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Autoantibodies to glucosylated proteins in the plasma of patients with diabetes mellitus

(low density lipoprotein)

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ABSTRACT **Nonenzymatic glucosylation interferes with recognition of low density lipoprotein (LDL) by its receptor and markedly decreases the rate of plasma clearance of glucosylated LDL, both in experimental animals and in normal human subjects. However, in selected diabetic subjects we have observed a paradoxical *increase* in the clearance of glucosylated LDL, suggesting the possibility of immune-mediated clearance. Immunoassay demonstrated antibodies specific for glucosylated LDL in the preinjection plasma of each of four such diabetic subjects studied. These antibodies cross-react with other glucosylated proteins and recognize specifically the glucosylated lysine epitope—i.e., glucitollysine. These data suggest that nonenzymatic glucosylation of plasma or structural proteins may render them immunogenic and result in production of autoantibodies that recognize not only the particular immunogen but also many other glucosylated proteins, including glucosylated tissue proteins. These findings may be relevant to the increased prevalence of immune complexes in plasma of diabetic subjects and the late complications of diabetes mellitus.**

Nonenzymatic glucosylation of low density lipoprotein (LDL) is enhanced in diabetics. In model systems, glucosylated LDL (Glc-LDL) is not recognized by the LDL receptor. In guinea pigs, rabbits, and some euglycemic human subjects, the clearance of autologous Glc-LDL is much slower than that of native LDL (1–5). However, we now report unusually rapid clearance from plasma of an injected Glc-LDL tracer in three of four diabetic subjects, in one subject with hypothyroidism, and in one euglycemic subject. The pattern of clearance suggested an immune mechanism. We have previously shown that nonenzymatic glucosylation of homologous LDL and albumin rendered these proteins immunogenic in the guinea pig and lead to rapid immune-mediated clearance of Glc-LDL (6). In the current report we extend these observations and document the presence in the plasma of human diabetic subjects of similar antibodies to Glc-LDL and other glucosylated proteins.

METHODS

Human Studies. Ten individuals who received simultaneous injections of radioiodinated autologous control LDL and Glc-LDL are the subject of this report. Pertinent clinical characteristics of the 10 individuals are given in Table 1. Subjects G–J have been the subject of a prior report (subjects 3–6 in ref. 4). These studies were approved by the Human Studies Committee of the University of California, San Diego.

Subjects were fed a standard diet for 2 weeks on a metabolic ward (4). LDL was isolated and one aliquot was used to prepare ^{131}I-labeled control LDL and another aliquot was used to prepare ^{125}I-labeled Glc-LDL (>40% of lysine residues derivatized with glucose) exactly as previously described (4). These two tracers were then simultaneously injected into each subject and the plasma decay curves were determined over the ensuing 2 weeks. The plasma decay curves for subjects G–J and E have been published (4).

Table 1. Clinical data and antibody titer

Subject	Clinical diagnosis*	Antibody titer† IgG	IgM	IgA	Glc-LDL tracer remaining in plasma after 10 days, %
A	AODM, insulin	—‡	—	8	40.0
B	AODM, insulin	—	—	16	0.8
C	AODM	32	8	64	0.8
D	AODM	—	128	>256	0.8
E	HT	16	—	—	0.1
F	Normal	—	—‡	—	3.0
G	Normal	—	—‡	—	30.0
H	Normal	—	—	—	46.0
I	Normal	—§	—	—	38.0
J	Normal	—	—	—	58.0

Subjects are all men, ages 35–62, except J, who is a 60-year-old woman.

*AODM, adult-onset diabetes mellitus (patients treated with insulin are indicated); HT, hypothyroidism (thyroxine = 4.2 μg/dl, thyrotropin = 69 microunits/ml, plasma cholesterol = 341 mg/dl). Normal indicates subject is normoglycemic.

†Antibody titer is defined as reciprocal of highest dilution of plasma showing level of antibody binding to Glc_{RED}LDL that is twice that of control plasma.

‡None of these plasma showed binding of IgG or IgM 2 times that of control plasma, but all showed competition for antibody binding by Glc_{RED}LDL in competition assays.

