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Monoclonal Antibodies for Detection of 4-Hydroxynonenal Modified Proteins

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A promising approach to study lipid peroxidation pathology is antibodies recognizing aldehydes which react with and became bound to amino acid side chains of proteins. We present in this study the characterization of several monoclonal antibodies which recognize 4-hydroxynonenal (HNE) modified proteins. Six out of 20 antibodies recognizing HNE modified BSA were able to detect HNE-protein adducts in peroxidized liver microsomes. Two of these antibodies were selected and characterized. Both antibodies could also detect HNE-protein adducts in oxidized low density lipoprotein. They exhibit no detectable cross reaction with proteins modified by malonaldehyde, nonanal, nonenal and 4-hydroxyhexenal. Protein bound 4-hydroxyoctenal and 4-hydroxydecenal were recognized to some extent. Further characterization revealed that the two antibodies are highly selective for HNE bound to histidine with only some cross reaction to HNE bound to lysine and cysteine. Preliminary quantitative ELISA-analysis showed that oxidized microsomes and oxidized LDL contain 12 nmol and 3 nmol HNE-histidine per mg protein respectively.

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; EDTA, ethylene-diamine-tetra-acetic acid; HNE, 4-hydroxynonenal; HNE-BSA HNE, modified bovine serum albumin; HNE-KLH HNE, modified keyhole limpet hemocyanin; HNE-His, HNE-αN-acetyl-histidine adduct; KLH, keyhole limpet hemocyanin; LDL, low density lipoprotein; MDA, malonaldehyde; PBS, phosphate buffered saline; TBS, tris buffered saline; TTBS, tween tris buffered saline

INTRODUCTION

Lipid peroxidation inevitably gives rise to a wide range of aldehydes.[1,2] 4-Hydroxyalkenals and in particular 4-hydroxynonenal (HNE) are the most significant products, because they are produced in relatively large amounts and are very reactive.[1,3] The large body of information (reviewed in[1,3,4]) concerning cytotoxic, cytostatic, genotoxic and other biological properties of HNE results to a large extent from investigations where whole cells, cell organelles or isolated proteins were treated with synthetic HNE. The question whether and to what extent these observations can be extrapolated to the situation of HNE generated in situ by peroxidizing membrane lipids has so far received little attention. To demonstrate convincingly the causal involvement of HNE and other aldehydes in the lipid peroxidation

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pathology, conclusive evidence for aldehyde modified proteins in peroxidized biological samples is of crucial importance. A highly promising approach for this is antibodies recognizing aldehydes which react with and become covalently linked to amino acid side chains of proteins.

Antisera raised against malonaldehyde allowed the immunochemical detection of malonaldehyde-epitopes in atherosclerotic lesions[5] and in fibroblasts treated with ascorbate/iron.[6] Antisera to HNE prepared by us and others led to the demonstration of HNE-protein conjugates in rabbit and human atherosclerotic lesions,[7,8] human renal carcinoma cells,[9] hepatocytes of carbon tetrachloride treated rats[10] and renal proximal tubules of iron/nitrilotriacetate treated rats.[11] Using gold-labeled HNE-antibodies as a probe HNE-protein conjugates were recently detected by electron microscopy in the phagosome of human polymorphonuclear neutrophils.[12]

We now report on a series of monoclonal antibodies recognizing HNE in peroxidized microsomes and oxidized low density lipoprotein (LDL). One of them, which has been fully characterized, is highly specific for HNE bound as a Michael adduct to histidine residues. When this work was at the stage of completion and in part presented as an abstract,[13] Uchida's group,[14] following independently the same line of research, reported on an antibody which also seems to be highly specific for HNE bound to histidine residues.

MATERIAL AND METHODS

Chemicals

4-Hydroxyalkenals were synthesized as described.[15] Malonaldehyde (MDA) was prepared from tetramethoxy-propane (Merck, Darmstadt, Germany) by saponification with sulfuric acid.[16] Nonanal and 2-nonenal were obtained from Aldrich (Vienna, Austria). Bovine serum albumin, fraction V (BSA), keyhole limpet hemocyanin (KLH) and N-acetyl-L-amino acids were purchased from Sigma (Vienna, Austria). All other chemicals and buffers were obtained either from Merck, from Sigma or Fluka (Vienna, Austria).

The phosphate buffered saline (PBS) pH 7.4 used in this study contained per liter: 1.43 g $Na_2HPO_4 \times 2\ H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl and 8 g NaCl.

Preparation of aldehyde conjugates

Solutions of the aldehydes, BSA and KLH were prepared in PBS. The aldehyde solution was mixed with the protein solution to give final concentrations of 1 mg protein/ml and 2 mM aldehyde. After 6 hours reaction time at 25°C excess aldehyde was removed by gel filtration using Biorad PD-10 columns.

