American Journal of Biomedical Sciences
ISSN: 1937-9080
nwpii.com/ajbms

Peroxynitrite-modified H3 Histone is Highly Immunogenic and Binds Circulating SLE Autoantibodies Better than Native DNA

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Received:7 September 2012; | Revised:20 December 2012; | Accepted: 30 December 2012

Abstract

Peroxynitrite is a bifaceted reactive species. It can oxidize and nitrate proteins/nucleic acids/lipids and is involved in inflammation, apoptosis, cytotoxicity and autoimmune disorders of unknown etiology, including SLE. In this study, H3 histone exposed to peroxynitrite caused loss of tertiary structure, nitration of tyrosine residues and dityrosine formation. Experimentally produced antibodies against peroxynitrite-modified H3 histone showed specificity for the immunogen. However, cross-reactions with other nitrated proteins were also observed. We further investigated the binding of SLE autoantibodies with native DNA, native H3 histone and peroxynitrite-modified H3 histone to explore the possible role of modified-H3 histone in the initiation and/or progression of SLE in a sub-group of patients. The results showed preferential binding of SLE anti-nDNA autoantibodies to peroxynitrite-modified H3 histone. The visual detection of immune complex formation with native and peroxynitrite-modified H3 histone reiterated preferential binding of SLE autoantibodies with modified H3 histone. It may be concluded that anti-histone autoantibodies seen in a sub-group of SLE patients might be due to the immunogenicity of peroxynitrite-modified H3 histone.

Keywords: Peroxynitrite, nitrotyrosine, SLE, H3 histone.

1. Introduction

Peroxynitrite is formed by rapid reaction of nitric oxide (NO) and superoxide anion [1]. It is a potent oxidant and nitrating species and can modify a variety of biomolecules, but possesses high affinity for tyrosine residues in proteins. Moreover, 3-nitrotyrosine is a relatively specific marker of peroxynitrite mediated damage to proteins [2]. The generation of peroxynitrite by

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activated macrophages, neutrophils and endothelial cells and presence of nitrotyrosine in human tissues and fluids and in animal models of various diseases, calls for a comprehensive study of protein-peroxynitrite interactions.

Histones are conserved proteins. They play a critical role in the proper packaging of DNA within the eukaryotic nucleus and are poor immunogens. However, histones were found to be immunogenic after acetylation [3] or binding with RNA [4]. A variety of post-translationally modified (including nitrated) proteins have been shown to accumulate in apoptotic or inflamed tissues [5]. The accumulation in inflamed tissue of nitrotyrosine-containing autologous proteins, that appear as foreign to the immune system, might induce an autoimmune response and sustain a chronic inflammatory response [6]. Elevated levels of anti-nitrotyrosine antibodies have been found in synovial fluids of patients with rheumatoid arthritis and osteoarthritis [7] as well as in the sera of SLE patients [7,8].

SLE is an autoimmune disease characterised by autoantibodies against nuclear antigens for which the nucleosome is considered a major autoantigen [9]. The nucleosome is comprised of an octamer of two copies each of H2A, H2B, H3 and H4 histones and a 146 bp DNA. The linker histone H1 is highly susceptible to modifications and might be central in autoimmunity. In SLE, the nucleosomes are released from apoptotic cells and not cleared completely [10,11]. This results in accumulation of DNA, histones and nucleosomes in the circulation and tissues [12].

Anti-histone antibodies (AHA) are part of the so-called antinuclear antibodies family. Certain AHA subsets recognize individual histones, whereas others preferentially react with histone complexes, associated with or without DNA [13]. In contrast to anti-nucleosome antibodies that are almost exclusively found in SLE, AHA are found in several systemic and organ-specific autoimmune diseases, including SLE, as well as in infectious and neurologic diseases. Therefore, identification of *in vivo* targets of protein tyrosine nitration may help in understanding the development and progression of SLE. Elevated levels of peroxynitrite in SLE patients have been reported [14,15].

In the present study, commercially available H3 histone was modified with varying concentrations of peroxynitrite. Immunogenicity of native and peroxynitrite-modified H3 histone was checked in rabbits and the binding specificity of the induced antibodies was assessed by inhibition ELISA. SLE autoantibodies binding with native DNA, native H3 and peroxynitrite-modified H3 histone were investigated to explore peroxynitrite-modified H3 histone as likely trigger for the origin of anti-histone autoantibodies or its involvement in the progression of the disease in a sub-group of SLE patients.

