Generation and initial characterization of a novel polyclonal antibody directed against homocysteine thiolactone-modified low density lipoprotein

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Abstract Elevated plasma homocysteine (homocysteinemia) are presumed to be responsible for the development of coronary artery disease, however, the precise etiology is unclear. We examined the possibility that the adduct formed from the reaction between homocysteine thiolactone, a metabolic product of homocysteine, and apolipoprotein B-100 lysyl residues of low density lipoprotein (LDL) was immunogenic. New Zealand White rabbits were immunized with this adduct at 6-week intervals. Antisera collected following the 3rd immunization was assayed for antibody titers using solid phase ELISA techniques. Titers (defined as the inverse of the greatest serum dilution in which there was a significant difference ($P < 0.05$) between the percentage antibody bound from the antiserum and the pre-immune serum) were approximately 10^5. In competition-based ELISAs, homocysteine thiolactone-treated LDL competed for binding with the antiserum, as the 50% inhibitory concentration was approximately 10 μg/ml. Neither homocysteine, homocystine (homocysteine disulfide), nor Cu^{2+}-oxidized LDL competed for binding. LDL in which lysyl residues were derivatized by acetylation or methylation were not recognized by the antiserum. Homocysteine thiolactone-treated plasma competed for binding to the antiserum, whereas native plasma did not. All lipoprotein fractions from the homocysteine thiolactone-treated plasma competed for binding to the antiserum.▪ We conclude that homocysteine thiolactone-modified LDL is highly immunogenic and specific for homocysteine thiolactone-modified lysines. The potential for using this antibody as a diagnostic tool for measuring plasma homocysteine concentrations and the implications for understanding diseases induced by homocysteinemia are discussed.—Ferguson, E., S. Parthasarathy, J. Joseph, and B. Kalyanaraman. Generation and initial characterization of a novel polyclonal antibody directed against homocysteine thiolactone-modified low density lipoprotein. *J. Lipid Res.* 1998. **39:** 925–933.
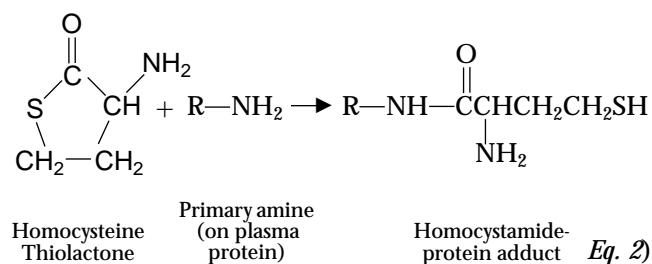
Supplementary key words homocysteinemia • atherosclerosis • apolipoprotein B-100 • lysyl residues • measurement of plasma homocystamide-protein adducts • enzyme-linked immunoadsorbent assay

Elevated levels of plasma homocysteine (homocysteinemia) are present in several pathologies including coronary artery disease (1–3). The cyclic thioester, homocysteine thiolactone, has been implicated as a compound that may be formed in conditions of homocysteinemia (equation 1):

$$\text{Eq. 1)}$$
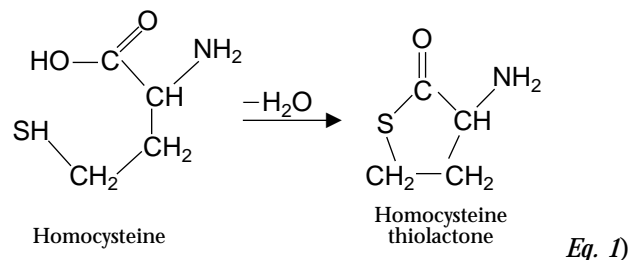
Homocysteine thiolactone has been shown to react with primary amines by forming an amide linkage (equation 2, ref. 4):

$$\text{Eq. 2)}$$

It has been reported that homocysteine thiolactone is capable of modifying LDL to a form that is capable of generating foam cells (5). While there is evidence that indicates homocysteine thiolactone may be involved in atherosclerosis and thromboembolic disorders, a method for the detection of the homocystamide adduct has been lacking.

Autoantibodies that recognize oxidized LDL, malondialdehyde-lysyl-LDL adduct or 4-hydroxynoneal-lysyl-LDL adduct have been found to occur in vivo (6). In addition, derivitization of albumin, fibrinogen, or LDL lysyl residues by carbamylation, acetylation, ethylation, and methylation has been shown to result in high affinity antibodies directed against these modifications in experimental animals. These studies show that LDL may be a good carrier molecule for the immunization of animals (7). It has been suggested that analogous biochemistry between homocysteine thiolactone and LDL, resulting in homocystamide-LDL adducts, could result in the formation of autoantibodies against these adducts (equation 3) (8):

