Protein-bound 4-Hydroxy-2-nonenal
AN ENDOGENOUS TRIGGERING ANTIGEN OF ANTI-DNA RESPONSE

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Several lines of evidence indicate that the nonenzymatic oxidative modification of proteins and the subsequent accumulation of the modified proteins have been found in cells during aging and oxidative stress and in various pathological states, including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis. Our previous work suggested the existence of molecular mimicry between antibodies raised against hydroxy-2-nonenal (HNE)-modified protein and anti-DNA autoantibodies, a serologic hallmark of systemic lupus erythematosus (SLE). In the present study, we investigated the possible involvement of HNE-modified proteins as the endogenous source of the anti-DNA antibodies. Accumulation of the antigen recognized by the antibody against the HNE-modified protein was observed in the nucleus of almost all of the epidermal cells from patients with autoimmune diseases, including SLE. The SLE patients also showed significantly higher serum levels of the anti-HNE titer than healthy individuals. To determine if a specific anti-DNA response could be initiated by the HNE-derived epitopes, we immunized BALB/c mice with the HNE-modified protein and observed a progressive increase in the anti-DNA response. Moreover, we generated the monoclonal antibodies, showing recognition specificity toward DNA, and found that they can bind to two structurally distinct antigens (i.e. the native DNA and protein-bound 4-oxo-2-nonenal). The findings in this study provide evidence to suspect an etiologic role for lipid peroxidation in autoimmune diseases.

Several lines of evidence indicate that the nonenzymatic oxidative modification of proteins and the subsequent accumulation of the modified proteins have been found in cells during aging and oxidative stress and in various pathological states, including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis. It has also been suggested that many of the effects of cellular dysfunction under oxidative stress are mediated by the products of nonenzymatic reactions, such as the peroxidative degradation of polyunsaturated fatty acids (3, 4). Lipid peroxidation leads to the formation of a broad array of different products with diverse and powerful biological activities. Among them are a variety of different aldehydes. The primary products of lipid peroxidation, lipid hydroperoxides, can undergo carbon-carbon bond cleavage via alkoxyl radicals in the presence of transition metals, giving rise to the formation of short chain, unesterified aldehydes of 3–9 carbons in length, and a second class of aldehydes still esterified to the parent lipid (5). These aldehydes generated during the lipid peroxidation have been implicated as causative agents in cytotoxic processes initiated by the exposure of biological systems to oxidizing agents.

Some of the lipid peroxidation products exhibit a facile reactivity with proteins, generating a variety of intra- and intermolecular covalent adducts. Such adducts could be the targets of B cell-mediated immune responses and induce T cell responses and add the potential of certain aldehydes to induce an autoimmunity by breaking the B cell tolerance to nonmodified proteins. It has been shown that the modification of self-proteins by lipid peroxidation products indeed results in a break of a tolerance to self-proteins (6). The fact that post-translational modification of proteins is enhanced in aging and stressed cells and arises under physiological conditions (1, 2) suggests the existence of an association between covalent modification of protein with lipid peroxidation products and autoimmune diseases.

Anti-DNA autoantibodies are a prime feature of human systemic lupus erythematosus (SLE)(7). The appearance of these antibodies in humans and in murine models of lupus correlates with the progression of the disease, and by comparison with all of the other lupus autoantibodies, those against the double-stranded DNA (dsDNA) are considered the major pathogenic agents in lupus. The lupus autoantibodies, particularly anti-dsDNA antibodies, exhibit a wide range of specificities, and it is possible that these specificities reflect the diversity of the modified antigens and the structure of the dsDNA molecule. The finding that anti-DNA antibodies can recognize a variety of different epitopes on DNA suggests the existence of molecular mimicry between antibodies raised against protein and antibodies raised against DNA. A similar phenomenon has also been observed in the murine model of lupus, where the monoclonal antibody 7H15, raised against protein modified with an aldehyde, can recognize the native DNA (8).
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Modification of Protein and DNA by Reactive Aldehydes—Modification of the protein by aldehydes was performed by incubating BSA (1.0 mg/ml) with 1–10 mM aldehydes in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. Modification of the DNA was performed by incubating calf thymus DNA (1.0 mg/ml) with 10 mM aldehydes in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 24 h.