2. Materials and methods

2.1 Materials

Calf thymus H3 histone, human serum albumin, low density lipoprotein, catalase, trypsin, tryptophan, phenylalanine, superoxide dismutase, 3-nitrotyrosine, Protein A-agarose affinity column, p-nitrophenyl phosphate, Tween-20, Freund's complete and incomplete adjuvants and diethylenepenta-acetic acid (DTPA) were obtained from Sigma Chemical Company, St. Louis, MO, USA. Yeast RNA was from BDH Chemicals Ltd, England. Sodium nitrite, hydrogen peroxide, silver nitrate, sodium hydroxide and ammonium persulphate were obtained from Qualigens, India. Flat bottom polysorp ELISA modules were purchased from NUNC, Denmark. All other reagents were of highest analytical grade available.

2.2 Methods

2.2.1 Modification of H3 histone by peroxynitrite

Peroxynitrite was synthesized in laboratory by rapid quenched flow process from sodium nitrite (Merck, Darmstadt, Germany) and acidified hydrogen peroxide (Merck, Darmstadt, Germany) [16] and stored in 1.2 M NaOH at -20°C. Before each use, the concentration of stored peroxynitrite was determined by recording absorbance at 302 nm using molar extinction

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coefficient of 1670 $M^{-1}cm^{-1}$. The modification was carried out by incubating 25 µM of H3 histone in a buffer (10 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl and 100 µM DTPA) with different amount of peroxynitrite (50, 100 and 200 µM) at 37°C for 30 min. The approximate pH of the incubation mixture was in the range of 10-11. Unmodified H3 histone served as a control.

2.2.2 Immunization schedule

Female New Zealand white rabbits weighing 1-1.5 kg were selected for immunization [17]. Briefly, rabbits (n=4; two each for native and peroxynitrite-modified H3 histone) were given intramuscular injections of H3 histone (200 µg) or its peroxynitrite-modified counterparts at multiple sites. Before injecting into animals, the respective immunogens were complexed with yeast RNA (one-third of immunogen concentration) and emulsified with equal volume of Freund's complete adjuvant (first injection). Subsequent injections of immunogen were given in Freund's incomplete adjuvant at weekly intervals. During the course of immunization, each animal received a total of 1200 µg of immunogen. A similar course of immunization was followed with RNA alone (66.5 µg). One week after the last dose, marginal ear veins of the animals were punctured and blood was carefully collected. The separated sera were heated at 56°C for 30 min to inactivate complement proteins and stored at -20 °C with 0.1% sodium azide as preservative.

2.2.3 Serum samples

SLE sera (n = 50) were collected from female patients (18–50 years of age) attending speciality clinic at the All India Institute of Medical Sciences, New Delhi. Consent was obtained from all patients before blood was collected. All SLE patients satisfied the American College of Rheumatology criteria [18] and showed high-titer anti-nDNA autoantibodies (>1:12,800). No patient had active infection, known malignancy, tuberculosis, pregnancy, or liver cirrhosis. Control sera were obtained from 25 age- and sex- matched non-smoking healthy female volunteers. All serum samples were decomplemented by heating at 56°C for 30 min and stored in aliquots at -80°C.

2.2.4 Enzyme linked immunosorbent assay (ELISA)

Direct binding ELISA was carried out on flat bottom polystyrene plates as described earlier [19] with slight modifications. Antibody specificity was ascertained by inhibition ELISA [20]. Percent inhibition was calculated as follows:

$$\text{Percent inhibition} = 1 - \frac{A_{\text{inhibited}}}{A_{\text{uniniited}}} \text{ X } 100$$

2.2.5 Purification of IgG on Protein A-agarose matrix

Immunoglobulin G (IgG) was affinity purified from preimmune and immune sera on a Protein A-agarose column [21]. The homogeneity of isolated IgG was ascertained on 7.5% SDS–PAGE.

