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Role of the Carbohydrate Moiety in the Antigenic Site(s) of Human Serum Low-Density Lipoprotein[†,‡]

Sonia Goldstein* and M. John Chapman

ABSTRACT: Radioimmunoassay techniques have been used to evaluate the contribution of the carbohydrate moiety to the immunological reactivity of human serum low-density lipoprotein (LDL). Low-density lipoprotein (d = 1.024–1.045 g/mL) was isolated from normolipidemic serum by ultracentrifugal flotation. Radioimmunoassay was performed with ^{125}I-labeled LDL and several homologous antisera, each corresponding to different periods (1–18 weeks) of immunization and thus containing various antibody populations. Unlabeled LDL and different monosaccharides characteristic to this particle, i.e., mannose, sialic acid, glucose, *N*-acetylglucosamine, galactose, *N*-acetylgalactosamine, and fucose, were used as competitors in the binding of the labeled antigen with antibody. In the reaction with antisera corresponding to the highest antibody titer, unlabeled LDL, sialic acid, and mannose inhibited the binding of labeled LDL up to 62%, 25%, and 16%, respectively; a low degree of inhibition (some 13%) was occasionally obtained with glucose. Galactose, galactosamine, glucosamine, and fucose failed to compete with labeled LDL. Studies with antisera corresponding to different periods of immunization (2, 4, and 8 weeks) indicated that antibodies reacting with mannose appeared early (maximum 31% inhibition at 2 weeks), disappearing at 6–8 weeks; in contrast, antibodies reacting with sialic acid augmented progressively (10% inhibition at 2 weeks, 20% at 4 weeks, and 35% at the end of the immunization). These data are consistent with the conclusion that sialic acid and mannose, the terminal residues of LDL glycopeptides I and II [Swaminathan, N., & Aladjem, F. (1976) *Biochemistry 15*, 1516–1521], are implicated in the antigenic site(s) of LDL.

The nature of the carbohydrate moiety of serum low-density lipoprotein is incompletely known, although several structural studies were described in the early sixties (Schultze & Heide, 1960; Ayrault-Jarrier, 1961; Marshall & Kummerov, 1962), and a renewal of interest has occurred in recent years (Kwiterovich et al., 1974; McConathy & Alaupovic, 1974; Swaminathan & Aladjem, 1976; Dawson et al., 1976; Chatterjee & Kwiterovich, 1976).

The carbohydrate components bound to the protein moiety of LDL[1] amount to 8–10% of its dry weight and are essentially mannose (4.8%), galactose (2.1%), sialic acid (1.7%), and glucosamine (0.9%) (Swaminathan & Aladjem, 1976). The latter authors succeeded in the separation and structural characterization of two types of glycopeptides, with sialic acid and mannose, respectively, as terminal residues and which together represented some 50% of the carbohydrate moiety of LDL.

Several monosaccharides are also present in the glycolipid fraction of LDL; they amount to ~10 μmol of glucose/g of phospholipid and are principally glucose, galactose, galactosamine, and sialic acid (Chatterjee & Kwiterovich, 1976; Dawson et al., 1976). In addition, Marcus & Cass (1969) have reported the presence of fucose and glucosamine in certain LDL glycosphingolipids apparently related to Lewis blood activity.

[†] From Unité 35, Unité de Recherches sur le Métabolisme des Lipides, INSERM, Hôpital Henri Mondor, 94010 Créteil, France. *Received June 12, 1980.* Part of this paper was presented at the 5th International Symposium on Atherosclerosis, Houston, TX, Nov 1979 (Abstract No. 330). S.G. was partially supported by C.R.L. 80 50 26 from INSERM and M.J.C. by Grant No. 78.7.2598 from the D.G.R.S.T.
[‡] With the technical assistance of Dominique Lagrange and Isabelle Tayeb.

[1] Abbreviations used: LDL, low-density lipoproteins of density 1.024–1.045 g/mL; Apo-B, apolipoprotein B; NANA, *N*-acetylneuraminic acid; Man, mannose; Fuc, fucose; Glu, glucose; Gal, galactose; GluNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; ID, immunodiffusion; IEP, immunoelectrophoresis; RIA, radioimmunoassay; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.

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Several investigations have concerned the role of the carbohydrate moiety in the molecular interactions and metabolism of LDL. Thus, the glucopyranosyl and mannopyranosyl residues of LDL are involved in its binding to concanavalin A (Harmony & Cordes, 1975; McConathy & Alaupovic, 1974). While sialic acid residues are involved in the interaction of several serum glycoproteins (i.e., ceruloplasmin and fetuin) with hepatic cells (Ashwell & Morell, 1978), controversial observations have been made on the role of carbohydrate in the cellular binding and metabolism of LDL. Thus, in contrast to the apparent lack of a role for the carbohydrate moiety in the metabolism of LDL in vivo (Attie et al., 1979) and in the binding to cultured fibroblasts in vitro (Shireman & Fisher, 1979), Filipovic et al. (1979) have described evidence consistent with the participation of sialic acid in the receptor binding and uptake of LDL by cultured cells, and Avila et al. (1978) contend that this residue is implicated in the degradation of LDL in vivo in the rabbit.

In previous reports (Chapman et al., 1978; Goldstein & Chapman, 1979) we showed that the peptide fraction obtained from LDL by limited tryptic digestion and containing ~30% of the sialic acid of LDL was highly immunogenic and exhibited competitive reactivity with ^{125}I-labeled LDL in binding with homologous antisera. The aim of the present study was therefore to evaluate the possible participation of the carbohydrate moiety in the antigenic site(s) of LDL and to subsequently provide information on its location in this particle.