ELISA—A 100-μl aliquot of the antigen solution was added to each well of a 96-well microtiter plate and incubated for 20 h at 4 °C. The antigen solution was then removed, and the plate was washed with phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS/Tween). Each well was incubated with 200 μl of 4% Blockace (Yukijirushi, Sapporo, Japan) in PBS/Tween for 60 min at 37 °C to block the unsaturated plastic surface. The plate was then washed three times with PBS/Tween. A 100-μl aliquot of a 10^2× dilution of serum was added to each well and incubated for 2 h at 37 °C. After discarding the supernatants and washing three times with PBS/Tween, 100 μl of a 5 × 10^3 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase in PBS/Tween was added. After incubation for 1 h at 37 °C, the supernatant was discarded, and the plates were washed three times with PBS/Tween. The enzyme-linked antibody bound to the well was revealed by adding 100 μl of 1,2-phenylenediamine (0.5 mg/ml) in a 0.1M citrate/phosphate buffer (pH 5.5) containing 0.003% hydrogen peroxide. The reaction was terminated by the addition of 2 μl 4% sulfuric acid (50 ml/well), and the absorbance at 492 nm was read using a micro-ELISA plate reader. In a competitive ELISA, the competitor was incubated with the antibody for 20 h at 4 °C to yield competitor/antibody mixtures containing the antibody at 0.8 μg/ml and variable concentrations of the competitor. A 100-μl aliquot of the competitor/antibody mixture was added to each well and incubated for 2 h at 37 °C. After discarding the supernatants...
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Cloning, Sequencing, and Analysis of Variable Heavy (\(V_H\)) and Light Chain (\(V_L\)) Genes—Immunoglobulin variable region genes were cloned and sequenced following amplification by PCR. Total RNA was prepared from 5 \(\times\) 10^6 hybridoma cells by the phenol-guanidine isothiocyanate method (TRIZol reagent; Invitrogen) according to the manufacturer’s protocol. The first strand cDNA synthesis was performed with recombinant Moloney murine leukemia virus reverse transcriptase (SuperScript II; Invitrogen) using the manufacturer’s protocol. A 5-\(\mu\)g sample of the total RNA was primed with 10 pmol of random primers. Variable region genes were amplified using degenerate sense primers homologous to the mouse heavy and light chain leader sequences and antisense constant primers (Novagen), as previously described (16). The amplification products were ligated into the pGEM-T Easy Vector (Promega) using standard protocols, and both strands of inserts were sequenced using an automated dye-chain termination DNA sequencer. The obtained sequences were analyzed using the DNAStar software (DNASTAR, Madison, WI). The Basic Local Alignment Search Tool (BLAST) protocol was used to search the GenBank data base to determine the homology with the \(V\) regions of other murine Abs that have been sequenced (17).

Serum Samples—The serum samples were prepared from 90 patients with SLE, 20 patients with progressive systemic sclerosis, and 30 patients with rheumatoid arthritis, all of whom met the criteria for diagnosis proposed by the American College of Rheumatology (18–20); 15 patients with polypositis/dERMAtomyositis, who met the criteria proposed by Bohan et al. (21); and 20 patients with Sjogren’s syndrome, who were diagnosed according to the criteria proposed by the European Community (22). The anti-DNA and anti-HNE titers in the serum samples were measured by ELISA using calf thymus DNA and HNE-modified protein, respectively, as the coating antigens.