The column effluent containing the aldehyde-BSA and aldehyde-KLH conjugate was used freshly or stored at –70°C until use. N-acetyl-L-cysteine, αN-acetyl-L-lysine and αN-acetyl-L-histidine (20 mM each) were reacted with 10 mM HNE in PBS for 12 hours at 37°C. As judged by the UV-spectra no free HNE was detectable after incubation. The reaction mixture was stored at –70°C until use. In addition, the HNE-αN-acetyl-histidine reaction mixture (HNE-His) was separated by HPLC on a ODS-2 column (5 μm, 4 × 250 mm) with methanol/10 mM phosphate buffer, pH 7.4 (25:75) at a flow rate of 1 ml/min and detection by a refractive index detector (Erma ERC 7512). Peak fractions (0.5 ml) were evaporated on a vacuum concentrator and resolubilized in 0.5 ml H_2O for performing competitive enzyme linked immunosorbent assay (ELISA). In preparative HPLC, a volume of 0.6 ml of the HNE-His reaction mixture was separated on an Ultrasphere ODS column (5 μm, 10 × 250 mm) with methanol/10 mM phosphate buffer pH 7.4 (35:65).

Immunization, fusion and screening

Female Balb-c mice were immunized according to standard protocols[17] with 25 μg HNE-KLH using complete Freund's adjuvant for the primary

immunization (day 0) and boosted with 25 μg HNE-KLH with incomplete Freund's adjuvant at day 7, 21 and 28. Starting at day 32 after primary immunization B-cell formation was stimulated by intravenous injection of 5 μg HNE-KLH without adjuvant on four consecutive days. At day 36 one mouse was sacrificed and the spleen removed to isolate activated B-cells. These cells were fused with myeloma cell line Sp2-Ag8 (American Type Culture Collection) using polyethylene-glycol. The immortalized B-cells were selected in hypoxanthine-aminopterin and thymidine containing medium.

Screening of clones was performed by indirect ELISA using HNE-BSA coated plates. After subcloning to assure monoclonality, positive clones showing good growth were cryopreserved. Production of monoclonal antibodies was performed by cultivating the clones in DMEM medium in the presence of 2–5% fetal calf serum. The tissue culture supernatant was used for the immuno assays as outlined below.

Indirect ELISA

Maxisorp 96 well plates (Nunc, Roskilde, Denmark) were coated with HNE-BSA in PBS at 4°C for 16 hours (100 μl/well, 1 μg/ml). After four washes with ELISA-wash (PBS plus 21.2 g NaCl/Liter plus 0.5 ml Tween 20/Liter) unspecific binding was blocked by incubation with PBS containing 1% BSA (ELISA-diluent, 200 μl/well). 200 μl of tissue culture supernatants diluted 1:5 to 1:78125 in ELISA-diluent were applied. After 2 hours incubation at 37°C the bound antibody was detected by horse-radish peroxidase conjugated goat anti-mouse IgG (AG181P Chemicon, Temecula, California) and tetramethylbenzidine/H_2O_2 as substrate. Absorbance was read at 450 nm using a microtiter plate-reader.

Competitive ELISA

The working dilution of the antibody (hybridoma supernatant) used in the competitive assay was established to give 25 to 50% of the maximal 450 nm absorbance judged by indirect ELISA. The antibody at this dilution was mixed with various amounts of competitors dissolved in ELISA-diluent and applied to Maxisorp 96 well plates coated and blocked as described above. Further incubation and detection of bound antibody was performed as described for the indirect ELISA. Binding is expressed as the ratio B/B_0, where B and B_0 are the 450 nm absorbance in the presence and absence of the competitor.

Dot-immunobinding test

The tests were performed with the dot-blot apparatus from Bio-Rad (Vienna, Austria) using 0.2 μm nitrocellulose membranes (Hoefer, Vienna, Austria). The membrane moistened with TBS (2.42 g Tris + 8.77 g NaCl per Liter, pH 7.5) and TTBS (TBS + 0.5 ml Tween 20/Liter) was fitted to the dot-blot apparatus and washed with TBS. Volumes of 100 μl microsomal suspension (100 μg protein/ml) were applied per dot. After 45 minutes, the membrane was washed with TBS and blocking solution (TBS + 1% BSA) followed by 15 minute blocking of endogenous peroxidase (7 ml blocking solution + 2.5 ml 30% H_2O_2 + 0.5 ml 10% sodium azide). The hybridoma supernatants were then added in dilutions of 1:10, 1:100 and 1:1000 and incubated for 60 minutes. After two washes, binding of the antibodies was determined with peroxidase labeled detection antibody in principle as described above for indirect ELISA. As control for complete blocking of endogenous peroxidase, we used dots to which no detection antibody was applied.