§Subject I showed no binding of IgG above normal plasma at dilutions less than 1:64; however, at dilutions of 1:64 and 1:128 binding more than twice normal was observed.

Preparation of Glucosylated Proteins. Nonenzymatic glucosylation of lysine residues of LDL initially occurs via a labile Schiff base, which in turn slowly undergoes the Amadori rearrangement to form ketoamine and hemiketal forms (1, 7). (See figure 1 of ref. 6.) We designate proteins glucosylated under nonreducing (NR) conditions as "Glc_{NR}protein." Even in the presence of high glucose concentrations (i.e., 80 mM) such preparations usually yield only 5–7% of lysine residues glucosylated (8). In contrast, glucosylation of LDL in the presence of the reducing agent $NaCNBH_3$ proceeds

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Abbreviations: LDL, low density lipoprotein; Glc-LDL, glucosylated LDL; Glc_{RED}LDL, Glc-LDL formed in presence of reducing agent; Glc_{NR}LDL, nonreduced Glc-LDL; HDL, high density lipoprotein; HSA, human serum albumin; GAHIgG, goat anti-human IgG; GAHIgM, goat anti-human IgM; GAHIgA, goat anti-human IgA; FCR, fractional catabolic rate.

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more rapidly and completely and yields glucitollysine as the glucose adduct (7). This product we designate "Glc_{RED}LDL." By varying the incubation times in the presence of $NaCNBH_3$, the degree of derivatization of lysine residues can be varied from 2% to greater than 50% (8, 9). Reduced and nonreduced forms of glucosylated human HDL and albumin were prepared in a similar fashion. Human serum albumin (HSA, Sigma) was first reisolated by chromatography on Sephacryl S-300. The extent of derivatization of lysine residues of the modified proteins was determined as described (1, 8), and the synthesis of glucitollysine was performed as described (1).

Determination of Specificity and Titers of Antibodies to Glc-LDL. Small aliquots of plasma had been collected from each subject prior to injection of the tracers, and from some at various times after injection of the tracers. To determine the titer and specificity of antibodies to a given antigen we used a solid-phase radioimmunoassay (RIA), similar to that previously described for assay of antibodies to Glc-LDL in immunized guinea pigs (6). In brief, 96-well polyvinylchloride microtiter plates were coated with antigen at 50 ng per well, and remaining adsorptive sites on the plastic wells were blocked by incubation with a "postcoat" buffer containing 3% nonimmune goat serum and 3% bovine serum albumin. To measure a subject's antibody titer, 50 μl of plasma at various dilutions was added to wells containing antigen and incubated for 18 hr at 4°C. Plasma was then aspirated, each well was washed four times, and the amount of human immunoglobulin bound (IgG, IgA, or IgM) was quantitated by use of ^{125}I-labeled goat anti-human immunoglobulin (10). Polyclonal goat anti-human IgG (GAHIgG) directed against both light and heavy chains (United States Biochemical, Cleveland, OH) was purified on staphylococcal protein A-Sepharose 4B (Pharmacia) and then absorbed with purified human IgA and IgM (Calbiochem) to remove cross-activity with non-IgG immunoglobulins. Heavy chain-specific goat anti-human-IgM (GAHIgM) and anti-IgA (GAHIgA) (United States Biochemical) were purified by protein A affinity chromatography. In this paper, a titer is defined as the reciprocal of the highest dilution that gave absolute binding of second antibody 2-fold greater than that of a control plasma shown to contain no antibodies specific for Glc-LDL. Because nonspecific immunoglobulin binding occurs at low plasma dilutions, we also required that a significant fraction of the bound radioactive material be displaceable by Glc-LDL in competitive inhibition studies to qualify as positive. Competitive inhibition studies were performed with plasmas by incubating 25-μl aliquots of fixed dilutions of plasma (as source of antibody) in each well simultaneously with 25 μl of various concentrations of competitor for 18 hr at 4°C and then the amount of first antibody bound was quantified as above.

