A Novel Method for Generating Region-specific Monoclonal Antibodies to Modified Proteins

APPLICATION TO THE IDENTIFICATION OF HUMAN GLUCOSYLATED LOW DENSITY LIPOPROTEINS

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ABSTRACT Modifications of plasma lipoprotein structure and function resulting from in vivo posttranslational nonenzymatic glycosylation may play a role in the premature atherosclerosis of patients with diabetes mellitus. This report describes the generation and characterization of six unique murine monoclonal antibodies that bind glucosylated human plasma lipoproteins, but do not react with normal plasma lipoproteins. This was accomplished by immunizing mice with homologous glucosylated low density lipoprotein. In competitive inhibition radioimmunoassays, the dominant epitope recognized by these antibodies on glucosylated low density lipoprotein was identified as glucitollysine, the reduced hexose alcohol form of glucose conjugated to the epsilon amino group of lysine. Each of these antibodies was capable of identifying glucitollysine epitopes on all reduced glucosylated proteins studied, including high density lipoprotein, albumin, hemoglobin, and transferrin. These antibodies were also capable of identifying and quantitating glucitollysine residues on the total plasma proteins and isolated lipoproteins of normal and diabetic individuals after reduction of the proteins with $NaBH_4$. Preliminary data suggest that diabetic total plasma proteins and isolated lipoproteins contain at least threefold more immunochemically detectable glucitollysine residues than nondiabetic plasma proteins and lipoproteins.

The technique described in this report should allow production of region-specific antibodies to any immunogenic modification of a protein.

INTRODUCTION

Nonenzymatic glycosylation of a variety of plasma and structural proteins has been demonstrated in normal and, to an even greater extent, in diabetic subjects. The modification of protein structure and function brought about by such posttranslational modifications has been implicated in a number of pathologic sequelae of diabetes, including cataract formation, neuropathy, connective tissue pathology, and atherosclerosis. Glycosylation of hemoglobin alters its affinity for oxygen (1). Lens crystallin glycosylation results in opacification and may contribute to cataract formation (2). Glycosylation of collagen alters the extent and perhaps the type of collagen cross-linking that leads to stiffening of tissues (3). Finally, glucosylation of lipoproteins alters their biologic behavior (4, 5). In particular, glucosylation of low density lipoproteins (LDL) alters their high affinity cellular uptake and degradation in vitro (4, 6–9), and reduces their fractional catabolic rate in animals (4, 8, 10) and man (11).

The glycosylation of hemoglobin and plasma proteins is of considerable clinical interest, since the extent of protein glycosylation provides a useful marker for monitoring blood glucose concentrations over time (12, 13). Measurements of glycosylated proteins are used

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to follow diabetic control and methods are readily available to measure glycosylated hemoglobin (HbA_{1C}).[1] However, since this protein has a long half-life, measurement of HbA_{1C} is relatively insensitive to recent changes in glucose concentration. In addition, labile forms and other chemical modifications alter hemoglobin's physical properties and complicate measurement (12–16). Thus, it would be useful to have an assay that measures the extent of glucosylation of specific plasma proteins with shorter half-lives and is specific for the glucose adduct. A radioimmunoassay (RIA) for HbA_{1C} has been described (17). However, the antibodies cross-reacted with nonglycosylated hemoglobin, and were of low affinity, since they were obtained by conventional approaches and involved extensive adsorption of a heterologous antisera (17).

LDL, with a half-life in the blood stream of ~3–5 d (18), could provide a useful marker for accurately assessing ambient blood glucose levels of diabetic individuals over short periods of time. In addition, since glucosylated LDL (glc-LDL) is present in higher concentrations in diabetic plasma and could be atherogenic (4, 19), it would be important to quantitate it readily. In this report, we describe a novel technique that allowed us to generate six murine monoclonal antibodies capable of identifying and quantitating glucosylated amino acid residues of lipoproteins as well as other plasma proteins.

METHODS

Lipoprotein isolation and characterization. Human plasma lipoproteins were isolated from pooled fresh fasting plasma that was obtained from normal healthy donors by plasmapheresis. LDL (d = 1.019–1.063 g/ml) and high density lipoprotein (HDL, d = 1.063–1.25 g/ml) were isolated by sequential ultracentrifugation using KBr for density adjustment in the presence of 0.1% (wt/vol) EDTA, 1 mg/ml gentamycin sulfate, 0.2% sodium azide, 1 mM benzamidine, 10 mM diisopropylfluorophosphate and 10 μg/ml soybean trypsin inhibitor as previously described (20). Lipoproteins were dialyzed thoroughly against 0.15 M NaCl containing 0.3 mM EDTA, 5 mM benzamidine, and 0.005% alpha tocopherol, pH 7.4, filter sterilized and stored at 4°C for ≤14 d. The total protein content of the lipoproteins was analyzed by a modification (21) of the method of Lowry, with a bovine albumin standard, and all lipoprotein concentrations are expressed on the basis of protein. The purity and apoprotein composition of each lipoprotein class were assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described (20).

Murine LDL, obtained from chow-fed Balb/c mice, was isolated from 50 ml pooled plasma, extensively dialyzed against phosphate-buffered saline (PBS) containing 1 mM EDTA and sterilized.

Preparation and characterization of glucosylated proteins and compounds. Proteins that were glucosylated in the presence of glucose and the reducing agent sodium cyanoborohydride ($NaCNBH_3$) are referred to as glc_{RED}-protein adducts; whereas proteins that were glucosylated in the absence of a reducing agent, "nonreduced" (NR), are referred to as glc_{NR}-protein adducts. Murine glc_{RED}-LDL was prepared by incubating 2 mg sterile mouse LDL at room temperature for 7 d in 5 ml sterile PBS, pH 7.4, containing 80 mM glucose (Mallinckrodt Inc., Science Products Div., St. Louis, MO) and 12.5 mg/ml of fresh $NaCNBH_3$ (J. T. Baker Chemical Co., Phillipsburg, NJ). Reduced (or nonreduced) forms of human glc-LDL and glc-HDL were prepared by incubating 5 mg protein for 3–168 h at 37°C in 2–5 ml sterile PBS, pH 7.4, containing 80 mM glucose in the presence (or absence) of 12.5 mg/ml $NaCNBH_3$. Polylysine-4000, human albumin, transferrin, and hemoglobin (Sigma Chemical Co., St. Louis, MO) were glucosylated in a similar manner. Reduced, mannosylated LDL (man_{RED}-LDL) was prepared in a similar fashion by substitution of 80 mM mannose (Mallinckrodt Inc.) for glucose. At the end of the incubations, all samples were exhaustively dialyzed against PBS and sterilized. The extent of glucosylation of each of the samples was assessed by amino acid analysis as described (4). Glucitollysine was synthesized according to the method of Schwartz and Grey (22), with alpha-T-boc-lysine (Vega Biochemicals, Div. of Vega Biotechnologies, Inc., Tucson, AZ) as substrate (4).