2.2.6 Band shift assay

For the visual detection of antigen–antibody interactions and immune complex formation, a gel retardation assay was performed [20]. Immune complex was prepared by incubating constant amount of native or peroxynitrite-modified H3 histone with varying amounts of affinity purified anti-peroxynitrite-modified H3 IgG in PBS for 2 h at 37°C and overnight at 4°C. One-fourth volume of sample dye buffer (0.1% glycerol, 0.5 M Tris, 10% SDS and 1% bromophenol blue, pH 6.8) was added to the mixture and electrophoresed on 10% non-reducing SDS-polyacrylamide gel for 4 h at 80 V. The bands were visualized by silver nitrate.

2.2.7 Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical significance of data was determined by Student's t test, and a *p* value of <0.05 was considered significant.

3. Results

3.1 Characterization of H3 histone modified by peroxynitrite

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Our previous study [22] has shown structural alterations in H3 histone following exposure to peroxynitrite. The changes were studied by UV and fluorescence spectroscopy, 8-anilino-1-naphthalenesulfonic acid binding, FT-IR, CD and PAGE. Analysis of the data revealed that carbonyl, nitrotyrosine, and dityrosine contents were significantly increased in peroxynitrite-modified H3 histone.

3.2 Immunogenicity of native and peroxynitrite-modified H3 histone

Sera of animals immunized with native and peroxynitrite-modified H3 were tested on polysorp wells coated with respective immunogens. Antiserum against 50 µM peroxynitrite-modified H3 showed an antibody titre of 1:3200 (Fig. 1). Modification by 100 µM peroxynitrite of H3 histone resulted in substantial enhanced immunogenicity with a titre of 1:12800. Native H3 induced moderate antibody response. Yeast RNA used as carrier was non-immunogenic. Preimmune serum, used as negative control, showed weak binding with the immunogen. As modification of H3 histone by 100 µM peroxynitrite gave better antibody response, further studies were carried out with this antibody. Our results suggest that modification of H3 histone by peroxynitrite generates highly immunogenic neo-epitopes on the H3 histone.

