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Plasma anti-α-galactoside antibody binds to serine- and threonine-rich peptide sequence of apo(a) subunit in Lp(a)

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Abstract Lipoprotein(a) immune complexes [Lp(a) IC] of varying particle density obtained by ultracentrifugation of plasma from normal healthy donors were markedly dominated by IgG. Lp(a) and immunoglobulins were liberated from plasma Lp(a) IC by treatment with melibiose, a sugar specific for circulating anti-α-galactoside antibody (anti-Gal). Upon incubation with plasma lipoprotein fraction anti-Gal but not the α-glucoside-specific antibody from human plasma formed *de novo* IC with Lp(a). Binding of Lp(a) sugar-reversibly enhanced the fluorescence of FITC-labeled anti-Gal as did binding of α-galactoside-containing glycoproteins. This effect apparently due to conformational shift in the Fc region of the antibody was also produced by apo(a) subunit separated from Lp(a) and de-O–glycosylated apo(a) but not by any other plasma lipoproteins or by Lp(a) pre-incubated with the O–glycan-specific lectin jacalin. O–Glycans and their terminal sialic acid moieties in apo(a) of circulating Lp(a)-anti-Gal IC, in contrast to those in pure Lp(a), were inaccessible to jacalin and anion exchange resin, respectively. Unlike other plasma lipoproteins, Lp(a) inhibited *Griffonia simplicifolia* isolectin B4 which also accommodates serine- and threonine-rich peptide sequence (STPS) as surrogate ligand to α-galactosides at its binding site. Results suggest that anti-Gal recognizes STPS in the O-glycan-rich regions of apo(a) subunit in Lp(a) which contains no α-linked galactose.

Keywords Lipoprotein(a) · Lp(a) · Apo(a) · Anti-α-galactoside antibody · Serine- and threonine-rich peptide sequence · Immune complex

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Abbreviations

TAG	Terminal α-linked galactose
STPS	Serine- and threonine-rich peptide sequence
APAG	Affinity-purified anti-Gal
GS I-B_4	G*riffonia simplicifolia* isolectin B4
TI	Soybean trypsin inhibitor
TIM	Soybean trypsin inhibitor-melibiose
DIg	Dextran-binding immunoglobulin
PBS	Potassium phosphate buffer (20 mM) containing 150 mM NaCl
PBS-T	PBS containing 0.05 % Tween 20
IC	Immune complex

Introduction

Plasma anti-α-galactoside antibody (anti-Gal) is an evolutionary milestone as its biosynthesis started with advanced mammals such as man, apes and old world monkeys along with suppression of synthesis of the corresponding antigenic epitope *viz*. terminal α-linked galactose (TAG) on their glycoconjugates [1]. Consequently anti-Gal is the major plasma antibody responsible for rejection of xenografts from lower animals into man [2] justifying the extensive investigations on its sugar specificity [3]. Consisting mostly of IgG, anti-Gal accounts for about 1 % of circulating immunoglobulins and its synthesis is proposed to be triggered by TAG epitopes in gut bacterial antigens [4]. We had reported earlier that anti-Gal-reactive epitopes are present on glycoproteins isolated from human brain grey matter [5]. Later atherosclerotic plaques in humans were found to contain antibodies reactive against TAG [6]. Recognition by anti-Gal of TAG epitopes presented on liposomes was reported to augment wound healing in an *in vitro* model [7]. Epitopes reported to be surrogate to TAG in occupying the sugar binding site of

anti-Gal are serine- and threonine-rich peptide sequence (STPS) present in MUC-1 family of peptides [8].

High concentration of plasma lipoprotein(a) [Lp(a)] is a positive risk factor for atherosclerosis, stroke and neurodegenerative disorders [9]. Though Lp(a) molecules are far fewer in circulation than LDL, Lp(a) outnumbers LDL in atherosclerotic plaques [10]. Incidentally anti-Gal and Lp(a) occur in the same animals and are reported to be of comparable evolutionary age [1, 11]. We have recently reported that all samples of affinity-purified anti-Gal (APAG) from plasma contained co-purified anti-Gal-Lp(a) immune complexes [12]. In this communication we provide evidence that the carbohydrate-binding site of anti-Gal is utilized to form IC with Lp(a). We also show that though Lp(a) lacks TAG epitopes STPS at O-glycosylation sites of apo(a) subunit in Lp(a) is surrogate to TAG as ligand for anti-Gal.

Materials

Polystyrene 96 well break-apart microplates (MAXISORB) were purchased from Nunc, Denmark. Antibodies to human IgA, IgM, IgG raised in goat and those to apo(a) and apoB raised in rabbit were obtained from Dako, Denmark. *Griffonia simplicifolia* isolectin I-B4 (GS I-B_4), Tween-20, bovine thyroglobulin, horse radish peroxidase (HRP), orthophenylene diamine (OPD), dithiothreitol (DTT), melibiose, 1-O-methyl-α-D-galactoside, soybean trypsin inhibitor (TI), biotin-3 sulfo-N-hydroxysuccinimide ester sodium salt, streptavidin, fluorescein isothiocyanate (FITC), DEAE-Sephadex A-50 and Sephadex G-50 were purchased from Sigma Aldrich, Bangalore, India. Standard Lp(a) (International Reference SRM 2B standardisation) [13] used was part of Lp(a) assay kit from APTEC Diagnostics nv, Belgium. All other reagents used were of analytical grade. Out-dated human plasma from healthy volunteers (18–40 years) was collected from Department of Blood Transfusion Services of this institute with Institutional Ethics Committee clearance (IEC-502).