Immunization. Two 16-wk-old female Balb/c mice were each injected with 20 μg i.p. of murine glc_{RED}-LDL emulsified in complete Freund's adjuvant. On days 14 and 28, they received secondary injections of 20 μg i.p. of murine glc_{RED}-LDL in incomplete adjuvant. 4 d before fusion (day 51), the mice were given injections of 20 μg i.v. of human glc_{RED}-LDL (46% of lysines glucosylated). Mice were bled on days 0, 38, and 55 and the serum antibody responses monitored by solid-phase RIA as described below.

Hybridomas. The spleens of the two mice were removed, pooled (3.2×10^8 total cells), and fused with P3 × 63-Ag8.653 mouse myeloma cells in the presence of 30% (wt/vol) polyethyleneglycol-1000 at a ratio of 10 myeloma cells/spleen cell as described (20). 3 d after fusion, viable cells were plated out in 96-well tissue culture plates at 2×10^4 cells/well (768 total wells). Growth was followed microscopically and culture supernatants collected on day 14 for assay of antigen-specific antibody production by solid-phase RIA. On day 14, 79.6% of the wells contained foci of viable cells, with 18.4% of these containing single foci. Of the 611 wells assayed, 187, or 31%, of the culture supernatants reacted with human glc_{RED}-LDL. These 187 wells were selected for differential screening (i.e., no reaction with normal human LDL, but good reaction with human glc_{RED}-LDL) and six were selected 21 d after fusion for recloning by limiting dilution. Cloned hybridomas were cultivated in medium containing 10% calf serum and stored frozen in liquid nitrogen (20).

Ig heavy and light chains of the antibodies secreted by the cloned hybridomas were typed using the MonoAB-ID EIA kit A (lot Number 20920, Zymed Laboratories, Inc., San Francisco, CA). The assays were performed with hybridoma culture supernatants exactly as described by the manufacturer.

RIA. Assays were performed in round-bottomed polyvinyl chloride microtiter plates (Falcon Labware, Micro Test III, Becton, Dickinson & Co., Oxnard, CA). The wells were

[1] *Abbreviations used in this paper:* glc-, glucosylated protein adducts; glc_{NR}-, nonreduced glucosylated protein adducts; glc_{RED}-, reduced glucosylated protein adducts; HbA_{1C}, glycosylated hemoglobin A; IDL, intermediate density lipoprotein; man_{RED}-LDL, reduced and mannosylated LDL.

coated with antigen by adding 0.05 ml antigen in PBS or lipoprotein buffer and by incubating the plates for 3 h at room temperature. All antigens were coated at 1 μg/ml. The wells were postcoated for 30 min with 0.25 ml PBS containing 3% bovine albumin and 3% normal goat serum to block remaining active sites. For assay, 0.05 ml of mouse serum or hybridoma culture supernatant, diluted in PBS containing 3% bovine albumin, 3% goat serum, and 0.05% Tween-20, were added and incubated for 18 h at 4°C. After washing, mouse antibody binding was detected by a 4-h incubation at 4°C with 10 ng/well of immunochemically purified and radioiodinated goat anti-murine Ig (3–4 μCi/μg) as described (20). Competitive assays were performed in an identical manner and contained 0.025 ml of competitor diluted in PBS (with 3% bovine albumin, 3% goat serum, and 0.05% Tween-20) and 0.025 ml of culture supernatants containing limiting amounts of monoclonal antibody. Nonspecific binding was determined by replacing specific hybridoma culture supernatants with similar dilutions of the culture supernatants of irrelevant hybridomas producing Ig of the same heavy chain type. The maximum amount of ^{125}I-second antibody bound by specific antibody (Bo) was determined in the absence of competitors. Data were calculated as B/Bo, where B equals the mean counts per minute bound at a given concentration of competitor. The monoclonal antibody B24 binds with high affinity to the apoprotein of human LDL (apoprotein B), has been extensively characterized (20, 23), and was used as one control. Monoclonal antibody AV45B6, an IgG_{2b} antibody that binds human albumin, was obtained from the fusion of immune spleen cells with the SP 2/0 myeloma and was used as a second control.

Identification of glucosylated human plasma proteins. Blood was drawn into EDTA by venipuncture from both normoglycemic control subjects and patients with insulin-requiring diabetes mellitus and immediately placed on ice. Plasma was separated by low speed centrifugation and the erythrocyte lysates assayed for HbA_{1C} in the laboratory of Dr. Helen Ranney by the Isolab column technique (Isolab, Inc., Akron, OH). Plasma glucose was determined by the glucose oxidase method. The plasma was adjusted to 1 mM EDTA and clarified by centrifugation at 11,000 *g* at 4°C and immediately stored at −70°C. To remove free glucose, 0.9 ml clarified plasma was chromatographed on a 0.5 × 20-cm column of Sephadex G-25 equilibrated in PBS containing 1 mM EDTA. The void volume (4 ml) was incubated at 37°C for 4 h in the absence or in the presence of either 12 mM $NaCNBH_3$ or 10 mM $NaBH_4$. After exhaustive dialysis against PBS, pH 7.2, plasma protein was determined (21). The plasmas were added undiluted as competitor (0.025 ml) to the solid-phase RIA described above, with human glc_{RED}-LDL (48.7% of lysines glucosylated) at 1 μg/ml for antigen coating and glucitollysine at 0.078–300 nmol/ml as standard.

RESULTS

Reaction scheme for glucosylation of LDL. The reaction scheme for nonenzymatic glucosylation of LDL is shown in Fig. 1. Glucose forms a labile intermediate Schiff base with the epsilon amino group of lysine, which undergoes an Amadori rearrangement to form a relatively stable ketoamine (24, 25). As a result of this reaction, a bulky glyco group is introduced onto lysine. The ketoamine in turn is in equilibrium with a hemiketal or ring form. These Amadori

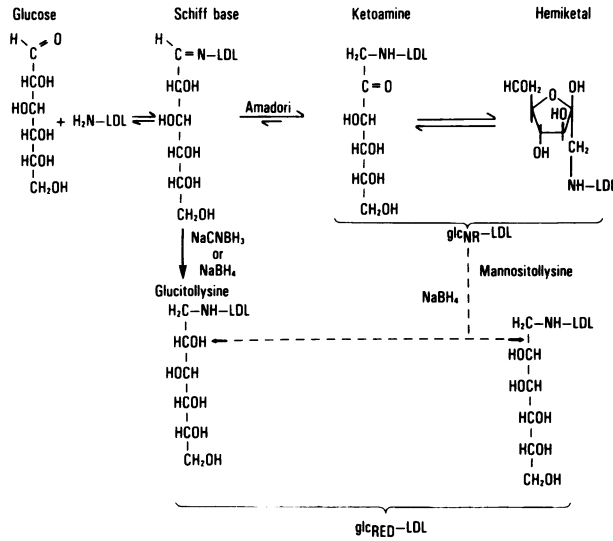

FIGURE 1 Reaction scheme for nonenzymatic glucosylation of the lysine residues of LDL. The product of the reaction of glucose with the epsilon amino group of lysine is a labile Schiff base. Glucosylation of LDL in vitro in the presence of $NaCNBH_3$ quantitatively reduces the labile Schiff base to glucitollysine. This reduced adduct is referred to as glc_{RED}-LDL. If glucosylation of LDL is carried out in vitro in the absence of a reducing agent (glc_{NR}-LDL), the labile Schiff base undergoes an Amadori rearrangement to form the more stable ketoamine and hemiketal adducts. The Schiff base, ketoamine, and hemiketal products can all exist in vivo. However, only the Schiff base is reduced to glucitollysine by $NaCNBH_3$ reduction, whereas each of these products can be reduced by $NaBH_4$. Reduction of the hemiketal by $NaBH_4$ can also result in the generation of a mannositollysine residue, the epimer of a glucitollysine residue.

