Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2757–2761, May 1983
Medical Sciences

Nonenzymatic glucosylation of homologous low density lipoprotein and albumin renders them immunogenic in the guinea pig

(glucosylated low density lipoprotein/autoantibodies/diabetes)

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Communicated by Daniel Steinberg, January 24, 1983

ABSTRACT **Nonenzymatic glucosylation of low density lipoprotein (LDL) and other plasma and structural proteins is enhanced in diabetics. Because conjugated carbohydrates are known to play an important role in the immunogenicity of proteins, we sought to determine if glucosylation of LDL (yielding Glc-LDL) and albumin would make them immunogenic. Therefore, we immunized guinea pigs with homologous glucosylated proteins and measured antibody response by solid-phase radioimmunoassay. Glucosylation of LDL in the presence of cyanoborohydride yields glucitol-lysine as the glucose adduct. Immunization with this Glc-LDL yielded a high-titered antiserum that reacted specifically with guinea pig Glc-LDL but not native LDL. Glucitol-lysine was an effective competitor for binding to the antibody, as were other reductively glucosylated human proteins. Glucosylation of LDL by incubation with glucose in the absence of a reducing agent yields fructosyllysine as the glucose adduct. This product, which has been demonstrated in human plasma, was also immunogenic, though the antiserum produced was of lower titer and affinity. Homologous glucosylated albumin was also immunogenic. These data suggest that nonenzymatic glucosylation of proteins could lead to autoantibody production and the formation of immune complexes in diabetic plasma and tissues.**

We have previously shown that nonenzymatic glucosylation of low density lipoprotein (LDL) blocks its recognition by the LDL receptor (1) and that in guinea pigs and rabbits the clearance of homologous glucosylated LDL (Glc-LDL) was much slower than that of native LDL (2). We also found that the clearance of autologous Glc-LDL in euglycemic human subjects was monoexponential and only 20% of that of native LDL (3). However, in several diabetic subjects we observed unusual biophasic decay curves for Glc-LDL. After injection of ^{125}I-labeled Glc-LDL tracer, there was an initial period of monoexponential decay, followed by a sudden break in the decay curve 4–8 days after injection, with subsequent rapid clearance from plasma of the Glc-LDL tracer. This suggested the possibility of an immunological basis for the observed phenomenon. Because conjugated carbohydrates are known to play an important role in the immunogenicity of proteins we sought to determine if the covalent addition of glucose to LDL would render it immunogenic. In this report we document that nonenzymatic glucosylation of homologous LDL and albumin renders them immunogenic in the guinea pig.

METHODS

LDL (guinea pig or human) was isolated between densities 1.019 and 1.063 g/ml (1) and was free of all but trace amounts of albumin as judged by NaDodSO$_4$/polyacrylamide gel electrophoresis. Glc-LDL was prepared as described (1, 2) by incubating LDL with 80 mM glucose in the presence (or absence) of sodium cyanoborohydride ($NaCNBH_3$) at 12.5 mg/ml for 7 days. In this report the product of the glucosylation of LDL in the presence of the reducing agent $NaCNBH_3$ is termed "Glc$_{RED}$-LDL," whereas the products of glucosylation in the absence of the reducing agent are termed nonreduced (NR) glucosylated LDL, "Glc$_{NR}$-LDL" (see Fig. 1). All preparations of Glc-LDL were extensively dialyzed and therefore contained none of the labile Schiff base intermediate. Control LDL (cLDL) was incubated with $NaCNBH_3$ but without glucose. After incubation, each lipoprotein was exhaustively dialyzed against phosphate-buffered saline, pH 7.4, containing 0.01% EDTA. Guinea pig albumin (United States Biochemical, Cleveland, OH) was purified on Sephacryl S-300 (Pharmacia) and gave a single band on NaDodSO$_4$/polyacrylamide gel electrophoresis. Guinea pig albumin as well as human albumin, hemoglobin, and transferrin (Sigma) were glucosylated in 80 mM glucose in the presence or absence of cyanoborohydride. Extent of glucosylation was determined by amino acid analysis after reduction with sodium borohydride ($NaBH_4$) (1).