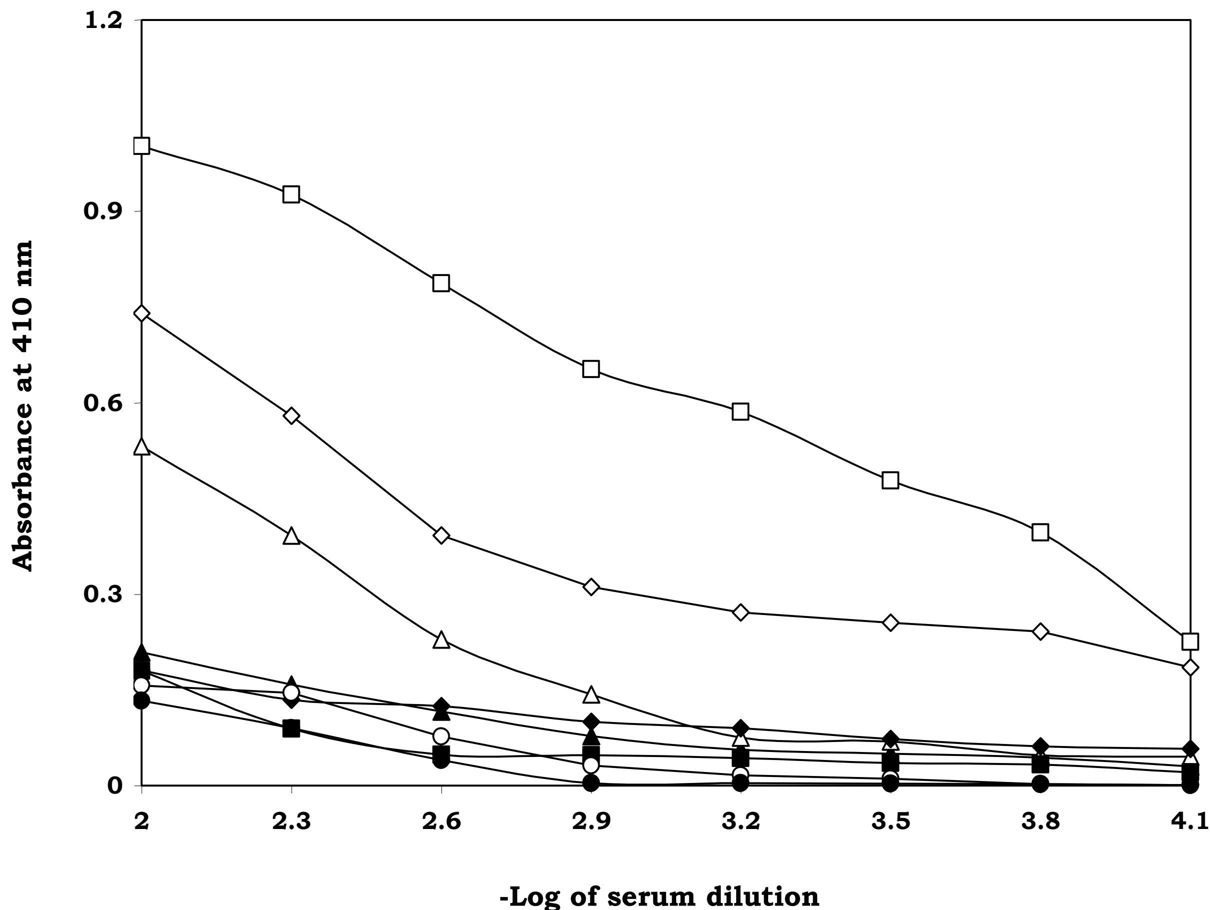

Figure 1. Direct binding ELISA of experimentally produced antibodies against 100 µM peroxynitrite-modified H3 (─□─), 50 µM peroxynitrite-modified H3 (─◇─), native H3 (─Δ─) and yeast RNA (─○─). The corresponding filled symbols show preimmune sera binding with respective immunogens coated on microtitre wells.

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3.3 Characterization of anti-100 µM peroxynitrite-modified H3 IgG

IgG purified from 100 µM peroxynitrite-modified H3 histone antisera were subjected to direct binding immunoassay on wells coated with 100 µM peroxynitrite-modified H3 histone. Anti-100 µM peroxynitrite-modified H3 IgG showed strong binding with coated antigen (Fig. 2). Preimmune IgG showed negligible binding under identitical conditions. Specificity of anti-100 µM peroxynitrite-modified H3 IgG was evaluated by inhibition ELISA using immunogen as inhibitor. The induced antibodies were highly specific for the immunogen (Fig. 3).

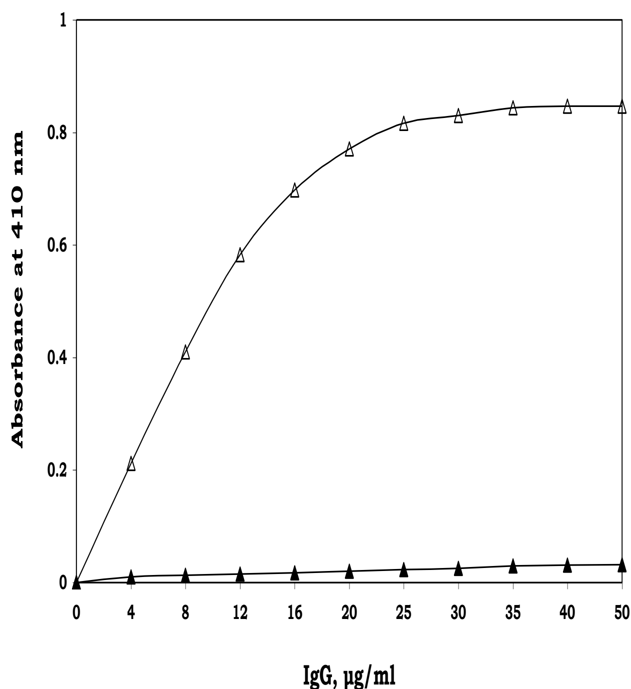

Figure 2. Direct binding of affinity purified immune IgG (─Δ─) and preimmune IgG (─▲─). The microtitre wells were coated with 100 µM peroxynitrite-modified H3.

3.4 Use of anti-100 µM peroxynitrite-modified H3 IgG antibodies to study epitope sharing by other proteins modified by peroxynitrite

Native H3 used as inhibitor of anti-100 µM peroxynitrite-modified H3 IgG showed 51% inhibition in antibody binding. Binding of anti-100 µM peroxynitrite-modified H3 IgG antibodies was also carried out with an array of peroxynitrite-modified proteins including H2A, H2B and H1 histones. Results of cross-reaction studies have been summarized in Table 1. The data suggests that nitrated epitopes of different proteins (or amino acids) share common properties. Furthermore, among peroxynitrite-modified amino acids, nitrated-tyrosine showed 68% inhibition in antibody binding followed by nitrated-phenylalanine and nitrated-tryptophan. Participation of nitrated-tyrosine (nitrotyrosine) as preferred substrate for induced antibodies points out the significance of nitrotyrosine in immunogenicity.