rearrangement products, which are formed in the absence of a reducing agent, are referred to as nonreduced (NR) glucosylated LDL (glc_{NR}-LDL). When glucosylation is carried out in vitro in the presence of a reducing agent such as $NaCNBH_3$, the labile Schiff base is immediately and quantitatively reduced to glucitollysine (25). The product of LDL glucosylation carried out in vitro in the presence of a reducing agent is referred to as reduced glucosylated LDL (glc_{RED}-LDL).

Generation of monoclonal antibodies. Previous experience has demonstrated that apoprotein B of human LDL is a good immunogen in Balb/c mice (20, 23). Therefore, to prevent an antibody response in the immunized mice to normal nonglucosylated epitopes on human LDL, homologous Balb/c LDL was isolated, glucosylated in the presence of $NaCNBH_3$ and used as the primary and secondary immunogen. Human glc_{RED}-LDL, the prefusion immunogen, was injected intravenously 4 d before the mice were killed, specifically to enhance the in vivo proliferative expansion

and differentiation of murine B cell clones producing antibodies that were capable of cross-reacting with human glc_{RED}-LDL. After immunization, specific serum antibody responses for human glc_{RED}-LDL, the prefusion immunogen, were detected by RIA at serum dilutions of $>10^{-6}$ (Fig. 2). No response to normal (nonglucosylated) human LDL could be detected in the sera of the immunized mice. Thus, the immune spleen cells used for fusion contained a highly restricted population of antigen-specific antibody-forming B cells. When these spleen cells were fused in vitro with mouse myeloma cells in the presence of polyethyleneglycol and the fused cells plated out in 96-well tissue culture plates, assay of the culture supernatants confirmed this. Of the 768 hybridoma cell cultures originally plated out, 187 contained hybridomas that produced antibody capable of binding human glc_{RED}-LDL. Of these 187 culture supernatants, 176 (94%) did not react with normal human LDL. Six hybridomas from this fusion were cloned and propagated for further study.

Antibody specificity. The specificity of the antibodies produced by the six cloned hybridomas was tested, and compared with two control mouse monoclonal antibodies obtained from other fusions (Table I). One of these, the B24 antibody, is an apoprotein B-specific antibody with high affinity ($K_a = 3 \times 10^9$ M^{-1}) for human LDL (20, 23). The second, AV45B6, reacts specifically with human albumin. In the solid-phase RIA, culture supernatants from these six cloned hybridomas were tested for their ability to react with the following human antigens bound to the microtiter plate: albumin, LDL, the glc_{RED}-LDL antigen used for prefusion immunization and for screening, as well as the nonreduced Amadori adducts of the glucosylation reaction (glc_{NR}-LDL). In contrast to the normal apoprotein B-specific antibody (B24), each of the antibodies from this fusion reacted only with glc_{RED}-LDL, and none of them reacted with either normal LDL or glc_{NR}-LDL (Table I).

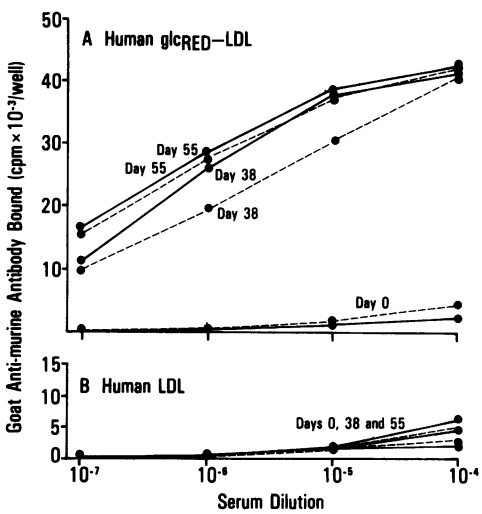

FIGURE 2 Solid-phase RIA of the murine serum antibody responses to human glc_{RED}-LDL and human LDL. Two mice (hatched vs. solid lines) were prebled, hyperimmunized with murine glc_{RED}-LDL as described, and bled on day 38. The mice were boosted with 20 μg i.v. of human glc_{RED}-LDL on day 51 and bled and killed on day 55, when the spleen cells were removed for fusion. (*A*) ^{125}I-goat anti-murine Ig binding to microtiter wells coated at 1 μg/ml with glc_{RED}-LDL (46% of lysines glucosylated) and incubated with dilutions of mouse serum as indicated. (*B*) ^{125}I-goat anti-murine Ig binding to microtiter wells coated at 1 μg/ml with native, nonglucosylated human LDL and incubated with identical dilutions of mouse serum.

To determine whether the inability of these antibodies to bind glc_{NR}-LDL reflected only the low degree of glucosylation of lysine residues (5.9% for glc_{NR}-LDL compared with 46% for glc_{RED}-LDL), we prepared glc_{RED}-LDL that contained between 3.3 and 28.9% of the total lysine residues modified, as assessed by amino acid composition analysis. These preparations of glc_{RED}-LDL were generated by incubating LDL with glucose in the presence of $NaCNBH_3$ for 3–75 h. Each of these preparations of glc_{RED}-LDL, as well as a preparation of LDL incubated for 75 h with $NaCNBH_3$ in the absence of glucose, was compared in a solid-phase RIA for its ability to compete with heavily glucosylated glc_{RED}-LDL for antibody binding. All preparations of

TABLE I
Specificity of the Cloned Hybridoma Culture Supernatants for glc_{RED}-LDL

Monoclonal antibody	Ig type‡	Mean ^{125}I-goat anti-murine Ig bound*			
		glc_{RED}-LDL	glc_{NR}-LDL	LDL	Albumin
		cpm/well			
G5H3	IgG_1k	20,512	237	230	349
G6C9	IgG_1k	15,750	471	287	445
G8C11	IgG_1k	20,721	236	213	357
G5E10	IgG_1k	21,299	262	267	501
G8H6	IgG_1k	17,070	218	219	330
G8G7	IgMk	20,434	240	268	287
B24	IgG_1k	9,042	10,681	11,454	394
AV45B6	IgG_1k	773	455	918	16,615

* All antigens were human proteins and were coated onto the microtiter wells at 1 μg/ml. 46 and 5.9% of the lysine residues of glc_{RED}-LDL and glc_{NR}-LDL, respectively, contained glucose by amino acid analysis. All hybridoma culture supernatants were used at 1:50. Antibody B24 binds apoprotein B and antibody AV45B6 binds human albumin.

