Original Contribution

Modification of lupus-associated 60-kDa Ro protein with the lipid oxidation product 4-hydroxy-2-nonenal increases antigenicity and facilitates epitope spreading

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Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with autoantibodies as a near universal feature of the disease. The Ro ribonucleoprotein particle, composed of a 60-kDa protein noncovalently associated with human cytoplasmic RNA, is the target of antibodies in 25–40% of lupus patients. Purified human 60-kDa Ro was found to be oxidatively modified. Earlier investigations from our laboratory revealed increased oxidative damage in SLE patients. Therefore we hypothesized that oxidation by-products, such as 4-hydroxy-2-nonenal (HNE), could lead to neoantigens like HNE-modified 60-kDa Ro, which could in turn initiate autoimmunity or drive epitope spreading. To test this hypothesis we immunized rabbits with either HNE-modified 60-kDa Ro or the unmodified Ro. Intramolecular epitope spreading within the Ro molecule and intermolecular epitope spreading to La, double-stranded DNA, nRNP, and Sm occurred preferentially in HNE–Ro-immunized animals. Nonspecific anti-HNE antibody, generated by immunization with HNE–keyhole limpet hemocyanin conjugate, did not significantly bind to these autoantigens. These data may suggest a hitherto unappreciated mechanism by which oxidative stress facilitates epitope spreading in SLE.

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Introduction

Systemic lupus erythematosus (SLE) is associated with the production of antibodies to self-constituents, particularly targeting certain specific ribonucleoprotein (RNP) particles [reviewed in 1]. Among these is the Ro RNP, composed of a 60-kDa protein (60-kDa Ro or SS-A) that is noncovalently associated with at least one of four short uridine-rich RNAs (the hY RNAs) [2,3]. The hY RNAs are associated with the La or SS-B (48 kDa) autoantigen, at least transiently. Anti-Ro is found in up to 50% of patients with SLE, whereas anti-La is found in substantially fewer patients [4,5]. Anti-Ro is associated with subacute cutaneous lupus, photosensitive skin rash, deficiency of early complement components, renal disease, neonatal lupus, lymphopenia, and neutropenia [6–8]. Anti-Ro is most likely the result of the interaction of HLA-DQα, HLA-DQβ, and the T cell receptor β [9–14].

The mechanism by which tolerance is lost in patients with SLE, such that the Ro RNP complex becomes targeted, is unknown. Studies have shown that if tolerance is broken to one component of an in vivo complex, the immune response can then generalize and expand, so that an entire
complex is no longer recognized as self by the immune system [15–18]. This acquisition of new autoreactivity during the course of disease is known as epitope spreading. Intramolecular epitope spreading occurs when the antigen-specific autoimmune response spreads to different epitopes on the one protein. Intermolecular epitope spreading occurs when the response spreads to epitopes on other structural/functional proteins.

Tolerance to self is maintained by removal of self-reactive lymphocytes in the thymus during the maturation of the immune system [19,20] and by making self-reactive T lymphocytes anergic in the periphery [21,22]. Autoimmunity arises from the emergence of both autoreactive T and B cells with an etiology that involves both genetic and environmental factors. Molecular mimicry of viral or bacterial antigens with self-determinants has been proposed as one of the pathogenic mechanisms for the appearance of autoreactive cells [24,25]. The diversification and amplification of autoimmunity in an individual might result from epitope spreading, which has been described as an important factor. The concept of epitope spreading was first described in experimental autoimmune encephalomyelitis [26] and the concept has been extended to other autoimmune diseases [16,17]. Several investigations based on immunization of nonautoimmune mice with self-peptides support the view that the highly diverse B and T cell autoimmune responses in SLE might originate from a single protein or even a single cryptic self-epitope without the need for foreign pathogens or molecular mimics [16,27–29].

Free radical-mediated damage has been implicated in the pathogenesis of SLE and other diseases [30–35]. Reactive lipid peroxidation products have been shown to form adducts with lysine, histidine, and cysteine targets [36–40]. One of the most common and reactive lipid oxidation products is 4-hydroxy-2-nonenal (HNE) [41]. Increased levels of HNE-modified proteins have been detected in the sera of children with autoimmune diseases [32]. HNE–protein adducts are potential neoantigens and so could be involved in the pathogenesis of autoimmune diseases.