Immunohistochemical Analysis—Skin specimens were obtained by biopsy from eight control subjects (sex: two males and six females; age: 33–49 (40.75 \(\pm\) 5.57) years), eight contact dermatitis patients (sex: two males and six females; age: 25–74 (50.13 \(\pm\) 17.60) years), three pemphigus vulgaris patients (sex: two males and one female; age: 21–55 (36.67 \(\pm\) 17.04) years), and five SLE patients (sex: one male and four females; age: 21–37 (26.80 \(\pm\) 8.90) years). Multiple 4-\(\mu\)m-thick sections were cut from formalin (20%)-fixed, paraffin-embedded skin materials of each case that were used for the hematoxylin-eosin staining and immunohistochemical staining. The sections were deparaffinized, rehydrated, quenched for 10 min at 4 °C with 3% hydrogen peroxide, rinsed in phosphate-buffered saline, pH 7.6, pretreated for 20 min at room temperature with 3% skim milk in PBS, and subsequently incubated overnight at 4 °C with the primary mAb HNEJ2 (23) at a final concentration of 0.1 \(\mu\)g/ml. The antibody binding was visualized by the avidin-biotin-immunoperoxidase complex method using the appropriate Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. The chromogen was 3,3’-diaminobenzidine tetrahydrochloride, and the counterstain was hematoxylin. Sections from which the primary antibody was omitted served as the negative reaction controls. The immunohistochemical localization of the protein-bound HNE was verified by comparison

and washing three times with PBS/Tween, the second antibody was added, and the enzyme-linked antibody bound to the well was revealed as already described.

Western Blot Analysis—BSA (1 mg/ml) was incubated with ONE (0–5 mM) in 1 ml of 50 mM sodium phosphate buffer, pH 7.4, for 24 h at 37 °C. The protein samples were electrophoresed through a 10% polyacrylamide gel. After electrophoresis, the gel was transblotted onto a nitrocellulose or polyvinylidene difluo-

... membrane (Amersham Biosciences), incubated with Blockace for blocking, washed, and then incubated with a primary antibody for detection of the ONE-modified protein. This procedure was followed by the addition of horseradish peroxidase-conjugated goat anti-rabbit IgG immunoglobulin and ECL reagents. The bands were visualized using a Cool Saver instrument.

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FIGURE 2. Immunohistochemical detection of HNE-modified protein in skin sections from patients with autoimmune diseases. We determined the presence of HNE-modified protein in the skin specimens obtained by biopsy from eight control subjects, eight contact dermatitis patients, three pemphigus vulgaris patients, and five SLE patients. The most representative data in each group are shown. A, control; B, contact dermatitis; C, pemphigus vulgaris; D, SLE. Original magnification was ×40.

with consecutive sections stained with the hematoxylin-eosin-stained consecutive sections.

Statistical Analyses—The statistical analysis was performed with the StatView software (version 4.0; Abacus Concepts, Berkeley, CA). Differences were analyzed by the unpaired two-tailed Student’s t test or Welch’s t test as appropriate, and p values of <0.05 were considered significant.

RESULTS

Presence of HNE-specific Epitopes in Human Autoimmune Diseases—To establish the association between the HNE modification of protein and autoimmune diseases, we first determined the presence of the HNE-specific epitopes in the contact dermatitis, pemphigus vulgaris, and SLE using the mAb against the HNE-modified proteins. Contact dermatitis is known to be a T cell-mediated immune reaction, whereas pemphigus vulgaris and SLE are based on T cell- and B cell-mediated autoimmune mechanisms with the appearance of antiepidermal cell membrane antibodies and antinuclear component antibodies, respectively. The hematoxylin-eosin-stained skin sections demonstrated the characteristic features of these diseases as follows. Briefly, (i) the contact dermatitis patients showed focal infiltration of the lymphocytes in the epidermis associated with spongiosis and in the dermis surrounding the superficial vessels, (ii) the pemphigus vulgaris patients exhibited suprabasilar acantholytic blister formation, resulting in bullous cavity formation, and (iii) the SLE patients indicated keratinization and liquefaction degeneration of the epidermis and fibrinoid degeneration of the dermis. No immunoreaction product deposits were detectable on sections with the omission of the primary antibody (data not shown). In the control subjects, the HNE-specific epitopes were obscure and weakly detectable in the superficial layer of the epidermis (Fig. 2A). In the chronic dermatitis patients, the immunoreactivity was distinct and focally seen in the nucleus of the epidermal cells restricted to regions adjacent to the lymphocytic infiltrates (Fig. 2B). In the patients with pemphigus vulgaris (Fig. 2C) and SLE (Fig. 2D), the immunoreactivity was distinct and diffusely seen in the nucleus of almost all of the epidermal cells. In addition, the HNE-specific epitopes were detectable in the cytoplasm of the epidermal cells in the examined cases, and the staining intensities varied from case to case, as shown in Fig. 2D. The HNE-specific epitopes were also localized in the nucleus of the endothelial cells of the capillary vessels surrounded by lymphocytic infiltrates in the dermis, whereas the epitopes were undetectable in the lymphocytes.