The preparation of monospecific antisera against a carbohydrate moiety of a glycoprotein or a glycolipid is generally restrained by the low antibody titer which is obtained. In contrast, however, the use of specific and sensitive immunochemical techniques, such as rocket immunoelectrophoresis and radioimmunoassay, and, more specifically, their inhibition by different carbohydrates, has allowed assessment of the contribution of carbohydrates to the antigenic site of several glycoproteins (Vreeland & Chapman, 1978; Zopf et al., 1978; Kieda et al., 1977) and glycolipids (Hruby et al., 1977). Using various antibody populations together with inhibition tests of the radioimmunoassay by several monosaccharides known to be present in the LDL particle, we presently demonstrate that certain carbohydrates are significantly involved in the antigenic site(s) of LDL.

Materials and Methods

Preparation of Serum Low-Density Lipoprotein. Low-density lipoprotein (d = 1.024–1.045 g/mL) was separated from normal human serum by sequential ultracentrifugation according to established procedures (Havel et al., 1955) and as previously described (Chapman et al., 1978).

The purity of LDL preparations was established by electron microscopy (Forte et al., 1968), immunoelectrophoresis (Scheidegger, 1955), and immunodiffusion (Ouchterlony, 1964; Mancini et al., 1965). For the immunological techniques, several commercial antisera were employed, i.e., antiserum to human whole serum (Institut Pasteur, Paris) and antisera to human serum albumin and to human serum β_2-glycoprotein I (Behringwerke, A.G., Marburg, West Germany); M-Partigen β_2-glycoprotein I plates were also purchased from Behringwerke. Antisera to human Apo-C I, Apo-C II, and Apo-C III were a gift from Dr. P. N. Herbert. Apo-LDL was examined in the $NaDodSo_4$–polyacrylamide gel system of Weber & Osborn (1969) as outlined earlier (Chapman et al., 1978).

Preparation of Apolipoprotein B. The preparation of apolipoprotein B from LDL was based on that of Herbert et al. (1973) and carried out as previously described (Chapman & Goldstein, 1976). The determination of protein content in LDL and Apo-B was performed according to Lowry et al. (1951) by employing appropriate controls and using bovine serum albumin as the standard.

Antisera Preparation. Five rabbits were injected intradermally at several sites in the back with 250–300 μg of LDL protein, and two others with 500 μg of Apo-B protein, as previously described (Goldstein et al., 1977). Booster injections with the same amount of antigen were given after 3 weeks for LDL and after 3 and 5 weeks for Apo-B.

All animals were bled by puncture of the marginal ear vein or of the central artery of the ear prior to immunization and subsequently at weekly intervals. Serum samples (0.5 mL) from each bleeding were stored separately at −20 °C, after addition of sodium azide (final concentration 0.01% w/v).

The presence of antibodies in the various antisera was detected by double immunodiffusion (ID) and immunoelectrophoresis (IEP) which were performed in triplicate with the antisera obtained from each bleeding. Individual slides were stained separately for protein (Amidoschwarz), lipid (Sudan black), and carbohydrate (Schiff). Titration of the different samples of antisera was performed on Ouchterlony plates; a constant amount of antigen was allowed to react with an equal volume of serially diluted antiserum (1:2–1:256).

Radioimmunoassay Procedure. The iodination of LDL was performed according to a modification (Bilheimer et al., 1972) of McFarlane's iodine monochloride technique (McFarlane, 1958). The radioimmunoassay was carried out according to Felber's double antibody method (Felber, 1974), under previously described conditions (Goldstein & Chapman, 1979).

The LDL was iodinated with Na^{125}I (Amersham). After dialysis against a glycine buffer at pH 10, 2 mg of LDL was reacted with Na^{125}I (1 mCi) for ~30 s in an ice bath. Iodine monochloride, prepared by diluting a stock solution with 2 M NaCl, was added in amounts to provide 1 atom of iodine/mol of LDL, with mild stirring. The labeled LDL was immediately diluted 10-fold with the same glycine buffer and free iodine removed by extensive dialysis against 10 L of solution containing 0.15 M NaCl, 10 mM Tris, and 0.01% EDTA, at pH 7.4 and 4° C, with 8–10 changes of the dialyzate.

The specific activity of the labeled LDL was calculated from the precipitate obtained after the addition of 10% trichloroacetic acid (final concentration 5%). The extent of labeling of the lipid moiety was determined by measurement of the radioactivity of the lipids extracted with ethanol–ether at 4 °C (Chapman et al., 1978).

After dialysis, the labeled LDL was diluted to ~1:10 in glycine buffer and then stored in aliquots of 0.5 mL at 4 °C; a final dilution giving 10000–12000 cpm in 100 μL of labeled LDL was performed immediately prior to radioimmunoassay.

For the competition studies, several unlabeled LDL and monosaccharides were employed. The monosaccharides were as follows: D(+)-galactose, purified and free of glucose, and *N*-acetylgalactosamine, grade I, free of glucosamine and *N*-acetylneuraminic acid, type IV, synthetic, crystalline, from Sigma; D(+)-glucose, grade III, anhydrous and *N*-acetylglucosamine pure, from Industrie Biologique Française, Gennevilliers, France; D(+)-fucose and D-mannose, A grade from Calbiochem.