We hypothesized that oxidative by-products, like HNE, would cross-link with 60-kDa Ro and help initiate autoimmunity. To test this hypothesis we immunized rabbits with either HNE-modified Ro or unmodified Ro and found that autoimmunity was established faster and more vigorously in the animals that were immunized with the modified Ro. Specific, rapid intra- and intermolecular epitope spreading occurred when animals were immunized with the HNE-modified Ro but not when immunized with either unmodified Ro or HNE–keyhole limpet hemocyanin (KLH).

### Materials and methods

#### Chemicals

4-Hydroxy-2-nonenal was from Cayman Scientific (Ann Arbor, MI, USA), immunofluorescent anti-nDNA test kits were from Helix Diagnostics (Sacramento, CA, USA) and ANA test kits were from Inova Diagnostics (San Diego, CA, USA). Fluorescein-conjugated anti-rabbit IgG was from Jackson Laboratories (Bar Harbor, ME, USA). All other chemicals were of reagent grade.

#### 60-kDa Ro antigen

Human 60-kDa Ro [42–44] was purified from human liver and spleen obtained after autopsy from two patients as described for purifying 60-kDa Ro from bovine tissue [4,45]. One of the patients died of metastatic cancer and the other from an undisclosed bacterial infection. The second patient also suffered from diabetes mellitus and schizophrenia. Neither of them had acquired immunodeficiency disease or any other sexually transmitted disease. Purified bovine 60-kDa Ro was a generous gift from Immunovision (Springdale, AR, USA).

#### Multiple antigenic peptides (MAPs)

The 21 MAPs were synthesized from the sequence of the 60-kDa Ro [42–44] at the Molecular Biology Resource Facility (University of Oklahoma Health Sciences Center) by a manual stepwise solid-phase procedure. MAPs contain multiple copies of the same peptide sequence (eight copies in this instance) coupled covalently to a heptalysine backbone. Two unrelated MAPs bearing the sequences PPPGMRPP [17] and PSQQVMT (Sm 115) from the Sm autoantigen were also synthesized.

#### Preparation of antigen for immunization

Purified 60-kDa Ro was modified by the addition of 10 mM 4-hydroxy-2-nonenal at room temperature for 24 h in the presence of 10 mM sodium cyanoborohydride and then dialyzed against 0.1 N NaCl using a 10-kDa molecular weight cut-off membrane. Unmodified 60-kDa Ro treated with sodium cyanoborohydride and dialyzed simultaneously with the HNE–Ro served as the control.

#### Rabbit immunization

Two New Zealand White rabbits were immunized with HNE-modified 60-kDa Ro, whereas four rabbits were immunized with unmodified 60-kDa Ro. On day 1, 500 μg of either 60-kDa Ro or HNE-modified 60-kDa Ro was emulsified in 0.5 ml of complete Freund’s adjuvant and injected ip and sc. Subsequent boosts, with 500 μg antigen in incomplete Freund’s adjuvant, were administered on days
26, 53, and 99 with a final intravenous boost on day 152. The animals were bled weekly and sera obtained were stored at −20°C. All animals had preimmunization sera collected from them.

Antibodies against HNE were made by immunizing animals with HNE coupled to KLH. This antiserum was provided by Dr. Luke Szweda, Case Western Reserve University (Cleveland, OH, USA), and was used as a control in our experiments. Anti-KLH monoclonal antibody was obtained from Sigma Chemical Co.

Protein carbonylation

Protein carbonylation was studied by overnight incubation of 60-kDa Ro (bovine or human) 1:1 with 20 mM N-morpholinoethane sulfonate, pH 5.5, to which was added 1 mM butylated hydroxy tolouene (in ethanol) and 5 mM biotin hydrazide. The protein was immunoblotted with a streptavidin–peroxidase conjugate and developed using chemiluminescence [46].

Enzyme-linked immunosorbent assay (ELISA)

Solid phase ELISAs for 60-kDa Ro, HNE–60-kDa Ro, Sm, and nRNP were performed as previously described [3,4].