Homocysteine Thiolactone   Low density lipoprotein   Homocystamide-LDL adduct   *Eq. 3)*
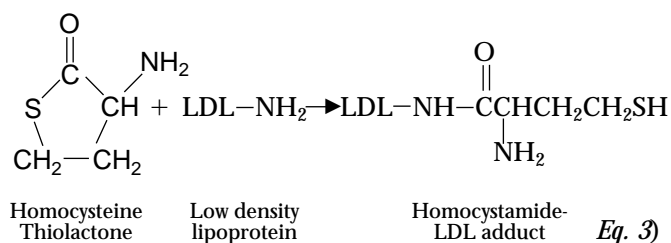
In this study, we assessed for the first time the possibility that the homocystamide-LDL adduct was immunogenic. We have raised in rabbits a polyclonal antibody directed against the homocystamide-LDL adduct. In this report, we have characterized the specificity of this polyclonal antiserum for the recognition of homocystamide-protein adducts.

MATERIALS AND METHODS

Materials

New Zealand White rabbits were supplied by New Franken Research Rabbits (New Franken, WI). Multiscreen plates (96-well) were obtained from Millipore (Bedford, MA). Horseradish peroxidase-labeled goat anti-rabbit IgG, 3,3′-5,5′-tetramethyl benzidine substrate solution (TMB), and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were supplied by Pierce (Rockford, IL). Freund's adjuvant was obtained from Gibco BRL (Grand Island, NY). Dithio-bis-2-nitrobenzoic acid (DTNB), potassium bromide, Folin and Ciocalteu's Phenol Reagent,

guanidine hydrochloride, sodium dodecyl sulfate (SDS), Sephadex G-25, polyoxyethylenesorbitan monolaurate (Tween-20), ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), sodium cyanoborohydride, materials for all phosphate-buffered saline (PBS, sodium phosphate (25 mm), sodium chloride (125 mm), pH 7.4), homocysteine thiolactone hydrochloride, and homocysteine were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium nitrite, sodium tartrate, sodium hydroxide, cupric sulfate, diethylenetriaminepentaacetic acid (DTPA), sulfuric acid, and methylene chloride were purchased from Fisher Scientific (Itasca, IL). Chelex-100 was obtained from Bio-Rad Laboratories (Hercules, CA). Ethanol was purchased from Quantum Chemical Corp. (Tuscola, IL). Formaldehyde and acetic anhydride were purchased from Aldrich Chemical Co. (Milwaukee, WI).

LDL, high density lipoprotein (1.063–1.21 g/ml, HDL), and lipoprotein-deficient serum proteins (>1.21 g/ml, LPDS) were isolated from plasma by sequential ultracentrifugation through a potassium bromide gradient (9). LDL protein concentrations were measured by the assay of Lowry et al. (10). The freshly prepared LDL was stored under argon at 4°C in PBS containing EDTA (1 mm) and was used within 2 weeks of preparation. The free base of homocysteine thiolactone was prepared from the hydrochloride salt. Typically, 100–500 mg (~1–5 mmol) of the hydrochloride salt was added to a separating funnel containing 2 ml NaOH (1.4 N) and 3 ml methylene chloride. The solution was shaken vigorously for 1 min and the bottom organic layer was separated and dried over anhydrous sodium sulfate. This solution was evaporated under a steady stream of nitrogen until a clear, colorless oil was obtained.

Protein modification

Acetylation of LDL was performed as described elsewhere (7). This modification was assessed qualitatively by agarose gel electrophoresis using a Paragon Lipogel electrophoresis apparatus (11). LDL (5 μg) was loaded onto the gel and electrophoresed for 30 min at 150 V. The mobility of native LDL was compared to acetylated LDL. Reductive methylation was done as described previously (7), and the extent of methylation was measured using the TNBS assay (12) with an extinction coefficient ($\epsilon = 3,800$ m^1 cm$^{-1)}$) determined from a standard curve using valine.

Thiobarbituric acid-reactive substances (TBARS) measurement

Aliquots of LDL were collected at the indicated times during Cu^{2+}-mediated oxidation, and BHT (500 μm in ethanol) and EDTA (1 mm) were added in order to

quench the reactions. TBARS were measured by incubating LDL (10–20 μg) with thiobarbituric acid (0.5% w/v in H_2SO_4, 50 mm) for 30 min at 100°C (13). Samples were centrifuged for 5 min, and the difference in absorbance ($A_{532nm}-A_{580nm}$) was determined. Concentrations of TBARS were calculated using an extinction coefficient ($\epsilon = 150,000$ $m^{-1}cm^{-1}$) as previously described (14).