‡ The Ig heavy and light chains were typed as described in Methods.

glc_{RED}-LDL were able to compete with the glc_{RED}-LDL (49.5% of lysines glucosylated) antigen bound to the plate for binding to each of the six monoclonal antibodies. Data obtained with the G5H3 antibody were representative and is shown in Fig. 3. In all cases, the degree of competition was directly related to the degree of glucosylation, and was independent of the LDL protein added. LDL incubated for 75 h with $NaCNBH_3$ in the absence of glucose did not compete with any of these antibodies. Therefore, these antibodies bound a new epitope on LDL that was created by in vitro glucosylation and reduction, and could bind glc_{RED}-LDL that contained as little as 3.3% of the lysine residues reductively glucosylated.

The data above suggested that these antibodies reacted with the reduced glucose adducts of lysine conjugates (glucitollysine), but not with the nonreduced glucose adducts (ketoamine or hemiketal forms) (Fig. 1). To define further the specificity of the epitope identified by these antibodies, we synthesized glucitollysine and assessed its ability to compete with glc_{RED}-LDL for binding to each of the antibodies. As shown in Fig. 4, glucitollysine could completely inhibit the binding of each of these antibodies. Glucitolpolylysine was prepared and also found to be an effective competitor. Thus, the reduced conjugate of glucose and lysine could occupy the antigen-binding sites of each of these antibodies.

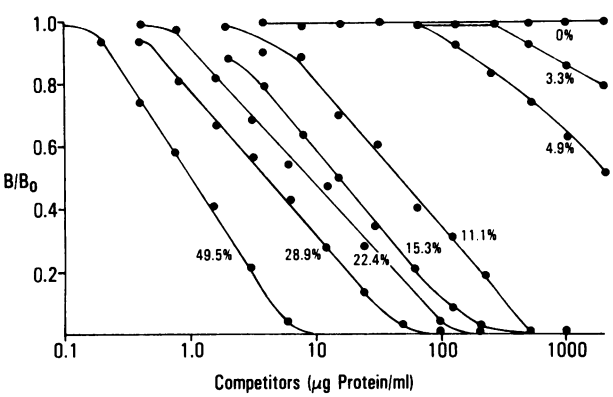

FIGURE 3 Competitive double-antibody RIA for glc_{RED}-LDL. Microtiter wells were coated at 1 μg/ml with glc_{RED}-LDL (49.5% of lysines glucosylated). A limiting amount of monoclonal antibody (0.025 ml) was incubated for 18 h at 4°C in the absence or presence of 0.025 ml of competing lipoprotein. Competitors included LDL incubated for 75 h with $NaCNBH_3$ in the absence of glucose (0% glucosylation), and LDL incubated with 80 mM glucose in the presence of $NaCNBH_3$ for 3 h (3.3% glucosylation), 6 h (4.9% glucosylation), 21.5 h (11.1% glucosylation), 33.5 h (15.3% glucosylation), 52 h (22.4% glucosylation), 75 h (28.9% glucosylation), or 168 h (49.5% glucosylation). Monoclonal antibody binding was detected with a second 4-h incubation with ^{125}I-goat anti-murine Ig. Data shown are qualitatively representative of each monoclonal antibody and was obtained with a 1:800 dilution of culture supernatant containing the G5H3 antibody.

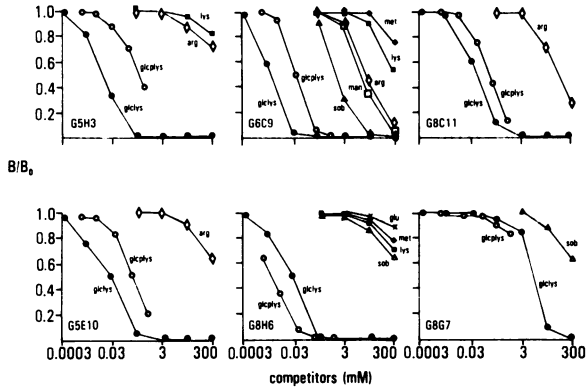

FIGURE 4 Specificities of the antigen-binding sites of individual antibodies. Microtiter wells were coated at 1 μg/ml with glc_{RED}-LDL that contained 48.7% of lysines glucosylated. Limiting amounts of each monoclonal antibody (0.025 ml) were incubated for 18 h at 4°C in the absence or presence of 0.025 ml of competitor. Competitors included glucitollysine (*glclys*, ●), glucitolpolylysine (*glcplys*, ○), sorbitol (glucitol) (*sob*, △), mannitol (*man*, □), lysine (*lys*, ■), arginine (*arg*, ◇), glutamine (*glu*, ×), and methionine (*met*, ◆). Only those compounds that were able to compete specifically for binding to individual antibodies are shown. Glucose and mannose failed to compete with any of the antibodies. Antibody binding to glc_{RED}-LDL was detected with a second 4-h incubation at 4°C with ^{125}I-goat anti-murine Ig. G5H3, G6C9, G8C11, G5E10, G8H6, and G8G7 antibodies were used at dilutions of 1:900, 1:1,000, 1:900, 1:1,000, 1:500, and 1:800, respectively.

Next, we assessed the ability of a number of other simple compounds to compete in the RIA. Included were the amino acids lysine, arginine, methionine, and glutamine; the aldosehexose epimers glucose and mannose; and the hexose alcohol epimers sorbitol (glucitol) and mannitol. Since each of these compounds was to be added as competitor at fairly high concentration, they were first screened for nonspecific effects by measuring the binding of antibody B24 (which is directed against native human apoprotein B) to glc_{RED}-LDL-coated plates in the presence of each of these compounds. This antibody fully recognized glc_{RED}-LDL, i.e., glucosylation did not interefere with the apoprotein B epitope recognized by B24 (Table I). With the exception of lysine and arginine, none of the compounds was able to inhibit the binding of this antibody to glc_{RED}-LDL at concentrations up to 300 mM. The levels of inhibition produced by lysine (20% at 300 mM) and arginine (20% at 100 mM) were considered to be nonspecific and only values that exceeded these levels were considered significant in the competitions performed with the antibodies that bound glc_{RED}-LDL.