SLE Patients Have Higher Titers of Anti-HNE Antibodies—Based on the detection of the HNE-specific epitopes in the patients with the autoimmune diseases, we speculated that the production of specific antibodies against the epitopes might also be accelerated. Hence, to evaluate the presence of these antibodies in the autoimmune diseases, we measured the serum antibody titers directed against the native DNA and HNE-modified serum albumin. Although it was not statistically significant, we observed a modest increase in the anti-DNA antibodies in the SLE patients (Fig. 2A). In contrast, the SLE patients exhibited significant increases in the anti-HNE antibodies compared with the controls (Fig. 2B). We also measured the serum anti-DNA and anti-HNE antibody titers in other autoimmune diseases, including progressive systemic sclerosis, rheumatoid arthritis, polymyositis/dermatomyositis, and Sjögren syndrome. Although there was no significant increase in the serum anti-DNA levels, we observed significant increases in the anti-HNE antibodies compared with the controls (Fig. S1). In addition, in our preliminary experiments, we have observed pronounced increases in anti-DNA and anti-HNE titers in MRL-lpr mice, a representative murine model of SLE.3 Thus, the antibody response against the HNE-specific epitopes may be an immunological characteristic common to various types of autoimmune diseases, and the serum anti-HNE titer has a potentially important diagnostic value in human autoimmune diseases. These clin-

3 K. Toyoda and K. Uchida, unpublished observation.
HNE-derived epitopes determined if the anti-DNA response could be initiated by the HNE-specific epitopes. The immunogen was prepared by incubating KLH (1.0 mg/ml) with 1 mCi HNE in 10 ml of 50 mm sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. Female BALB/c mice were immunized on day 1 with complete Freund adjuvant and 0.5 mg of immunogen (HNE-modified KLH) and boosted on days 7, 17, and 27 with incomplete Freund adjuvant by emulsifying and intraperitoneal injection. Antibody response was examined by an ELISA employing pairs of wells in microtiter plates on which were absorbed calf thymus DNA (red diamond), BSA (black triangle), and HNE-treated BSA (blue square) as antigens. B, cross-reactivity of the mAbs raised against HNE-specific epitopes. Coating antigens were DNA (red bar), HNE-modified BSA (blue bar), and native BSA (black bar). The affinity of the antibodies was determined by a direct antigen ELISA, employing pairs of wells in microtiter plates on which were absorbed calf thymus DNA, BSA, and HNE-treated BSA as antigens.

Antigenic and animal data offer an attractive hypothesis that the HNE-specific epitopes may represent immunologic triggering antigens for the human autoimmune diseases.

HNE-specific Epitopes Elicit the Anti-DNA Response in BALB/c Mice—Since HNE physiologically arises in cells, we determined if the anti-DNA response could be initiated by the HNE-derived epitopes in vivo. To this end, female BALB/c mice were immunized every 2 weeks with the HNE-modified KLH emulsified with complete Freund’s adjuvant, and both the anti-HNE and anti-DNA responses were examined. Not only anti-HNE response but also anti-DNA response began to appear after the third immunization, during week 8 (Fig. 4A), although BALB/c mice are not the strain with spontaneous autoimmune disease. All of the HNE-modified protein-immunized mice developed an IgG anti-DNA response, which was comparable with the anti-HNE response. BALB/c mice immunized with KLH alone did not induce any significant anti-DNA response (data not shown). Four hybridoma clones (D1H3, D2A3, D3A8, and D3A11) producing the antibodies, showing recognition specificity toward native DNA, and two hybridoma clones (P2G11 and P4E6) producing the anti-HNE mAbs were prepared from mice immunized with the HNE-modified protein after screening based on specific binding to each corresponding antigen, DNA, or HNE-modified protein. As shown in Fig. 4B, the antibodies, showing recognition specificity toward native DNA, did not react with the HNE-modified BSA at all, whereas the anti-HNE mAbs did not show any cross-reactivity toward the native DNA. Thus, it appeared that animals immunized with the HNE-modified protein progressively developed two distinct populations of B cell clones, which are specific to either the HNE-modified proteins or the native DNA.