Male Hartley guinea pigs (Charles River Breeding Laboratories) were immunized with guinea pig Glc-LDL, cLDL, Glc-albumin or control albumin. For each preparation, 125 μg of protein, emulsified in complete Freund's adjuvant, was injected into footpads and intradermally into multiple sites. On days 14 and 28 animals received subcutaneous booster injections of 125 μg of protein in Freund's incomplete adjuvant. On day 38 blood was obtained for immunological studies or animals were used for turnover studies described below.

To document the titer and specificity of antibodies, we established solid-phase radioimmunoassays, using techniques similar to those described by Curtiss *et al.* (4). To titer the antisera, we coated 96-well polyvinylchloride microtiter plates (Dynatech Laboratories, Alexandria, VA) with 50 ng of antigen per well. For example, for guinea pig Glc-LDL, this was achieved by incubating 500 ng of Glc-LDL dissolved in 50 μl of phosphate-buffered saline/EDTA for 2 hr at 37°C. Each well was then aspirated and washed four times with buffer A (phosphate-buffered saline/0.01% EDTA/0.02% NaN_3/0.05% Tween 20/0.1% bovine serum albumin/0.001% Trasylol). To block all remaining active sites on the plastic, each plate was then incubated with buffer B (phosphate-buffered saline/0.01% EDTA/3% normal goat serum/3% bovine serum albumin) at room temperature for 30 min and then aspirated to dryness. Plates were stored at −20°C and could be used for several months without apparent loss of antigenicity of the protein coating the wells. To determine the antibody titer of various guinea pig

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Abbreviations: LDL, low density lipoprotein; Glc-LDL, glucosylated LDL; Glc$_{RED}$-LDL, Glc-LDL formed in the presence of a reducing agent; Glc$_{NR}$-LDL, nonreduced Glc-LDL; cLDL, control LDL; GAGPIgG, goat anti-guinea pig IgG.

antisera, 50 μl of antiserum at various dilutions was added to each microtiter well and the plates were incubated for 18 hr at 4°C. The serum was aspirated from each well, and the wells were washed four times with buffer A. To quantitate the amount of guinea pig IgG bound, we prepared ^{125}I-labeled goat anti-guinea pig IgG (^{125}I-GAGPIgG). The GAGPIgG (United States Biochemical) was purified by DEAE-cellulose chromatography and iodinated by solid-phase lactoperoxidase (Enzymobeads, Bio-Rad). Fifty microliters of ^{125}I-GAGPIgG (approximately 0.5 μg/ml and 2.0–3.5 × 10^5 cpm) was added to each well and the plates were incubated for 4 hr at 4°C. The liquid was then aspirated from each well, and the wells were washed four times with buffer A and dried. Individual wells were isolated and their radioactivities were measured on an LKB 1275 Minigamma spectrometer. For this report a titer is defined as that dilution of antiserum that yields ^{125}I-GAGPIgG binding 3 times greater than that of nonimmune serum. Competitive inhibition studies were performed by incubating fixed and limiting dilutions of antiserum (25 μl) in each well simultaneously with various doses of competitor (25 μl). All competitive incubations were for 18 hr at 4°C and then the amount of first antibody bound was quantitated as described above.

To determine the effect of immunizations on turnover of the various immunogens, we prepared ^{125}I-labeled Glc-LDL (^{125}I-Glc-LDL) and ^{131}I-labeled cLDL (^{131}I-cLDL) as described (1, 2). Iodinated control and Glc-albumin were prepared in a similar manner. The turnover of the various tracers in control and immunized guinea pigs was determined as described (1, 2).

RESULTS

The reaction scheme for the glucosylation of LDL is shown in Fig. 1. When glucose is incubated with LDL, it initially forms a Schiff base with the ε-amino groups of lysine. Although glucose will also react with free NH_2-terminal amino groups, we could find no evidence for such a reaction with LDL (1). In the absence of a reducing agent, this intermediate undergoes an Amadori rearrangement to yield a stable ketoamine, which in turn is in equilibrium with the hemiketal ring form, 1-deoxyfructosyllysine (5). However, in the presence of a reducing agent, the Schiff base intermediate is quantitatively reduced to give a glucitol-lysine adduct (6). Thus the reduced form of Glc-LDL is not chemically equivalent to the nonreduced, Amadori rearrangement products. Only the latter, however, are believed to occur *in vivo* (5, 6). We will designate the reduced form Glc_{RED}-LDL and the nonreduced Amadori rearrangement forms Glc_{NR}-LDL.