The ability of all 10 compounds to compete with glc_{RED}-LDL for binding to each of the antibodies was tested in the experiment shown in Fig. 4, but only those compounds which competed are shown. Despite the fact that glucitollysine and glucitolpolylysine were effective competitors for each of the antibodies, even 300 mM amounts of glucose or mannose did not compete with any of these antibodies. This suggests that the hemiketal or ring form of the hexoses could not occupy the antigen binding site of any of these antibodies. However, the glucose alcohol sorbitol (glucitol) competed for binding to antibodies G8H6 and G8G7 at concentrations of ≤300 mM and was a good competitor of antibody G6C9 (50% inhibition at 1 mM). Therefore, the open-chain hexose alcohol was able to occupy the antigen binding site of three of these antibodies. The observation that mannitol, the alcohol of mannose, could compete for binding to antibody G6C9, and was equally effective at a 10-fold higher concentration relative to sorbitol, suggests a relaxed specificity of the G6C9 antibody for this epimer of sorbitol. At least one of the amino acids, lysine or arginine, was able to compete significantly with all antibodies except G8G7. Only arginine competed with G8C11 and G5E10, whereas both amino acids competed with antibodies G6C9 and G5H3. Arginine was the most effective amino acid competitor of G6C9 and was able to inhibit binding 50% at 20 mM compared with lysine, which required 300 mM for 50% inhibition. Lysine, but not arginine, significantly inhibited antibody G8H6; and this antibody, unlike all the others, was also inhibited by high concentrations of methionine and glutamine. Thus, the amino acid portion of glucitollysine also contributed to the individual specificity of some of these antibodies.

Glc_{RED}-LDL containing 48.7% of its lysine residues conjugated with glucose was included also in the competitive RIA shown in Fig. 4 (data not shown). When the antibodies were compared for their relative ability to quantitate the estimated number of glucitollysine residues per mole of glc_{RED}-LDL, one antibody was quite good. This comparison was accomplished by assuming a protein molecular weight for glc_{RED}-LDL of 6.5×10^5 and a glucitollysine/LDL molar ratio of 156. (See legend of Table II for calculation of molar ratio.) As shown in Table II, the smallest difference among the six antibodies in the amount of glucitollysine and glucitollysine residues on glc_{RED}-LDL required for 50% inhibition of antibody binding was obtained with antibody G6C9, and this difference was less than threefold.

We also prepared reductively mannosylated LDL by incubating LDL with mannose in the presence of $NaCNBH_3$. Man_{RED}-LDL (79% of lysines mannosylated) was a poor competitor for the binding of each

TABLE II

Relative Ability of Each Antibody to Measure the Estimated No. of Glucitollysine Residues on glc_{RED}-LDL

Antibody	Concentration of competitor required for 50% inhibition of binding		
	Glucitollysine*	Glucitollysine-LDL‡	Difference§
		μM	
G5H3	10	1.4	0.93
G6C9	3	1.1	0.28
G8C11	100	1.8	1.99
G5E10	10	2.2	0.89
G8H6	30	1.1	1.46
G8G7	10,000	12.1	4.0

* Quantitated directly from the competitive RIA shown in Fig. 4.
‡ Estimated from the competitive RIA shown in Fig. 4. Glc_{RED}-LDL was added as competitor to this assay at concentrations of 0.3–50 μg/ml. The μM concentration of glucitollysine on glc_{RED}-LDL was calculated with an LDL protein, mol wt of 6.5×10^5, and an estimated 320 lysine residues/mol of LDL (26). Amino acid composition analysis of this preparation of glc_{RED}-LDL indicated that 48.7% of the lysine residues were glucitollysine.
§ Log_{10} of the difference between the concentration of glucitollysine required for 50% inhibition and the concentration of glucitollysine residues on LDL required for 50% inhibition.

of the antibodies to glc_{RED}-LDL (49.5% of lysines glucosylated) and >1,000 times more man_{RED}-LDL protein was required for comparable levels of inhibition (Fig. 5). Thus, each of the antibodies also displayed a striking degree of specificity for the glucitol epimer conjugate of LDL.

Identification of glucitollysine residues in other proteins. The observation that glucitollysine was a dominant determinant of the epitope recognized by each of these antibodies suggested that these antibodies would be able to quantitate this residue in other plasma proteins. The competitive RIA was used to identify reductively glucosylated human HDL, albumin, transferrin, and hemoglobin. Each of these proteins competed for binding to each of the six monoclonal antibodies (Fig. 5), although the amount of each glc_{RED}-protein required for comparable levels of inhibition differed. In contrast, neither the native proteins nor the same proteins glucosylated in the absence of a reducing agent (glc_{NR}-protein adducts) competed with glc_{RED}-LDL for binding to any of the antibodies, despite the fact that they all contained at least 6% of their lysine residues conjugated to glucose in the Amadori forms (data not shown).

Evidence concerning the specificity of the individual antigen combining sites of each antibody also was obtained from these competitive assays. As illustrated in Fig. 5, different quantitative differences in binding were observed among the six different antibodies. For

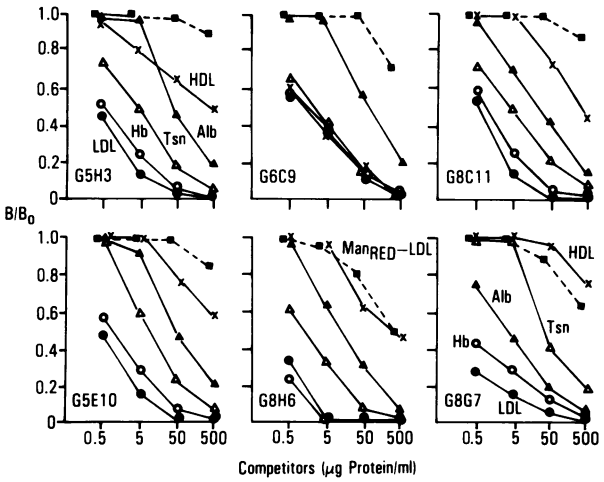

FIGURE 5 Identification of reduced mannosylated epitopes on LDL, and reduced glucosylated epitopes on human LDL, HDL, hemoglobin, albumin, and transferrin. Microtiter wells were coated at 1 μg/ml with glc_{RED}-LDL (49.5% of lysines glucosylated). Limiting amounts of each monoclonal antibody (0.025 ml) were incubated for 18 h at 4°C in the absence or presence of 0.025 ml of competitor. The percentage of lysines glycosylated are given in parentheses after each protein. Competitors included man_{RED}-LDL (79.6%) (■), glc_{RED}-LDL (49.5%) (●), glc_{RED}-HDL (29.2%) (×), glc_{RED}-hemoglobin (>85%) (*Hb*, ○), glc_{RED}-albumin (57.1%) (*Alb*, ▲), and glc_{RED}-transferrin (51.4%) (*Tsn*, △). Only those proteins that were able to compete for binding to individual antibodies are shown. The following glucosylated and nonreduced proteins also were tested and none was able to compete: glc_{NR}-LDL (6.2%), glc_{NR}-HDL (5.1%), glc_{NR}-hemoglobin (6.4%), glc_{NR}-albumin (6.1%), and glc_{NR}-transferrin (5.7%). Monoclonal antibody binding was detected with a second 4-h incubation with ^{125}I-goat anti-murine Ig. G5H3, G6C9, G8C11, G5E10, G8H6, and G8G7 antibodies were used at dilutions of 1:800, 1:1,000, 1:800, 1:800, 1:350, and 1:400, respectively.

example, (*a*) glc_{RED}-hemoglobin was a better competitor than glc_{RED}-LDL for binding to antibody G8H6, (*b*) glc_{RED}-albumin was a better competitor than glc_{RED}-transferrin for all antibodies except G8G7, and (*c*) all the glc_{RED}-proteins except albumin were equally good competitors for binding to only antibody G6C9.