Preparation of oxidized microsomes and LDL

Rat liver microsomes prepared as described[18] were incubated at 37°C in oxygen saturated 25 mM Tris buffer pH 7.4, 90 mM KCl by shaking with 0.5 mM ascorbate plus 0.02 mM $FeSO_4$ for 2 hours. The malonaldehyde determined colorimetrically as TBARS[16] after 15, 30, 60 and 120

minutes gave typical values of 45, 70, 70 and 70 nmol/mg protein. The concentration of free HNE was 2 nmol/mg protein after 120 minutes incubation. HNE was detected by HPLC with detection at 223 nm as previously described.[16] Samples (0.5 ml) of the 2 hours microsomal reaction mixture were stored at –70°C for up to 45 days until use for dot-blot assays. One sample was assayed after 12 hours storage at 4°C. For the dot-blots the microsomal suspension was thawed at 37°C and diluted 10 fold with TBS.

Human plasma LDL samples (3 independent preparations) prepared as described[19] were oxidized with Cu^{2+}ions. In brief, LDL (50 μg protein/ml) in PBS was incubated with 1.66 μM $CuCl_2$ at 25°C. The oxidation kinetics followed by the conjugated diene method[19] showed the typical profiles with a lag-phase of 60 to 90 minutes and diene peaks at 150–240 minutes depending on LDL. After 6 hours oxidation was stopped by addition of EDTA (1 mg/ml) and the sample was concentrated by ultrafiltration (cut-off 10.000 Da) to 800 μg protein/ml. Aliquots of this concentrated sample were stored at –70°C until use for ELISA assays.

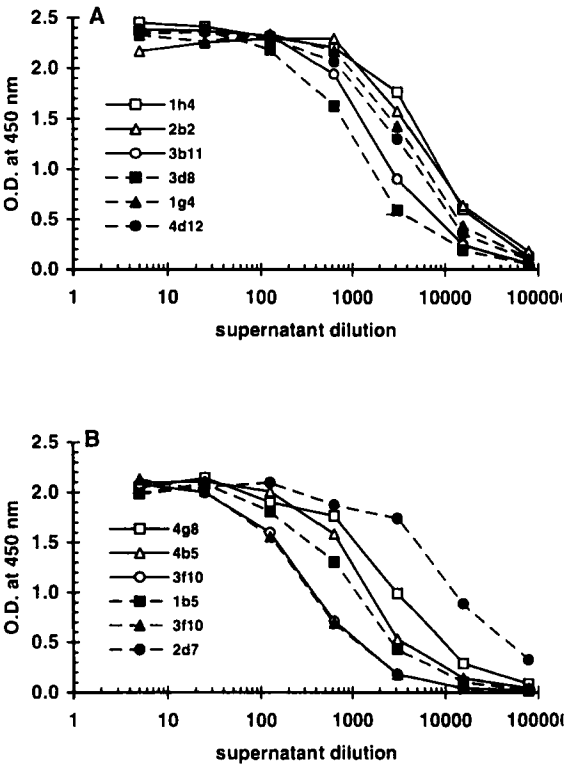

FIGURE 1 Determination of titers of hybridoma supernatants by indirect ELISA with HNE-BSA. Microtiter plates were coated with HNE-BSA (1 μg protein/ml). Serial dilutions (1:5 to 1:78125) of the hybridoma supernatants were added and incubated for 2 hours at 37°C. The bound antibodies were detected with peroxidase labeled goat anti-mouse IgG and tetramethylbenzidine/H_2O_2. The absorbance (O.D.) at 450 nm was measured with a microtiter plate reader. Each curve represents one clone, the code numbers for the clones are given next to the symbols. The antibody of clone 1g4 (▲ in Figure 1A) was fully characterized.

RESULTS

Screening of hybridomas with HNE-BSA conjugate and peroxidized microsomes

Hybridomas were prepared by fusion of myeloma cells with spleen cells of mice immunized with HNE-KLH. Initial screening of hybridoma supernatants by indirect ELISA on plates coated with HNE-BSA revealed about 800 positive colonies. Forty-eight colonies with relatively high color signal and good growth were selected and cryopreserved. They were subcloned by repeated limiting dilution to assure monoclonality and their antibody titers were determined by serial dilutions of the supernatants (Figure 1). In total, 20 of the clones had titers (i.e. supernatant-dilution giving half maximal 450 nm absorbance) between 500 to 2000. The remaining 28 clones had lower titers and were discarded. Plates coated with BSA only served as negative control and none of the antibodies showed a binding to BSA. The intra-assay variation for titration curves measured in parallel on the same plates was less than 2% (Figure 1A, clone 3f10), the inter-assay variation on different plates and different days was in the range of 5 to 10%.