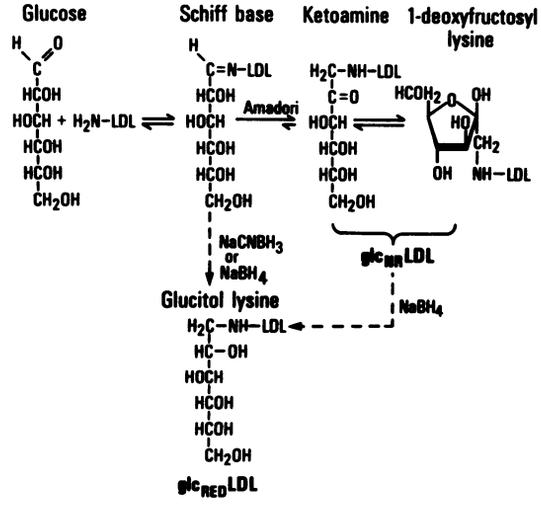

FIG. 1. Reaction scheme for the nonenzymatic glucosylation of LDL, showing the products obtained after reduction with $NaCNBH_3$ or $NaBH_4$. Glc_{RED}-LDL represents the product obtained when nonenzymatic glucosylation of LDL occurs in the presence of a reducing agent; Glc_{NR}-LDL refers to products of the glucosylation that occur when LDL is incubated with glucose alone. It should be pointed out that when Glc_{NR}-LDL is reduced with $NaBH_4$, in addition to glucitol-lysine as a product, mannositol-lysine can also form because the C-2 carbon can epimerize during the reduction reaction. However, $NaCNBH_3$ reduction of the Schiff base should yield only glucitol-lysine.

Immune Response to Glc_{RED}-LDL. In initial experiments, we immunized guinea pigs with a Glc_{RED}-LDL preparation in which 61% of the lysines were derivatized as glucitol-lysine. With this immunogen a high titer (1:130,000) of anti-Glc-LDL antibodies was found in the plasmas of immunized guinea pigs (Fig. 2). In a guinea pig immunized with control LDL no antibody was demonstrable. The potency of Glc_{RED}-LDL as an immunogen was shown by the fact that a 1:800 titer of anti-Glc-LDL antibodies was demonstrable in the plasma of a guinea pig 5 days after the intravenous injection of only 100 μg of Glc_{RED}-LDL, the tracer amount used in metabolic studies (Fig. 2). To define the specificity of these antisera, a 1:2,000 dilution of one antiserum, no. 14, was added to microtiter wells coated with guinea pig Glc_{RED}-LDL and then increasing amounts of guinea pig Glc_{RED}-LDL and other compounds were tested for their ability to compete for antibody binding. As expected, guinea pig Glc_{RED}-LDL was an efficient competitor for the antibody. On the other hand, native guinea pig LDL, free glucose, or free glucitol was not capable of competing, even when added in large excess. However, glucitol-lysine was a specific and highly effective competitor (data not shown). The observation that glucitol-lysine was an important part of the epitope recognized by antiserum no. 14 suggested that other reduced glucosylated proteins might also react with this antiserum. As shown in Fig. 3, human LDL, albumin, hemoglobin, and transferrin that had been glucosylated in the presence of cyanoborohydride all reacted.

To determine if the presence of antibodies in the immunized guinea pigs would lead to immune clearance, equal amounts of homologous ^{125}I-Glc_{RED}-LDL and ^{131}I-cLDL were mixed and injected intravenously into immunized guinea pigs. In animals immunized with cLDL, the turnovers of both the control and