Preparation of homocystamide adducts and immunization of animals

Homocystamide-LDL adduct was prepared by the addition of homologous LDL ($\sim$2–10 mg in phosphate buffer containing 100 μm DTPA) to homocysteine thiolactone free base. The mixture was incubated for 30 min with gentle stirring on ice and passed through a Sephadex G-25 column in order to separate the unreacted homocysteine thiolactone. The percentage of apoB lysyl residues that reacted with homocysteine thiolactone was quantified by measuring the increase in protein thiols using the DTNB assay (15, 16) with an extinction coefficient ($\epsilon = 11,000$ $m^{-1}cm^{-1}$) determined from a standard curve using a reduced cysteine, as the reaction between homocysteine thiolactone and a primary amino group generates a thiol (equation 2). Using these conditions, consistently 30–40% of apoB lysyl residues reacted with homocysteine thiolactone to form homocystamide-LDL adducts (assuming 500,000 molecular weight for apoB and 180 lysyl residues per apoB molecule). The immunogen was prepared as described within 24 h of each of the immunizations. Homocystamide adducts of BSA and of hemoglobin were prepared using the same conditions that were used for the preparation of homocystamide-LDL adduct.

Two 1.8-kg female New Zealand White rabbits were immunized using standard protocols (17) and in accordance with the guidelines of the Medical College of Wisconsin Animal Care Committee. Prior to the first immunization, 10 ml serum was collected from each rabbit to serve as negative control. In an equal volume of complete Freund's adjuvant, 0.5 mg homocystamide LDL adduct was emulsified so that the final volume was $\sim$1 ml. This was injected subcutaneously. Serum was collected 7 days later and was tested for antibody titers. Subsequent immunizations were spaced at 6-week intervals after the injection, but incomplete Freund's adjuvant was used for emulsification of the immunogen.

Solid phase ELISA techniques

Solid phase indirect antibody capture techniques were used in all assays and have been described elsewhere (17). Briefly, Millipore Multiscreen 96-well plates were initially coated with homocystamide-LDL adduct (100 μl, 10 μg/ml). For antibody titer determinations,

the antisera were diluted at concentrations as indicated in the Results section. We defined antibody titers as the inverse of the greatest serum dilution in which a significant difference ($P < 0.05$) between the percentage antibody bound from the antiserum and the pre-immune serum was observed. In order to determine antibody specificity, competition experiments were used, using a limiting dilution (70%) of antiserum. Horseradish peroxidase-labeled goat anti-rabbit IgG was used as a substrate, and the reaction was quenched by the addition of H_2SO_4 (100 μl, 1.8 m). The absorbance was measured at 405 nm in a 96-well plate reader.

RESULTS

Determination of antibody titers

Prior to immunization procedures, pre-immune sera were obtained from two New Zealand White rabbits in order to determine nonspecific binding in future assays. Rabbits were immunized as described in Materials and Methods. Seven days after the 2nd immunization, blood was collected from the animals and serum was obtained. The antiserum and the pre-immune serum from each animal were assayed for antibody titers. Homocysteine thiolactone-treated rabbit LDL and native LDL (10 μg/ml) were coated onto 96-well plates. After blocking with BSA and washing, antiserum (1:10–1:10^6) was added. Antibody binding was measured as described above. Titers from this immunization were approximately 10^5 (titers defined in Materials and Methods section). Binding of the pre-immune serum was nonexistent. Human LDL was used in the remaining assays reported here, as titers were the same when using either rabbit LDL or human LDL. The immune responses of both rabbits following the 3rd immunization roughly mirrored the 2nd immunization. As shown in **Fig. 1**, antibody titers 7 days after the 3rd immunization were approximately 10^5 (serum dilution curve shown from one animal only). Maximum binding was reached at an antiserum dilution of 1:100, as the percentage of the total antibody bound was not significantly different ($P < 0.05$) in less dilute samples (Fig. 1).

We sought to determine whether or not homocystamide adducts of other proteins could also be used in order to measure antibody titers. Both hemoglobin and BSA were treated with homocysteine thiolactone, as described in Materials and Methods. Solid-phase ELISA techniques, as described for Fig. 1, were used in order to obtain a serum dilution curve for homocystamide-BSA adduct and for homocystamide-hemoglobin adduct (**Fig. 2**, top and bottom, respectively). This curve
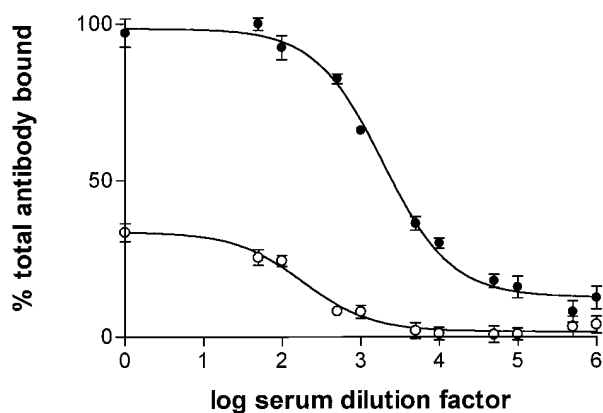
Fig. 1. Antibody dilution curve for antiserum to rabbit homocystamide-LDL adduct. New Zealand White rabbits were initially immunized with homologous homocystamide-LDL adduct (0.5 mg LDL, 30% lysinyl residues reacted with homocysteine thiolactone) emulsified in complete Freund's adjuvant. Later immunizations were spaced at 6-week intervals, but incomplete Freund's adjuvant was used in order to emulsify the LDL. Serum was collected 7 days after the 3rd immunization. Serial dilutions of the antiserum were assayed by solid-phase ELISA techniques in which LDL (100 μl, 10 μg/ml in PBS) was bound to Millipore 96-well plates. Horseradish peroxidase-labeled goat anti-rabbit IgG was used as a secondary antibody, and TMB was used as a substrate; (●) immune serum after the 3rd immunization; (○) pre-immune serum (n = 3 for each data point). Values are the mean ± SEM.