All the dilutions for LDL and for the monosaccharides employed as competitors were made in a standard buffer containing 0.05 M potassium phosphate and 0.01 M NaCl (PBS), at pH 7.5, to which 0.01 sodium azide, 0.01% EDTA, and 1% bovine serum albumin were added. The determination of the dilution of antiserum to be used was performed with

0.1 mL of each antiserum in serial dilutions from 1:50–1:25 600 and with radiolabeled LDL (in a dilution corresponding to approximately 50–100 ng of protein and 10 000–12 000 cpm). Standards and samples, made up to a total volume of 0.5 mL, were incubated for 48 h at 4 °C. For the precipitation of the antigen–antibody complexes, the second antibody, a sheep antiserum to rabbit γ-globulin (Institut Pasteur, Paris), was then added in a dilution of 1:10 and volume of 0.1 mL. After 24 h at 4 °C and subsequent washings with 1 mL of ice-cold PBS containing 0.5% bovine serum albumin, the tubes were centrifuged at 2000*g* for 20 min at 4 °C. After the supernatant was discarded, precipitates were counted for 1–2 min in an Autogamma spectrometer (Model CG 4000, Intertechnique). All experiments were performed in duplicate or triplicate. The results were plotted to give an antigen binding curve; the dilution of antiserum corresponding to a 45–50% binding of radiolabeled LDL was retained for the competition assay.

In a second step, competition assays with progressive amounts of unlabeled LDL were performed for various incubation periods. Thus, aliquots of 0.1 mL of antiserum (at the chosen dilution) were reacted with nonlabeled LDL, i.e., 60 ng of LDL protein. After incubation for 1–14 days, 0.1 mL of labeled LDL (10 000–12 000 cpm) was added; the second antibody was introduced after a further 24-h incubation at 4 °C, and the experiment was completed as described above.

The period which corresponded to the highest degree of inhibition was retained for subsequent competition studies with the various monosaccharides. For such inhibition assays, increasing amounts of LDL (5–600 ng of protein, i.e., 0.002–0.24 pmol) and of monosaccharides (i.e., 0.5 nmol to 1.2 μmol), in 0.1 mL of PBS buffer containing 1% bovine serum albumin, were reacted with 0.1 mL of antiserum, and the radioimmunoassay was carried out as outlined above.

Controls (1) for the nonspecific binding of labeled LDL alone or of this LDL in the presence of the corresponding unlabeled fraction and (2) for the various carbohydrates, which lacked specific antisera, were included; they typically represented about 3–5% of the total reactivity detectable in each assay and were subtracted from the corresponding experimental values.

The results of the competition assay were plotted as B/B_0 (B = cpm in precipitate of unlabeled antigen; B_0 = cpm in precipitate in the absence of unlabeled antigen, against the protein concentration of the unlabeled antigen, or against the amount of each monosaccharide, on a semilog scale). The intraassay variability was ~5%, and the interassay difference did not exceed 10–12%. The significance of the results which were obtained in the different experiments was assessed on the basis of the Wilcoxon test for coupled differences (Wilcoxon, 1963).

Results

Purity of LDL Preparations. Thorough evaluation of the purity of the LDL fractions was needed in order to avoid misinterpretation due to the presence of contaminating lipoproteins, apolipoproteins, or serum proteins (trace amounts of Apo-A I, Apo-C, Apo-D, or Apo-E may be present (Lee & Alaupovic, 1972; Alaupovic et al., 1972; Huang & Lee, 1979). Minute amounts of albumin (D. M. Lee, personal communication) or of β_2-glycoprotein I (Polz et al., 1979) may also be present as actual constituents or as contaminants of the LDL particle.

The immunodiffusion and immunoelectrophoretic patterns of the LDL preparations reacting with antisera to human whole serum showed a single precipitation line characteristic for β-lipoprotein in the β region. No reactivity was observed against antisera to Apo-C I, Apo-C II, Apo-C III, albumin, or β_2-glycoprotein. The absence of albumin was ascertained by reaction of different amounts of LDL (5–40 μg of LDL protein) with 80 μL of serial dilutions of antiserum to human albumin (1:1–1:10).Furthermore, β_2-glycoprotein was absent in the radial immunodiffusion assays performed with the M-Partigen plates impregnated with antiserum to β_2-glycoprotein I, the amount of LDL applied to the plates being in the range 25–60 μg of protein LDL.

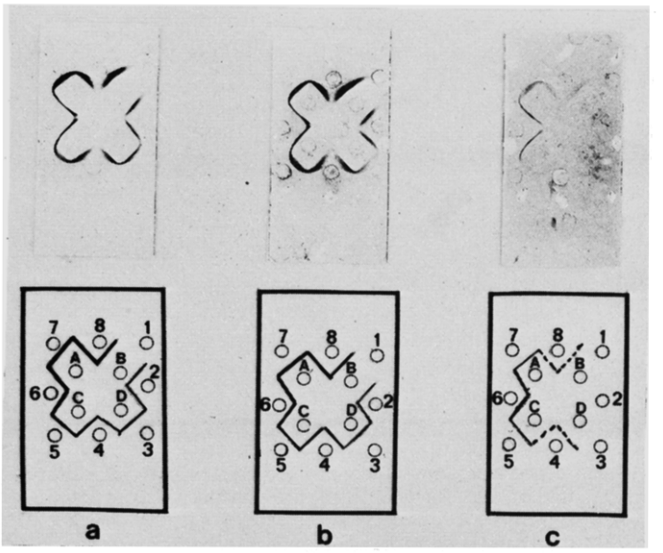

FIGURE 1: Evolution of antibodies during the course of immunization. Immunodiffusion patterns of an antiserum to LDL (antiserum no. 132), from different bleedings, reacting with LDL. (Wells A, B, C, and D) LDL (same preparation, 45 μg of LDL protein); (well 1) rabbit serum before immunization; (wells 2–8) antiserum corresponding to the first to seventh week after immunization. (a, b, and c) Slides stained for protein, lipid, and carbohydrate, respectively.