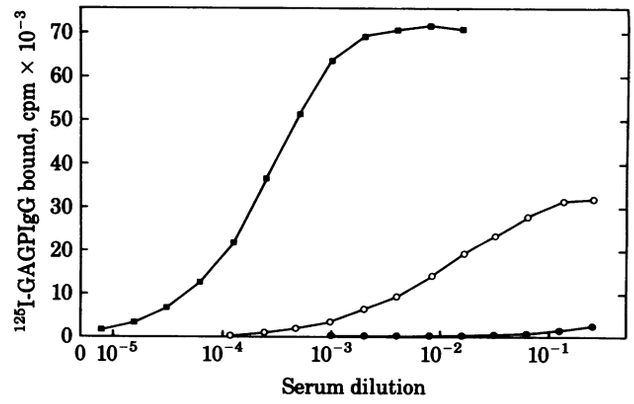

FIG. 2. Titer of anti-Glc_{RED}-LDL antibodies. Fifty nanograms of guinea pig Glc_{RED}-LDL (61% lysines glucosylated) was attached to each well and 50 μl of various dilutions of guinea pig serum was added. After 18-hr incubation at 4°C the amount of guinea pig IgG bound was quantitated by using ^{125}I-GAGPIgG. ●, Serum from a guinea pig immunized with control guinea pig LDL; ■, serum obtained from guinea pig no. 14 immunized with guinea pig Glc_{RED}-LDL; ○, serum from a guinea pig 5 days after intravenous injection of 100 μg of Glc_{RED}-LDL. Each point is the mean of triplicate determinations.

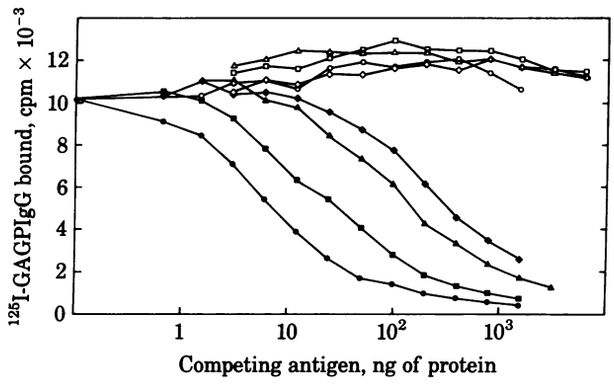

FIG. 3. Competition of guinea pig Glc_{RED}-LDL versus glucosylated human proteins. Antiserum no. 14, at a final dilution of 1:2,000, was added to wells coated with 50 ng per well of Glc_{RED}-LDL in the presence of the indicated quantity of the following proteins. The percent lysines glucosylated are given in parenthesis after each protein. ●, Human Glc_{RED}-LDL (49.5%); ■, human Glc_{RED}-hemoglobin (85%); ▲, human Glc_{RED}-transferrin (51.4%); ◆, human Glc_{RED}-albumin (57.1%); ○, human Glc_{NR}-LDL (6.2%); □, human Glc_{NR}-hemoglobin (6.4%); △, human Glc_{NR}-transferrin (5.7%); ◇, human Glc_{NR}-albumin (6.1%). In each case the Glc_{RED}-protein is protein glucosylated in the presence of cyanoborohydride and Glc_{NR}-protein is the protein glucosylated in the absence of cyanoborohydride. Each point is the mean of triplicate determinations.

Glc_{RED}-LDL tracers were similar to turnovers in normal, nonimmunized animals (Fig. 4*A*). In animals immunized with Glc_{RED}-LDL, the turnover of cLDL remained unchanged, but beginning immediately after injection there was rapid clearance from plasma of the ^{125}I-Glc_{RED}-LDL, so that 92% had disappeared within the first hour (Fig. 4*B*). When human ^{125}I-Glc_{RED}-LDL and human ^{131}I-cLCL were injected into a guinea pig immunized with homologous Glc_{RED}-LDL, the turnover of the human control LDL was similar to that observed in nonimmunized guinea pigs, but the decay of the human Glc_{RED}-LDL was again extremely rapid (Fig. 4*C*). Finally, when guinea pig control and Glc_{RED}-albumin tracers were injected into a guinea pig immunized with Glc_{RED}-LDL, there was rapid clearance of the Glc_{RED}-albumin tracer, whereas the turnover of the control albumin was similar to that found in nonimmunized animals (Fig. 4*D*).