Identification of glucitollysine residues in plasma proteins of control and diabetic subjects. The above data documented that these monoclonal antibodies recognized the reduced glucose protein adduct, glucitollysine. It is believed that most of the glucose adducts present in vivo are present only in the Amadori forms (12). If this is true, nonreduced glucosylated plasma proteins from normal or diabetic subjects should have little reactivity with these monoclonal antibodies, whereas glucosylated plasma proteins subsequently reduced in vitro (postreduced) should react. The specific reducing agent, $NaCNBH_3$, reduces Schiff base forms, but not the stable Amadori forms. $NaBH_4$, which is a more general reducing agent, converts both the Schiff base and the Amadori forms (Fig. 1). Thus, quantitation of glucitollysine residues of native plasma proteins should measure the minimum content of this adduct naturally occurring in plasma; quantitation of glucitollysine residues in plasma proteins after reduction with $NaCNBH_3$ (after removal of free glucose) should give the minimum content of glucose adducts originally present in the Schiff base form; and quantitation of glucitollysine residues in plasma proteins reduced with $NaBH_4$ (after removal of glucose) should yield the minimum content of total glucose adducts, including the labile Schiff base and the Amadori forms.

To test this hypothesis and to assess the ability of these antibodies to identify and quantitate these glucose adducts, plasmas were initially obtained from seven individuals (designated A–G in Fig. 6). Each plasma was depleted of free glucose by chromatography on Sephadex G-25 and divided into three aliquots. One aliquot was not reduced, a second was reduced with 12 mM $NaCNBH_3$, and a third was reduced with 10 mM $NaBH_4$. With glucitollysine as standard in a competitive RIA, the moles of glucitollysine residues per milligram of plasma protein were quantitated with five of the six antibodies. The sixth antibody, G8G7, was not studied further, since it was an IgM antibody (Table I) and was only inhibitable by high concentrations of glucitollysine (Table II). As expected, only very small amounts of glucitollysine residues were found in the seven individual native (nonreduced) plasmas (Fig. 6). However, some of the antibodies were capable of identifying larger amounts of glucitollysine residues in the $NaCNBH_3$-reduced plasmas of some of the subjects, and each of them identified substantially more residues in the $NaBH_4$-reduced plasmas. A comparison of the relative amounts of glucitollysine residues in the $NaCNBH_3$-reduced plasma aliquots with the $NaBH_4$-reduced plasma aliquots suggested that the majority of the glycosylated proteins in plasma existed as Amadori products rather than in the more labile Schiff base form.

Although the number of glucitollysine residues quantitated in the $NaBH_4$-reduced plasmas can only be considered relative rather than absolute, considerable variation was apparent among the seven individuals tested (Fig. 6). To determine whether the level of glucitollysine residues was increased in diabetics, we assayed the $NaBH_4$-reduced plasmas of five normals and 10 patients with diabetes mellitus. As shown in Table III, a significant increase in the moles of glucitollysine residues per milligram of $NaBH_4$-reduced protein was detectable in plasmas of the diabetics with each of the five antibodies. Even though the number of glucitollysine residues quantitated by each of the

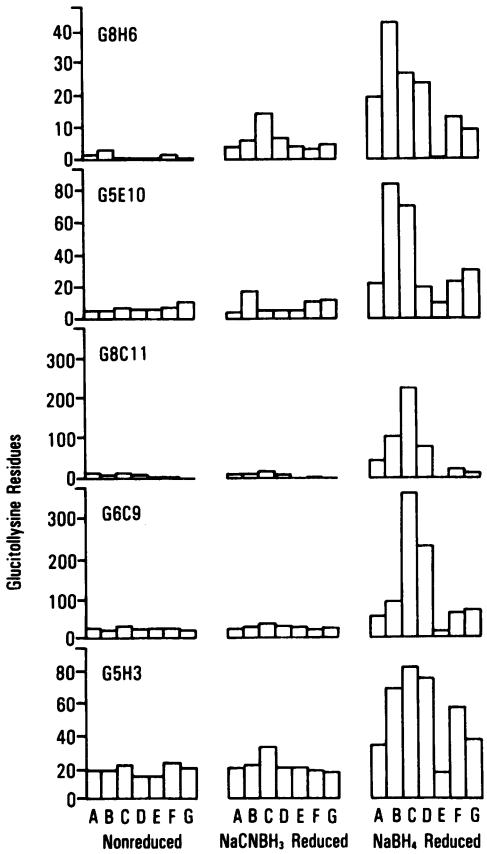

FIGURE 6 Immunochemical identification of glucitollysine residues in plasma proteins (picomoles per milligram of plasma protein). Plasma samples were obtained from seven individuals designated A–G and included a random sample of normoglycemic control as well as diabetic individuals. The plasmas were freed of glucose and separate aliquots incubated for 4 h at 37°C in the absence of a reducing agent, in the presence of 12 mM $NaCNBH_3$, or in the presence of 10 mM $NaBH_4$. A nonreduced, $NaCNBH_3$-reduced, and $NaBH_4$-reduced plasma sample from each individual was added as competitor to the five different RIA. The antigen used for coating the wells was glc_{RED}-LDL (48.7% of lysines glucosylated). Antibodies G8H6, G5E10, G8C11, G6C9, and G5H3 were used at dilutions of 1:400, 1:900, 1:800, 1:1,000, and 1:1,000, respectively. The moles of glucitollysine residues per milligram of plasma protein were obtained by reference to a standard curve with glucitollysine as competitor, and therefore should be considered only a relative rather than an absolute measure of the extent of glucosylation. The estimates for the Schiff base form represent the minimum amount of this adduct, since a portion of the glucose could have dissociated during chromatography.

antibodies differed, each antibody was capable of identifying at least two- to threefold increases in the moles of glucitollysine residues present in the diabetic plasmas relative to normal plasmas.

To demonstrate that the antibodies also could recognize glucitollysine residues in isolated lipoprotein fractions, we isolated very low density lipoproteins (VLDL) plus intermediate density lipoproteins (IDL), LDL, and HDL fractions from seven normal and 13 diabetic plasmas, and measured their content of glucitollysine with antibody G6C9 (Table IV). The mean content of glucitollysine was almost 10-fold higher in the total plasma protein of these diabetics, although their HbA_{1C} was increased only 2.5-fold. There was an approximate three- to fourfold increase in glucitollysine content of diabetic LDL and HDL preparations. Of particular surprise, was the eightfold increase in glucitollysine residues in the isolated VLDL plus IDL fraction of the diabetics. Including normals and diabetics, the correlation coefficient between the plasma glucose level and total plasma glucitollysine content was $r = 0.834$ ($P < 0.001$), and of HbA_{1C} and total glucitollysine content was $r = 0.824$ ($P < 0.001$). The correlation between HbA_{1C} and plasma glucose was $r = 0.929$ ($P < 0.001$). There were also significant correlations between plasma glucose and glucosylated VLDL plus IDL, and plasma glucose and glucosylated HDL of $r = 0.703$ ($P < 0.001$) and 0.818 ($P < 0.001$), respectively.