Having established a number of clones with good titers towards HNE-BSA, the next stage of screening aimed to reveal clones which additionally are able to detect HNE-protein conjugates in

rat liver microsomes oxidized with ascorbate/iron. Indirect ELISA on plates coated with the oxidized microsomes and antibody dilutions of 1:10 gave with few exceptions only a weak 450 nm absorbance, presumably because of the relatively low binding capacity of the plates. We therefore used for this type of screening dot-blot assays with nitrocellulose membranes to which 10 μg microsomal protein per dot was applied. The antibodies were used in dilutions up to 1:1000. Among the twenty HNE-BSA positive clones there were seven which showed no reactivity, seven clones showed weak reactivity at dilution of 1:10 and 1:100. Six clones (1g4, 1h4, 1b5, 4b5, 4d12, 2d7) were highly positive and produced strongly colored dots with a 1000 fold supernatant dilution. These clones, being superior regarding recognition of HNE in peroxidized microsomes, were selected for further characterization. All HNE-BSA positive clones were also examined for possible binding to native microsomes which were protected against oxidation with EDTA, (1 mg/ml final concentration) and butylated hydroxytoluene (10 μM final concentration). No or only a very weak reaction was observed with a 10 fold antibody dilution, higher dilutions were negative for all antibodies. Artefactual color formation owing to endogenous peroxidase activity of microsomes was prevented by a blocking step with sodium azide plus H_2O_2 prior to addition of the peroxidase-labeled detection antibody. Dots where the detection antibody was omitted were not colored, indicating that endogenous peroxidases were completely blocked.

Determination of cross reactivity and aldehyde specificity

The six antibodies showing high reactivity with both HNE-BSA as well as oxidized microsomes were investigated by indirect ELISA with regard to cross reaction with aldehydes other than HNE. Figure 2 shows as a typical example titration curves of the antibodies 1g4 and 1h4 respectively using plates coated with various aldehyde-BSA

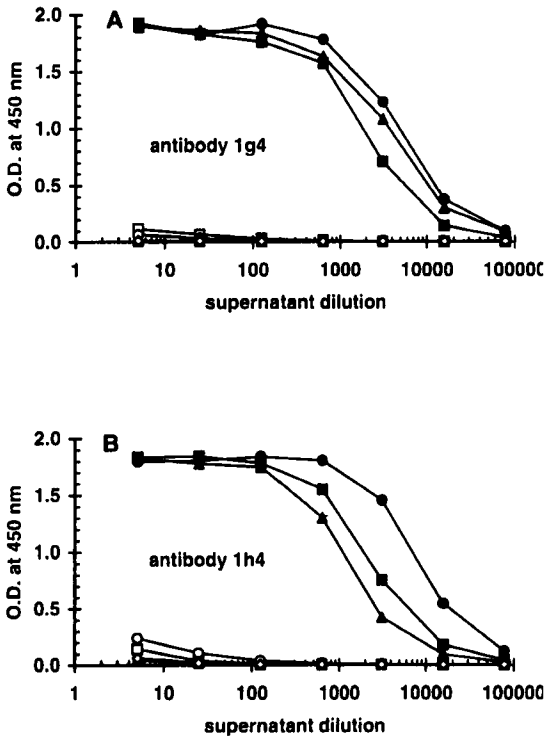

FIGURE 2 Cross reactivity of monoclonal antibody 1g4 (A) and 1h4 (B) with various aldehyde-BSA conjugates judged by indirect ELISA. The aldehyde-BSA conjugates (1 μg protein/ml) were coated onto microtiter plates. Serial dilutions of the hybridoma supernatants (1:5 to 1:78125) were added and the bound antibody was detected as in Figure 1. The aldehyde conjugates were prepared as described in Methods by reacting BSA with 4-hydroxynonenal (●), 4-hydroxyoctenal (▲), 4-hydroxydecenal (■), 4-hydroxyhexenal (□), 2-nonenal (○), nonanal (△) and malonaldehyde (◊).

conjugates. The antibodies did not show any cross reaction with 2-nonenal, nonanal, malonaldehyde and 4-hydroxyhexenal. The latter aldehyde is a homologue of HNE with three CH_2-groups less. On the other hand, both antibodies strongly cross reacted with 4-hydroxyoctenal and 4-hydroxydecenal, which differ from HNE by only one CH_2-group each. Titration curves similar as those shown in Figure 2 were obtained with the other four antibodies. The cross reaction of all six antibodies expressed as titer, is summarized in Table 1. In addition to indirect ELISA, the specificity of the antibodies 1g4 and 1h4 was elucidated by competitive ELISA assays on plates coated