MAP ELISA

A solid-phase immunoassay for the MAPs was performed as described earlier [15]. Fifty microliters of MAPs was coated onto the wells of 96-well Costar vinyl plates (Cambridge, MA, USA) at 10 μg/ml in 0.5 M carbonate buffer, pH 9.5. Coating was carried out at 4°C overnight and followed by blocking with 175 μl 3% milk in phosphate-buffered saline (PBS), pH 7.4, containing 0.1% BSA. After being washed with PBS–Tween 20 (PBS-T), the wells were then charged with 50 μl of rabbit serum (1:100 dilution) and incubated at room temperature for 2 h or overnight at 4°C. After six washes with PBS-T, 50 μl of 1:5000 dilution of appropriate alkaline phosphatase-conjugated secondary antibody was added and color developed with p-nitrophenyl phosphate.

HeLa cell lysate

Freshly cultured HeLa cells were harvested and washed with phosphate-buffered saline, pH 7.4. The cells were lysed by sonication in SDS-lysis buffer. The samples were then used for SDS–PAGE and immunoblotting.

SDS–PAGE and immunoblotting

Gradient (4–20%) and 10% SDS–PAGE was carried out according to Laemmli [47] and the gel was transferred to nitrocellulose. Standard immunoblotting was carried out with alkaline phosphatase-conjugated IgGs or chemiluminescence as indicated for specific experiments [48,49].

Detection of antibodies to double-stranded DNA by immunofluorescence ELISA

Plasmid DNA or sonicated calf thymus DNA was bound to polylysine-coated plates at 5 μg/ml of phosphate-buffered saline, pH 7.4. The remaining steps were carried out essentially as for the ELISA described above. The primary rabbit sera were used at a dilution of 1:10. Anti-rabbit IgG conjugated to fluorescein isothiocyanate at a dilution of 1:500 was used as the secondary antibody. After being washed, the plates were read spectrofluorometrically after the incubation with the secondary antibody.

Results

We have observed increased oxidative damage in lupus patients, in a previous study [50]. Building on these findings, we sought to determine whether purified 60-kDa Ro from a human source (tissue obtained at autopsy) was a target of oxidative modification in vivo. Human 60-kDa Ro was found to be HNE modified (Fig. 1), thus implying the potential for 60-kDa Ro to be a target for oxidative modification in SLE patients.

Therefore, we embarked on modifying purified bovine 60-kDa Ro with HNE in vitro for use as an immunological reagent in animal experiments. The modified 60-kDa Ro was characterized by SDS–PAGE and immunoblotting using anti-HNE–KLH antibody (data not shown). Our previous immunization with unmodified purified 60-kDa Ro resulted in rabbits with limited autoimmunity but without clear-cut clinical evidence of SLE [15]. We hypothesized that immunization of rabbits with HNE-modified 60-kDa Ro would result in an accelerated autoimmune response.

Fig. 1. Immunoblot of anti-HNE staining of 60-kDa Ro isolated from the liver of a human subject after autopsy. Purified human 60-kDa Ro was immunoblotted with anti-HNE serum. The blot on the left was probed with serum from an animal immunized with HNE–KLH. The blot on the right was probed with serum from an animal immunized with HNE–Ro. Animals immunized with unmodified Ro did not generate antibodies that would strongly recognize Ro protein in regular Western blots.
Four rabbits were immunized with 60-kDa Ro (the unmodified bovine Ro was not found to be modified when analyzed by immunoblot—data not shown) and two rabbits were immunized with HNE-modified 60-kDa Ro. First, the time course was determined for the response against 60-kDa Ro and HNE–60-kDa Ro in these experimental animals. The 60-kDa Ro and HNE-modified 60-kDa Ro were coated onto ELISA plates and the binding of these antigens by each rabbit serum was determined. Anti-Ro response began by bleed 1 and bleed 2 in the two HNE–Ro-immunized animals, whereas it began only by bleed 7 and bleed 5 in Ro rabbits 1 and 2. The anti-Ro response began by bleed 1 and 4, respectively, for Ro rabbits 3 and 4. Sera from both the 60-kDa Ro- and the HNE–Ro-immunized rabbits responded to 60-kDa Ro or HNE–Ro as early as the third bleed (Fig. 2). The anti-HNE–Ro response in the rabbits was higher than the anti-Ro response at this time point, against both modified and unmodified Ro, and it remained higher against HNE–Ro through the bleeds tested (Fig. 2). Anti-Ro antibody levels were higher in Ro rabbits 3 and 4 by the fourth bleeds, but these rabbits did not have anti-HNE antibodies. Thus the animals immunized with modified Ro made a vigorous antibody response against both forms of Ro, whereas the animals immunized with unmodified Ro made a very strong response only against the unmodified Ro.