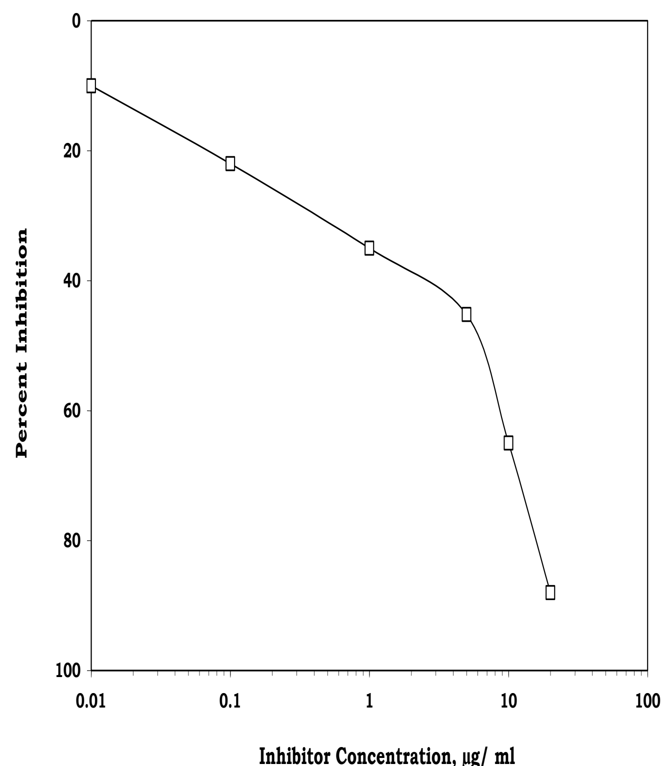

Figure 3. Inhibition of binding of anti-peroxynitrite-modified H3 IgG by 100 µM peroxynitrite-modified H3. The microtitre wells were coated with H3 modified by 100 µM peroxynitrite.

3.5 Direct binding ELISA of SLE sera with native DNA, native H3 and 100 µM peroxynitrite-modified H3

All 50 SLE sera included in this study were pre-tested for anti-native DNA autoantibodies and found to be antibody positive. Age- and sex-matched sera from apparently healthy individuals served as controls. The diluted (1:100) sera were subjected to direct binding ELISA on microtiter wells separately coated with equal amounts of

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native DNA, native H3 histone, and 100 μM peroxynitrite-modified H3 histone (Fig. 4). Approximately 60% of tested SLE sera showed higher binding with peroxynitrite-modified H3 histone compared to native DNA or native H3 histone. No appreciable binding was observed with the pooled sera of normal subjects. These observations are important in view of the immunogenicity of peroxynitrite-modified H3 histone and the reported incidence of hypernitrotyrosinemia and anti-histone antibodies in a sun-group of SLE patients (7).

Table 1. Cross reaction of peroxynitrite-modified H3 IgG antibodies#

Inhibitors	Maximum percent inhibition at 20 μg/ml
Nitrated-H3	88
Native H3	51
Nitrated-H2A	70
Native H2A	39
Nitrated-H2B	62
Native H2B	44
Nitrated-H1	58
Native H1	27
Nitrated-human IgG	66
Native human IgG	31
3-nitrotyrosine	68
Tyrosine	33
Nitrated-SOD	29
SOD	10
Nitrated-HSA	64
HSA	22
Nitrated-Catalase	58
Catalase	28
Nitrated-LDL	59
LDL	27
Nitrated-Trypsin	56
Trypsin	19
Nitrated-Tryptophan	13
Tryptophan	06
Nitrated-Phenylalanine	20
Phenylalanine	07

Wells were coated with 100 μM peroxynitrite-modified H3 histone.