resembled the serum dilution curve obtained in Fig. 1, as the antiserum recognized both homocystamide-BSA and homocystamide-hemoglobin adduct, indicated by antiserum dilution-dependent increase in the absorbance at 450 nm (Fig. 2, top and bottom). Neither native BSA nor native hemoglobin was recognized by the antiserum (Fig. 2, top and bottom, respectively) (n = 2 for each measurement).

Antigenicity of modified LDL lysyl residues

We sought to examine the specificity of the antiserum with respect to various lysine modifications, including the immunization compound, the homocystamide-LDL adduct. Homocysteine thiolactone-treated LDL (10 μg/ml) was coated onto 96-well plates. As shown in **Fig. 3**, after blocking with BSA and washing the plates, homocystamide-LDL adduct (0.01–500 μg/ml) was added to the wells in the presence of a constant limiting dilution (1:700) determined from Fig. 1. As shown in Fig. 3, a dose-dependent inhibition of antiserum binding to the solid phase was observed for the homocystamide-LDL adduct. The concentration of homocystamide-LDL adduct that was required to cause a 50% decrease of % B/B_0 (IC_{50}) (in which B_0 was defined as the total binding in the absence of competitor
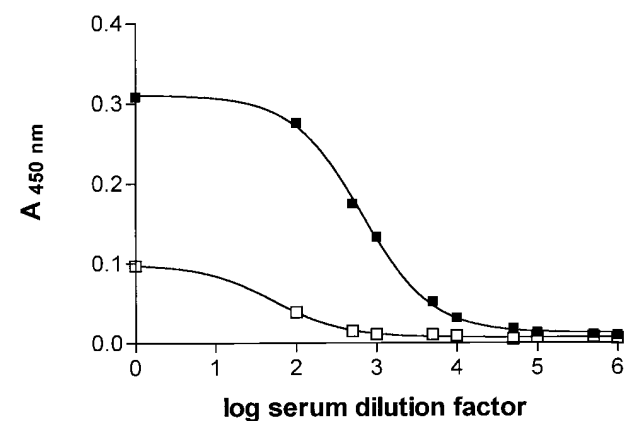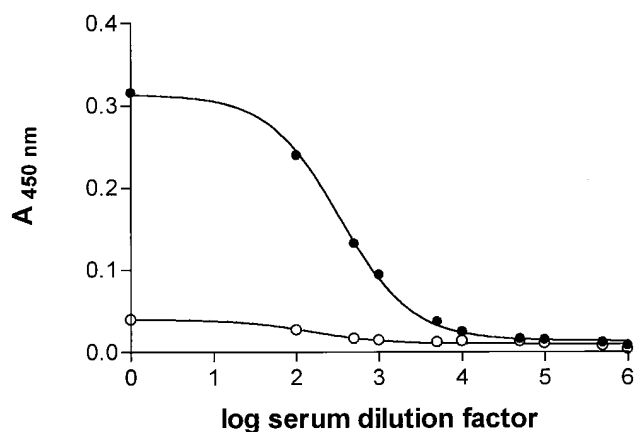

Fig. 2. Antibody dilution curve for antiserum raised against rabbit homocystamide-LDL adduct. Antiserum was obtained as described in Fig. 1. Serial dilutions of the antiserum were assayed by solid-phase ELISA techniques in which homocystamide-BSA adduct (top) (100 μl, 10 μg/ml in PBS) and homocystamide-hemoglobin adduct (bottom) (100 μl, 10 μg/ml in PBS) were bound to Millipore 96-well plates. Horseradish peroxidase-labeled goat anti-rabbit IgG was used as a secondary antibody, and TMB was used as a substrate; (●) homocystamide-BSA adduct; (○) native BSA; (■) homocystamide-hemoglobin adduct; (○) native hemoglobin (n = 2 for each data point).