The chemical and physical properties of our LDL preparations of *d* 1.024–1.045 g/mL have been described in detail elsewhere (Chapman & Goldstein, 1976; Chapman et al., 1978); those employed in the present studies did not differ in any significant way from the aforementioned.

Testing and Titration of Antisera. All five rabbits immunized with LDL, together with a further two animals immunized with Apo-B, developed antibodies to the respective immunogens. All the antisera showed sharp precipitation lines, typical of β-lipoprotein upon immunoelectrophoresis against human whole serum. The presence of antibodies was detected by immunodiffusion. Figure 1 shows an LDL reacting with antiserum taken from the same rabbit at eight different bleedings and corresponding to preimmunization and then to weeks 1–7. The presence and sharpness of the precipitation line reveals a weak reactivity from the seventh day of immunization onward (well 2); this reactivity reaches a maximal level in the midst of the immunization (well 6), and declines after 2 months (well 8). Such findings are evident on the slides stained for protein (a) and for lipid (b). The third slide (c), stained for carbohydrate, shows a faint immunological reactivity only after 2 weeks of immunization (well 3); this reactivity became more marked after 5 weeks of immunization (well 6, corresponding to the strongest line evident in the slides stained for protein and lipid) and began to decline the week after (wells 7 and 8).

When antisera were allowed to migrate during electrophoresis and subsequently reacted with LDL, antisera corresponding to the early course of immunization were found to consist almost entirely of IgM antibodies (the precipitation line was located near the well in which the sample of antiserum

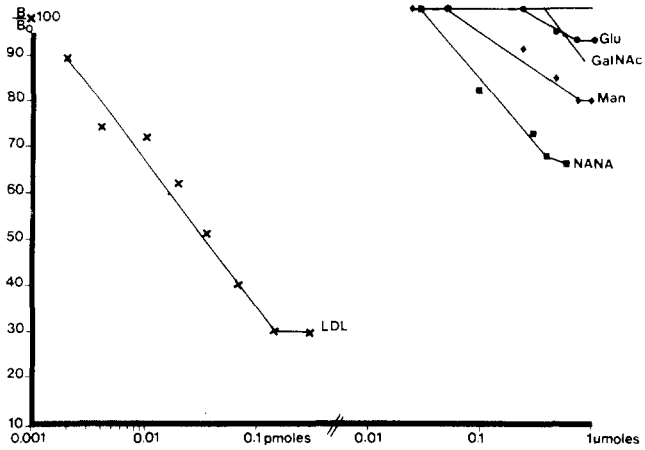

FIGURE 2: Displacement of radiolabeled LDL by unlabeled LDL and by monosaccharides. Representative curves obtained with an antiserum to LDL (no. 147, dilution 1:5000), reacting with unlabeled LDL (0.002–0.28 pmol) and with several monosaccharides (0.5 nmol–1.1 μmol). Data are plotted as $B/B_0 \times 100$ (see Materials and Methods) against concentration of ligand on a semilog scale.

had been applied), whereas all the antisera derived from later bleedings contained IgG primarily, i.e.; the precipitation line exhibited an anodal mobility typical for IgG (not shown).

The titers of the different antisera to LDL, determined by double immunodiffusion, varied from 1:2–1:4 after the first week of immunization to 1:64–1:128 after the booster injection. The highest titer of the Apo-B antisera did not exceed 1:16–1:32.

Radioimmunoassay. (*a*) *Assessment of Quality of Labeled LDL.* Four different LDL prepartions were labeled with ^{125}I; the incorporation of ^{125}I into LDL was some 29–34%. The specific activity of the ^{125}I-labeled LDL ranged between 130 and 250 cpm/ng of protein; after addition of 10% trichloroacetic acid, 3–6% of the radioactivity remained in the supernatant. The extent of lipid labeling did not exceed 8%. The immunodiffusion and immunoelectrophoretic patterns of labeled LDL were identical with those of the native molecule, and when incubated with antibody excess, 95–98% of the radiolabeled LDL was precipitated; the integrity of the labeled molecule was thus established. The labeled LDL diluted 1:10 in glycine buffer was found to be stable for 4–6 weeks when conserved at 4 °C.

(*b*) *Radioimmunoassay for Determination of Antiserum Dilution.* Dilution curves were established for all the antisera; the dilution of each antiserum corresponding to a 40–50% binding of radiolabeled LDL was retained for the subsequent competition assays. A 50% binding of labeled LDL was obtained with a 1:4000 dilution of the antiserum to LDL and with 1:500 dilution for the antiserum to Apo-B. Similar results were obtained with the other antisera to LDL and to Apo-B.

(*c*) *Kinetics of Binding of Radiolabeled LDL.* In order to establish the optimal period of incubation for the competition assay, we performed several kinetic studies of the binding of labeled LDL to the homologous antiserum. These studies revealed that an incubation in excess of 5 days was necessary for maximum binding (35%, 48%, 60%, 72%, and 75% at 1, 2, 3, 4, and 7 days, respectively). We therefore adopted a 7–9-day incubation period in the present study.