The binding of these animal sera to Ro and HNE–Ro was then determined by immunoblot. By the eighth bleed, there was only minimal binding to denatured 60-kDa Ro by the animals immunized with unmodified 60-kDa Ro (Fig. 3). However, antibodies from HNE–Ro-immunized rabbits bound the purified 60-kDa Ro antigen very strongly (Fig. 3).

The specificities of the anti-Ro and anti-HNE–Ro antibodies were then determined by inhibition studies. Using...
solution-phase antigen as the inhibitor in an ELISA, it was found that binding of serum from the 60-kDa Ro-immunized rabbit to unmodified 60-kDa Ro could be inhibited 60 or 50% by either purified 60-kDa Ro or HNE-modified 60-kDa Ro, respectively. Also, the binding of serum from a HNE–60-kDa Ro-immunized rabbit to unmodified Ro could be inhibited by either unmodified Ro or HNE-modified Ro. Meanwhile, the binding of serum from a HNE–60-kDa Ro-immunized rabbit to HNE-modified Ro could be inhibited by HNE-modified Ro but not by purified 60-kDa Ro (Fig. 4). There was no significant binding of serum from the unmodified 60-kDa Ro-immunized animal to HNE-modified Ro. These data demonstrate that the anti-Ro and anti-HNE–Ro antibodies are not cross-reactive (at the eighth bleed) and that anti-HNE–Ro was probably an entity separate from anti-60-kDa Ro.

The anti-HNE antibody levels in the animals immunized with HNE–Ro were determined next. For this, the Ro- and HNE–Ro-immunized animals were assayed for binding to KLH and HNE–KLH in an ELISA system. Anti-HNE antibodies were found only in the HNE–Ro-immunized animals and these antibodies were found to peak by the eighth bleed (Fig. 5). Ro rabbits 3 and 4 also had low levels of anti-HNE antibodies (data not shown).

In order to investigate intramolecular spreading, animal sera were then tested by ELISA for binding to 60-kDa Ro MAPs [15]. Control MAPs from other unrelated autoantigens were also used. MAPs were coated onto ELISA plates and the binding of antibodies from these animals was determined [15]. Only two multiple antigenic peptides spanning the amino acid sequences 126–137 and 212–219 on the 60-kDa Ro antigen were bound strongly by Ro-immunized animals as early as the fourth bleed, whereas at least 16 of 21 60-kDa Ro multiple antigenic peptides [44] were bound by HNE–Ro-immunized animals by the fourth bleed. These data indicate that the immunological response in HNE–Ro-immunized rabbits had spread to almost the whole protein. Anti-HNE–KLH antisera did not bind to the MAPs, showing the binding specificity of anti-HNE–Ro sera (Fig. 6). The average optical densities of preimmune values of normal and test animals were averaged to obtain the cut-off value shown in Fig. 6.

Intermolecular spreading was then determined in these animals by analyzing the binding to La, Sm, and nRNP autoantigens. Recombinant La was immunoblotted with the sera from the six animals and we found that HNE–Ro-immunized rabbits bound La much more strongly and earlier compared to the Ro-immunized animals. Recombinant human La was also coated onto ELISA plates and the binding of antibodies from the six animals was determined (Fig. 7A). A representative graph is shown (Fig. 7B). Sera from both HNE–60-kDa Ro- and 60-kDa Ro-immunized animals bound recombinant human La. The response in the HNE–Ro-immunized animal had spread to La at the first bleed and the binding was much stronger than the binding of the Ro-immunized animal up until the eighth bleed. Thus, immunization with the modified 60-kDa Ro brought about