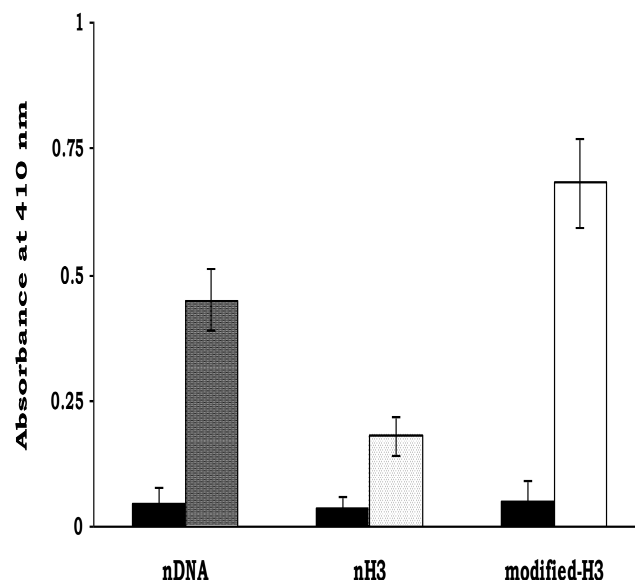

Figure 4. Direct binding of 1:100 diluted SLE sera with native DNA (▓), native H3 (░) and peroxynitrite-modified H3 (□). The histograms represent mean ± S.D. of 50 SLE sera. Filled bars represent mean ± S.D of 35 control human sera binding with corresponding antigens.

3.6 Inhibition ELISA of SLE IgG with native DNA, native H3, and 100 μM peroxynitrite-modified H3

IgG were purified from 20 active SLE sera that showed higher binding with peroxynitrite-modified H3 histone in direct-binding ELISA. The fine antigenic specificity of isolated SLE IgG was evaluated using native DNA, native H3, and 100 μM peroxynitrite-modified H3 as inhibitors. Purified SLE IgG was separately mixed with DNA, H3, and 100 μM peroxynitrite-modified H3 (0–20 μg/mL) and incubated for 2 h at 37°C and overnight at 4°C. The resulting complex was coated instead of IgG. The remaining steps were the same as described for direct-binding ELISA. The interaction of inhibitors with different SLE IgG has been represented as the percentage inhibition in antibody binding (Table 2). The peroxynitrite-modified H3 histone emerged as the most effective inhibitor, followed by native DNA and native H3. Inter comparison of the data using a statistical method yielded a p value <0.001 for peroxynitrite-modified H3 versus native DNA or native histone, which indicates that the data are statistically significant. The immunogenic nature

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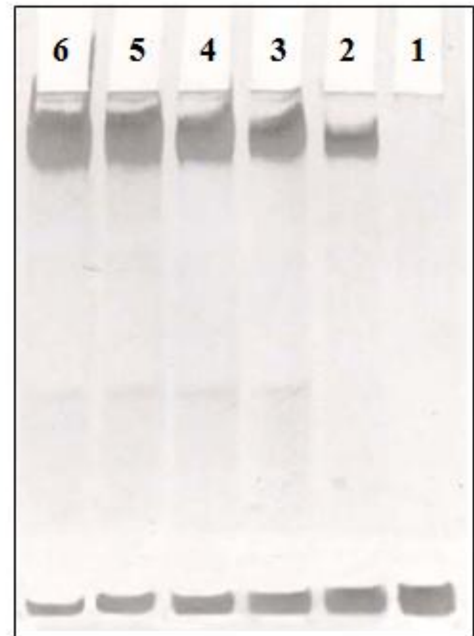
of peroxynitrite-modified H3 and the reported incidence of anti-histone antibodies and hypernitrotyrosinemia in SLE patients advocate a possible role of peroxynitrite-modified H3 histone in SLE initiation, at least in a subpopulation of patients.

Table 2. #Inhibition of SLE IgG binding by native DNA, native H3 and peroxynitrite-modified H

SLE IgG	Maximum percent inhibition at 20 μg/ml		
	nDNA	nH3	Peroxynitrite-modified H3
01 (2)	50.8	20.0	70.3
02 (3)	57.4	30.0	69.2
03 (4)	56.0	28.0	73.7
04 (15)	49.8	32.0	65.4
05 (16)	45.8	34.0	66.5
06 (17)	46.5	26.0	69.7
07 (21)	53.5	27.0	75.2
08 (22)	47.0	30.0	70.0
09 (23)	45.8	24.0	68.7
10 (24)	49.3	31.0	69.2
11 (25)	51.7	22.0	68.7
12 (26)	54.6	21.0	70.2
13 (29)	58.0	37.0	72.4
14 (30)	50.0	20.0	64.0
15 (33)	44.0	18.0	64.5
16 (34)	52.0	24.0	76.1
17 (35)	56.0	26.0	75.4
18 (42)	58.8	32.0	82.3
19 (49)	52.0	36.0	60.0
20 (50)	43.2	28.0	63.7
Mean ± SD	**(51.11 ± 4.75)**	**(27.30 ± 5.49)**	**(67.77 ± 5.79)**