Sequence Analysis of the mAbs Raised against HNE-specific Epitopes—To further confirm that the mAbs raised from animals immunized with HNE-specific epitopes represent the anti-DNA antibodies, the identity of their V region genes was determined for homologies to the known V region genes using the BLAST protocol (17). A homology search of the GenBank™ revealed that D2A3 shared a high degree of sequence identity with the anti-DNA and anti-polysaccharide antibodies. The V(1) domain of D2A3 was highly homologous (85.0–91.6% identity) to the anti-DNA mAbs (JeI466, VH30, and BW2.18-2) and to the anti-polysaccharide mAbs (HmenB3 and mAb 735) (Fig. 5A). In addition, the sequence identity of greater than 93% for the V(1) domain of D2A3 was shared with the antibodies, anti-CD3 (HuM291 and OKT3) and the anti-α(1–6) dextran antibodies (Fig. 5B). The V genes of D3A8 and D3A11 also revealed a sequence similarity with the anti-DNA mAbs. Clonally related V(1) genes of D3A8 and D3A11 were weakly homologous to D2A3 and its homologous anti-DNA and anti-polysaccharide antibodies: JeI466, VH30, BW2.18-2, HmenB3, and mAb 735 (78.5–85.0%). The V(1) domains of D3A8 and D3A11 were homologous to the anti-DNA mAbs, ZB2G10 (95.4%) and DP7VK (94.4%), respectively.

On the other hand, the V(1) genes of the anti-HNE mAbs P4E6 and P2G11 revealed a weak sequence similarity with the anti-DNA mAb ZB2F12 (78.5–81.3%) and anti-HNE mAb RS17 (78.5–79.4%), whereas their identities were significantly lower than the identity of D2A3 with the anti-DNA mAbs (Fig. 5A). The V(1) domain of P4E6 was homologous to the anti-DNA mAb (5.9-1, ZA1H3) and anti-HNE mAbs R310 and RS17. No sequence similarity with the anti-DNA mAbs was observed in the V(1) genes of P2G11 (Fig. 5B). In addition, the V(1) domain of rheumatoid factor RFA28-A showed a high similarity with R310 and DNA-1 (89.7% identity). The V(1) domain of rheumatoid factor 6-19 was almost identical with R310 and DNA-1 (89.7% identity). The V(1) domain of rheumatoid factor 6-19 was almost identical with R310 and DNA-1 (89.7% identity). The V(1) domain of rheumatoid factor 6-19 showed a weak sequence similarity with the anti-DNA, including the anti-polysaccharide mAbs.

Dual Specificity of the mAbs Raised against HNE-specific Epitopes—We have previously shown that the mAb raised against the modified protein with the HNE enantiomer ((R)-HNE) shows only a slight cross-reactivity with the native DNA, whereas the immunoreactivity is dramatically enhanced by the modification of DNA with ONE, an analog of HNE (12). Hence, we examined the immunoreactivity of the mAbs raised from animals immunized with the HNE-specific epitopes toward the aldehyde-modified DNA. Consistent with our previous findings, the immunoreactivity of the anti-HNE mAb P4E6 with the native DNA was dramatically enhanced by the modification of the DNA with ONE. It was also revealed that ONE, among the
tested lipid peroxidation-derived reactive aldehydes, was the only source of the immunoreactive structures recognized by the anti-HNE mAb (Fig. S2A). Of interest, the immunoreactivity of mAb D2A3 with the native DNA was also enhanced by the modification of the DNA with ONE (Fig. S2B).