(*d*) *Competition Studies with Unlabeled LDL and with Different Monosaccharides in Binding of Labeled LDL to Antisera with High Antibody Titers.* In this series of experiments, competition studies were carried out with samples of antisera at their highest titer. After determination of the dilution to be employed, each antiserum was reacted with at least two separate labeled LDL and with up to five different unlabeled LDL. Seven to nine competition experiments were thus performed with unlabeled LDL and with the different monosaccharides.

Table I: Summary of Inhibition of Binding of Labeled LDL by Unlabeled LDL and Several Monosaccharides[a]

competitors for labeled LDL binding	amount of competitor (μmol)	% inhibition (±SD)	Wilcoxon test, $p_{2\alpha}$
LDL	0.002–0.24[b]	9.8–64.1 (±4.5–19)	≤0.01
mannose	0.05–1.1	0–19 (±0–12.2)	≤0.01
sialic acid	0.03–0.6	0.2–28.2 (±0.6–18)	≤0.02
glucose	0.05–1.1	0–1	ns[c]
N-acetylglucosamine	0.04–0.9	1–7 (±0.6–6.2)	≤0.10
galactose	0.05–1.1	0–3.7 (±0–7.6)	ns
N-acetylgalactosamine	0.04–0.9	0–9.3 (±0–11.4)	≤0.05
fucose	0.05–1.0	0–5.2 (±0–8.9)	ns

[a] The results represent means (±SD) from nine experiments with five different antisera to LDL, collected in the middle of the immunization course. [b] In picamoles. [c] ns = not significant.

Figure 2 shows such representative competition curves. Under the same experimental conditions, some monosaccharides compete with labeled LDL for binding; at the highest concentrations of competitors, sialic acid inhibited the binding of labeled LDL to ~35%, mannose to 18%, and glucose to ~11%. The inhibitions of 12% and 5% obtained with *N*-acetylgalactosamine and fucose, respectively, are difficult to interpret as they fall within the variability (10%) of the assay; galactose and *N*-acetylglucosamine do not show any inhibitory activity. It is noteworthy that significantly higher amounts of monosaccharides (the order of micromoles) than of unlabeled LDL (<1 pmol) are needed in order to exert a competitive effect.

The results obtained with five different antisera corresponding to the middle of the immunization period are summarized in Table I. Unlabeled LDL competed with labeled LDL up to a level of 64%; mannose and sialic acid inhibited the binding of labeled LDL up to 19% and 28%, respectively. These results were highly significant by the Wilcoxon test ($p_{2\alpha} \leq 0.01$ and ≤ 0.02, respectively). The other monosaccharides inhibited the binding of labeled LDL to a level of <10%. It may be noted, however, that galactosamine, which inhibited to the extent of 9.3%, presented a large standard deviation (up to 11.4%); considered together with the Wilcoxon test ($p_{2\alpha} \leq 0.05$), this suggests that such results, though not conclusive, may be considered indicative of some degree of competitiveness. The inhibition curves with mannose and sialic acid were not parallel (three experiments using five different antisera to LDL) with that for unlabeled LDL (data not shown), suggesting that only certain determinants are recognized in the monosaccharides as compared to the total LDL.

The interpretation of the above assays raises the question of the specificity of the inhibition exerted by the monosaccharides. The possibility of a nonspecific steric hindrance of the antibody in its reacting site had to be taken into consideration. In order to further validate the specificity of monosaccharide inhibition, we performed several control experiments in addition to those usually included for labeled LDL and for competitors and LDL incubated in the absence of specific antisera. A series of experiments was therefore performed with this aim with the same antisera but with samples obtained at the beginning of the immunization course, i.e., 7 days after the first injection of immunogen. The immuno-

Table II: Summary of Percent Inhibition Obtained with Unlabeled LDL and Different Monosaccharides Reacting as Competitors in Binding of Labeled LDL with Five Different Homologous Antisera[a]

	2–3 weeks of immunization			4–6 weeks of immunization			7–8 weeks of immunization		
	mean	SD	$p_{2\alpha}$	mean	SD	$p_{2\alpha}$	mean	SD	$p_{2\alpha}$
LDL (0.24 pmol)	46.6	22.5	≤ 0.01	61.4	26.2	≤ 0.01	61.8	24.7	nd[c]
Man (1.1 μmol)	31.8	20.6	≤ 0.02	16.3	16.9	≤ 0.05	11.8	13.6	nd
NANA (0.6 μmol)	3.2	6.5	ns[b]	25.0	17.1	≤ 0.01	36.4	14.7	nd
Glu (1.1 μmol)	2.0	6.0	ns	13.5	4.6	≤ 0.02	1.9	2.2	nd
GluNAc (0.9 μmol)	2.5	7.0	ns	3.0	6.0	ns	6.0	1.2	nd
Gal (1.1 μmol)	6.2	10.0	ns	9.2	3.9	≤ 0.10	3.4	4.5	nd
GalNAc (0.9 μmol)	4.1	7.0	ns	7.3	9.3	≤ 0.10	4.6	4.6	nd
Fuc (1.0 μmol)	2.5	6.8	ns	9.0	1.0	ns	9.4	4.8	nd

[a] The results are expressed as means (±SD) at the highest amount of competitor, i.e., 600 ng of unlabeled LDL and 200 ng of the different carbohydrates. For the periods 2–3 weeks and 4–6 weeks of immunization, seven to nine RIA experiments were performed, and the significance was calculated according to the Wilcoxon test; only four to five experiments were done for the last period of immunization, and no significance test could be applied. [b] ns = not significant. [c] nd = not determined.