and B was total binding in the presence of competitor) was approximately 10 μg/ml (Fig. 3). Native LDL did not compete for binding, as a dose-dependent decrease in % B/B_0 was not observed (Fig. 3). Similarly, neither BSA nor homocysteine disulfide (homocystine) was recognized, as a dose-dependent decrease in % B/B_0 was not observed (data not shown). In order to determine whether the antibody was directed against homocystamide-LDL adduct or derivatized lysine in general, LDL was acetylated, as described in Materials and Methods. Acetylation was assessed qualitatively by measuring REM, as described in Materials and Methods. The REM of acetylated LDL was 6.13 ± 0.04 (n = 3). When acetylated LDL was used as a competitor, a dose-dependent decrease in % B/B_0 was not observed,
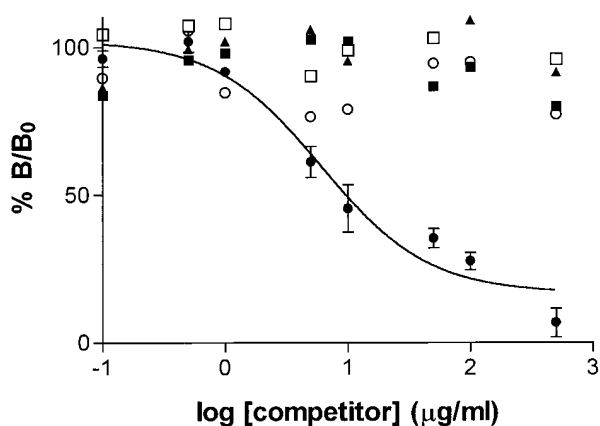
Fig. 3. Specificity of antiserum to homocystamide-LDL adduct with respect to various lysine modifications. Antiserum obtained as described in Fig. 1 was added to Millipore 96-well plates containing pre-adsorbed homocystamide-LDL adduct (100 μl, 10 μg/ml) at a limiting dilution (1:700) in the indicated concentrations of competitors (●, homocystamide-LDL adduct; ○, native LDL; ▲, acetylated LDL; □, methylated LDL; ■, methylated LDL treated with homocysteine thiolactone). After a 12-h incubation, antibody bound was quantified as described in Fig. 2. Results are presented as % B/B_0, in which B is the amount of antibody bound in the presence of a given concentration of competitor and B_0 is the amount bound in the absence of competitor. This experiment is representative of three independent experiments (n = 3 for each data point). Data points are the mean ± SEM).

which provided further evidence that the antiserum was specific for the homocystamide-LDL adduct (Fig. 3). In order to determine whether the antigenicity of the homocystamide-LDL adduct was due to the adduct itself or conformational changes in apoB induced by homocysteine thiolactone, LDL was reductively methylated. A 93 ± 4% decrease in apoB lysyl residues was observed, as measured by the TNBS assay (assuming 180 lysines per apoB, and a 500,000 molecular weight, n = 3 for each measurement). A portion of the methylated LDL was treated with homocysteine thiolactone using conditions identical to the formation of homocystamide-LDL adducts. Neither methylated LDL nor homocysteine thiolactone-treated methylated LDL competed for binding (Fig. 3). (This experiment is representative of three independent experiments; n = 3 for each measurement). This demonstrates that the antigenicity of homocystamide-LDL adduct is due to direct effects of homocysteine thiolactone on lysyl residues and not due to nonspecific modifications of LDL by homocysteine thiolactone.

The specificity of the antiserum for the homocystamide-LDL adduct was investigated further by examining the effect of Cu^{2+}-mediated LDL oxidation on its antigenicity. Homocystamide-LDL adduct was prepared as described in Fig. 3. Homocystamide-LDL adduct (0.5

mg/ml) and native LDL (0.5 mg/ml) were dialyzed in EDTA-free, Chelex-treated PBS for 24 h. After dialysis, Cu^{2+} (100 μm) was added to each of the samples, which were incubated at 37°C for 6 h. After incubation, BHT (500 μm) and EDTA (1 mm) were added to quench the reactions. TBARS were measured at this time, as described in Materials and Methods. TBARS concentrations were 68.4 ± 0.5 nmol/mg in homocysteine thiolactone-treated LDL and 62.1 ± 0.2 nmol/mg in native LDL (n = 3 for each measurement). As in Fig. 3, competition assays were performed in order to determine specificity of the antiserum for the above modifications. As shown in **Fig. 4**, homocysteine thiolactone-treated LDL that was oxidized with Cu^{2+} competed for binding in a dose-dependent manner, with an IC$_{50}$ of approximately 100 μg/ml. This level of competition is less than for the homocystamide-LDL adduct (IC$_{50}$ = 10 μg/ml) (Fig. 3). Two possibilities could explain this observation. First, the oxidized product could be a less potent competitor. Second, this extent of Cu^{2+} oxidation could have incompletely destroyed the antigens on homocysteine thiolactone-treated LDL. Cu^{2+}-oxidized LDL that was not pre-treated with homocysteine thiolactone did not compete (Fig. 4). Also, free homocysteine was tested as a competitor and did not compete for binding (Fig. 4).
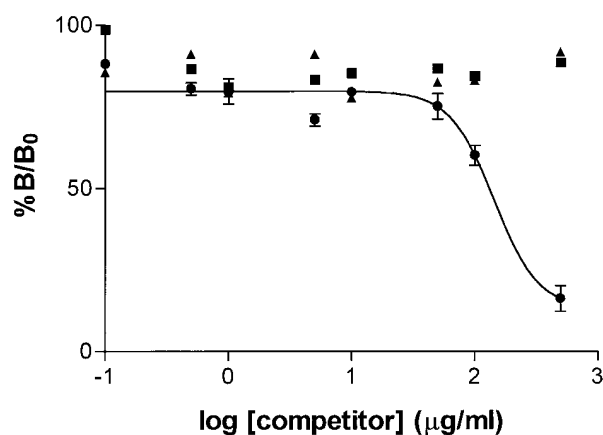