The lectins, jacalin from jackfruit seed (*Artocarpus integrifolia*) and concanavalin A from jack beans (*Canavalia ensiformis*) were prepared by affinity chromatography on cross-linked guar galactomannan (CLGG) and Sephadex G-50, respectively [14, 15]. Dextran-binding immunoglobulin (DIg) from human plasma was prepared by affinity chromatography on Sephadex G-200 [16]. Following treatment of 2 ml plasma with 4 ml CLGG and 4 ml Sephadex G-200 gels in succession in PBS at 4 °C for 4 h, the unbound protein was used as plasma proteins minus anti-Gal and DIg. Soybean trypsin inhibitor-melibiose (TIM) was prepared by coupling melibiose to trypsin inhibitor by reductive amination [17]. Proteins were biotinylated using sulfo-NHS-biotin as described by Anu Paul *et al.* [18] in presence of 1-O-methyl-α-D-galactose or dextrose (50 mM) for anti-Gal and DIg respectively. Purified anti-Gal and jacalin were labeled with FITC as described by Hudson and Hay [19].

Methods

Ultracentrifugation to separate plasma lipoproteins and their IC

Plasma samples were adjusted to density 1.24 g per ml by addition of KBr and centrifuged at 535,000×*g* (1 ml tubes) or 202,000×*g* (4 ml tubes) for 4 h at 4 °C in HITACHI CS150GXL ultracentrifuge. Specified volumes were collected from the top by aspiration and dialysed to remove KBr.

Preparation of purified Lp(a) and Lp(a)-free plasma lipoproteins

Purified Lp(a) was prepared using affinity precipitation with lectin followed by electrophoresis by a method developed in this laboratory [Kalaivani.V and Appukuttan. P S; communicated]. Briefly, plasma and jacalin solution (0.8 mg per ml PBS) were mixed in the ratio 1:1, protein precipitate obtained collected by centrifugation, redissolved in 100 mM 1-*O*-methyl-α-D-galactoside by 2 h incubation at 4 °C. After ultracentrifugation at 535,000×*g* as above lipoproteins that segregated to the top 20 % of the volume were collected and subjected to Tris-borate EDTA electrophoresis at pH 8.7. Lipoproteins from crushed gel bands were passively eluted into PBS. Lipoprotein band containing both apo(a) and apoB subunit as determined by ELISA was identified as Lp(a) and band containing only apoB as LDL. For identification of subunits in JL1 this sample was subjected to SDS-PAGE and Western blotting according to the method of Towbin *et al.* [20] using 3.5 % acrylamide and PVDF membrane for transfer. Transfer strips were stained with HRP conjugates of antibodies to apo(a) or apoB. The supernatant after removal of jacalin-precipitated proteins was subjected to another ultracentrifugation at 535,000×*g* (Ultracentrifugation to separate plasma lipoproteins and their IC section) and the top 20 % layer constituted Lp(a)-free lipoproteins.

Preparation of anti-Gal samples

Affinity purified anti-Gal (APAG) containing co-purified Lp(a)-anti-Gal IC was prepared from outdated human plasma by a single step affinity chromatography on CLGG as described earlier [21]. To prepare Lp(a)-free purified anti-Gal 70 ml plasma treated overnight at 4 °C with 0.2 M galactose was subjected to ultracentrifugation at 202,000×*g* as described above. Bottom 30 % volume from all tubes were pooled, dialysed against PBS and applied on a 60 ml CLGG column to prepare anti-Gal as above.

Assay of Lp(a), immunoglobulins and Lp(a)-IC

For enzyme-linked immunosorbent assay (ELISA), proteins were coated on 96 well microtitre wells by incubating their defined dilutions in 200 μl PBS with the wells at 4 °C overnight or at 37 °C for 3 h. Wells were then blocked by 30 min incubation with PBS containing 0.5 % Tween 20 and washed twice with PBS-T. Unless otherwise mentioned Lp(a) was assayed by the J-a assay reported recently [22]. Briefly, Lp(a) sample dilutions in PBS-T (200 μl) was incubated with jacalin-coated wells (1 μg per well) for 2 h and wells were washed as above. After 2 h incubation at 4 °C with anti-apo(a)-HRP (1.5 μg antibody per ml) in PBS-T and washing, bound HRP was assayed by incubating for 15 min with 200 μl OPD (0.5 mg/ml) in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.03 % H_2O_2, stopping the reaction with 50 μl 12.5 % H_2SO_4 and reading the plates at 490 nm in BIOTEK ELISA reader, USA. To assay immunoglobulin content samples coated on microwells as above were probed with a mixture of HRP conjugates of antibodies to human IgG, IgM and IgA (all 1.5 μg antibodies per ml) in PBS-T and bound HRP activity was assayed. Lp(a) IC was assayed by capturing dilutions on wells coated with anti-apo(a) (1 μg per well), washing with PBS-T and probing with a mixture of HRP conjugates of antibodies to human IgG, IgA and IgM as above.

Sugar-mediated release of Lp(a) and antibodies from plasma IC

Following ultracentrifugation at 535,000×*g* the fractions in 20–70 % volume from top was used as source of plasma Lp(a) IC . After dialysis in PBS this fraction was treated with or without 100 mM melibiose for 2 h at 4 °C. After a further 535,000×*g* ultracentrifugation in 1 ml tubes top 20 % volume was used for Lp(a) assay by coating its 40 times dilution in PBS on polystyrene wells and probing with HRP-labeled anti apo(a)-HRP as described above. Bottom 30 % was dialysed against PBS, diluted 3 times in PBS and coated on polystyrene plates. Bound antibody was assayed using a mixture of HRP conjugates of antibodies to human IgG, IgA and IgM as described before.

Determination of de novo IC formed between purified anti-Gal and plasma Lp(a)

Since top 20 % volume after 202,000×*g* ultracentrifugation of plasma contained >90 % of total plasma lipids without presence of free antibodies (data not shown), 950 μl of this sample in PBS was mixed with biotin-labeled antibody or protein (anti-Gal, DIg or plasma proteins minus anti-Gal and DIg; 100 ng in 50 μl) at 4 °C overnight. After ultracentrifugation at 535,000×*g* as described earlier (Ultracentrifugation to separate plasma lipoproteins and their IC section) top 70 % volume containing no free antibodies was diluted 5 times with PBS-T and 200 μl added to polystyrene wells coated with 2 μg streptavidin to capture lipid-bound biotinylated antibody. The latter was then assayed by probing with a mixture of HRP conjugates of anti-human IgA, IgG and IgM. To assay Lp(a) IC in bottom 30 %, 5 times dilution of this layer (200 μl) was incubated with anti-apo(a)-coated wells (2 μg per well), at 4 °C for 2 h and bound IC determined by incubation with HRP-labeled avidin (0.375 μg avidin per ml) and assay of bound HRP.