DISCUSSION

Since the possibility exists that nonenzymatic glucosylation of LDL could significantly alter the metabolism of this lipoprotein through interference with receptor-mediated endocytosis, we sought an immunochemical method that would permit identification and quantitation of human glc-LDL. We therefore wished to generate monoclonal antibodies that would recognize glc-LDL and not native LDL. A conventional approach would have been to immunize mice with human glc-LDL, anticipating that among the population of antibody-producing cells, some would be specific for glc-LDL. However, this selection could be accomplished only after fusion and differential analysis of antibodies produced by hybridomas initially cloned at limiting dilution. Our previous experience in generating human LDL-specific murine monoclonal antibodies provided evidence that human LDL was a potent immunogen in Balb/c mice (20), and thus the majority of hybridomas could be expected to synthesize antibodies to epitopes of native LDL. Identification of hybridoma antibodies to an epitope specific for glucosylation would be arduous. However, if the immune response was limited only to epitopes on LDL that were specific for glucosylation, a high percentage of the desired hybridomas could be anticipated. To restrict the primary immune response, we used homologous murine glc_{RED}-LDL as primary and secondary immunogens, with the rationale that only the mod-

TABLE III
Glucitollysine Residues in $NaBH_4$-Reduced Plasmas

Plasma source	Plasma glucose	HbA_{1c}	Glucitollysine residues*				
			G5H3	G6C9	G8C11	G5E10	G8H6
	mg/dl	%	*pmol/mg protein*				
Normal individuals (n = 5)	79±11	6.8±0.4	<22	53±8	36±6	25±3	24±4
Diabetics (n = 10)	245±113‡	11.2±2.0‡	54±17‡	156±53‡	110±64§	60±28§	41±22

* Fasting plasmas, freed of glucose by chromatography, were incubated for 4 h at 37°C in the presence of 10 mM $NaBH_4$. In preliminary experiments, it was demonstrated that this concentration of $NaBH_4$ was optimal for reducing between 0.2 and 20 mg/ml protein. Total plasma proteins were quantitated after dialysis against PBS, pH 7.4, and added to RIA using the five antibodies shown. Glc_{RED}-LDL (48.7% of lysines glucosylated) was used for antigen coating and glucitollysine was used as the standard. For each plasma, the mean of quadruplicate measurements was used to calculate a mean±SD for each group.
‡ $P < 0.006$, diabetic vs. normal.
§ $P < 0.025$, diabetic vs. normal.

ified portions of homologous LDL would be immunogenic. To propagate specifically in vivo those clones of cells making antibodies against murine glc_{RED}-LDL that also cross-reacted with human glc_{RED}-LDL, we used human glc_{RED}-LDL for the final intravenous immunization 4 d before fusion. This short time interval was sufficient to allow proliferation of specific memory B cell clones, but insufficient to allow significant primary stimulation and proliferation of clones recognizing epitopes on native human LDL. Thus, from a single fusion, we obtained a large number of hybridomas that secreted antibody capable of binding human glc_{RED}-LDL. 94% of these did not produce antibody reactive with normal human LDL. We should also emphasize that this experimental strategy produced specific murine antiserum as well. In a similar manner, immunization of guinea pigs with guinea pig glc_{RED}-LDL (or with other homologous glucosylated proteins) produced antisera that were specific for glc_{RED}-protein adducts (27).

The elicited hybridoma antibodies were not specific for glc_{RED}-LDL, since they also were capable of binding to reduced glucosylated derivatives of a variety of other proteins, including HDL, albumin, hemoglobin, and transferrin. However, several lines of evidence suggested that each of these monoclonal antibodies was specific for a novel epitope that resulted directly from the covalent attachment of glucose. First, no reactions were observed with nonglucosylated human proteins, including LDL, HDL, albumin, hemoglobin, and transferrin. Second, LDL incubated with $NaCNBH_3$ in the absence of glucose was devoid of epitopes recognized by any of the antibodies. Third, the number of glucosylation-derived epitopes on glc_{RED}-LDL obtained with each of the antibodies was directly proportional to the percentage of lysine residues of LDL that had been glucosylated. Finally, the binding of each of the antibodies to glc_{RED}-LDL was completely inhibited by glucitollysine, the only demonstrable glucosylated amino acid residue of LDL (4).

In spite of the observation that glucitollysine completely inhibited the binding of each of the antibodies to glc_{RED}-LDL, there was reasonable evidence that the individual specificities of the antibody-combining sites for antigen were not strictly identical. Evidence for differences in the antigen-binding sites of different hybridoma antibodies was obtained by comparing the ability of single amino acids and hexoses to inhibit binding. Whereas it can not be strictly determined that the ability of these compounds to inhibit antibody binding resulted only from their ability to occupy the antigen-binding sites of the antibodies, the observation that the degree of inhibition of individual antibodies differed from one another with respect to both amino acids and sugars suggested that this may be true. With this in mind, we were able to identify subtle differences in each of the antibody sites. For example, sorbitol and mannitol, but not glucose or mannose, could completely inhibit the binding of antibody G6C9. Therefore, the open-chain hexose portion of glucitollysine played a major role in the antigenic determinant of this antibody. This was the same antibody that had comparable reactivity for each of the glc_{RED}-proteins (Fig. 4) and suggested that the local environment may have less influence on the ability of this antibody to bind glucitollysine residues. In contrast, neither sorbitol nor mannitol inhibited the binding of antibodies G5E10, G8C11, or G5H3, but high concentrations of lysine or arginine did. Therefore, the primary amino acid sequence of the protein adjacent to the modified lysine may be more important in the antigen-combining sites of these antibodies.

