosine, 3-Nitrotyrosine, Anti-DNA Autoantibodies, SLE Autoantibodies

HPLC analysis. Separation of 3-nitrotyrosine was achieved by HPLC (Biologic Duo Flow System, BioRad, USA) and its concentration was calculated in the sera of immunized animals. Serum samples were diluted 1:1 (v/v) with eluant buffer and filtered through 0.42 µm Mirflex, disposable syringe filter before loading onto a C-18 reversed phase column (25 cm x 4.6 mm) from Vydac, USA. The eluant buffer was 500 mM potassium phosphate (pH 3.0) with 10% methanol (v/v). Samples were eluted at a flow rate of 0.8 ml/min. 3-Nitrotyrosine (absorbance maxima-274 nm, at pH 3.0) in various samples was detected at four wavelength ranges (274, 214, 280 and 410 nm) by UV-visible multiple wavelength detector. Identification of the peak was carried out on the basis of the retention time of standard 3-nitrotyrosine and spiking experiments. The absorbance of two series of known concentrations of standard 3-nitrotyrosine at acidic pH (pH < 3.5) was used as reference to determine the concentration of 3-nitrotyrosine in the serum samples (Kaur and Halliwell, 1994).

Purification of immunoglobulin G. Immunoglobulin G was isolated from preimmune, immune, pooled normal human sera, and from sera of various SLE patients, on a Protein-A Sepharose CL 4B affinity column. The homogeneity of isolated IgG was checked by performing 7.5% polyacrylamide gel electrophoresis (Dixit et al., 2003).

Modification of various polymers for crossreactivity studies. BSA, calf thymus DNA and chromatin (Mansoor et al., 2005) were nitrated according to Ohshima et al. (1990) as mentioned above. Poly L-tyrosine was also exposed to peroxynitrite by its incubation in 100 mM sodium phosphate buffer, pH 7.4, containing 1 mM diethylenetriaminepentaacetic acid (DTPA), 1.0 mM nitric oxide releasing compound [diethyamine NONOate (DEA-NO)] and 1.0 mM 1,4-hydroquinone at 37°C for 1 h (Yoshie and Ohshima, 1997).

Enzyme linked immunosorbent assay. Antibodies were detected by ELISA using polystyrene microtitre plates as solid support (Ali and Adam, 2002). One hundred microlitre of 2.5 mg/ml antigen in protein coating buffer (15 mM NaCO3, 55 mM NaHCO3, pH 9.6) was coated in test wells of microtitre plates, incubated for 2 h at 37°C and subsequently overnight at 4°C. The antigen-coated wells were washed three times with TBS-T and antibody (100 ml/well) to be tested was diluted in TBS and added to each well. After 2 h incubation at 37°C and overnight at 4°C, the plates were washed four times with TBS-T and an appropriate dilution of anti-immunoglobulin alkaline phosphatase conjugate was added to each well. After incubation at 37°C for 2 h, the plates were washed four times with TBS-T and developed using p-nitrophenyl phosphate as substrate. The absorbance was recorded at 410 nm on an automatic microplate reader. Each sample was run in duplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as a mean of Auninhibited - Auninhibited

Results

The UV absorption spectra of nitrated poly L-tyrosine showed peak shift of 4 nm towards shorter wavelength and 2% hypochromicity at 280 nm (Fig. 1). Another broad peak was observed at a wavelength of 420 nm, which is characteristic of 3-nitrotyrosine. The modifications incurred on poly L-tyrosine were also analyzed by UV-difference spectroscopy (Fig. 1 inset) reiterating the results of UV spectra. The concentration of 3-nitrotyrosine in the nitrated homopolymer was calculated from the absorbance spectrum of a series of known concentrations of standard 3-nitrotyrosine at basic pH used as reference to determine the concentration of 3-nitrotyrosine in nitrated protein samples as well as by competitive ELISA. By these methods of quantification, the concentration of 3-nitrotyrosine was found to be 0.39-mg/0.5 mg protein and 0.35-mg/0.5 mg protein, respectively. The average concentration of nitrotyrosine determined was 0.37 ± 0.08 mg/0.5 mg protein solution of poly L-tyrosine. This data indicated at 74% nitration of the native polymer.