Isolation and de-O-glycosylation of apo(a)

After ultracentrifugation of 8 ml plasma at 535,000×*g* in 1 ml tubes (Ultracentrifugation to separate plasma lipoproteins and their IC section), the combined yellow layer (about 700 μl) containing plasma lipoproteins only was collected, diluted to 2 ml in PBS and treated with 4 mM dithiothreitol for 15 min at 37 °C followed by a similar ultracentrifugation. The apo(a) separated from Lp(a) was collected in the bottom 20 % volume and dialysed against PBS. One half of the sample was de-O-glycosylated by treatment with 1 M sodium borohydride in 0.05 N NaOH for 24 h at 37 °C and dialysed against PBS. To check the integrity of peptide chains after alkaline borohydride treatment, de-O-glycosylated apo(a) [DOG apo(a)] or apo(a) (1.3 μg in 20 μl PBS) was injected into BIOSEP-SEC-S-2000 size exclusion column in Varian Prostar HPLC system run at 1 ml per min and eluate monitored for peptide absorption at 230 nm. To assess the O- and N- glycans, apo(a) or DOG apo(a) coated on microtitre wells (1.3 μg per well) were treated with HRP conjugates of jacalin (0.15 μg lectin per ml) or concanavalin A (15 μg lectin per ml) respectively for 2 h at 4 °C and bound HRP activity assayed (Assay of Lp(a), immunoglobulins and Lp(a)-IC section). Homogeneity of apo(a) was examined by probing polystyrene well-coated apo(a) with HRP conjugates of anti-apo(a) and anti-apoB (1.5 μg antibody per ml).

Fluorescence enhancement in FITC-anti-Gal

FITC-anti-Gal (6 μg antibody in 25 μl) was pre incubated with (+S) or without (−S) 50 mM melibiose in PBS for 1 h at 4 °C before mixing with 25 μl purified Lp(a) (6 μg) in PBS at 4 °C for 18 h. After dilution to 3 ml in PBS fluorescence was measured in Horiba Jobin Yvon FluroMax-4 spectrofluorometer using excitation at 485 nm and emission at 520 nm. Alternatives used for Lp(a) were apo(a), de-O-glycosylated apo(a), TIM, TI and Lp(a)-free plasma lipoproteins. To verify the involvement of sugar-binding site of anti-Gal in accommodating Lp(a), FITC-labeled antibody pre-incubated with graded concentrations of melibiose was used in the above trials. Further, to confirm the contribution of STPS of Lp(a)

towards binding to anti-Gal Lp(a) pre-incubated with jacalin was also employed as this lectin could cover the STPS region by binding to O-glycans characteristic of this sequence. FITC-jacalin was also used instead of FITC-anti-Gal.

Accessibility of O-glycans in free and IC Lp(a)

Lp(a) in Lp(a) IC present in APAG was not assayed by the J-a assay described earlier due to the possibility of its O-glycans being masked by bound anti-Gal. Alternatively apoB content was assayed by coating APAG on polystyrene wells (4 μg protein per well) and probing with HRP-labeled anti-apoB antibody (1.5 μg antibody per ml). ApoB content of known amount of pure Lp(a) from the same plasma sample was also determined by the same method coating 500 ng Lp(a) per well. From this the Lp(a) content of APAG was calculated. Accessibility of O-glycans in free and Lp(a) IC was measured in terms of their binding to DEAE-Sephadex A-50 and plate-coated jacalin. For DEAE-Sephadex A-50 treatment 400 ng purified Lp(a) or APAG Lp(a) containing the same amount of pure Lp(a) as determined by the above assay in 300 μl Tris–HCl buffer, pH 7.9 was treated overnight separately with 100 μl gel at 4 °C. Sephadex G-50 was used as control. For jacalin treatment purified Lp(a) or APAG Lp(a) each containing the same amount of pure Lp(a) (200 ng) in 200 μl PBS was added to polystyrene wells coated with jacalin (1 μg per well) and incubated overnight at 4 °C in Tween 20-free PBS . Uncoated polystyrene wells were used as control. To assay unbound fraction in trials with pure Lp(a) the lipoprotein in supernatant (200 μl of gel-treated and 180 μl of microwell-treated) was coated again on microwells and probed with HRP conjugate of anti-human apo(a) (1.5 μg antibody per ml). In trials with APAG the Lp(a) IC in aliquots of supernatants as above was measured as described earlier (Assay of Lp(a), immunoglobulins and Lp(a)-IC section). Binding of Lp(a) to DEAE-Sephadex or jacalin-coated wells was expressed as percentage reduction in their supernatants compared to control supernatants.

Lp(a) binding to MUC-1-specific lectin GS I-B_4

Biotinylated GS I-B_4 lectin (2 μg) was incubated overnight at 4 °C with varying dilutions of LDL purified by density separation method [23], purified Lp(a) or Lp(a)-free lipoproteins (Preparation of purified Lp(a) and Lp(a)-free plasma lipoproteins section). The mixture was added to wells coated with 1 μg thyroglobulin and incubated for 2 h at 4 °C. After washing, the bound lectin was assayed using HRP-labeled avidin (750 ng avidin per ml). Lp(a) was determined by J-a assay [22] and Lp(a)-free lipoproteins and LDL by Bradford assay [24].

Statistical Analysis

Data analysis was done using Microsoft Excel 2000 version.