TABLE IV

Glucitollysine Residues in $NaBH_4$-reduced Plasmas and Isolated Lipoproteins Using Antibody G6C9

Plasma source	Plasma glucose	HbA_{1C}	Glucitollysine residues*			
			Plasma	VLDL + IDL	LDL	HDL
	mg/dl	%	*pmol/mg protein*			
Normal						
6	67	3.9	9	53	<47‡	40
7	91	6.8	28	168	<41	36
8	69	5.6	8	200	<24	39
9	74	5.2	24	ND§	<34	49
10	75	5.2	35	107	<39	52
11	66	5.9	19	ND	<29	39
12	86	5.9	32	ND	<26	57
Mean±SD	75±10	5.5±.9	22±11	132±65	<47	45±8
Diabetic						
11	222	10.1	196	268	67	101
12	286	14.4	285	1,450	118	133
13	314	12.5	181	2,323	53	96
14	275	15.3	92	969	53	241
15	238	8.5	56	241	193	87
16	303	13.9	136	724	243	142
17	245	15.8	201	1,304	65	172
18	299	13.9	432	2,088	136	254
19	271	11.3	174	1,173	190	155
20	320	15.5	320	794	106	381
21	276	15.4	329	1,049	102	211
22	396	16.3	432	1,001	180	361
23	110	—‖	49	222	43	67
Mean±SD	273±66¶	13.5±2.5¶	222±129¶	1,047±650**	169±65¶	186±102¶

* Plasma was collected in EDTA and immediately kept at 4°C. Free glucose was removed by chromatography on Sephadex G-25 and lipoproteins isolated by sequential flotation. Fractions isolated included VLDL plus IDL (d < 1.019 gm/ml), LDL (d = 1.019–1.063 g/ml), and HDL (d = 1.063–1.21 gm/ml). As judged by SDS-polyacrylamide gel electrophoresis, the LDL and HDL fractions contained <2% contaminating albumin, whereas the VLDL plus IDL fractions had <7% contamination. Plasma and isolated lipoproteins were incubated with 10 mM $NaBH_4$ for 4 h at 37°C, dialyzed against PBS and 0.025 ml (1–13 mg/ml protein) added to the RIA. Antibody C6C9 was used at 1:4,000, the glucitollysine standard at 10–0.05 nmol/ml and glc_{RED}-LDL (48.7% of lysines glucosylated) for antigen coating at 9 μg/ml.

‡ For all seven normal LDL fractions assayed, no competition was observed at the level of protein added (1.70–3.28 mg/ml). Data given were calculated as the maximal number of glucitollysine residues that could have been detected. For statistical purposes, all normals were considered to have 47 pmol glucitollysine/mg protein.

§ ND, not detectable; no binding was detected at the level of protein added.

‖ Not assayed.

¶ $P < 0.002$, diabetic vs. normal.

** $P < 0.02$, diabetic vs. normal.

Posttranslational nonenzymatic glucosylation of the lysine residues of protein initially involves the attachment of glucose to the epsilon amino group of lysine or the free alpha amino group of *N*-terminal amino acids to form a Schiff base (24, 25). In the absence of a reducing agent, as presumably occurs in vivo, the Schiff base undergoes an Amadori rearrangement to yield a ketoamine, which in turn is in equilibrium with a hemiketal form (12). In this study, these products of nonreductive glucosylation have been referred to as glc_{NR}-protein adducts. None of the monoclonal antibodies selected for detailed analyses bound glc_{NR}-LDL (Table I) or the glc_{NR}-adducts of HDL, albumin, hemoglobin, or transferrin (data not shown), even though ~6% of the lysine residues of each of these proteins contained covalently attached glucose. In contrast, glc_{RED}-LDL that contained only 3.3% of the lysine residues glucosylated did compete (Fig. 3). As predicted, nonreduced plasmas containing ketoamine and hemiketal Amadori protein adducts had little reac-

tivity with these antibodies (Fig. 6). However, as demonstrated in this report, these plasma protein Amadori adducts can be measured immunochemically by incubating plasma with $NaBH_4$ (after removal of free glucose) and by reducing the ketoamine and hemiketal adducts to glucitollysine, which these antibodies specificially recognize. The Schiff base forms also can be measured by incubating plasma with $NaCNBH_3$, which converts only the Schiff base to glucitollysine (25).

HbA_{1C} measurements have been useful for both research applications and for the management of patients with diabetes mellitus (12–16). More recently, chromatographic and colorometric measurements of plasma or urine proteins have demonstrated that nonenzymatically glycosylated protein levels are approximately two to three times higher in diabetic than in nondiabetic subjects, and suggest that the extent of glycosylation of total plasma or urine proteins is a good measure of short-term glycemia in diabetics (15, 28). In this report, we have demonstrated that glycosylated plasma proteins can also be identified and quantitated immunochemically.

Previous estimates of the amount of glucose bound to total plasma proteins in diabetics have been in the range of 500–2,000 picomol/mg protein (16, 19, 29), whereas our five antibodies measured means of between 44 and 156 picomol/mg protein. Thus, the levels of glucitollysine quantitated in our studies must be considered relative rather than absolute. However, each of the antibodies tested was capable of quantitating a two- to fourfold increase in the moles of glucitollysine residues in diabetic compared with nondiabetic plasmas (Table III). In the limited survey of diabetics shown in Table IV, antibody G6C9 saw up to 10-fold higher levels of glucitollysines in diabetic plasmas. Glucosylated albumin probably constitutes the bulk of glucosylated protein in whole plasma, and this antibody identifies glucitollysine residues on albumin less well than other proteins tested (Fig. 5). Thus, measurements made with this antibody could preferentially reflect glucitollysine content of more rapidly turning over proteins and thus may be more sensitive to acute changes in glucose level. Correlative clinical studies will be needed to test this hypothesis. Efforts are currently underway to optimize assay conditions to allow a more quantitative assessment of glucitollysine content, e.g., pretreatment of plasma with agents to unfold protein may expose more glucosylated epitopes.

Antibody G6C9 was able to detect two- to fourfold increases in the degree of glucosylation of LDL and HDL, plasma lipoproteins with half-lives considerably shorter than that of hemoglobin (Table IV). In addition, the levels of glucitollysine appeared to be almost eightfold higher in the VLDL-plus-IDL fraction of diabetic plasmas compared with normals. This finding was surprising, since the half-lives of these lipoproteins are considerably shorter than those of LDL and HDL. The sites of glucosylation in VLDL are not known, but it seems reasonable that in part this represents glucosylation of apoprotein E and apoprotein C, apoproteins that are known to transfer between VLDL and HDL. Whether nonenzymatic glucosylation of VLDL and IDL interferes with their normal metabolism and plays a role in the hypertriglyceridemia characteristic of the diabetic state is currently under study.

An immunoassay for glucosylated proteins and lipoproteins using monoclonal antibodies offers a number of advantages including sensitivity, specificity, and the ability to handle a large number of samples. In addition, as outlined in this report, the present hybridoma antibodies allow the potential to differentiate between Schiff base forms and the Amadori rearrangement products. Although these antibodies are capable of identifying and quantitating glucitollysine residues in plasma, they are not specific for glucosylated LDL or any other glucosylated protein when used as outlined in this study. The RIA described here can be used to quantitate glucositollysine residues on any isolated protein but the isolation of particular proteins may be infeasible or too time consuming for routine investigations. However, preliminary studies using these antibodies in tandem immunoassays indicate that these antibodies can be used to quantitate directly, without prior isolation, the glucitollysine content of any protein of interest and suggest that these antibodies will prove very useful for identifying and quantitating all nonenzymatically glucosylated plasma and structural proteins that have been implicated in the pathologic sequelae of diabetes.

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