On the other hand, the immunoreactivity of the mAbs with the aldehyde-modified proteins was also examined. As shown in Fig. 6A, the anti-HNE mAb P4E6 cross-reacted with the modified proteins with HNE, regardless of its chirality. Strikingly, mAb D2A3, showing specificity toward native DNA, was found to cross-react with the ONE-treated protein (Fig. 6B). The other mAbs, D1H3, D3A8, and D3A11, showing specificity toward native DNA, also cross-reacted with the modified protein (data not shown). Polyacrylamide gel electrophoresis under nonreducing and nondenaturing conditions followed by immunoblot analysis revealed that the ONE-modified protein electrophoresed as the multiple protein bands was detected by mAb D2A3 (Fig. 6C). However, no immunoreactive protein bands were detected by the SDS-polyacrylamide gel electrophoresis/immunoblot analysis (data not shown), suggesting the fragility of the epitope structure(s). These data suggest that the HNE-specific epitopes may serve as an early immunologic stimulus that gives rise to the production of dual specific antibodies against the native DNA and ONE-modified proteins.

Identification of an Epitope Recognized by the mAb D2A3—ONE is a lipid peroxidation product, which has been recently established to be formed by the free radical-initiated degradation of polyunsaturated fatty acids (15, 24). It has been suggested that, upon reaction with protein, ONE covalently modifies the arginine, cysteine, histidine, and lysine residues (25). However, due to the instability of the adducts, the structures of the ONE-modified amino acid adducts have not been determined except for the ONE-arginine adduct (26). We characterized the specificity of the mAb D2A3 using the reaction mixtures of the ONE/amino acids. As shown in Fig. 7A, binding of the ONE-modified protein to the antibody was hardly inhibited by the reaction mixtures of ONE/arginine, ONE/histidine, and ONE/lysine, but significantly inhibited by the reaction mixture of ONE/cysteine, suggesting that the mAb might recognize an ONE-cysteine adduct as the epitope. To identify the ONE-cysteine adduct recognized by the mAb D2A3, the immunoreactivity with the reaction products of ONE with N′-acetylcysteine was characterized. As shown in Fig. 7B, the reaction of N′-acetylcysteine with ONE mainly pro-

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**DISCUSSION**

SLE is a potentially fatal systemic autoimmune disease, characterized by the increased production of autoantibodies, immune complex deposition in the microvasculature, leukocyte infiltration, and, ultimately, tissue damage in a range of organs. Of the multiple autoantibodies described in this disease, antibodies against the native DNA are among the most characteristic, yet the triggering antigen in the disease is still unknown. We have shown here that the antigen recognized by the antibody against HNE-modified protein was accumulated in the nucleus of almost all of the epidermal cells from patients with SLE and other autoimmune diseases. The increase in the levels of the anti-HNE antibodies was also observed in SLE patients. Based on these observations, we have made the following two major discoveries in this study (Fig. 8): (i) the HNE-specific epitopes can be a triggering antigen of anti-DNA response, and (ii) the mAbs raised against HNE-specific epitopes can bind to two structurally distinct antigens (i.e. native DNA and ONE-modified proteins). These findings provide evidence to suspect an etiologic role for the lipid peroxidation in autoimmune diseases.