RESULTS

Turnover Studies. The rate of clearance of an iodinated Glc-LDL tracer in guinea pigs and rabbits is only 25% of that of a native LDL tracer. When ^{131}I-labeled control LDL and ^{125}I-Glc_{RED}LDL were simultaneously injected into euglycemic subjects G–J, the clearance of the Glc-LDL tracer was very slow, and the fractional catabolic rates (FCRs) averaged 0.11 pool per day and were only 20% of the rate for control LDL (see decay curves in ref. 4). When Glc-LDL was injected into diabetic subject A, a similar slow clearance of the Glc-LDL tracer was noted (Fig. 1*A*). In subject A the FCR for Glc-LDL was 0.09 pool per day versus an FCR for control LDL of 0.59 pool per day. However, in the three other diabetic subjects studied (B, C, and D), the clearance of Glc-LDL was slow for 4–8 days, and was then followed by rapid disappearance of the Glc-LDL tracer from plasma

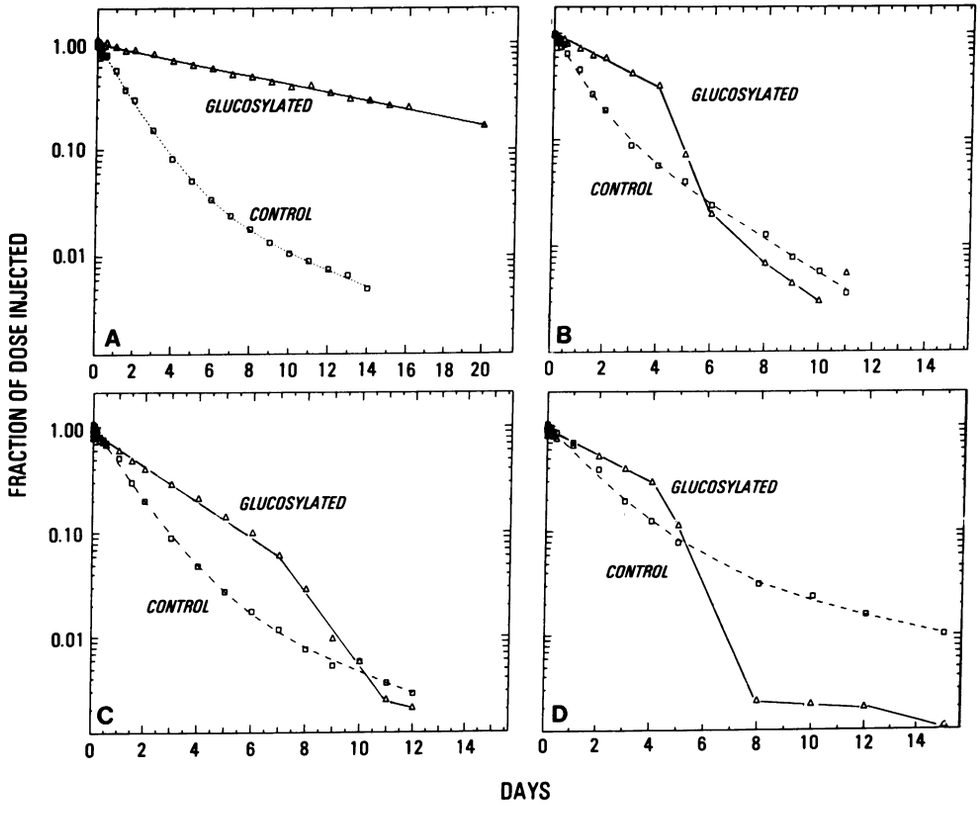

FIG. 1. Turnover of control LDL (□) and glucosylated LDL (△) in experimental subjects. For each subject equal amounts of ^{125}I-Glc-LDL and control ^{131}I-LDL were injected intravenously and plasma decay curves were determined over the ensuing 14–20 days. *A–D* are for subjects A, B, C, and E, respectively.