#Microtitre plates were coated with native DNA (2.5 μg/ml)
nDNA = native DNA; nH3 = native H3 histone
Numbers in parenthesis indicates the SLE sera that showed higher binding with peroxynitrite-modified H3 histone in direct binding ELISA.

(A)



(B)

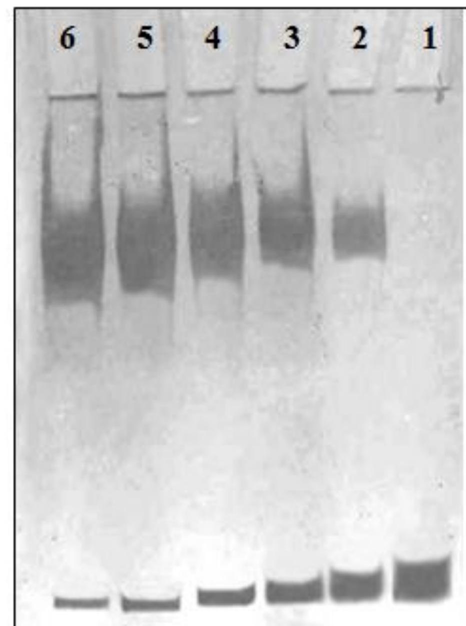


Figure 5. Gel retardation assay of an SLE IgG with native and peroxynitrite–modified H3 histone in SDS-polyacrylamide gel. Electrophoresis was performed on 10% SDS–polyacrylamide gel for 4 h at 80 Volts. **(A)** Native H3 (25 μg, lane 1) was incubated with 20, 30, 40, 60 and 80 μg of SLE IgG (lanes 2–6) and incubated for 2 h at 37°C and overnight at 4°C. **(B)** 100 μM peroxynitrite–modified H3 (25 μg, lane 1) was incubated with 20, 30, 40, 60 and 80 μg of SLE IgG (lanes 2–6) and incubated under identical conditions.

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3.7 Gel retardation assay with SLE IgG

The antigen–antibody interaction was also visualized in non-reducing SDS-polyacrylamide gel. The formation of immune complex between 100 µM peroxynitrite-modified H3 histone and SLE IgG was clearly evident from the retarded mobility of the complex with increasing concentrations of SLE IgG (Fig. 5B) as compared to antigen alone (Lane 1; Fig. 5B). The proportional decrease in free antigen intensity was accompanied by increase in the mass of immune complex which reiterates the specific antigen-antibody interaction. Under identical conditions interaction of native H3 histone with SLE IgG was moderate (Fig. 5A). Formation of large immune complex of SLE IgG and 100 µM peroxynitrite-modified H3 histone was clearly seen as compared to native H3.

4. Discussion

SLE is a complex autoimmune inflammatory disease. Despite decades of extensive research on SLE, the exact nature of antigen stimulus for initiation and progression of the diseases remains unknown. Endogenous proteins are normally not immunogenic due to immunological tolerance. However, post-translational modifications of self-proteins may lead to the generation or unmasking of epitopes, resulting in the triggering of immune response, which may induce autoimmunity [5]. Studies on the recognition of tyrosine nitrated proteins by T-cells showed that protein nitration of a tyrosyl residue(s) in a T-cell receptor (TCR) contact position may result in the formation of an immunogenic neoepitope [24,6]. Moreover, it was shown that nitration of tyrosine residues located in non-TCR-contact positions can have an indirect yet major impact on stimulation of the immune system by affecting interactions of the TCR with the peptide-loaded major histocompatibility complex [23].

Autoantibodies against intracellular proteins and nucleic acids are serological hallmark of the systemic rheumatic diseases such as SLE, Progressive systemic sclerosis (PSS), Sjogren's syndrome (SS), mixed connective tissue diseases (MCTD) and polymyositis (PM) [24]. Each of these diseases is identified by the unique autoantibodies. Antibodies to dsDNA serve as an immunological marker for the diagnosis of SLE [25]. These autoimmune diseases are associated with multiple antinuclear antibody specificities, suggesting a role for both generalized as well as antigen-specific immune abnormalities in their etiology [26,27].