The modification induced by NO on poly L-tyrosine was confirmed by the use of radical quenchers, i.e., ascorbate, carboxy-PITO and uric acid. Decrease in percent nitration to the extent of 91%, 84% and 73% was seen in the case of carboxy-PITO, uric acid and ascorbic acid, respectively.

Alkaline agarose gel electrophoresis of native and nitrated poly L-tyrosine was performed on 0.6% agarose gel. The
native sample migrated as a single band whereas the nitrated sample showed decrease in intensity with a broad band indicating formation of high and low molecular weight species (Fig. 2). The migration of BSA was hindered as the electrophoresis was performed in alkaline medium.

NO-poly L-tyrosine was found to be highly immunogenic inducing high titre (ELISA titre >1:12,800) antibodies in rabbits (data not shown). The IgG purified from immune serum showed higher binding with immunogen than with the native form (Fig. 3a). Preimmune IgG showed negligible binding under identical conditions. In competition ELISA (Fig. 3b) anti-NO-poly L-tyrosine IgG showed preference for immunogen. A maximum of 83% inhibition in antibody binding was observed using immunogen as competitor. Fifty percent inhibition was seen at immunogen concentration of 12 µg/ml. An array of polymers and their modified forms when exposed to reactive nitrogen species, were used as inhibitors to study the binding diversity of anti-NO-poly L-tyrosine antibodies (Table 1). The maximum inhibitions achieved were 74, 72 and 79% with the nitrated forms of BSA, chromatin and ONOO⁻ poly L-tyrosine, respectively. Inhibition with native forms of BSA, PLT, DNA, and chromatin, was 25%, 29%, 17% and 35% respectively. Moderate inhibition was observed in the case of nitrated DNA (52%).

HPLC of standard solution of 3-nitrotyrosine (0.01 µM) dissolved in the eluant buffer (500 mM potassium phosphate, pH 3.0) was performed. A well defined peak of 3-nitrotyrosine at retention time of ~7.2 minute was observed (Fig. 4a). 3-Nitrotyrosine was detected in the sera of immunized animals. The concentration of 3-nitrotyrosine in poly L-tyrosine immune sera was negligible (0.018 µM) as compared to 0.90 µM in nitrated poly L-tyrosine immune sera (Fig. 4b,c).

To probe the possible role of nitrated poly L-tyrosine in the etiopathogenesis of SLE, sera showing high titre anti-DNA...
autoantibodies (1: 6400) were selected for binding to native and nitrated poly L-tyrosine. The sera showed substantially higher binding with the nitrated forms relative to native form of DNA and poly L-tyrosine. The same sera were subjected to competitive-inhibition assay using native DNA, native and nitrated poly L-tyrosine as competitors. The microtitre plates were coated with calf thymus DNA. The average percent inhibition (±SD) of 24 SLE sera binding to native DNA showed significantly higher inhibition with NO-PLT (64 ± 9) than both native DNA (49 ± 8) and PLT (35 ± 11). There was a statistically significant difference on comparing the inhibition values of native DNA, PLT and nitrated poly L-tyrosine (p-value of <0.001) (Table 2).

Gel retardation assay was carried out in polyacrylamide gel to visualize the formation of immune complexes. As clearly evident from the data (Fig. 5a), with increasing concentrations of NO-PLT (0, 50, and 100 mg) there was a corresponding increase in the formation of high molecular weight immune complexes with immune IgG which resulted in decrease in the intensity of unbound IgG. Decrease in intensity of IgG band and increase in immune complex formation was also seen between NO-PLT and SLE IgG (Fig. 5b). The concentration of IgG was kept constant (20 mg).

**Discussion**

Protein nitration has been suggested to be a final product due to interaction 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/nitration 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 in vivo in diverse pathological conditions.