There is increasing evidence that lipid peroxidation plays a role in SLE. (i) SLE patients have an enhanced urinary excretion of isoprostanes, the well established biomarkers of lipid peroxidation (27), (ii) the levels of the lipid peroxidation-derived short chain aldehydes are significantly elevated in children with a high disease activity of SLE (28), and (iii) there are elevated levels of the oxidized low density lipoprotein together with elevated levels of autoantibodies related to the oxidized low density lipoprotein in female patients with SLE (29). The involvement of lipid peroxidation was further suggested by the detection of the HNE-specific epitopes in tissues from the SLE patients. The immunohistochemical studies clearly demonstrated the accumulation of the HNE-specific epitopes in the dermis of the patients (Fig. 2). The immunoreactivity was diffusely seen in the cytoplasm of the epidermal cells and in the nucleus of the endothelial cells of capillary vessels surrounded by lymphocytic infiltrates in the dermis of the patients. These observations verified for the first time the intracellular accumulation of the HNE-specific epitopes in human SLE and raised the possibility that the enhanced lipid peroxidation, through its pivotal role in oxidative stress, followed by the generation of immune complex deposition in the microvasculature, leukocyte infiltration, and, ultimately, tissue damage in a range of organs. Of the multiple autoantibodies described in this disease, antibodies against the native DNA are among the most characteristic, yet the triggering antigen in the disease is still unknown. We have shown here that the antigen recognized by the antibody against HNE-modified protein was accumulated in the nucleus of almost all of the epidermal cells from patients with SLE and other autoimmune diseases. The increase in the levels of the anti-HNE antibodies was also observed in SLE patients. Based on these observations, we have made the following two major discoveries in this study (Fig. 8): (i) the HNE-specific epitopes can be a triggering antigen of anti-DNA response, and (ii) the mAbs raised against HNE-specific epitopes can bind to two structurally distinct antigens (i.e. native DNA and ONE-modified proteins). These findings provide evidence to suspect an etiologic role for the lipid peroxidation in autoimmune diseases.

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specific epitopes, may be involved in the pathogenesis of autoimmune disorders.

One recent study demonstrated that elevated oxidative stress in erythrocytes due to a superoxide dismutase 1 deficiency triggered autoantibody production and that an antioxidant, N-acetylcysteine, significantly suppressed the inflammatory response (30). More intriguingly, our preliminary study demonstrated a significant increase in the levels of the anti-HNE titer in the superoxide dismutase 1-deficient mice. These observations also suggest that, although the potential role in pathogenesis needs to be further explored, lipid peroxidation may not simply be an associated side effect of disease progression but a possible etiology of SLE and other autoimmune diseases.

It is well known that the immunoreactivity for anti-DNA antibodies is the most characteristic serological finding in SLE (31). At the clinical level, however, we showed that the anti-HNE antibodies could be an important serological marker for SLE, showing a higher specificity than the anti-DNA antibodies (Fig. 3). In addition, a similar increase in the levels of anti-HNE titer has also been observed in the SLE-prone MRL-lpr mice. These data, showing the existence of a close association between the anti-DNA and anti-HNE antibody titers, significantly emphasized the relevance of HNE and its epitopes for the anti-DNA response in SLE. Moreover, since all of these patients had an active disease and some patients receiving no steroids had high anti-HNE levels, the increased anti-HNE antibodies may reflect some form of manifestation of SLE. Therefore, the HNE-specific epitopes may be used as the antigenic probe for detecting specific autoantibodies that can serve as reliable biomarkers for the practical evaluation of the disease activity in a subpopulation of SLE patients.

Although the anti-DNA antibodies are the hallmark of SLE, it has been difficult to identify an antigen that will elicit this specificity upon immunization. Based on the findings that (i) the sequence of an anti-HNE mAb was highly homologous to the anti-DNA autoantibodies (12), (ii) the HNE-specific epitopes were detected in tissues from patients with human SLE (Fig. 2), and (iii) the SLE patients with elevated serum levels of the anti-DNA titer were also positive for anti-HNE antibodies (Fig. 3), which is a significantly higher frequency than in patients without the elevated serum anti-HNE antibodies, we speculated that the HNE-specific epitopes could be an endogenous source of the anti-DNA antibodies. Hence, to determine if the HNE-specific epitopes could induce an anti-DNA response, we attempted to raise mAbs from BALB/c mice immunized with the HNE-modified KLH and successfully prepared the mAbs D1H3, D2A3, D3A8, and D3A11, showing recognition specificity toward native DNA, in addition to the anti-HNE mAbs, P2G11 and P4E6 (Fig. 4). Specificity studies demonstrated that the mAbs D1H3, D2A3, D3A8, and D3A11 strongly cross-reacted with DNA but did not cross-react with the HNE-modified BSA, whereas the anti-HNE mAbs did not show any cross-reactivity toward native DNA, which revealed that animals...
immunized with the HNE-specific epitopes progressively developed two distinct populations of mAbs that cross-reacted with either the HNE-modified proteins or native DNA. Antibodies cross-reacting with both the HNE epitopes and native DNA were not raised. Specificity studies also revealed that, consistent with our previous findings (12), the anti-HNE mAb P4E6 cross-reacted with the ONE-modified DNA (Fig. S2). This dual cross-reactivity of the anti-HNE mAb was previously proposed to arise through molecular mimicry between the HNE-histidine and the 1N2-etheno-type ONE-2'-deoxyribonucleoside adducts (12).