Results

Anti-Gal-specific sugar releases free Lp(a) and immunoglobulins from circulating Lp(a) IC

Ultracentrifugation of 1 ml plasma samples at 535,000×*g* and density 1.24 g per ml employed to stack components according to their particle density showed that Lp(a) IC was present in all layers below the top 20 % volume which is known to contain all free lipoproteins (Fig. 1). For further investigations 20–70 % volume from top after ultracentrifugation was used as source of Lp(a) IC since high concentration of free immunoglobulins in bottom 30 % volume would interfere in assays. Treatment of the above Lp(a) IC fraction with anti-Gal-specific sugar followed by further ultracentrifugation at 535,000×*g* resulted in significantly greater release of Lp(a) to free lipoprotein zone (top 20 %) as well as of immunoglobulins to the free antibody zone (bottom 30 %) (Fig. 2a and b), underlining the involvement of anti-Gal in formation of Lp(a) IC.

IC formation by Lp(a) depends on sugar specificity of antibody

De novo ICs formed upon incubating purified biotin labeled plasma antibodies with components of

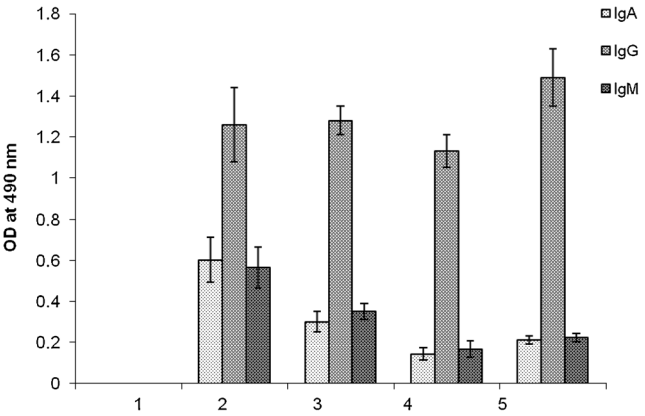

Fig. 1 Lp(a) IC is distributed in all layers of ultracentrifuged plasma except the top 20 %. Plasma (1 ml) subjected to ultracentrifugation at 535,000×*g* separated into 200 μl fractions (numbered 1 to 5 from top). Fifty times dilution of each fraction (200 μl in PBS-T) was added to microtitre wells coated with 2 μg anti-apo(a) antibody. The bound Lp(a)-IC was detected using HRP- labeled anti-human IgA, IgG and IgM individually. HRP activity was assayed as described (Assay of Lp(a), immunoglobulins and Lp(a)-IC section). Values are mean ± S.D of six consecutive plasma samples

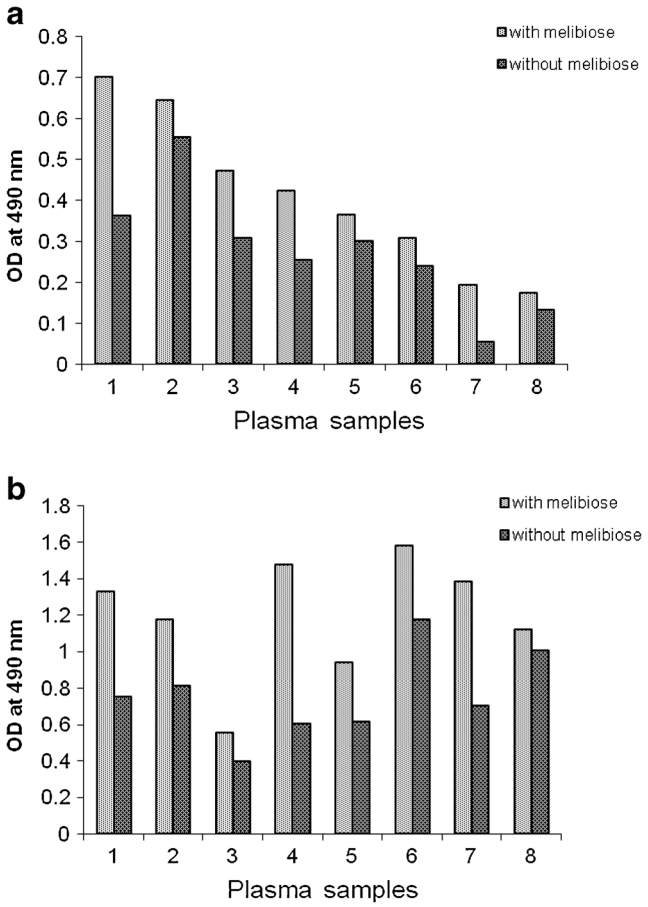

Fig. 2 **a** Anti-Gal-specific sugar releases Lp(a) from plasma immune complex layer. Following ultracentrifugation of plasma at 535,000×*g* Lp(a) IC free from non-lipoprotein IC was obtained in the 20–70 % volume from top. This layer was treated with or without 100 mM melibiose for 2 h at 4 °C. After further ultracentrifugation at 535,000×*g* top 20 % volume containing free Lp(a) was diluted 40 times, 200 µl coated on microplate wells and probed with 200 µl HRP-labeled anti-apo(a) (1.5 µg antibody per ml). P value of Lp(a) released by sugar= 0.0053; n=8. b Anti-Gal-specific sugar releases immunoglobulins from plasma immune complex layer. Antibodies liberated from Lp(a) IC-containing fraction of plasma by melibiose treatment were collected as bottom 30 % following ultracentrifugation at 535,000×*g* (Fig. 2a). Three times dilution of this sample was coated (200 µl) on microplate wells and probed with a mixture of anti-human IgA, IgG and IgM (1.5 µg antibody per ml). P value of antibody released by sugar=0.00195; n=8

lipoprotein-rich layer of plasma were examined after ultracentrifugation at 535,000×*g*. In the upper 70 % layer where free immunoglobulins are not found, the response in the assay protocol employed indicated level of antibody bound to any lipoprotein. In the denser bottom 30 % Lp(a) IC with labeled antibody was specifically measured. In both cases the response of the α-glucoside-specific DIg which had no affinity for Lp(a) was no better than that of total plasma proteins minus anti-Gal and DIg and significantly less than that of anti-Gal suggesting that anti-Gal is the chosen plasma antibody for IC formation by Lp(a) (Fig. 3).