The Amadori rearrangement forms of glucosylated LDL (Glc_{NR}-LDL) are more likely to occur *in vivo* than Glc_{RED}-LDL. We tested the reactivity of Glc_{NR}-LDL with antiserum no. 14 in terms of its ability to compete with Glc_{RED}-LDL for antibody binding (Fig. 5). Although this Glc_{NR}-LDL (prepared by incubation of LDL and glucose in the absence of $NaCNBH_3$) had 5.6% of its lysines glucosylated, no competition was seen even at high concentrations. Even though antiserum no. 14 was produced with a Glc_{RED}-LDL preparation in which 61% of its lysines were derivatized, it does fully recognize a Glc_{RED}-LDL preparation in which only 5% of its lysines are present as glucitol-lysine (data not shown). Therefore, this antiserum is specific to the glucitol-lysine moiety and does not recognize glucose conjugated to protein in the Amadori rearrangement forms present in Glc_{NR}-LDL (see Fig. 1). However, when the same Glc_{NR}-LDL preparation was incubated with $NaBH_4$, which re-

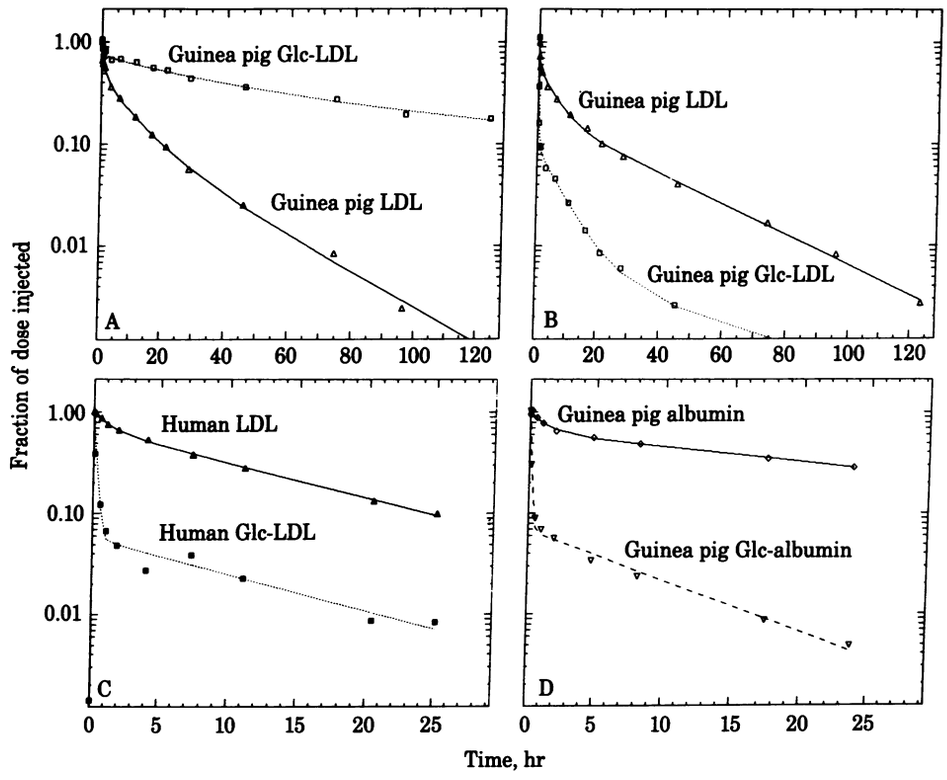

FIG. 4. Turnover of glucosylated and control proteins in guinea pigs immunized with various proteins. For each animal shown a simultaneous injection of the two indicated tracers was made and serial determinations of plasma radioactivity were made over the time periods indicated (note different time scales for each panel). (*A*) Turnover of guinea pig control and Glc_{RED}-LDL in a guinea pig immunized with control guinea pig LDL. The turnover of both tracers is similar to that observed in nonimmunized animals (1, 2). (*B*) Turnover of the same tracers in an animal immunized with homologous Glc_{RED}-LDL. (*C*) Turnover of human control and Glc_{RED}-LDL in a guinea pig immunized with guinea pig Glc_{RED}-LDL. The turnover of human control LDL is similar to that in nonimmunized animals (1, 2), whereas clearance of Glc_{RED}-LDL is very rapid. (*D*) Turnover of homologous control and Glc_{RED}-albumin in a guinea pig immunized with guinea pig Glc_{RED}-LDL. Turnover of control albumin is similar to that in nonimmunized guinea pig, whereas that of Glc_{RED}-albumin is extremely rapid.