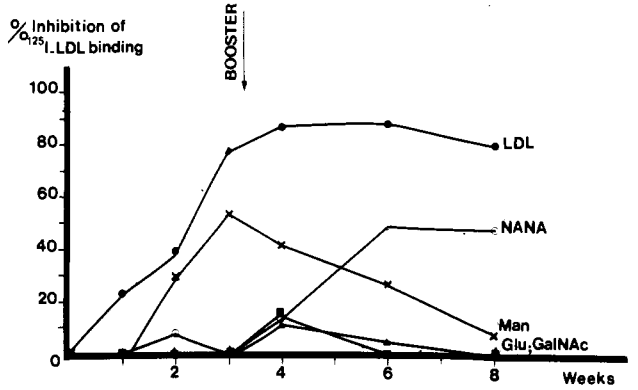

FIGURE 3: Competition curves of unlabeled LDL and of different monosaccharides in the reaction of labeled LDL with samples of the same antiserum to LDL taken at different periods of immunization. Samples of antiserum (0.1 mL) in a dilution of 1:4000 were allowed to react with the maximal amounts of competitor (i.e., 0.24 pmol of unlabled LDL or 1.1 μmol of the different monosaccharides) prior to addition of labeled LDL.

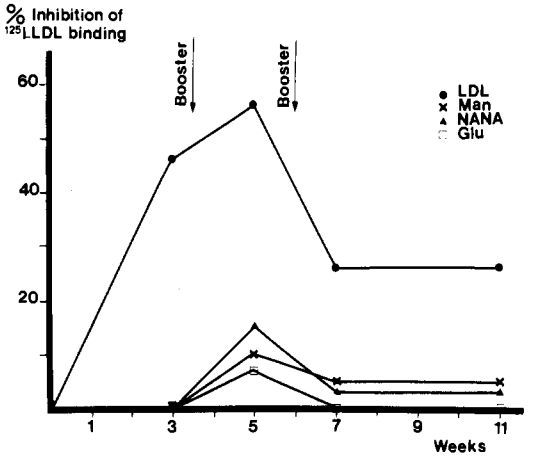

FIGURE 4: Inhibition of binding of labeled LDL to samples of antiserum to Apo-B, corresponding to different immunization periods, by unlabeled LDL and by several monosaccharides. Aliquots (0.1 mL) of an antiserum to Apo-B (no. 78), at a dilution of 1:500, were allowed to react for 7 days with the maximal amounts of competitors, i.e., unlabeled LDL (600 ng) and different monosaccharides (200 μg), before the addition of labeled LDL. (●) LDL; (×) Man; (▲) NANA; (□) Glu.

diffusion patterns had indeed shown (see above) the presence of a precipitation line staining with Amido black and Sudan black (but not with Schiff's reagent). At this period competition studies with antisera derived from early bleedings (Figure 3) showed that unlabeled LDL competed with labeled LDL for binding antiserum up to the level of 24% but that the various monosaccharides failed to inhibit this binding. The immunological specificity of the previous inhibition reaction exerted by the monosaccharides thus appeared to be established.

(*e*) *Competition Studies with Unlabeled LDL and with Monosaccharides in Reaction of Labeled LDL with Different Antibody Populations from the Same Antiserum.* As previously mentioned, antibodies reacting with the carbohydrate moiety of LDL did not appear at the commencement of immunization. We therefore investigated the inhibition exerted by the monosaccharides on the binding of labeled LDL to the different antibody populations produced during the immunization course. Figure 3 shows a representative inhibition which was obtained in the competition of unlabeled LDL and of the different monosaccharides with samples of antiserum corresponding to different immunization periods, i.e., between the first and the eight week following the initial immunization. Antibodies to LDL are present after the first week of immunization and rise regularly; they are maximal after the booster injection and begin to decline 8 weeks after the first injection of immunogen. Mannose acts as an inhibitor in the presence of antibodies derived from the early course of the immunization and less with antibodies corresponding to the end of the immunization period. In contrast, antibodies which react with sialic acid appear more slowly but persist longer; it is, however, noteworthy that a low degree of inhibition is present from the second week of immunization. Some inhibition was also apparent in this experiment with glucose and glucosamine. Similar inhibition curves were observed with the other antisera to LDL.

Table II presents a summary of the results which were obtained with five different antisera to LDL; the results refer to samples corresponding to the beginning of the immunization (weeks 2–3), to the middle of the immunization (i.e., after the booster injection, weeks 4–6), and to the end of the immunization period (weeks 7–8) and are in accordance with those reported in Figure 3.

Similar inhibition assays were performed for the binding of labeled LDL to two antisera to Apo-B; the development of the different antibodies was evaluated in one (Figure 4). Mannose and sialic acid (and to a lower degree glucose) inhibited the binding of labeled LDL only in the antiserum sample corresponding to the highest antibody titer; the maximum inhibition which was obtained with unlabeled LDL was ~37.8% (SD ± 14.6; $p_{2\alpha} \leq 0.01$). Sialic acid inhibited to the level of 9.3% (SD ± 14.1; $p_{2\alpha} \leq 0.10$) and mannose to ~11.0% (SD ± 13.2; $p_{2\alpha} \leq 0.05$); the large standard deviations probably arose from the use of only two antisera, which differed

markedly in their degree of reactivity. Thus, in the case of sialic acid, one showed <10% inhibition, whereas the other constantly revealed higher levels (10–20%). (Fucose showed an inhibitory activity of ~6%, but with an elevated SD (±10), a result which was at the limit of significance ($p_{2\alpha} \leq 0.10$) by the Wilcoxon test.)