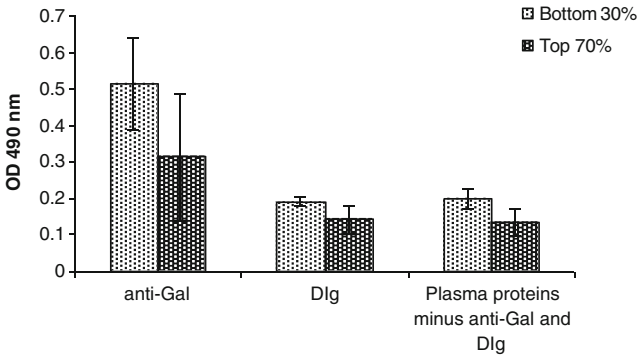

Fig. 3 Purified anti-Gal forms IC with Lp(a). Lipoprotein-rich plasma fraction was incubated overnight with biotinylated antibodies. Resulting ICs separated by ultracentrifugation into top 70 % and bottom 30 % were analysed by ELISA (Determination of de novo IC formed between purified anti-Gal and plasma Lp(a) section). Values are mean ± S.D of six consecutive plasma samples

De-O-glycosylation of apo(a) does not fragment its polypeptide chain

Limited reduction of plasma lipoproteins with 4 mM DTT effected detachment of apo(a) alone from Lp(a) and its migration on ultracentrifugation to the bottom 20 % volume where free proteins get sequestered. This reduction appears not to detach other apolipoproteins since the most prominent among them *viz*. apoB which outnumbers apo(a) several fold among plasma lipoproteins is undetectable in the apo(a) sample collected (Fig. 4a). Antigenicity of apo(a), however was lost during de-O-glycosylation (data not shown). Protocol for de-O-glycosylation of apo(a) destroyed both O- and N-glycans since DOG-apo(a) was not recognized by jacalin or concanavalin A (Fig. 4a). To examine possible fragmentation of polypeptide backbone of apo(a) its elution volume was recorded before and after de-O-glycosylation in a size exclusion HPLC column. The column employed (BIOSEP-SEC-S-2000) had separation range upto 300 kDa so that it would exclude intact apo(a) subunits as they varied in size between 300 and 800 kDa among Lp(a) samples. Result (Fig. 4b) shows that apo(a) as well as its de-O-glycosylated derivative was excluded by the column and emerged at identical elution volumes suggesting that no fragmentation of apo(a) accompanied de-O-glycosylation.

Lp(a) isolated by jacalin precipitation and electrophoresis is homogeneous

Jacalin-precipitated lipoproteins (JL1) of plasma isolated as described (Preparation of purified Lp(a) and Lp(a)-free plasma lipoproteins section) contained only two protein-containing bands as shown by electrophoresis in