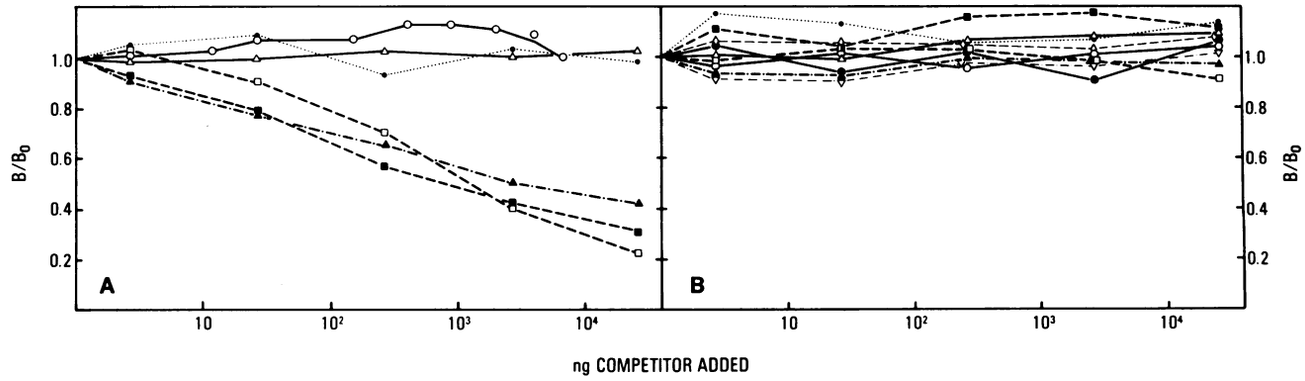

FIG. 2. Competition for binding to IgG of subject C and of a normal control. Dilutions (1:6) of plasma of a normal control (JW) (*B*) and of subject C (*A*) were added to wells containing 50 ng per well of $Glc_{RED}LDL$ adsorbed to plastic. Increasing amounts of competitor were added and after 18-hr incubations the amount of human IgG bound was quantified. Data are expressed as the ratio B/B_0, in which B represents the amount of binding of GAHIgG in the presence of competitor and B_0 that bound in the absence of competitor. The various competitors added were as follows: •, lysine; ●, HSA; ○, native LDL; ▽, native high density lipoprotein (HDL); □, glucitollysine. The following glucosylated proteins were also added (the percentage of lysines derivatized is given in parentheses): ■, $Glc_{RED}LDL$ (50%); ▲, $Glc_{RED}HDL$ (50%); ◇, $Glc_{RED}HSA$ (50%); △, $Glc_{NR}LDL$ (5%).

(Fig. 1). It is important to note that even the initial clearance rate in these three subjects was more rapid than in the other subjects (FCR for this component 0.26 ± 0.1 pool per day). Fig. 1*D* shows the decay curves for subject E, who was euglycemic but hypothyroid at the time of these studies. The FCR for the initial phase of his Glc-LDL tracer was 0.28 pool per day. The FCR for the first component in subject F was 0.33 pool per day (data not shown).

Presence and Specificity of Antibodies to Glc-LDL. To determine if antibodies that bind Glc-LDL were present in the plasma of these subjects, we assayed for the presence of antibodies to Glc-LDL in *preinjection* plasma samples. Five of the 10 subjects had one or more class of immunoglobulins that bound $Glc_{RED}LDL$ with titers from 8 to >256. Because significant levels of nonspecific binding can occur with plasmas used at low dilutions, we analyzed the specificity of binding by competition. Fig. 2*B* shows the result of a competition study for IgG binding using the control plasma as antibody source. In the absence of competitor, 3000 cpm of ^{125}I-GAHIgG was bound; however, even a large excess of $Glc_{RED}LDL$, as well as numerous other potential competitors, failed to compete. This level of binding was observed in control wells lacking antigen and demonstrates that the normal plasma lacked specific binding of IgG. In contrast, when a similar study was performed with plasma from subject C, (Fig. 2*A*) 13,000 cpm was bound in the absence of competitor, and almost 80% of that amount could be displaced by $Glc_{RED}LDL$, $Glc_{RED}HDL$, and even by glucitollysine itself. Native LDL, $Glc_{NR}LDL$, and lysine failed to compete.