Discussion

Knowledge of the participation of carbohydrates in the antigenic determinants of both glycoproteins and glycopeptides has been obtained from numerous studies [see Horowitz (1978) for a review]. Although it has been claimed that antibodies are not formed against carbohydrates such as sialic acid present in glycoproteins (Gottschalk et al., 1971), because they constitute "immunological self", the existence of important examples to the contrary indicates, according to Horowitz (1978), that this concept is an oversimplification. It may indeed appear at first sight surprising that antibodies to such a common component as carbohydrates can be produced in a host animal, and this problem has given rise to some degree of controversy (Horowitz, 1978). The important question as to the origin of antibodies directed to immunological-self substances in general has been approached in recent years by Kazim & Atassi (1977). These authors have shown that rabbits can make, for example, antibodies to regions of the sperm whale myoglobin molecule which are identical with the corresponding regions in rabbit myoglobin; however, such antibodies cannot be detected by precipitation reactions (rabbit myoglobin does not precipitate with antiserum to sperm whale myoglobin) but may be revealed by more sensitive reactions such as inhibition of the precipitation; thus, rabbit myoglobin may inhibit the precipitin reaction of sperm whale myoglobin with its homologous antiserum.

The role of carbohydrates in the antigenic determinants of several glycoproteins and the importance of tertiary structure in their specificity have been established by Kabat (1966, 1978). Thus, recent studies have demonstrated the production of antibodies against defined oligosaccharides bound to proteins (Kieda et al., 1977; Zopf et al., 1978; Ramanathan et al., 1979) and to phospholipids (Marcus & Schwarting, 1976); moreover, the immunological specificity of neutral glycosphingolipids is considered as being primarily determined by their carbohydrate residues (Rapport & Graf, 1967).

The major difficulty in studies concerning the involvement of the carbohydrate moiety in the antigenic determinants of glycoproteins and glycolipids lies in the technological approach, since carbohydrates may play the role of a hapten in both these glycoconjugates (Horowitz, 1978). It is therefore necessary to resort to the most sensitive immunological techniques, such as radioimmunoassay and electroimmunoassay, and moreover to employ the carbohydrates as inhibitors (Kieda et al., 1977; Hruby et al., 1977; Zopf et al., 1978; Vreeland & Chapman, 1978; Ramanathan et al., 1979; Zalc et al., 1979). In addition, elevated amounts of monosaccharides are needed in such competition studies. Thus, in order to obtain 50% inhibition of antimyelin antibodies, Hruby et al. (1977) used 0.5–1 M galactose, whereas Zopf et al. (1978) utilized 1 mM mannose for 50% inhibition of anti-mannotetraose antibodies and Kieda et al. (1977) used 2 mM sialic acid for a 10% inhibition of antibodies to *N*-diacetylchitobiose.

To evaluate the contribution of carbohydrates to the antigenic structure of LDL, we took recourse to competitive radioimmunoassay techniques. Thus in examining the binding of labeled LDL with homologous antisera, we employed relatively large amounts (with respect to the carbohydrate content of LDL) of different monosaccharides (i.e., up to 1.1 μmol of mannose, glucose, and galactose, 1.0 μmol of fucose, 0.9 μmol of *N*-acetylglucosamine and *N*-acetylgalactosamine, and 0.6 μmol of sialic acid). Our inhibition studies of the binding of labeled LDL with several homologous antisera, each corresponding to the maximum titer seen during the immunization period, indicated that sialic acid and mannose compete with labeled LDL to give 28% and 19% of inhibition, respectively, in the radioimmunoassay (Figure 2 and Table I). The competition studies were performed with antisera containing different antibody populations and corresponding to different periods of immunization. Mannose competed effectively with antibodies present during the early course of the immunization; such antibodies declined progressively, whereas sialic acid was more competitive with antibodies which appeared later during the immunization (Figure 3 and Table II) and which lasted longer.

Radioimmunoassay studies with antisera to apolipoprotein B (Figure 4) indicated a lower competitiveness of both carbohydrates and unlabeled LDL as compared to antisera to LDL; moreover, a parallelism was present in the inhibition course with mannose and sialic acid. As mentioned earlier, one of two antisera, of greater reactivity with apo-B itself, revealed levels of inhibition by both mannose and sialic acid which were clearly above that of experimental error. These data supplement those obtained with antisera to LDL.

Further evidence for the implication of sialic acid in the antigenic reactivity of LDL may be derived from the unpublished observations of S. Goldstein, C. de Blic, and M. J. Chapman. These studies indicate that LDL, deficient in some 60% of its sialic acid after (highly purified) neuraminidase treatment under well-defined conditions, inhibits the binding of labeled LDL in RIA some 10–15% less than the corresponding native preparation.