TABLE 1 Titers of hybridoma supernatants to various aldehyde-BSA conjugates as judged by indirect ELISA. Microtiter plates were coated with aldehyde-BSA conjugated as described in Methods. Serial dilutions of the hybridoma supernatant (1:5 to 1:78125) were added and the bound antibodies were detected as described in Figure 1. The titers are the dilutions giving 50% of the maximal absorbance at 450 nm i.e. about 1.0 to 1.2 AU. Mean and standard deviation (s.d.) of four experiments are given. (n.d. = not determined)

	Titers of antibodies[1]						
Antibody code	4-Hydroxynonenal-BSA mean	s.d.	4-Hydroxyoctenal-BSA mean	s.d.	4-Hydroxydecenal-BSA mean	s.d.	Other aldehydes-BSA[2]
1g4	5 200	300	4000	320	2000	120	<5
1h4	9 000	540	1500	120	2500	230	<5
1b5	1 000	80	250	20	70	n.d.	<5
4b5	400	35	200	20	150	12	<5
4d12	300	28	200	18	100	9	<5
2d7	10 000	570	6000	390	6000	470	<5

[1]Hybridoma supernatant dilution giving half-maximal absorbance.
[2]4-hydroxyhexenal-BSA, 2-nonenal-BSA, nonanal-BSA, malonaldehyde-BSA.

with HNE-BSA. The strongest competitor was HNE-BSA, followed by the 4-hydroxydecenal-BSA and 4-hydroxyoctenal-BSA conjugates. No competition was obtained even at the highest concentration of 0.25 mg protein/ml with the BSA conjugates of 4-hydroxyhexenal, 2-nonenal, nonanal and malonaldehyde. The results of the competitive assays are fully consistent with the indirect ELISA and confirm that the antibodies preferentially bind to HNE-conjugates with some cross reactivity to 4-hydroxyoctenal and 4-hydroxydecenal, but no cross reactivity to other aldehydes.

Characterization of the HNE-epitopes recognized by the 1g4 and 1h4 antibodies

The reaction of HNE with proteins can give rise to conjugates where the aldehyde is bound to the side chains of cysteine, lysine and histidine residues. To elucidate the preferred conjugates, the antibodies were examined by competitive ELISA using low molecular-mass competitors as probes. The competitors were prepared by reaction of HNE with αN-acetyl-histidine, αN-acetyl-lysine and N-acetyl-cysteine. The competition studies demonstrated that the preferred epitope is HNE bound to histidine (Figures 3, 4). Both antibodies displayed a weak cross reaction with the HNE-lysine conjugate and to a lesser extent with the HNE-cysteine conjugate. Neither the parent N-acetyl-amino acids nor free HNE showed a noteworthy competition at the highest tested concentration of 40 μM. Regarding affinity and specificity, the antibody 1g4 appears superior to antibody 1h4. First, the 1g4 antibody possesses an approximately 40 fold higher affinity to the HNE-histidine conjugate as a 50% competition was obtained at a concentration of 0.1 μM (Figure 3),

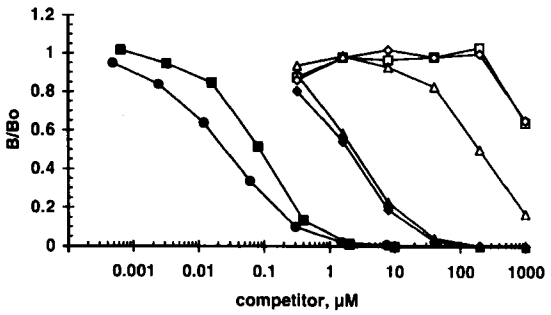

FIGURE 3 Characterization of the HNE-epitope recognized by the antibody 1g4 judged by competitive ELISA with various competitors. Microtiter plates were coated with HNE-BSA (1 μg protein/ml). A fixed, appropriate dilution of 1g4 antibody was mixed with various concentrations of competitors (final concentrations 1 nM to 1 mM) and added. The bound 1g4 antibody was detected as in Figure 1. B_0 and B are the absorbances at 450 nm in absence (B_0) and presence (B) of competitor. The competitors were prepared as described in Methods. HNE-BSA (●), HNE-His (■), HNE-Lys (♦), HNE-Cys (▲), αN-acetyl-histidine (□), αN-acetyl-lysine (◊) and N-acetyl-cysteine (△).

