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Isolation of a melibiose-binding protein from human spleen

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A melibiose-binding protein was isolated from human spleen by serial affinity chromatography on lactose-, mannose-, and melibiose-Sepharose. The purified protein agglutinated rabbit erythrocytes and re-bound to melibiose, but did not bind to murine nor human laminin. The protein was composed of ~58 kDA, 32 kDa and 26 kDa polypeptides. The polypeptides were detected in buffy coat cell extracts and they were synthesized *in vitro* by B lymphoblastoid cells. The polypeptides did not react with anti-galaptin, anti-C-reactive protein, anti-amyloid P, anti-keratin, and anti-rat lung lectin 29 sera. The 58 kDa polypeptide reacted very weakly with anti-core-specific lectin serum and reacted with anti-IgG serum. The data suggest that the major protein isolated is an anti-Gal $\alpha 1 \rightarrow 6$ immunoglobulin.

Keywords: lymphoid lectin, galactoside-binding, melibiose-binding.

Abbreviations: ME, mercaptoethanol; PMSF, phenylmethylsufonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanosulfonate; PBS, 0.01 M PO_4, 0.12 M NaCl, pH 7.3; TBS, 0.1 M NaCl, 0.05 M Tris, 0.05% NaN_3, 0.01 M $CaCl_2$, 0.001 M $MgCl_2$, pH 7.3; BSA, bovine serum albumin; GSI, Griffonia simplicifolia I; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Introduction

The isolation and characterization of vertebrate lectins have received considerable attention over the past several years [1–3]. Drickamer has classified the endogenous lectins into two broad categories based upon their calcium dependency. They were termed C-type (Ca^{2+}-dependent) and S-type (Ca^{2+}-independent, thiol dependent) lectins [4, 5].

The S-type lectins, also termed S-Lac lectins [6] or galectins [7], have been proved to be composed of numerous polypeptide species exhibiting various degrees of homology [8–10]. The C-type lectins are also represented by a diverse group of proteins and generally share a homologous carbohydrate-recognition domain [4, 5]. While the galectins are generally β-galactoside-specific [6], the C-type lectins exhibit binding specificities for Man, L-Fuc, and Gal/GalNAc residues as well as a complex binding specificity for Man/L-Fuc/GlcNAc residues [4, 5, 11].

We have previously reported the presence in human lymphoid tissues of several galectins with β-galactoside specificity [12,13]. Here we report our investigation of the possible presence of an α-galactoside binding lectin in human lymphoid tissue.

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Materials and methods

Materials

Laminin from murine tumour, CNBr activated Sepharose 4B, and *Griffonia simplicifolia* (GS-1) agglutinin were from Sigma Chemical Co., St Louis, MO. Human placental extracellular matrix containing 30% laminin was from Collaborative Research, Bedford, MA. Iodo-Beads were from Pierce Chemical Co., Rockford, IL. Carrier-free $Na^{125}I$, 2516 Ci $mmol^{-1}$ and [2,3,4,5-^{3}H] L-leucine, 115 Ci $mmol^{-1}$ were from ICN, Irvine, CA. [^{35}S] L-Methionine, 1125 Ci $mmol^{-1}$ was obtained from NEN, Boston, MA.

Rabbit anti-galaptin (galectin-1) serum was prepared as described [14]; the rabbit anti- [58 kDa polypeptide] serum was prepared by us by the same method [14]. Anti-[buffy coat cell] serum was obtained by immunization of rabbits with 0.8 ml dialysed human buffy coat cell extract (0.13 ml packed cell) in Freund's complete adjuvant followed by booster injections of 0.8 ml of cell extract in Freund's incomplete adjuvant at 3 and 6 weeks. Sera were obtained at 7 weeks following the first injection. Rabbit anti-(C-reactive protein) serum, anti-[amyloid P component] serum, and anti-[epidermal keratin] serum were products of Accurate Chemicals, Westbury, NY. Rabbit anti-human IgG (γ chain) was obtained from Sigma Chemical Co., St Louis, MO. Anti-[human core-specific lectin] serum was kindly donated by Dr J. U. Baenziger, Washington University School of Medicine, St Louis, MO. Anti-[rat lung lectin 29] serum was the gift of Dr S. Barondes, Langley Porter Psychiatric Institute, San Francisco, CA.

Lactose, mannose, melibiose and mercaptoethanol[1] were coupled separately to Sepharose 6B via divinyl sulfone according to Allen and Johnson [15]. Laminin from murine tumour was coupled to CNBr-activated Sepharose according to the Pharmacia protocol.