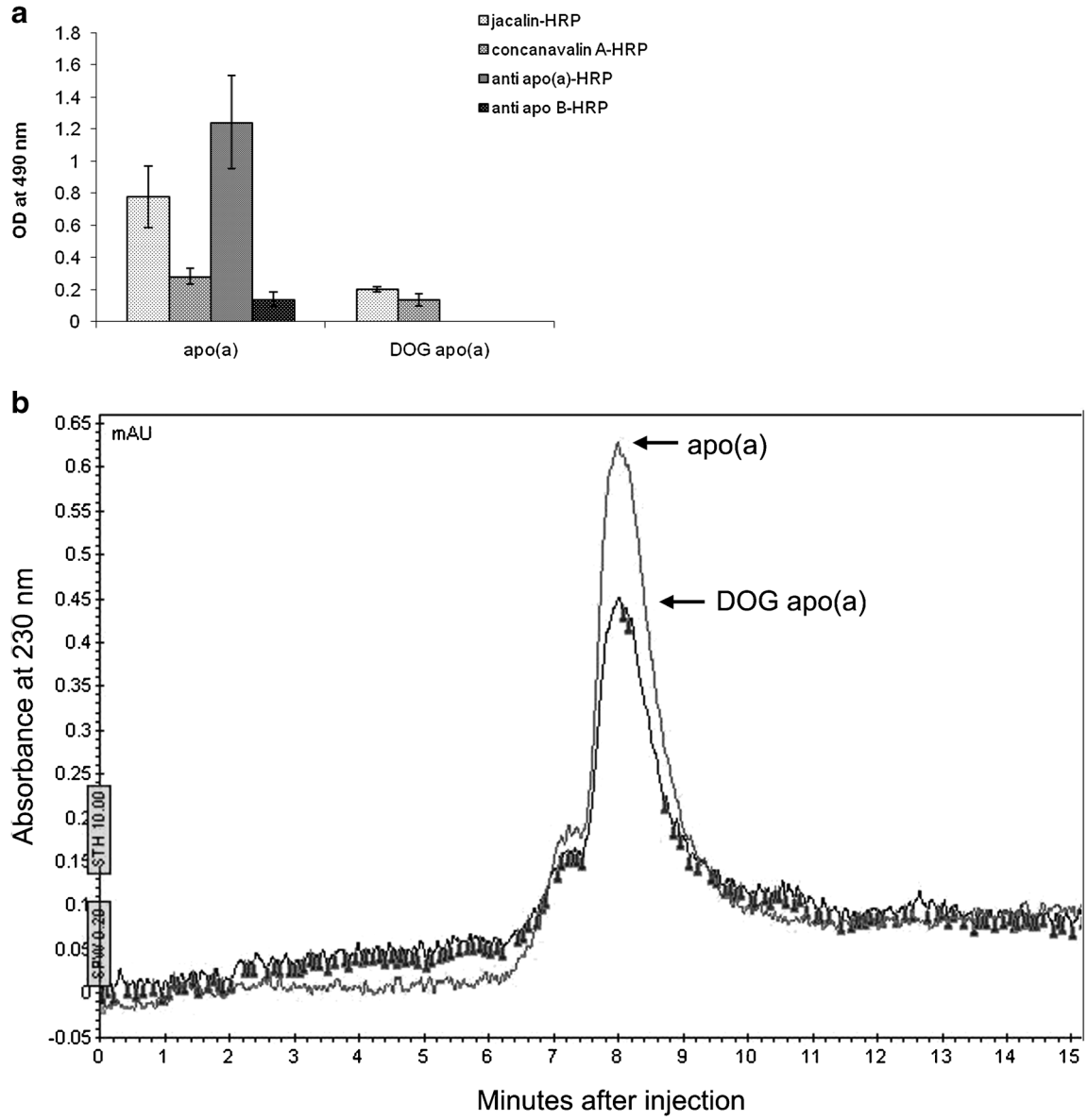

Fig. 4 **a** O- and N-glycans in apo(a) and DOG-apo(a). Microplate-coated apo(a) and DOG apo(a) (1.3 μg per well) probed with HRP conjugate of jacalin or concanavalin A. Apo(a) coating was also probed with HRP conjugates of antibodies to apo(a) and apoB (1.5 μg antibody per ml; Isolation and de-O-glycosylation of apo(a) section). Values are mean ± SD of three trials. **b** De-O-glycosylation does not fragment apo(a) polypeptide. Elution volume in size exclusion HPLC which is a function of protein size compared between apo(a) and DOG apo(a) by a superimposition of 230 nm absorbance curve of eluate (Isolation and de-O-glycosylation of apo(a) section)

Tris-borate-EDTA buffer (Fig. 5a) which were identified by ELISA as LDL (fast moving band) and Lp(a), respectively. Western blot of JL1 after SDS-PAGE probed with HRP-labeled antibodies showed apoB and apo(a) bands (Fig. 5b). Some samples (data not shown) contained an additional minor apo(a) band due to the reported size polymorphism of Lp(a). Presence of LDL in JL1 despite LDL being inert towards jacalin [22] could be due to a major fraction of Lp(a) in most plasma samples being in adduct form with non-covalently associated LDL [25, Kalaivani *et al.*, communicated]. Lp(a) eluted from gel in Fig. 5a was thus homogeneous.

Peptide sequence of apo(a) at its O-glycosylation site is surrogate ligand for carbohydrate binding site of anti-Gal

It has been recently observed in this laboratory that binding of appropriate carbohydrate ligands as part of macromolecules, but not as mono- or disaccharide to FITC-labeled carbohydrate-binding antibodies enhanced their fluorescence (George G *et al.* manuscript in preparation). Since the sugar binding sites of the antibodies were protected from getting labeled with FITC this effect could be a manifestation of conformational changes in the Fc region reported to accompany antigen occupation at binding sites [26]. Results in Fig. 6

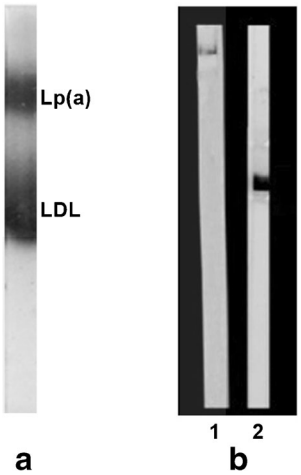

Fig. 5 Electrophoresis and immunoblotting of jacalin-precipitated lipoproteins. **a** JL1 (50 μg) run in 3.5 % acrylamide tube gel electrophoresis using Tris-borate-EDTA buffer and stained with Coomassie Brilliant blue R250. Bands were identified after passive elution and ELISA. **b** JL1 resolved by SDS-PAGE in 3.5 % slab gel and Western blotted to PVDF membrane probed with HRP conjugates of anti-apo(a) (1) and anti-apoB (2) [15 μg antibody per ml] (Preparation of purified Lp(a) and Lp(a)-free plasma lipoproteins section)

show that upon interaction with FITC-anti-Gal purified Lp(a) considerably increased the fluorescence of this conjugate as did the melibiose-conjugated protein TI that bears TAG groups whereas TI alone, Lp(a)-free lipoprotein or Lp(a) in presence of melibiose was ineffective. Though binding of jacalin to Lp(a) is much stronger than that of anti-Gal, Lp(a) binding did not enhance the fluorescence of FITC-jacalin underlining antigen-mediated conformational shift unique to immunoglobulins as reason for the enhanced fluorescence in FITC-anti-Gal.

Results in Fig. 6 also showed that apo(a) is responsible for anti-Gal recognition of Lp(a) and that removal of glycans only enhanced apo(a) binding to the antibody indicating that glycans of apo(a) *per se* are not involved in anti-Gal recognition. However, occupation by anti-Gal rendered O-glycans of apo(a) inaccessible to the O-glycan-specific lectin jacalin as shown by the inability of Lp(a) in anti-Gal-Lp(a) IC of APAG to bind jacalin (Table 1). Blocking of O-glycans of apo(a) following its occupation by anti-Gal is also testified by the inability of the negatively charged terminal sialic acid moieties of Lp(a) in APAG to bind to DEAE-Sephadex A-50 (Table 1). Amount of APAG used for treatment with anion exchange gel or jacalin wells was not in excess since optical density in Lp(a) IC assay of undiluted supernatant was below 1.0 in controls. Non-Lp(a) components in APAG (mostly IgG) had no affinity for DEAE group of jacalin (data not shown).

Prior incubation of FITC-anti-Gal with graded concentrations of its inhibiting sugar (melibiose) before addition of Lp(a) resulted in lowering of Lp(a)-mediated enhancement of fluorescence in proportion to the sugar concentration (Fig. 7) confirming that Lp(a) occupied the sugar-binding site of anti-Gal. Alternatively when STPS-containing region of Lp(a) was engaged by pre-incubation with jacalin which occupies the same region due to its high specificity for O-glycans that are hallmarks of the region, anti-Gal binding to the lipoprotein was fully abolished (Fig. 7) showing again that STPS is the ligand in Lp(a) recognized by anti-Gal. The total abolition of Lp(a)-mediated effect on FITC-anti-Gal could not be attributed to any interaction of the lectin with anti-Gal since the latter contains negligible amount of IgA, the only immunoglobulin recognized by jacalin [27].