One unanticipated result of this study is the finding that the mAb D2A3, showing recognition specificity toward DNA, significantly cross-reacted with the ONE-modified protein (Fig. 6). Our inhibition studies also demonstrated that the mAb D2A3 recognized the ONE-cysteine Michael adduct as the major epitope (Fig. 7). Of interest, this dual specificity toward DNA and the ONE-modified protein is not unique to this antibody raised from BALB/c mice immunized with the HNE-specific epitopes. We have indeed observed that the anti-DNA mAb BV 16-13, whose specificity toward DNA has been well characterized (32), also cross-reacts with the ONE-modified protein. In addition, we have examined the affinity of the anti-DNA mAbs D431 and D466 obtained from the 56R and CD40L double transgenic mice (33, 34), a spontaneous murine model of SLE, with the ONE-modified protein and have found that, of the two anti-DNA mAbs, mAb D466 cross-reacted with the ONE-modified BSA.

Several different antigenic cross-reactivities have been identified for the anti-DNA antibodies (35). These antibodies share structural similarities with antibodies against bacterial polysaccharide, and some cross-react with the bacterial polysaccharide and protect mice against a lethal bacterial infection (36, 37). Other studies have also demonstrated a cross-reactivity of the anti-DNA antibodies with microbial protein antigens, nucleic acid autoantigens, cell membranes, and extracellular matrix components (38–42). Thus, at least some of the anti-DNA antibodies seen in autoimmune disease are likely to arise from antigens associated with modification with HNE and ONE, whereas some related adducts arising from other lipid oxidation products or possibly some epitopes that are of quite distinct origin are also likely to be involved. Although the detailed mechanisms for the dual cross-reactivity of the mAb D2A3 toward the native DNA and the ONE-cysteine adduct remain unclear, there are several possible explanations for the antibody multispecificity: (i) the antibody-combining site has more than one contact region for unrelated epitopes (43); (ii) the antigen is a molecular mimic of DNA (commonly referred to as molecular mimicry, which is characterized by an immune response to an environmental agent that cross-reacts with a host antigen, resulting in disease) (44, 45); and (iii) finally, an intriguing possibility is an antibody conformational isomerism, in which the antibody has two structurally dissimilar binding site conformations and can bind to two structurally distinct antigens (one site has a deep hole that binds to aromatic haptons, and the other binds to an unrelated protein or DNA antigen) (46, 47). Among these possibilities, we speculate that the ONE-associated dual cross-reactivity of the antibodies may be ascribed, at least in part, to the highest reactivity of this major lipid hydroperoxide-derived bifunctional electrophile toward biomacromolecules, such as protein and DNA (24–26). Upon reaction with protein and DNA, ONE generates a variety of different types of adducts on protein and DNA molecules, some of which are known to have the same or very similar chemical structures as those originated from other aldehydes (4, 48, 49). It is likely that some of the adducts may serve as epitopes of anti-HNE and anti-DNA antibodies through molecular mimicry. A further study is required to establish the mechanism for the unexpected cross-reactivities of the anti-DNA mAbs toward covalently modified proteins with the lipid peroxidation products.

In conclusion, our data demonstrated that the HNE-specific epitopes could be an endogenous triggering antigen for the production of the antibodies specific to DNA. In addition, a subset of the antibodies, showing recognition specificity toward DNA, cross-reacted with the alternative protein-bound epitope. These findings contribute to a paradigm of lipid peroxidation products causing at least some autoimmune diseases.

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