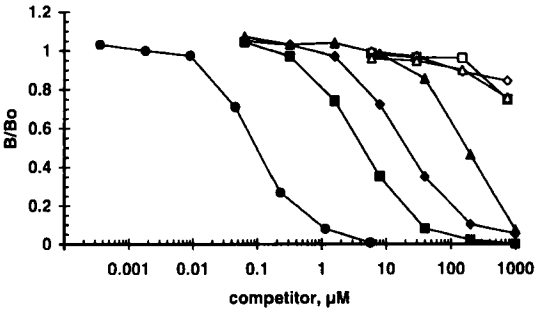

FIGURE 4 Characterization of the HNE-epitope recognized by the antibody 1h4 judged by competitive ELISA with various competitors. Experimental design and symbols as in Figure 3.

while a concentration of 4 μM was required in case of antibody 1h4 (Figure 4). Second, the 1g4 antibody is highly specific for the HNE-histidine epitope showing less than 5% cross reaction with the respective lysine and cysteine conjugate (Table 2). Subtype determination revealed that 1g4 antibody belongs to subtype IgG1κ. Regarding the precise chemical structure which is recognized by the 1g4 antibody we refer to the work of Tsai *et al.* (20) who clearly demonstrated that the reaction product of HNE with αN-acetyl-histidine is a Michael-adduct with a cyclic hemiacetal structure

TABLE 2 The HNE-His is the preferred epitope recognized by the antibody 1g4 and 1h4. For the indicated competitors the concentration (μM) giving $B/B_0 = 0.5$ was determined by competitive ELISA as shown in Figures 3, 4. The percent cross reaction is expressed relative to HNE-His (= 100%). The 1g4 antibody gave with HNE-His and HNE-Lys $B/B_0 = 0.5$ at 0.1 and 2 μM respectively, this gives a cross reaction with HNE-Lys of: $100 \times 1.5/2 = 5\%$.

	% Cross reaction	
Competitor	1g4	1h4
HNE-His[1]	100	100
HNE-Lys[1]	5	20
HNE-Cys[1]	4	2
free HNE	<0.05	<0.05
αN-acetyl-histidine	<0.05	<0.05
αN-acetyl-lysine	<0.05	<0.05
N-acetyl-cysteine	<0.05	<0.05

[1]Chemical structure is shown in Figure 5

of the parent aldehyde (Figure 5). Separation of our HNE-histidine conjugate by HPLC revealed four peaks with nearly equal area suggesting that the product consists of four compounds. It is reasonable to assume that they represent diastereomers as they are expected to be formed by the reaction of αN-acetyl-L-histidine with the racemic HNE. Investigation of the four HPLC-separable

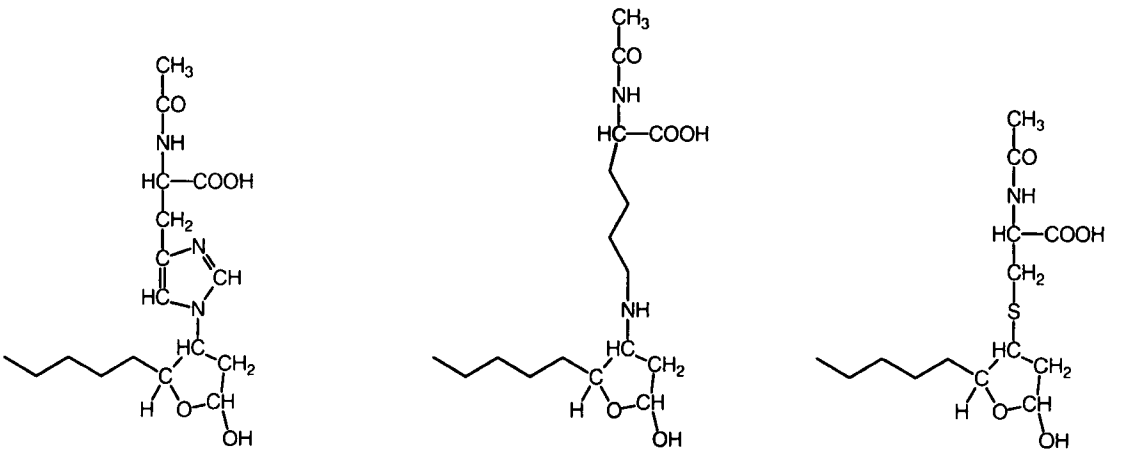

FIGURE 5 Suggested structures of the products formed by the reaction of HNE with αN-acetyl-histidine (A), αN-acetyl-lysine (B) and N-acetyl-cysteine (C). The products are formed by nucleophilic addition of the imidazole ring nitrogen (A), the epsilon amino nitrogen (B) and the sulfur (C) to the carbon atom 3 of HNE. The open chain form of the Michael adduct cyclizes to the cyclic hemiacetal. Note that the furanoid-ring of the hemiacetal contains 3 asymmetric carbon atoms i.e. C_1, C_3 and C_4.

compounds by competitive ELISA on HNE-BSA coated plates revealed that about 80% of the immunoreactivity was contained in peak no. 4, i.e. the last eluting peak. The exact stereochemistry of this compound is currently under investigation.