Serine- and threonine-rich peptide sequence surrogate to α-linked galactose are present in Lp(a)

Anti-Gal had been shown to accommodate serine- and threonine-rich peptide sequences (STPS) ligands surrogate to TAG moiety at its sugar binding site [8]. GS I-B_4, a lectin with

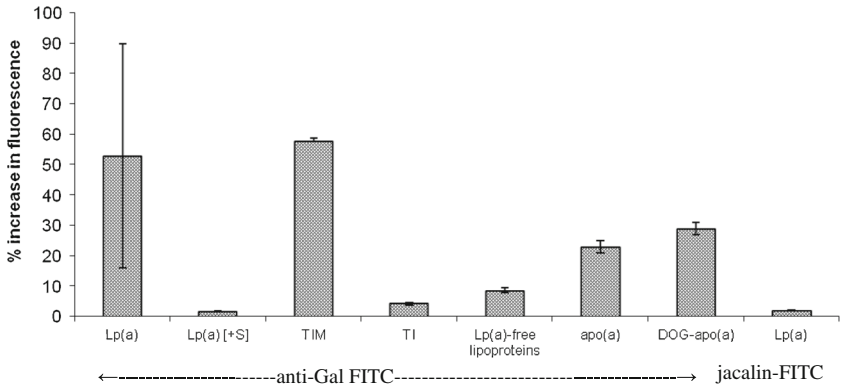

Fig. 6 Enhancement of fluorescence of FITC-labeled anti-Gal on binding to Lp(a). Purified Lp(a) [6 μg] (assayed by J-a method; Assay of Lp(a), immunoglobulins and Lp(a)-IC section) was incubated at 4 °C for 18 h with 6 μg FITC-labeled anti-Gal pre-incubated with (+S) or without (−S) 50 mM melibiose at 4 °C for 1 h or with FITC-labeled jacalin in a volume of 50 μl. Volume was made upto 3 ml with PBS just before fluorescence measurement by excitation at 485 nm and emission at 520 nm. Controls ligands used instead of Lp(a) were TIM, TI (125 μg) and Lp(a)-free plasma lipoproteins (6 μg). The fluorescence of ligand-free FITC-labeled anti-Gal or jacalin was used to calculate the percentage increase in fluorescence on addition of the ligands. Values are mean ± S.D of six consecutive plasma samples

Table 1 Reduced accessibility of O-glycans and their sialic acid residues in Lp(a) IC

Adsorbent for Lp(a)	Reduction of Lp(a) in supernatant after adsorption (%)	
	Pure Lp(a)	APAG Lp(a)
Microplate-coated jacalin	90±3.125	0
DEAE-Sephadex	70±23.3	4±10.2

Availability of O-glycans in pure Lp(a) and Lp(a) IC [APAG Lp(a)] isolated from same plasma were compared in terms of percentage reduction in Lp(a) content after treatment with DEAE-Sephadex A-50 gel and jacalin-coated polystyrene wells at 4 °C (Accessibility of O-glycans in free and IC Lp(a) section). Values are mean ± S.D. of six plasma samples

nearly identical specificity as anti-Gal also accommodates STPS in place of TAG at its sugar binding site [8]. Table 2 shows that all Lp(a) samples tried inhibited GS I-B_4 binding to thyroglobulin at sub-microgram concentrations while LDL or Lp(a)-free plasma lipoproteins was not inhibitory at concentrations several fold higher than that of Lp(a). Binding of GS I-B_4 lectin to thyroglobulin is exclusively mediated by the TAG groups of this glycoprotein [28]. Since apo(a) is devoid of α-galactoside moiety and its heavily O-glycosylated regions are rich in serine and threonine results suggest that STPS in Lp(a) accounts for binding of the lipoprotein to the sugar binding site of anti-Gal.

Discussion

When plasma Lp(a) IC treated with anti-Gal-specific sugar was subjected to ultracentrifugation at 535,000×*g* top 20 % volume (Fig. 2a) contained Lp(a) even in sample not treated with the sugar, apparently as IC, since free lipoproteins enough to occupy this volume are lacking in the sample employed. However sugar treatment of IC resulted in sequestration of significantly m ore Lp(a) into the top 20 % layer. The same

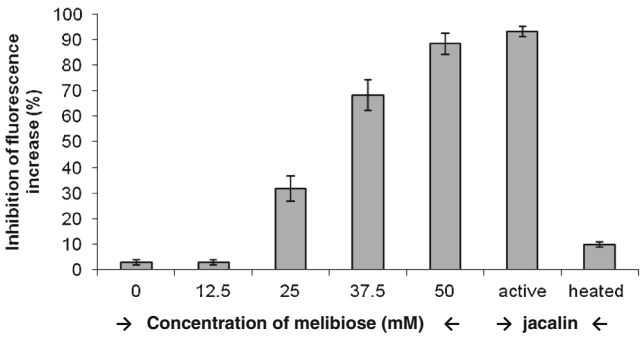

Fig. 7 STPS in Lp(a) occupies sugar binding site of anti-Gal. FITC-anti-Gal samples (6 μg) pre-incubated with varying concentrations of melibiose were treated with Lp(a) (6 μg) and fluorescence measured as described in Fig. 6. Alternatively Lp(a) pre-incubated with 20 μg active or heat-inactivated jacalin was used for interaction with FITC-anti-Gal. Mean ± SD of percentage of reduction of fluorescence increase was plotted (n=3)

Table 2 Lp(a) inhibits binding of GS I-B_4 to thyroglobulin

Standard Lp(a)	538 ng/ml[a]
Lp(a)[1]	495 ng/ml[a]
Lp(a)[2]	40 ng/ml[a]
Lp(a)[3]	143 ng/ml[a]
Lp(a)[4]	730 ng/ml[a]
Lp(a) free lipoproteins	>5 μg/ml[b]
LDL	>20 μg/ml[b]

Biotinylated GS I-B_4 lectin (2 μg) was incubated overnight at 4 °C with or without varying dilutions of purified Lp(a) [1 to 4] or LDL purified by density separation method or Lp(a)-free lipoproteins before adding to thyroglobulin-coated wells and bound lectin assayed using avidin-HRP as described (Lp(a) binding to MUC-1-specific lectin GS I-B4 section)

[a] Determined by J-a assay

[b] Determined by Bradford protein assay

effect of sugar was seen for liberation of antibodies to bottom 30 % volume (Fig. 2b) indicating the involvement of an α-galactoside-binding antibody in IC formation with Lp(a). This was further confirmed in results of Fig. 3 showing that while the antibody specific to the glucose analogue of α-linked galactose was only as good as any serum protein in combining with Lp(a), anti-Gal was significantly more effective. Involvement of anti-Gal, unlike other antibodies, in natural IC formation with Lp(a) in circulation was also supported by the remarkable dominance of IgG in Lp(a) IC (Fig. 1) since other known carbohydrate-binding natural plasma antibodies such as DIg [16] and anti-T [29] are dominated by IgM.