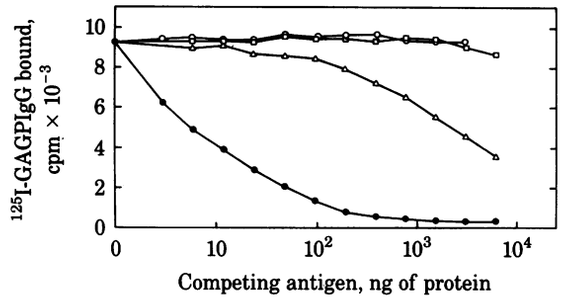

FIG. 5. Competition of Glc_{RED}-LDL with other glucosylated LDL preparations. Conditions for assay are the same as described in the legend to Fig. 3. ●, Guinea pig Glc_{RED}-LDL (61% lysines glucosylated); ○, guinea pig Glc_{NR}-LDL (5.6% lysines glucosylated); □, same guinea pig Glc_{NR}-LDL after incubation with $NaCNBH_3$; △, same guinea pig Glc_{NR}-LDL after incubation with $NaBH_4$. Each point represents the mean of triplicate determinations.

duces the Amadori rearrangement forms to yield glucitol-lysine, it developed antigenic sites of identity with Glc_{RED}-LDL and competed to the degree expected for a preparation containing 5.6% of its lysines as glucitol-lysine (Fig. 5). In contrast, when Glc_{NR}-LDL was incubated with $NaCNBH_3$, which will *not* reduce the Amadori rearrangement forms, it remained nonreactive (Fig. 5). (It should be noted that the Glc_{NR}-LDL preparation contained no labile Schiff base form because it had been extensively dialyzed.) In a similar manner, human LDL, hemoglobin, albumin, and transferrin, glucosylated by using glucose alone, failed to bind to antiserum no. 14, despite the fact that 6% of their lysines contained glucose conjugated in the Amadori rearrangement forms (Fig. 3).

Immune Response to Glc_{NR}-LDL. Clearly, Glc_{RED}-LDL is immunogenic. To determine if the Amadori rearrangement forms (Glc_{NR}-LDL) that occur *in vivo* are also immunogenic, we immunized guinea pigs with guinea pig Glc_{NR}-LDL (5.6% lysines glucosylated). Fig. 6*A* shows the level of antibody binding to Glc_{NR}-LDL before immunization and 7 days after a second and third booster. The titer of antibodies produced was lower (1:512) than when Glc_{RED}-LDL was the immunogen. In competition studies Glc_{NR}-LDL competed for over 80% of antibody binding, demonstrating the specificity of the antiserum (Fig. 6*B*). Partial crossreactivity was seen with guinea pig Glc_{RED}-LDL, and at high concentrations native guinea pig LDL competed slightly, presumably reflecting the presence of a small amount of Glc_{NR}-LDL (2% of lysines were glucosylated in the native preparation).

To determine if the low titer of anti-Glc_{NR}-LDL antibodies would result in immune clearance of Glc_{NR}-LDL, we injected guinea pig ^{125}I-Glc_{NR}-LDL and ^{131}I-cLDL into a guinea pig immunized with Glc_{NR}-LDL. In a nonimmunized guinea pig, the turnover of Glc_{NR}-LDL is 20–30% slower than that of cLDL. This was also true in the immunized guinea pig, suggesting that the presence of these low-titer anti-Glc_{NR}-LDL antibodies did not result in enhanced immune clearance of this tracer.

Immune Response to Glc-Albumin. In other studies, we demonstrated that guinea pig Glc-albumin (prepared in the presence of $NaCNBH_3$) was also an effective immunogen in the guinea pig. A moderately high-titered antiserum (1:32,000) reacted specifically with Glc_{RED}-albumin but, of interest, did not crossreact with guinea pig Glc_{RED}-LDL. The presence of these antibodies also did not lead to immune clearance. In animals immunized with either control or Glc_{RED}-albumin, the turnover of both ^{125}I-labeled control albumin and ^{131}I-labeled Glc_{RED}-albumin was the same.