Histones are small, cationic proteins which bind DNA. They are weak immunogen because of their conserved nature. Histones are major constituent of cells' chromatin and remain confined to nucleus. However, after apoptosis they may appear in circulation as nucleosomes. Anti-histone antibodies are autoantibodies that are found in 50%-70% of patients with SLE and in more than 95% of patients with drug-induced SLE [28].

Our study demonstrated that modification of H3 histone by peroxynitrite resulted in tyrosine nitration, formation of protein carbonyl, dityrosine and cross-linking. The structural changes appear to have favoured polymerization of native epitopes of H3 histone into potent immunogenic neo-epitopes. Earlier studies have shown that acetylation, alterations in amino acid structure or sequence can generate neo-epitopes on self proteins causing autoaggressive immune attack [29]. Interpretation of our results in the light of above suggests that oxidative and nitrative action of peroxynitrite has conferred additional immunogenicity on H3 histone and probably there is a direct correlation between nitration and immunogenicity. The H3 histone modified by 100 µM peroxynitrite was a potent immunogen and induced high titre antibodies in rabbits. Although anti-100 µM peroxynitrite-modified H3 antibodies showed specificity for the immunogen but cross reacted with native H3. It indicates that not all epitopes typical of H3 histone have been converted into neoepitopes upon nitration. In another words, peroxynitrite-modified H3 still has some original epitopes which are scattered among neo-epitopes. Hence, immunization with peroxynitrite-modified H3 may produce polyspecific antibodies which can recognize both original and neo-epitopes or altogether there are two types of antibodies, one recognizing nitrated neo-epitopes and other

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binding exclusively with old epitopes. Accumulation of a variety of post-translationally modified self-proteins during inflammation may lead to generation or unmasking of new antigenic epitopes that in turn activate B-and ⁄or T-cells, thereby impairing or bypassing immunological tolerance [30]. Against this backdrop, peroxynitrite-modified H3 histone could act as an autoantigen leading to generation of anti-H3 histone antibodies.

We further investigated the binding characteristics of naturally occurring SLE anti-DNA autoantibodies to native calf thymus DNA and native and peroxynitrite-modified-H3 histone. Sera of SLE patients, having high titre anti-DNA antibodies, showed preference for peroxynitrite–modified H3 histone compared to native H3 histone or native DNA. This suggests that peroxynitrite–modified H3 histone is an effective inhibitor of native DNA-anti-DNA antibody interaction. Band shift assay and inhibition ELISA with SLE IgG substantiated the preferential binding of peroxynitrite–modified H3 histone with SLE autoantibodies. It may be interpreted that nitration of tyrosine residues resulted in generation of neoantigen(s) with explicit immune response compared to native H3 histone. The body's immunosurveillance may prove ineffective if the generation of peroxynitrite is enhanced tremendously, as seen in chronic inflammation and in injured tissues. Once peroxynitrite level increases, the damage and nitration would be inevitable and the immunoregulatory network would be activated resulting in the production of autoantibodies. Cellular metabolism and ionization radiation produces both reactive nitrogen and oxygen species. These radicals and their subsequent intermediates may react with cellular macromolecules and induce a variety of chemical alterations leading to autoantibodies [31]. In SLE, reactive nitrogen intermediates are over produced due to over expression of inducible nitric oxide synthase (iNOS) which may lead to tissue injury and increase carbonyl groups in a sub-group of SLE patients. The mechanism through which peroxynitrite can be pathogenic in the setting of SLE is through the generation of neo-epitopes on self antigens. Serum from SLE patients exhibited increased binding to peroxynitrite–modified H3 histone compared to native DNA or native H3 histone. The present studies suggest that peroxynitrite modification of H3 histone can generate highly immunogenic neo-epitopes generating autoantibodies recognizing native H3 histone as seen in a sub-group of SLE patients.

Acknowledgement

This study was supported by a research grant (52/11/2003-BMS) to K.A. from the Indian Council of Medical Research, New Delhi. Authors also acknowledge the infrastructure facilities of DST-FIST.

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