![Fig. 3. Binding of anti-HNE–60-kDa Ro and anti-60-kDa Ro to purified 60-kDa Ro (prebleed and eighth bleed). 60-kDa Ro on blocked nitrocellulose strips was incubated with either anti-Ro or anti-HNE–Ro rabbit antiserum. Then the strips were incubated with appropriate alkaline phosphatase conjugate and then developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The two lanes on the right show a negative control and a positive control, respectively. The arrow shows the 60-kDa Ro band after immunoblotting.](image)

![Fig. 4. Inhibition of anti-HNE–60-kDa Ro and anti-60-kDa Ro binding to 60-kDa Ro (top) and HNE–60-kDa Ro (bottom). Unmodified Ro or HNE-modified Ro was coated onto ELISA plates and the binding of anti-HNE–Ro or anti-Ro rabbit antiserum to these antigens was determined in the presence or absence of Ro/HNE–Ro. Inhibition of binding to respective antigens is expressed as percentage of the optical density of the control–uninhibited sample.](image)
greater and more rapid intermolecular epitope spreading to La than did immunization with unmodified 60-kDa Ro.

Then, we determined whether immunization with an unrelated protein coupled to HNE could mediate the effects demonstrated using HNE-modified 60-kDa Ro. It was found that anti-HNE–KLH did not bind 60-kDa Ro, La, or the MAPs, thus demonstrating the importance of the moiety coupled to HNE (Fig. 8).

A HeLa cell lysate immunoblot confirmed the La binding observation obtained with ELISA. The La antigen was not bound on immunoblot by the serum from the 60-kDa Ro-immunized animal, whereas a band is clearly seen in all the bleeds tested from the HNE–Ro-immunized animal (Fig. 9). This shows that the intermolecular epitope spreading to the La autoantigen occurred preferentially in the HNE–Ro-immunized rabbits. Prototype anti-Ro 60 sera do not bind denatured 60-kDa Ro on tissue lysate immunoblot and therefore we are unable to see the 60-kDa Ro band on the immunoblot.

ELISA plates were then coated with either Sm or nRNP antigens obtained from a commercial source. The autoimmune response in both unmodified and modified Ro-immunized animals spread to Sm and nRNP (data not shown) at almost the same rate. Anti-RNP antibodies arose as early as the third bleed, with a stronger response in the HNE–Ro-immunized animals. Anti-Sm antibodies arose as early as the fifth bleed, with a stronger response observed in the 60-kDa Ro-immunized animals. This response dropped by the next bleed, at which the animals immunized with HNE–Ro predominated. After this the response in the animals fluctuated against both Sm and RNP. Western blot analyses showed that sera from the animals immunized with HNE–Ro bound strongly to the autoantigens 70 K and SmB/B, demonstrating intermolecular epitope spreading to these autoantigens.

Antibodies to double-stranded DNA, as determined by a modified ELISA, were observed in both the Ro- and the HNE–Ro-immunized animals as early as the 8th bleed, with increases by the 24th bleed. The titer almost doubled for one of the animals immunized with HNE–Ro compared to one of the unmodified Ro-immunized animals. The second
HNE–Ro animal had similar levels of anti-DNA antibodies, compared to the second animal immunized with unmodified Ro (data not shown).

Discussion

This work describes a novel oxidative mechanism by which epitope spreading could occur. Oxidative modification of SLE-associated proteins such as 60-kDa Ro might result in the formation of chemical adducts which could serve as neoantigens that the immune system has probably not been exposed to. This modified Ro might be more readily internalized, on account of its neoconformation, than the unmodified Ro, by antigen-presenting cells, such as dendritic cells or macrophages. These in turn present novel self-peptides to T cells, which can provide help to autoreactive B cells to bring about intramolecular spreading. B cells specific for either the modified or the unmodified Ro could internalize the antigen, along with associated antigens, by means of its cell surface Ig receptor. Epitopes from each of these proteins could then be presented to naive T cells, in the context of major histocompatibility complex class II, resulting in a diversification of autoreactive T cells, which assist a diversified B cell response that can recognize separate B cell antigenic determinants from the different antigens resulting in autoreactivity to numerous antigens.