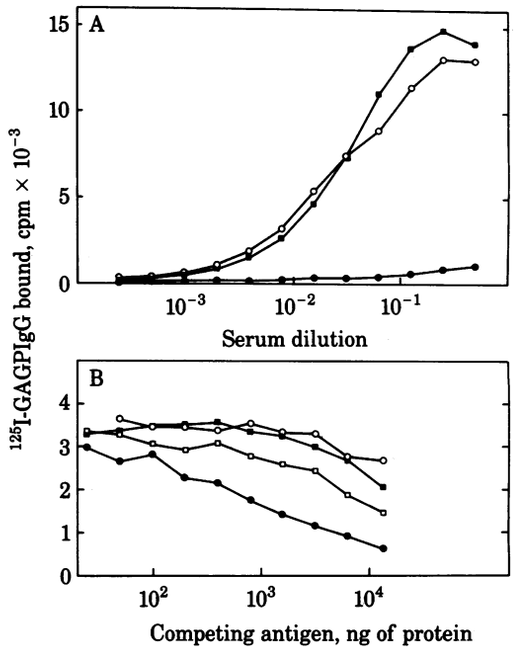

FIG. 6. (*A*) Titer of anti-guinea pig Glc_{NR}-LDL antibodies. Guinea pig no. 19 was immunized with guinea pig Glc_{NR}-LDL (5.6% lysines glucosylated) and boosters were given at 2-week intervals. Titers of antibodies were determined as described in the legend to Fig. 1 except Glc_{NR}-LDL (5.6% lysines derivatized) was bound to plastic wells. ●, Preimmune plasma; ○, plasma obtained 7 days after the second booster; □, plasma obtained 7 days after the third booster. (*B*) Competition of Glc_{NR}-LDL versus other guinea pig proteins. Antiserum no. 19 at a final dilution of 1:100 was added to wells containing 50 ng per well of guinea pig Glc_{NR}-LDL in the presence of the indicated concentrations of the following proteins: ●, guinea pig Glc_{NR}-LDL; □, guinea pig Glc_{RED}-LDL; ■, guinea pig native LDL (contains 2% lysines glucosylated); and ○, whole guinea pig plasma. Points in both *A* and *B* are the average of duplicate determinations.

DISCUSSION

Posttranslational, nonenzymatic glucosylation of hemoglobin (7) as well as a variety of plasma and structural proteins has been shown to occur in normoglycemic and to a much greater extent in hyperglycemic diabetic subjects (1, 5–8). In this reaction, glucose forms a Schiff base intermediate with ε-amino groups of lysine, or free amino groups of NH_2-terminal amino acids. The Schiff base intermediate undergoes an Amadori rearrangement to form a ketoamine and subsequent hemiketal formation. For some proteins even further, more complicated, rearrangements have been proposed (8). In preliminary studies, we found that 1–2% of lysines of LDL isolated from normoglycemic individuals, and 2–6% of lysines of LDL isolated from diabetic subjects were glucosylated (1). In this report, we demonstrate that such posttranslational, nonenzymatic modifications of autologous LDL and albumin render these proteins immunogenic in the guinea pig. When Glc_{RED}-LDL, which contained 61% of its lysines as a glucitol-lysine adduct, was the immunogen, a high titer of antibodies to the modified LDL occurred. This guinea pig antiserum did not crossreact with native guinea pig LDL, reflecting tolerance to the unmodified epitopes of the homologous protein. To our knowledge there is no reductase *in vivo* capable of reducing the Schiff base form of the glycosylation reaction. Thus the reductive glucosylation of LDL generated novel antigenic sites and led to the production of high titers of region-specific antibodies.

These antibodies crossreacted with other reductively glucosylated proteins, suggesting that glucitol-lysine itself is a crit-