Fig. 4. Specificity of antiserum to homocystamide-LDL adduct after Cu^{2+}-mediated oxidation. Antiserum obtained as described in Fig. 1 was added to Millipore 96-well plates containing pre-adsorbed homocystamide-LDL adduct (100 μl, 10 μg/ml) at a limiting dilution (1:700) in the indicated concentrations of competitors (●, Cu^{2+}-oxidized homocystamide-LDL adduct; ■, Cu^{2+}-oxidized LDL; ▲, homocysteine). After a 12-h incubation, antibody bound was quantified as described in Fig. 1. Results are presented as % B/B_0, in which B is the amount of antibody bound in the presence of a given concentration of competitor and B_0 is the amount bound in the absence of competitor. This experiment is representative of three independent experiments (n = 3 for each data point). Data points are the mean ± SEM.

Antigenicity of homocysteine thiolactone-treated plasma

In order to determine whether protein homocysta-mide-lysyl adducts could be detected in plasma proteins, freshly drawn human plasma was treated with homocysteine thiolactone and compared to native plasma. The plasma solution was added to homocysteine thiolactone free base (500 mg). This solution was incubated 30 min on ice with gentle stirring and was passed through a Sephadex G-25 column in order to remove unreacted homocysteine thiolactone. Subsequently, LDL, HDL, and LPDS were isolated by sequential ultracentrifugation in a potassium bromide gradient. The presence of homocystamide-lysine adducts was determined by using competition assays. As shown in **Fig. 5**, protein homocystamide-lysine adducts were present in LDL, HDL, and in the LPDS fractions of the homocysteine thiolactone-treated plasma, as indicated by their dose-dependent inhibitory effects. In contrast, inhibition was not observed for untreated plasma (Fig. 5). None of the three fractions (LDL, HDL, LPDS) isolated from untreated plasma showed an inhibitory effect (data not shown).
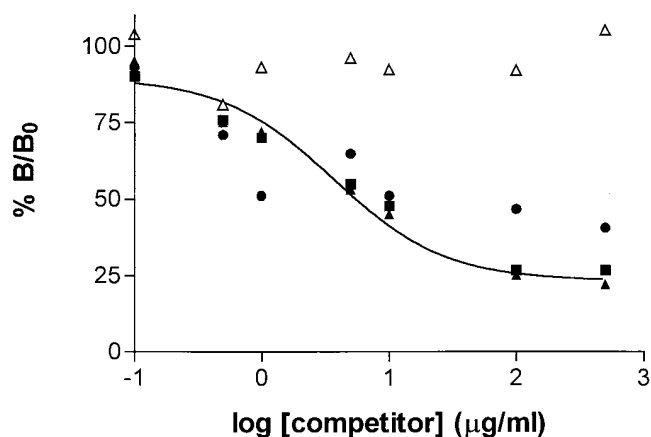

Fig. 5. Identification of specific homocystamide-adducts in homocysteine thiolactone-treated plasma. Antiserum obtained as described in Fig. 1 was added to Millipore 96-well plates containing pre-adsorbed homocystamide-LDL adduct (100 μl, 10 μg/ml) at a limiting dilution (1:700) in the indicated concentrations of competitors (▲, ●, ■, homocystamide-LDL, -HDL, and -lipoprotein-deficient serum proteins isolated from homocysteine thiolactone-treated plasma, and freshly drawn plasma without homocysteine thiolactone treatment, △). After a 12-h incubation, antibody bound was quantified as described in Fig. 2. Results are presented as % B/B_0, in which B is the amount of antibody bound in the presence of a given concentration of competitor and B_0 is the amount bound in the absence of competitor. This experiment is representative of three independent experiments. Data points are the mean of duplicate measurements.