To confirm that IgG in the plasma of patient C was responsible for binding to $Glc_{RED}LDL$ we isolated IgG from a small aliquot of preinjection plasma by affinity chromatography on protein A. Competition studies using this purified IgG gave results comparable to those observed with whole plasma (Fig. 3). Glucitollysine, $Glc_{RED}LDL$, and another reductively glucosylated protein, $Glc_{RED}HSA$, competed for antibody binding, while native LDL, $Glc_{NR}LDL$, and $Glc_{NR}HSA$ did not. The $Glc_{RED}LDL$ and $Glc_{RED}HSA$ preparations were extensively derivatized (approximately 50% lysines glucosylated) and the possibility remained that the $Glc_{NR}LDL$ (5% lysines glucosylated) failed to compete simply because the extent of derivatization was limited. However, a $Glc_{RED}LDL$ preparation in which only 5% of lysines were derivatized was also able to compete. This suggests that the epitope recognized by this IgG was the reduced glucose conjugate, glucitollysine.

Results of the titrations of all 10 subjects for IgG antibodies, using the same normal control, are shown in Table 1. Of the five subjects showing rapid clearance, one had an IgG titer of 16, and one had a titer of 32. Of the five not showing rapid clearance, none had specific binding twice that of the control plasma. To determine the specificity of the antibody binding, competition assays against $Glc_{RED}LDL$ were done with each patient's plasma. Significant competition, indicating specificity for $Glc_{RED}LDL$, was demonstrated for subjects C and E.

We also determined the presence and titer of IgM and IgA antibodies that bind $Glc_{RED}LDL$. Only subjects C and D had IgM antibodies (i.e., binding twice that of normal control). To demonstrate the fine specificity of IgM binding of subject D (Fig. 4), a competition assay utilizing his preinjection plasma as the source of antibody was performed. His IgM antibodies specifically recognized $Glc_{RED}LDL$, $Glc_{RED}HDL$, $Glc_{RED}HSA$, and glucitollysine, but not $Glc_{NR}LDL$ (5%) or $Glc_{RED}LDL$ (5%), possibly reflecting a lower affinity for glucitollysine than in the case of the IgG from subject C. Plasma samples from all 10 subjects were tested in competition assays. In addition to subject D, antibodies from subject C showed equivalent specificity. Although plasma of subjects F and G did not have absolute binding twice that of

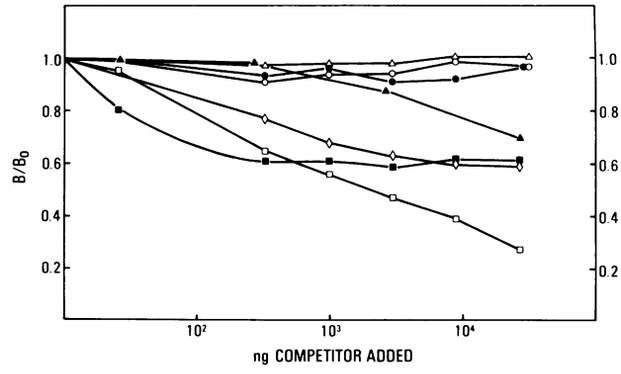

FIG. 3. Competition for binding to protein A-purified IgG of subject C. A 1:6 dilution of IgG isolated from 2 ml of plasma of subject C by affinity chromatography was used as antibody source in a competition RIA as explained in legend to Fig. 2. The competitors added were as follows: ○, native LDL; and □, glucitollysine. The following glucosylated proteins were also added (the percentage of lysines derivatized is given in parentheses): ■, $Glc_{RED}LDL$ (50%); ◇, $Glc_{RED}HSA$ (50%); ▲, $Glc_{RED}LDL$ (5%); △, $Glc_{NR}LDL$ (5%); and ●, $Glc_{NR}HSA$ (5%).