Since the different controls for any nonspecific competition by the different monosaccharides were negative, our data suggest that sialic acid and mannose participate in the antigenic determinant(s) of LDL. Such findings are consistent with the data of Swaminathan & Aladjem (1976) and with those of Harmony & Cordes (1975) and McConathy & Alaupovic (1974). Indeed, Swaminathan & Aladjem have shown that 50% of the carbohydrate moiety of LDL is represented by two glycopeptides, i.e., glycopeptide I, containing 6 residues of mannose and 2 residues of *N*-acetylglycosamine per mole of glycopeptide and which presents mannose as terminal carbohydrate, and glycopeptide II, containing 2 residues of sialic acid, 5 residues of mannose, 3 residues of glucosamine, and 2 residues of glucose per mole of glycopeptide and in which sialic acid represents the terminal carbohydrate. At the same time, the studies of McConathy & Alaupovic (1974) and those of Harmony & Cordes (1975) demonstrated the specific binding of LDL to concanavalin A, thereby suggesting the presence of mannose (the principal carbohydrate involved in the binding of glycoproteins to Con A) at the surface of LDL.

The elevated amounts of mannose in the LDL particle, its location at the surface of the molecule, and its presence as a terminal residue may explain the early appearance of antibodies which react with this carbohydrate. Concomitantly, the decline of these antibodies during the course of immunization may indicate that only those residues of mannose which are exposed at the surface of LDL or are located in terminal positions in glycopeptides (e.g., in Swaminathan's glycopeptide I), or both, are involved in the immunological reactivity of LDL; mannose residues located more toward the core of the glycopeptide (e.g., in glycopeptide II) may be less involved in

the immunological site(s) of LDL.

Sialic acid, which is also known to be present at the surface of LDL (Margolis & Langdon, 1966) and the terminal carbohydrate residue of glycopeptide I (Swaminathan & Aladjem, 1976), is more competitive with antibodies occurring toward the end of immunization. The apparition of antisera constituted of various antibody populations may be a result of the booster injection (i.e., augmented and repeated stimulation) but also of the in vivo activity of certain endoglycosidases which may liberate new oligosaccharide fragments from the intact glycoprotein; such fragments may subsequently stimulate antibody formation. The above findings prompt us to suggest that mannose and sialic acid may be located not only in surface-exposed regions of the LDL particle, but also in less superficial areas, a proposal which is not inconsistent with the structure of glycopeptides I and II (in the case of mannose) (Swaminathan & Aladjem, 1976) and with the inability of proteases to remove sialic acid and mannose-containing fragments from this lipoprotein (Margolis & Langdon, 1966; Harmony & Cordes, 1975).

The inhibitory reactivity of both mannose and sialic acid probably depends not only on the sequence of the oligosaccharide chain, but also on the general configuration, charge, and dimension (Horowitz, 1978) of the antigenic site of LDL; this suggestion is consistent with the lower reactivity observed in the reactions with the antisera to Apo-B. The conformational changes occurring in Apo-B subsequent to the delipidation of LDL may ellicit antibodies which differ from those resulting from immunization with the native molecule.

Galactose, galactosamine, glucosamine, and fucose did not exhibit inhibitory reactivity in the reactions with any of the antisera to LDL or to Apo-B. This was especially interesting for fucose, since this monosaccharide represents the carbohydrate residue specific for human erythrocyte Lewis antigens (Horowitz, 1978). Lewis antigens are glycosphingolipids (Hakomory & Kobata, 1974) which are not synthesized in situ but which are acquired by red cells from circulating lipoproteins, and especially from LDL (Marcus & Cass, 1969). It was therefore of interest to determine whether fucose also contributes to the antigenic site of LDL, the latter ensuring their transport. This was not the case, and fucose did not appear to participate in the antigenic site(s) of LDL. The serum LDL contains some 60% of the glycosphingolipids of the total lipoproteins of $d < 1.21$ g/mL (Dawson et al., 1976). As reported by these authors, and also by others (Chatterjee & Kwiterovitch, 1976), such glycosphingolipids contain in addition to fucose, glucose, galactose, galacosamine, and sialic acid. A slight degree of inhibition (mean 13.5%, SD ± 4.6%) was seen only with glucose ($p_{2\alpha} \leq 0.02$); it is difficult to assess whether this is related to an involvement of glucose in the antigenic site of LDL. Moreover, if glycosphingolipids are involved in the antigenic site(s) of LDL, then the high degree of inhibition obtained with sialic acid may also derive from the presence of this component (and particularly of GM_3 ganglioside).

Antibodies to carbohydrate determinants may cross-react with components bearing the same nonreducing terminal residues, regardless of other differences in structure (Kabat, 1966). It was thus important to establish whether any cross-reactivity existed between LDL and other glycoproteins, especially β_2-glycoprotein I, which has been reported to be present in human serum lipoproteins (especially in VLDL, but also in LDL) (Polz et al., 1979). In studies with several LDL preparations and antiserum to β_2-glycoprotein I, no cross-reactivity was detected upon either ID or IEP. Moreover, use of antisera to different glycoproteins (i.e., antiserum to β_2-glycoprotein I and antiserum to β_1SP_1-glycoprotein) in the radioimmunoassay of labeled LDL failed to detect any binding of LDL (unpublished data).

Briefly then, we conclude that mannose and sialic acid participate in the antigenic site(s) of LDL, a finding which is concordant with earlier suggestions of Giblett (1969) and of Utermann & Wiegandt (1971), which indicated an involvement of the carbohydrate moiety of LDL in the Ag system and in Lp(a) specificity, respectively. Finally, the involvement of mannose and sialic acid in the antigenic site(s) of LDL does not preclude their possible role(s) in the metabolism of the LDL particle, which remains controversial.

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