The fact that 60-kDa human Ro was found to be oxidatively modified in a human liver points to the possibility that 60-kDa Ro in lupus patients may be subject to modification, especially because we and others have found increased oxidative damage in SLE [30–34,50]. Such a scenario proposes developing antibodies to 60-kDa Ro and thus autoimmunity to the entire Ro ribonucleoprotein particle after an initial immune response to oxidized 60-kDa Ro. This effect was seen under experimental conditions when we immunized rabbits with HNE-modified 60-kDa Ro. Distinct intramolecular and intermolecular epitope spreading effects were seen in these animals.

Three different responses were seen when we analyzed intramolecular spreading. First there was the response against 60-kDa Ro peptides that are not antibody targets in human or immunization-induced anti-Ro [15,16]. Second, there was an enhanced response in these animals toward peptides that were bound by the unmodified Ro-immunized animals. Third, there were certain peptides against which no response was generated in either group. Three distinct responses were observed when we studied intermolecular spreading between these two groups of animals. First, there was an accelerated and enhanced response against 60-kDa Ro in the HNE–Ro-immunized animals. Second, tolerance was abrogated with respect to La and double-stranded DNA mainly in the animals that were immunized with the modified Ro. Third, autoimmunity against nRNP and Sm...
was observed in both groups of animals. Importantly, immunization with HNE–KLH failed to generate antibodies that bind to Ro, La, or the Ro MAPs, demonstrating the importance of the antigen that is oxidatively modified. The fact that an anti-KLH monoclonal antibody did not bind to any of these antigens also showed the specificity of Ro 60 immunization. Also, another critical observation is that immunization with either unmodified Ro or HNE–Ro led to lupus-like autoimmunity, not antibodies binding antigens unrelated to human lupus, as demonstrated in the tissue extract immunoblot (Fig. 9).

The fact that antibodies were found against 60-kDa Ro MAP 401, which has not been shown to be an epitope hitherto, raises the possibility that neoepitopes are generated by oxidative modification, which could be the reason for the increased anti-60-kDa Ro titer seen in the animals immunized with modified Ro. Autoimmunity is initiated at an accelerated pace in those animals that were immunized with HNE–Ro. Epitope spreading to La and other antigens is not a result of contamination with La in the Ro preparation, because the commercial source from which we obtained the antigen is free of La. Moreover, we have observed the spreading to La by immunizing with peptides from 60-kDa Ro [15,16].

There are other examples of an immune response against oxidatively modified self-antigens. Aldehyde-modified proteins have been found to be highly immunogenic and autoantibodies directed against epitopes in malondialdehyde- (MDA) and HNE-modified low-density lipoproteins (LDL) have been demonstrated in the plasma of rabbits and mice immunized with oxidized LDL (ox-LDL) [37,51]. Antibodies against ox-LDL or MDA-LDL have been demonstrated in atherosclerotic plaques [52–55]. Itabe et al. [56] have shown that MDA-cross-linked proteins are actually present in vivo and that their production is stimulated by oxidative stress. Recently Chang et al. [57] demonstrated oxidation-specific antigens on the surface of apoptotic cells. Antigens modified by oxidative by-products have been shown to induce immune responses in alcoholic liver disease [58]. In type 1 diabetes mellitus, autoantibodies are present in patient sera that bind oxidatively modified islet cell proteins [59].

If all the lysines (47), histidines (13), and cysteines (13) were to be modified, the Ro protein has a hypothetical molecular weight of 71.4 kDa instead of 60 for the unmodified protein. Owing probably to the changes in structural conformation of Ro 60 or formation of aggregates, the HNE-modified Ro was not amenable to analysis on SDS–PAGE.

As far as we know this is the first report demonstrating accelerated development of autoimmunity after immunization with an oxidatively modified autoantigen. This report also underscores the importance of a hitherto unappreciated mechanism for epitope spreading, which may have clinical implications. If in fact oxidative processes are the important factors that induce autoimmunity in the clinical scenario, it may be possible to prevent the development of SLE in genetically susceptible individuals by administration of antioxidants or other dietary manipulations to decrease formation of HNE/oxidative by-products.

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