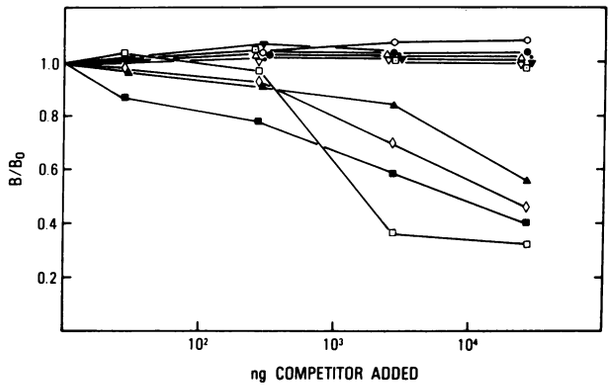

FIG. 4. Competition for binding to IgM of subject D. A 1:6 dilution of plasma from subject D was used as antibody source in a competition assay as outlined in the legend to Fig. 2. Amount of IgM bound was quantified by the amount of binding of iodinated GAH-IgM. Competitors added include the following: •, lysine; ●, native LDL; □, glucitollysine; △, native HDL; ▼, native HSA. The following glucosylated proteins were added (the percentage of lysines glucosylated is given in parentheses): ■, Glc_{RED}LDL (50%); ⊡, Glc_{RED}LDL (5%); ▲, Glc_{RED}HDL (50%); ◇, Glc_{RED}HSA (50%); ○, Glc_{NR}LDL (5%); and ▽, Glc_{NR}HSA (5%).

normal plasma, their plasma did show small degrees of IgM binding that was displaced by Glc_{RED}LDL. When plasma taken 7 days after injection of the Glc-LDL tracer was titrated, subject C showed a rise in titer (128 versus 8 preinjection), suggesting an anamnestic response to the injection of the Glc-LDL tracer.

Similar studies were also done to test for the presence of IgA antibodies. Three of the subjects who had shown rapid clearance of the Glc_{RED}LDL tracer had antibodies of this class, as did diabetic subject A. In all four of these subjects, Glc_{RED}LDL was capable of competing for IgA antibody binding to the antigen-coated wells.

DISCUSSION

We recently showed that nonenzymatic glucosylation of homologous LDL and albumin rendered these proteins immunogenic in the guinea pig (6). The resultant antisera were specific for the modification, and did not react with the native proteins. For example, when homologous Glc_{RED}LDL (with 61% of lysines modified) was used as immunogen, the guinea pig antiserum that resulted from the immunization was of high titer and specifically recognized the reduced glucose adduct, glucitollysine, on a variety of homologous as well as heterologous glucosylated proteins. However, this antiserum did not recognize glucose conjugated in the Amadori forms—i.e., Glc_{NR}LDL. When a ^{125}I-Glc_{RED}LDL tracer was injected into such immunized animals, clearance of the tracer was extremely rapid.

Glc_{NR}LDL, produced by incubation with glucose alone, in which only 5% of lysines were glucosylated but in which all of the modifications presumably existed in Amadori rearrangement forms, also was immunogenic (6). However, the antibodies were of lower titer and lower affinity and their presence was not associated with a rapid clearance of an injected ^{125}I-Glc_{NR}LDL tracer. Of great interest, this antiserum cross-reacted with Glc_{RED}LDL, possibly due to antibodies that recognized both open-chain ketoamine and glucitol adducts.

In this report we document the presence of antibodies to Glc_{RED}LDL in human plasma. In all four diabetic subjects, at least one class of immunoglobulins could be demonstrated with specificity to Glc_{RED}LDL, and in two definition of the fine specificity of the antibodies was possible. In subject C, glucitollysine itself was an effective competitor for IgG binding, as were glucitollysine adducts in LDL, HDL, and HSA. Native LDL, HDL, and HSA were not competitors, nor was Glc_{NR}LDL (5% lysine glucosylated). Thus this subject's IgG antibodies were specific for glucitollysine and bear a striking resemblance to the specificity observed in guinea pig antiserum produced by immunization with homologous Glc_{RED}LDL (6) as well as murine monoclonal antibodies produced against murine Glc_{RED}LDL (9). This suggests that glucitollysine adducts of LDL or of other proteins must have existed in this individual, and that they were immunogenic. A reductase capable of generating such glucitollysine adducts *in vivo* has not yet been described. However, using monoclonal antibodies specific for glucitollysine, we have observed small amounts of immunoreactivity in untreated plasmas of diabetic subjects (9). An alternative explanation is that the open-chain ketoamine form was the actual immunogen but that the elicited antibodies cross-reacted with the open-chain glucitol adduct, which is abundantly present in Glc_{RED}LDL, but present in limited quantity in Glc_{NR}LDL. The latter interpretation would be consistent with the observation that, in the guinea pigs, antibodies produced as the result of immunization with Glc_{NR}LDL did in fact react with Glc_{RED}LDL.