DISCUSSION

Possible role of homocysteine in the etiology of vascular disease

At present, there is minimal insight into the etiology of homocysteinemia-associated cardiovascular disease. Animal models of homocysteinemia have shown that chronic homocysteine infusion causes endothelial cell injury and intimal proliferation (18). Oxidative modification of LDL has been implicated in the pathogenesis of atherosclerosis (6, 19, 20). It has been suggested that reactive oxygen species produced by homocysteine modify low density lipoprotein (LDL) to an oxidized, atherogenic form. According to this mechanism, autoxidation of homocysteine to the homocysteinyl radical, with the concomitant generation of superoxide anion, hydrogen peroxide, and hydroxyl radical, has been attributed to vascular injury that is associated with homocysteinemia (21–23). The issue of whether or not homocysteine autoxidation could facilitate oxidation of LDL is still controversial, as there are a number of conflicting reports on the role of thiol autoxidation in the susceptibility of LDL to oxidative modification (24–29). Homocysteine and cysteine exhibit very similar mechanisms of autoxidation. As cysteine has not been identified as a risk factor for the development of atherosclerosis, it is likely that pathways of homocysteine biochemistry other than autoxidation are responsible for any proatherogenic effects.

One situation for which the biochemistries of homocysteine and cysteine are clearly different occurs during the biosynthesis of proteins. Jakubowski (30) has recently elucidated a mechanism in human cells by which homocysteine thiolactone is formed during protein synthesis when homocysteine levels are elevated. Homocysteine thiolactone reacts with primary amines, forming an amide bond (equation 2). This reaction will readily occur in aqueous solution. Furthermore, reactions between primary amino groups and homocysteine thiolactone could occur readily and favor the formation of homocystamide adducts. It has been suggested that homocysteine thiolactone could react with apoB lysyl residues of LDL, forming homocystamide-LDL adducts (equation 3) (1, 2). In the plasma from hypercholesterolemic individuals, homocysteine exists in all major protein-containing fractions (i.e., LDL, VLDL, HDL, LPDS). The highest concentrations of total homocysteine, however, are contained in LDL (31). A portion of this total homocysteine is thought to originate from homocystamide adducts. Homocysteine thiolactone has been reported to cause morphological changes and aggregation of LDL (32). In addition, it has been reported that the aggregation of LDL in-

duced by homocysteine thiolactone treatment induces uptake by macrophages in culture (5).

Immunogenicity of the homocystamide-LDL adduct

Lysine modifications of LDL have been shown to generate antibodies in a number of species (6, 7). It was suggested several years ago that if homocysteine thiolactone existed in vivo, autoantibodies directed against homocystamide adducts would possibly be generated, although the notion of homocysteine thiolactone in vivo was not considered to by physiologically relevant (8). The present study is the first ever to investigate the possibility that the homocystamide-LDL adduct could be immunogenic. We have demonstrated that antiserum against homocystamide-LDL adduct raised in New Zealand White rabbits is specific not only for the homocystamide-LDL adduct but also for homocystamide-lysyl residues of other plasma proteins (Fig. 1, 3, and 5). Our results are in agreement with previous observations demonstrating that homologous LDL is an excellent carrier molecule for the generation of antiserum directed toward specific protein lysyl residue modifications (7). This specificity could be an important property in order to utilize this antiserum as a research tool for which atherosclerotic tissues of animals could be examined for the presence of homocystamide-adducts. Atherosclerotic lesions contain LDL that is oxidatively modified (6). The antiserum that we
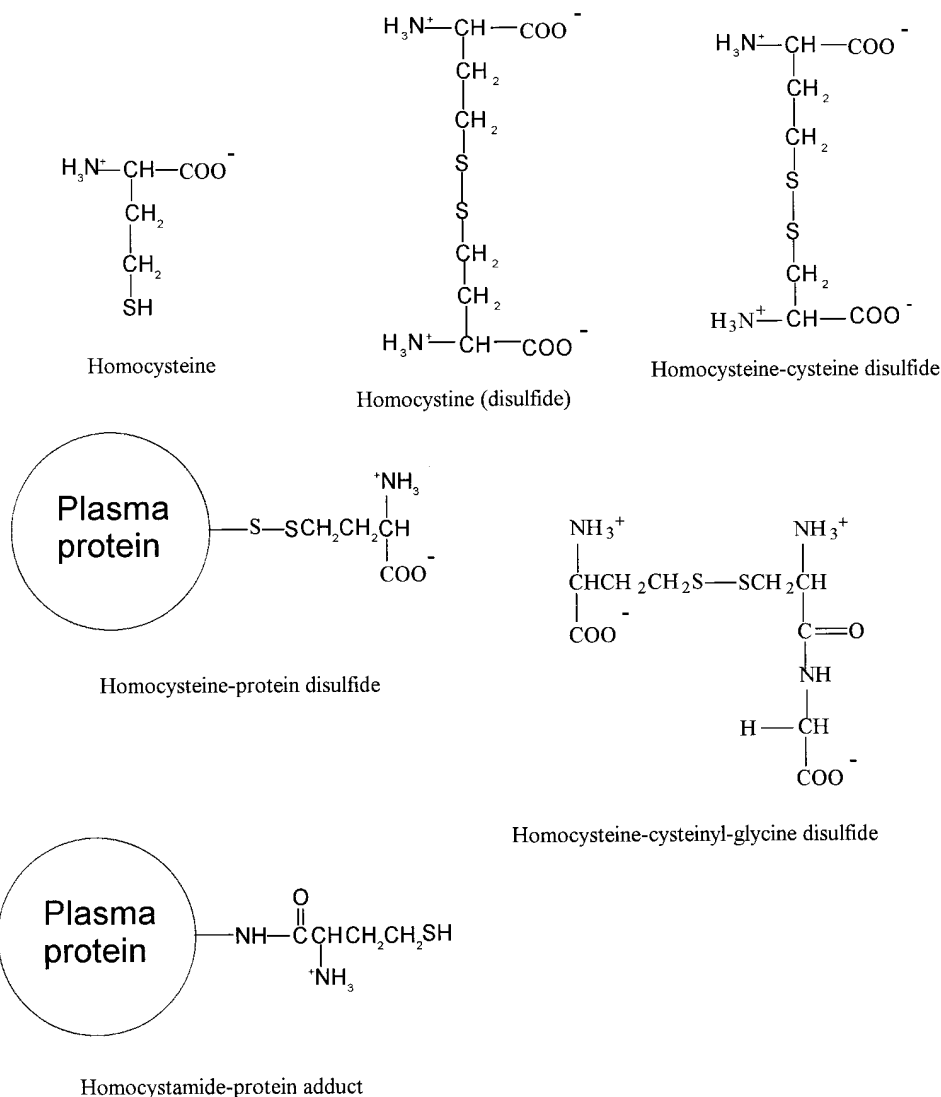