ical component of the antigenic region. This was confirmed by competition studies showing that glucitol-lysine effectively competed with Glc_{RED}-LDL for antibody binding. Antiserum produced against Glc_{RED}-LDL, however, did not react with any of the proteins glucosylated by incubation with glucose alone, in which the glucose was conjugated to protein in the Amadori rearrangement forms—i.e., a ketoamine or hemiketal form. Cyanoborohydride selectively reduces only the Schiff base and will not reduce Glc_{NR}-LDL. In fact, when Glc_{NR}-LDL was incubated with $NaCNBH_3$ reactivity of Glc_{NR}-LDL with antiserum no. 14 was not changed. However, when Glc_{NR}-LDL was reduced with $NaBH_4$, a nonselective reducing agent that will reduce the Amadori rearrangement forms to glucitol-lysine, the Glc_{NR}-LDL was converted to a form capable of competing against Glc_{RED}-LDL. This latter observation confirms the specificity of antiserum no. 14 against glucitol-lysine adducts and suggests that such an antiserum might be useful in quantitating either the Schiff base form of glucosylated proteins (after reduction with $NaCNBH_3$) or total glucosylated protein (after reduction with $NaBH_4$). Indeed, using an approach similar to that described above, we have generated monoclonal antibodies capable of making such measurements (9).

Glc_{NR}-LDL, produced by incubation of LDL with glucose alone, in which only 5.6% of lysines were modified but in which all of the modifications presumably existed in the naturally occurring Amadori rearrangement forms, was also immunogenic, but the antibodies produced were of low titer and low affinity. This may reflect partial tolerance to this naturally occurring posttranslational modification. Precedent for such low-titer antibodies directed against a naturally occurring posttranslational modification of a native protein exists in the example of the antibodies directed against neoantigens of fibrinogen produced by cleavage of fibrinogen by plasmin (10).

When Glc_{RED}-LDL was the immunogen, the circulating antibodies were capable of producing rapid immune clearance of the modified LDL, as well as of similarly modified albumin. When Glc_{NR}-LDL or Glc_{RED}-albumin was the immunogen, the antibodies produced did not lead to enhanced immune clearance of injected antigens. Whether this was simply due to the lower titer of the latter antibodies, a lower affinity, or a different class of antibodies (not capable of binding complement, for example) is not known at present. However, the failure to induce rapid immune clearance suggests that such antibodies could circulate in plasma as immune complexes with a variety of glycosylated proteins. A complete characterization of these antibodies will be of importance in elucidating whether or not they could have any pathophysiological consequences. In particular, it will be important to determine the site of deposition and degradation of the presumed antibody–Glc-LDL complexes (or complexes with other glucosylated proteins). Such complexes could deposit in and damage tissues such as arterial endothelium or renal glomeruli. Also, if complexes containing LDL were ingested by macrophages located in the arterial wall, lipid deposition in such cells might occur. In this regard, it is noteworthy that a number of recent reports have documented the presence of an increased incidence of circulating immune complexes in diabetics, particularly in diabetics with small vessel disease (11–13). Furthermore, the presence in diabetics of immunoglobulins within vessel walls and sites such as the renal glomeruli is well known (14, 15). Of particular interest also are the recent reports of an increased incidence in diabetics of autoantibodies directed against LDL (16, 17).

These studies were initiated by our finding in human subjects of rapid clearance of a Glc_{RED}-LDL tracer in three of four diabetics and in two of six euglycemic subjects (3). In experiments to be reported elsewhere we have been able to demonstrate in two of these patients the presence of antibodies directed against Glc_{RED}-LDL but not native LDL. These antibodies were present in the patients' plasma *before* the Glc_{RED}-LDL tracer was injected. Our finding in the guinea pig that antibodies produced against the Glc_{NR}-LDL partially cross-reacted with the reduced Glc_{RED}-LDL suggests at least one mechanism for the formation of such antibodies. Further studies are required to establish the existence and prevalence of such autoantibodies directed against Glc-LDL or other glucosylated proteins, as well as to determine if such antibodies exist in plasma as immune complexes. Our data suggest a novel consequence of nonenzymatic glucosylation of proteins that could lead to autoantibody formation, with a variety of pathophysiological consequences.

The authors are deeply grateful to Dr. Daniel Steinberg for his generous support, encouragement, and insightful advice. Dr. L. Curtiss provided expert advice in setting up the immunoassays and Dr. T. Edgington provided helpful criticism. We thank Richard L. Elam for excellent technical assistance and Anita Fargo for secretarial help. This work was supported by Grant HL-14197 awarded by the National Heart, Lung, and Blood Institute. U.P.S. is a Fellow of the Medical Research Council of Canada and A.K. was a Visiting Scientist from the Second Department of Medicine, University of Helsinki, Finland.

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