Binding of the 1g4 Antibody to Oxidized LDL and Microsomes Judged by the Competitive ELISA.

To compare the binding of the 1g4 antibody to oxidized LDL and microsomes competitive ELISAs were performed with HNE-BSA coated plates (Figure 6). Compared to HNE-BSA, which served as positive control and competed 50% at a concentration of 2 μg protein/ml, oxidized microsomes and oxidized LDL gave 50% competition at concentrations of 12 and 55 μg protein/ml. Native microsomes and LDL did not or only minimally compete at the highest tested concentration of up to 500 μg/ml. Antibody 4d12 (see Table 1) showed a similar preference for oxidized LDL which exhibited in the same assay a 50% competition by oxidized LDL at 40 μg protein/ml. Two other antibodies (clones 2b2 and 1h4) examined by this assay showed only weak binding to oxidized LDL with 50% competition at the relatively large dose of about 250 μg protein/ml and no pronounced difference to native LDL.

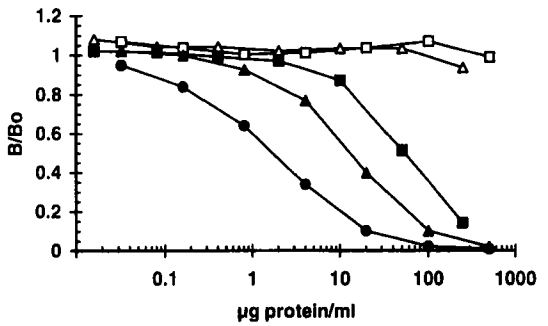

FIGURE 6 The antibody 1g4 binds to oxidized LDL and microsomes as judged by competitive ELISA. Microtiter plates were coated with HNE-BSA (1 μg protein/ml). A fixed appropriate dilution of 1g4 antibody was mixed with the competitor and added. The bound 1g4 antibody was detected as in Figure 1. B_0 and B are the 450 nm absorbances measured in absence and presence of competitor. The competitors were: HNE-BSA (●), microsomes oxidized by ascorbate/iron (▲), LDL oxidized by Cu^{2+} ions (■), native microsomes (△) and native LDL (□).

DISCUSSION

The reaction of lipid derived aldehydes with proteins is implicated in causing part of the damage associated with lipid peroxidation.[1,3,4] Measurement of increase in total protein carbonyls gives a global marker for protein modification, but does not discriminate between lipid-aldehydes and carbonyls resulting from direct oxidation of amino acid residues.[21] Uchida and Stadtman[22] have developed a procedure for detection of HNE-histidine and HNE-lysine conjugates in proteins by amino acid analyses. Immunological studies using antibodies as a probe for malonaldehyde- or HNE-modified proteins are a significant development in identification and localization of proteins which became altered by these aldehydes during the course of a lipid peroxidation process. Several previous studies employed antisera, i.e. polyclonal antibodies, to demonstrate malonaldehyde- and HNE- conjugates, most likely resulting from apolipoprotein B, in atherosclerotic lesions.[5,7,8] Antisera have also enabled the detection of HNE-conjugates in oxidatively stressed hepatocytes,[10,23] fibroblasts,[6] renal proximal-tubule[11] and polymorphonuclear neutrophils.[12]

Binding of HNE to proteins can give rise to multiple epitopes including Schiff's bases, cross links and Michael adducts with cysteine, lysine, and histidine side chains.[1,22,24,25] Polyclonal antibodies, even if purified by affinity chromatography, exhibit a relatively broad specificity and are therefore of limited value for unequivocal characterization of HNE-positive epitopes in complex biological samples, such as cells, subcellular fractions or lipoproteins. We have produced in this study a series of monoclonal antibodies recognizing HNE modified BSA (HNE-BSA). One of them, antibody 1g4, proved to be highly selective for HNE bound to histidine (HNE-His) as its cross