Subject D had IgM antibodies shown to be specific for glucitollysine. His antibodies bound glucitollysine adducts of several different glucosylated proteins. The possibility that these antibodies also recognize glucose adducts in the Amadori forms cannot be dismissed, since these antibodies did not react with Glc_{RED}LDL with 5% lysine derivatization.

This report demonstrates that some diabetic subjects have antibodies that bind to reductively glucosylated proteins. Four of the five subjects who showed rapid clearance of the ^{125}I-Glc_{RED}LDL tracer had demonstrable, preexisting antibodies, of at least one class, directed against Glc_{RED}LDL. The observation that the clearance of Glc_{RED}LDL in these individuals was more rapid than the clearance of Glc_{RED}LDL in normal subjects immediately after injection is consistent with accelerated clearance of soluble immune complexes formed in the presence of low levels of preexisting antibodies. The much more rapid decay that occurred 4–8 days later probably reflects newly recruited antibodies leading to formation of larger complexes and a further acceleration of clearance. Consistent with this hypothesis, in subject C we documented an increase in IgM titer in a plasma sample taken just before the rapid disappearance of the glucosylated tracer on day 7. In the five subjects not showing this rapid clearance we were able to demonstrate unequivocal evidence for specific antibodies in only one (diabetic subject A), and this was of low titer.

Our previous demonstration that *in vitro* nonenzymatic glucosylation of LDL and albumin rendered these proteins immunogenic in guinea pigs, and the finding of antibodies with almost identical specificity in the plasma of diabetic subjects, strongly suggests that these post-translational modifications are recognized as foreign by the immune system of at least some individuals. In turn, the ability to recognize such a modification may be under genetic control (10, 11). The prevalence and specificity of such antibodies directed against glucosylated proteins in diabetic patients (and others) remains to be determined. However, it is of interest to note that a number of recent reports have documented an increased incidence of circulating immune complexes in diabetics, particularly in diabetics with small vessel disease (11–16). Furthermore, the marked increase in the incidence of immunoglobulins within renal glomeruli and vessel walls in diabetics has long been known (17, 18). Of note also are recent reports of an increased incidence in diabetics and older people of autoantibodies directed against LDL (19–21), including a significant number of autoantibodies of the IgM or IgA class (19–24).

We have previously shown that the extent of glucosylation of LDL is enhanced 2- to 4-fold in plasma of diabetic subjects (9), raising the question of whether such a modification could play a role in the accelerated atherosclerosis characteristic of the diabetic state. Our preliminary evidence indicates that the extent of glucosylation of LDL that occurs in some diabetics could inhibit LDL clearance 5–25% (8). Because of the longer residence time in plasma, the LDL particle would be further glucosylated and would be more susceptible to additional modifications (25). For example, Stevens *et al.* have identified ethylated glucitollysine in protein adducts of alcoholic subjects (26). As well, proteolytic cleavage can generate neo-antigens and low-titer autoantibodies (27). Such modifications of LDL may lead to autoantibody formation because LDL is a particularly effective immunogen. Recent studies in our laboratory have shown that subtle modifications of lysine residues of LDL, such as acetylation, ethylation, carbamoylation, or methylation, render the modified LDL immunogenic and that the resulting antibodies cross-react with other similarly modified proteins (unpublished results).

The autoantibodies described here recognized glucitollysine itself, as well as glucitollysine adducts of many proteins. Glucosylation of many tissue proteins has been described, including proteins of aorta, lens, kidney, and nerve. Generalizing the present results, autoantibodies directed againt Glc-LDL (or other glucosylated proteins) could react with a variety of glucosylated structural or membrane proteins. Such autoantibodies could explain some of the heterogeneous so-called tissue complications of diabetes mellitus.

Post-translational nonenzymatic modifications of plasma and structural proteins occur in a number of conditions, including uremia (28), galactosemia (29), and secondary to chronic alcohol consumption (26). In addition, specific and nonspecific proteolytic cleavage of many proteins occurs in plasma. It is possible that along with nonenzymatic glucosylation secondary to hyperglycemia, some of these other modifications may also be immunogenic and in susceptible individuals lead to autoantibody formation with a variety of pathophysiological consequences.

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