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. 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. The UV absorption spectra of nitrated poly L-tyrosine showed peak shift of 4 nm towards shorter wavelength

### Table 1. Antigenic Binding Characteristics of Anti-Nitrated Poly L-Tyrosine IgG

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Maximum % inhibition at 20 µg/ml Anti-NO-poly L-tyrosine IgG</th>
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<tr>
<td>Native BSA</td>
<td>25</td>
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<tr>
<td>Nitrated BSA</td>
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<tr>
<td>Native poly L-tyrosine</td>
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<tr>
<td>ONOO-poly L-tyrosine</td>
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<tr>
<td>Native DNA</td>
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<td>Nitrated DNA</td>
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<td>Nitrated chromatin</td>
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![Figure 4](image-url)
and 25% hypochromicity at 280 nm. Another broad peak was observed at a wavelength of 420 nm, which is characteristic of 3-nitrotyrosine. The observed change could be attributed to structural alterations as well as damage of chromophoric groups. 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 results 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. Formation of high and low molecular weight species indicating aggregation and peptide bond cleavage of nitrated poly L-tyrosine was seen in the alkaline agarose gel electrophoresis.

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. The subsequent release of altered proteins during various cell functions may enable it to act as an antigen inducing antibodies crossreacting with self-proteins. At the very least, the presence of 3-nitrotyrosine in biological samples indicate that reactive nitric oxide derived species were produced in vivo, although the exact nature of these species remains to be determined (Crow, 1999). The immunoreactivity of 3-nitrotyrosine has been reported in several human pathological conditions as mentioned earlier. In addition, numerous other disease states using non-human models have been shown to involve formation of 3-nitrotyrosine (Crow, 1999).

Nitrated poly L-tyrosine was found to be immunogenic in rabbits as revealed by the result of direct binding and inhibition ELISA. Band shift assay clearly demonstrated the specificity of the purified immune IgG towards the immunogen. Antigenic specificity of purified IgG was confirmed by inhibition ELISA. A maximum of 83% inhibition in the binding of anti-nitrated poly L-tyrosine antibody with immunogen as inhibitor was observed at 20 μg/ml and, 50% inhibition was achieved at 12 μg/ml. These results suggest that the nitration of tyrosine results in the generation of neo-epitopes, making it a potential immunogen. The antibody developed can be used as a probe for detecting the presence of 3-NT, demonstrating NO-mediated pathology and testing the effectiveness of therapeutic aids in preventing damage by reactive nitrogen species.

The separation and quantitation of 3-nitrotyrosine was also

<table>
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<th>SLE serum</th>
<th>Native DNA</th>
<th>Native PLT</th>
<th>Nitrated PLT</th>
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Table 2. Inhibition of SLE Autoantibodies Binding to Native DNA by Native and Nitrated Poly L-Tyrosine

Maximum percent inhibition at 20 μg/ml

Poly L-tyrosine and native DNA: t = 4.88, p < 0.001; Nitrated poly L-tyrosine and native DNA: t = 6.05, p < 0.001; Nitrated and native poly L-tyrosine: t = 9.79, p < 0.001
Nitric Oxide, Poly L-Tyrosine, 3-Nitrotyrosine, Anti-DNA Autoantibodies, SLE Autoantibodies

carried out by HPLC. The sera from the animal’s 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 elevated level of nitrated free or protein bound tyrosine (neoantigen) play a major role in the production of SLE autoantibodies.

Most lesions in murine and human systemic lupus erythematosus (SLE) are considered to be associated with the presence of anti-double-stranded DNA (dsDNA) antibodies that are spontaneously produced in large amounts (Jacob et al., 1986). SLE anti-DNA autoantibodies exhibit polyspecificity with respect to antigen binding (Wani and Alam, 2004). It has been reported that self-proteins bearing 3-nitrotyrosine are generated in inflamed tissues where activated inflammatory cells may release peroxynitrite, a naturally occurring nitrating generated in inflamed tissues where activated inflammatory cells may release peroxynitrite, a naturally occurring nitrating.

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. 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 (Oates et al., 1999; Gilkeson et al., 1999). The level of 3-nitrotyrosine was found elevated in immunized rabbits. 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 competition ELISA. All sera showed stronger binding to nitrated poly L-tyrosine when compared to native poly L-tyrosine and nDNA. The band shift assay reiterated the results obtained by competition ELISA. No detectable antibody activity was found in the pooled normal human sera.

These studies demonstrated that nitration of tyrosine resulted in the formation of neoantigen(s) thus inducing explicit immune response. When compared with dsDNA and native poly L-tyrosine, nitrated-PLT level correlated better with SLE subjects. The data confirms the overproduction of NO and intermediates 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.

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