Presence of specific sugar during FITC conjugation of anti-Gal or jacalin protects their binding sites from labeling so that the Fc region might contain most of the FITC label in case of anti-Gal. Conformational change in FITC-labeled region could be expected to alter the microenvironment around the FITC ligands and increase their degree of excitability. Close to 60 % enhancement of fluorescence of anti-Gal by TIM, but not by TI alone or even by melibiose suggested conformational change in Fc region induced by a macromolecular antigenic ligand as a requirement for increase in fluorescence and that this effect could be used to evaluate recognition of cognate macromolecules by an antibody. Fluorescence of FITC-anti-Gal but not that of FITC-jacalin was enhanced by Lp(a) binding supporting the above conclusion and proving that Lp(a) is a binding site-specific macromolecular ligand for anti-Gal capable of activating its Fc region. Induction of fluorescence increase in FITC-anti-Gal by apo(a) and its de-O-glycosylated derivative but not by Lp(a)-free lipoproteins of plasma suggested that lipid component of Lp(a) had no role in anti-Gal binding. Concentration-dependent inhibition by melibiose of Lp(a) binding to anti-Gal despite absence of α-linked galactose moiety in Lp(a), inability of jacalin-bound Lp(a) to act as anti-Gal ligand and increase in anti-Gal binding activity of apo(a) after de-O-glycosylation suggested peptide

sequence of apo(a) as ligand for anti-Gal which had been shown to accommodate STPS as alternative ligands at its sugar binding site [8].

Lp(a) IC co-purified with anti-Gal in APAG is obviously anti-Gal partially occupied by Lp(a). Non-availability of O-glycans and their terminating sialic acid moieties of apo(a) of Lp(a)-anti-Gal IC in APAG from binding to jacalin and diethyl aminoethyl ion, respectively, could arise from binding of anti-Gal at or near the O-glycan-rich region in apo(a), though O-glycans *per se* are not ligands for anti-Gal [3]. Sustained anti-Gal binding of apo(a) even after its de-O-glycosylation and inhibition of GS I-B4 by Lp(a) suggested serine- and threonine-rich peptide sequences (STPS) where O-glycosylation occurs as ligand for anti-Gal in Lp(a). Apo(a) in Lp(a) can contain upto 40 inter-kringle O-glycosylated regions, each carrying six O-glycan chains.

The above results imply that larger Lp(a) molecules with extended O-glycosylated regions should be more reactive towards anti-Gal. However, analysis of different plasma samples has shown that in circulation most of the Lp(a) in all individuals attach additional LDL molecules non-covalently and that the number of extra LDL molecules attached increases with but faster than the increase in size of the apo(a) chain. It was also observed that extra LDL molecules occupy the densely O-glycosylated kringle 4 type 2 repeat region of apo(a) (Kalaivani *et al.*; manuscript in preparation). Present results therefore predict that in circulation smaller Lp(a) molecules will be relatively more available for recognition by anti-Gal. Another factor affecting Lp(a)-anti-Gal IC formation is specific activity (α-galactoside binding activity per unit antibody mass) of anti-Gal which has been found to vary considerably among individuals (our unpublished results).

Lp(a) and anti-Gal may owe their molecular complementarity to their similar evolutionary age and presence in same animal species [1, 11]. Apo(a) being a plasma protein possessing a uniquely long heavily O-glycosylated region STPS in it may be evolutionarily too young to be fully tolerated unlike the apoB subunit. Notably IgA1 the most numerous O-glycosylated protein in man is poorly O-glycosylated or polymerized in non-primates [30]. Anti-Gal, though proposed to be induced by gut bacterial antigens [4] consists almost entirely of IgG in contrast to the IgM domination in most carbohydrate-binding antibodies [27]. Continued presence in circulation of antigenic epitopes is known to induce affinity maturation of the corresponding antibody making it more specific to the antigen and shifting immunoglobulin type composition to IgG dominance [31]. Comparison of O-glycan availability in circulating Lp(a) with specific activity of anti-Gal may reveal if STPS regions in Lp(a) can attenuate anti-Gal activity by affinity maturation, if not trigger it. Whether lipid association, a factor known to enhance antigenicity of proteins, helps Lp(a) in this function is also a relevant question.

Size of the Lp(a)-anti-Gal IC (above 4 million kDa) may dictate its pathological potential since larger ICs are more damaging at the blood vessel wall [32]. At least a section of Lp(a)-anti-Gal IC is known to possess unoccupied binding site on the antibody capable of recognizing antigen on other macromolecules. Therefore, quality of these ICs may also matter in blood vessel pathology since a) occupation of one binding site may increase affinity of the other [33], b) Fc regions of anti-Gal in IC may be variably activated by occupation of binding site by Lp(a) of differing size. Circulating and cell-surface located proteins containing variable number of tandem repeats of STPS and implicated in vascular disorders include LDL receptor, LDL receptor-related proteins, apo E receptor [34] and tau proteins [35]. Whether binding of Lp(a)-anti-Gal IC to these molecules could trigger an inflammatory cascade different from that of free anti-Gal or binding of Lp(a)-anti-Gal IC to STPS-rich receptors is a possible route for Lp(a) entry into cells like macrophages prior to atherosclerosis are questions posed by the present results.

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