reaction with HNE bound to lysine or cysteine was negligible. Apart from that, antibody 1g4 does not cross react with epitopes produced by 2-nonenal, nonanal or malonaldehyde. The reaction of HNE with histidine is a Michael type nucleophilic addition reaction,[20,22,23] where the imidazole ring nitrogen atom adds to the α,β-unsaturated double bond of HNE, yielding a 4-hydroxynonanal derivative with the imidazole ring attached to carbon atom 3 of the aldehyde. Owing to the hydroxy-group the primary adduct forms a cyclic hemiacetal with a five membered furanose ring (Figure 5A). Similar chemical reactions occur with lysine[24,25] and cysteine[1] and the final products differ only in the linkage of the amino acid to the aldehyde (Figure 5B, C). The selectivity of the antibody 1g4 for HNE-His suggests that the recognized epitope includes at least a part of the imidazole ring, the furanose ring and the tail of the parent HNE, i.e. CH_3-$(CH_2)_4$-. The furanose ring appears to be essential, as the 2-nonenal adduct, which has a similar chemical structure but does not give a cyclic hemiacetal, is not recognized. Elongation or shortening of the tail by one CH_2-group only, i.e. 4-hydroxyoctenal, 4-hydroxydecenal had little influence on binding of the 1g4 antibody. However, shortening the tail by three CH_2-groups, i.e. 4-hydroxyhexenal completely abolished the binding of the antibody. HNE results from peroxidation of ω-6 polyunsaturated fatty acids while 4-hydroxyhexenal is formed from ω-3 poly-unsaturated fatty acids.[1] As membranes contain both types of fatty acids the two 4-hydroxyalkenals are commonly formed simultaneously, although HNE is usually in excess owing to the larger proportion of ω-6 fatty acids. The high specificity of the 1g4 antibody allows the selective immunochemical identification of proteins modified by HNE without interference by 4-hydroxyhexenal modifications. The cross-reactivity with 4-hydroxydecenal and 4-hydroxyoctenal does not limit the usefulness of the antibody, as these two aldehydes are only formed in minute amounts.[1]

The stereochemistry of the HNE-His adducts (Figure 5A) is rather complex owing to the four chiral centers, i.e. one at the histidine moiety and three at the furanose ring. We were able to separate the product by HPLC into four components which are present in approximately equal amounts; they likely represent different diastereomers. As judged by competitive ELISA, the antibody 1g4 recognized preferentially one of these putative diastereomers, its precise structure is currently investigated by NMR. Uchida's group[14] has recently also reported on the development of a monoclonal antibody (code mAb HNE J-2) with a relatively high specificity for HNE-histidine conjugates as judged by indirect and competitive ELISA. The usefulness of this antibody for selective detection of HNE-His in complex biological samples is, however, uncertain, because only model compounds (HNE-modified glyceraldehyde-3-phosphate dehydrogenase, HNE-BSA and HNE-amino acids) were tested as antigens. The current study demonstrates that our antibody 1g4 is able to detect HNE-His epitopes in peroxidized rat liver microsomes and oxidized LDL. A striking result of our screening was that only a few (6 out of 20) of the antibodies, showing a strong binding to HNE-BSA, also recognized peroxidized microsomes. This suggests that HNE generated in situ in a complex sample such as microsomes, produces a more specific product pattern than the reaction of relatively highly concentrated synthetic HNE with isolated model proteins. This also demonstrates that screening of antibodies as probes for in situ peroxidatively modified proteins should not be limited to model antigens but include putative biological antigens.

The question of the number of histidine residues modified by HNE and recognized by the 1g4 antibody has not been investigated in detail in this study. Based on the HNE-His standard yet not considering the stereoisomer distribution it can be estimated from the competition curves (Figure 3) that HNE-treated bovine serum albumin (HNE-BSA) contained about four moles HNE-His epitopes per mole (= 60 nmol/mg protein), this number is close to the 6.1 moles HNE-histidines

found by Uchida and Stadtman[22] by amino acid analyses after adduct stabilization with sodium borohydride. As BSA contains 13 histidine residues/mol in total, this suggests that only a fraction of them is HNE-reactive. Similar estimations based on competition curves (Figure 6) gave about 12 nmol and 3 nmol HNE-His per mg protein for ascorbate/iron oxidized microsomes and Cu^{2+} oxidized LDL. These values are again in good agreement with the 2.5 to 38 nmol aldehydes per mg microsomal protein found by Benedetti *et al.*[26] employing the dinitrophenyl hydrazone method and the 7 to 9 mol HNE-His per mol LDL (1.4 to 1.6 nmol per mg protein) found by Uchida *et al.*[27] using a polyclonal antibody.

The assessment of lipid peroxidation in tissue and single cells has been difficult due to the lack of sensitive and selective probes. We believe that well characterized antibodies such as the antibody 1g4 are useful probes with a great promise for lipid peroxidation research.

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