Fig. 6. Forms of homocysteine in the plasma. Homocysteine can exist in the plasma as free homocysteine, homocysteine disulfide (homocystine), or as a mixed disulfide. The homocystamide adduct is thought to occur in homocysteinemia.

have generated could be used in this situation, as it is unlikely that the presence of oxidized LDL would obscure the detection of homocystamide adducts. This assertion is supported by the observation that Cu^{2+}-oxidized LDL was not recognized by our antiserum, whereas homocystamide-LDL adduct was recognized after Cu^{2+}-mediated oxidation (Fig. 4). From a clinical standpoint, our immunological approach may have implications for the measurement of plasma homocysteine.

Techniques for the measurement of homocysteine concentrations: possible future directions

Homocysteine is known to exist in the plasma as free homocysteine, homocystine (homocysteine disulfide), or as a mixed disulfide with cysteine, cysteinyl-glycine, or a protein thiol (Fig. 6) (33, 34). It remains unknown whether or not homocystamide-protein adducts (Fig. 6) exist in plasma. Current HPLC methodology uses a reductive step in which disulfides are reduced to thiols. This is followed by derivatization of thiol groups with a fluorescent substrate (e.g., monobromobimane), acid (e.g., perchloric acid) precipitation of proteins, HPLC separation of amino acids using either gradient or isocratic conditions, and measurement of post-column fluorescence (see reference 35 for a review). The amide bond linking the homocystamide-protein adduct is resistant to the chemical reduction step, and therefore the adduct is lost at the precipitation step. In order to break the amide bond, homocystamide adduct must be hydrolyzed using strong acid which will destroy thiols beyond the point at which they are amenable to reduction. Therefore, it is impossible to measure homocystamide-protein adducts using current HPLC techniques. Evidence suggests that homocysteine thiolactone formation may play a key role in atherogenesis (1–3). Inasmuch, failure to detect homocysteine thiolactone-modified proteins may grossly underestimate the atherogenic potential of homocysteine thiolactone. An antibody could possibly measure the homocystamide-adduct levels in plasma. Due to the mechanism by which homocysteine thiolactone is formed in cells (i.e., during protein synthesis), it is likely that reactions between homocysteine thiolactone and proteins occur in the mammalian liver, which has a high level of protein synthesis. As the mechanism for the formation of homocystamide adducts involves biosynthesis of proteins, it follows that protein turnover would also affect levels of homocystamide adducts. For these reasons, levels of homocystamide adducts in plasma may be an important measurement of chronic homocysteine concentrations. The use of an immunological assay for the detection of homocystamide-adducts in plasma may be more useful than HPLC measurement of homocysteine, as the other forms of homocysteine in plasma (Fig. 6) do not necessarily rely on active protein synthesis. Furthermore, levels of these forms of homocysteine are dependent upon a host of other factors such as disease and daily changes in nutritional states (36). The development of methodology for the measurement of homocystamide-adducts in vivo is an area of active investigation in our laboratory. At present, no other technique is available that can precisely identify the homocystamide-lysine adduct.

The polyclonal antibody reported here is specifically directed against homocystamide-lysine adducts. It is concluded that using this immunological approach, it may be possible to investigate the exact role of elevated homocysteine levels in atherosclerosis.▰

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