Galectin-1-peroxidase conjugate was prepared as described [16]. B-142 lymphoblastoid cells were previously described [17] and are polyclonal Epstein-Barr virus-immortalized human B lymphoblasts.

Methods

Isolation of melibiose-binding protein Frozen human spleen (1 kg) was cut into small pieces with a hacksaw and homogenized in ice cold acetone/0.01 M ME (10 ml g^{-1}). The homogenate was filtered on a Buchner funnel and the residue was dried in a vacuum dessicator at −20°C. The dry acetone powder (325 g) was homogenized in cold extraction buffer, 0.1 M NaCl, 0.05 M Tris, 0.05% NaN_3, 0.01 M $CaCl_2$, 0.001 M $MgCl_2$, 0.5% Triton X-100, 0.1 M melibiose, pH 7.3 (4 ml buffer per g) with a Brinkman Polytron. The homogenate was immediately made 0.25 mM PMSF and stirred in the cold for 2.5 h. The homogenate was centrifuged at 15 000 × **g** at 4°C for 30 min and the supernate was dialysed against 0.1 M NaCl, 0.05 M Tris, 0.05% NaN_3, 0.01 M $CaCl_2$, 0.001 M $MgCl_2$, pH 7.3. The dialysate was passed through a ME-Sepharose column to remove irrelevant proteins that bind to the affinity matrix followed by serial affinity chromatography on lactosyl-Sepharose (2.4 × 4.5 cm), mannosyl-Sepharose (2.4 × 4.5 cm) and melibiosyl-Sepharose (2.4 × 3.1 cm) equilibrated with 0.6 M NaCl, 0.05 M Tris, 0.05% NaN_3, 0.01 M $CaCl_2$, 0.001 M $MgCl_2$, 0.05% Triton X-100, pH 7.3. The columns were disconnected and washed with 15 bed volumes of the preceding buffer. The bound protein from each column was eluted with 0.1 M respective sugar in the preceding buffer. The protein obtained from melibiose-Sepharose was dialysed and repurified by chromatography on a fresh melibiose-Sepharose column (0.8 × 4.0 cm). For estimation of protein, Triton X-100 was removed by isoamyl alcohol extraction and the resulting solution was assayed with a Coomassie blue dye binding reagent.

Isolation of melibiose-binding protein from $^{35}S/^{3}H$ labelled B-142 lymphoblastoid cells Lymphoblastoid cells (50 × 10^6) were washed twice with leucine/methionine-free RPMI 1640 medium containing 25 mM HEPES and 2 mM glutamine. Cells were suspended in 25 ml of above medium containing dialysed fetal calf serum, and ^{35}S-methionine (2.5 mCi) and ^{3}H-leucine (2.5 mCi) were added. After incubation for 8 h at 37°C in 5% CO_2 atmosphere, the cells were washed twice with PBS and extracted in 0.1 M NaCl, 0.05 M Tris, 0.05% NaN_3, 0.01 M $CaCl_2$, 0.001 M $MgCl_2$, 0.5% Triton X-100, 0.1 M melibiose, pH 7.3 by sonication. Finally, melibiose-binding protein was obtained from a melibiose-Sepharose column as described for spleen.

Haemagglutination and haemagglutination inhibition assays These assays were carried out with glutaraldehyde-fixed, trypsinized rabbit erythrocytes suspended at 0.5–1% in TBS, 1% BSA [15].

SDS-PAGE SDS-PAGE [18] and fluorography [19] were carried out. Samples were alkylated before electrophoresis [14]. Standard proteins were run in parallel for construction of a standard curve.

Western blot analyses Western blot analyses were carried out as described [14].

Dot blot assay Two-fold serial dilutions of melibiose-binding protein (200 ng to 12.5 ng) were blotted on to nitrocellulose paper. After blocking the nitrocellulose with 1% BSA, 0.05% Tween 20, the blotted protein was immunodetected either by anti-human IgG (γ chain) or anti-58 kDA followed by goat anti-rabbit IgG-peroxidase as described [14]. Human IgG and human serum were also blotted as controls.

Iodination of melibiose-binding protein The melibiose-binding protein was radioiodinated with carrier free $Na^{125}I$ using Iodo-Beads according to the Pierce protocol. Four Iodo-Beads were washed with TBS containing 0.5% Triton X-100 and treated with $Na^{125}I$ (1 mCi) for 5 min at room temperature in a fume hood. The melibiose-binding protein (0.8 nmol based on a MW of 53 kDa) in 500 μl of TBS containing 0.5% Triton X-100 and 0.1 M melibiose was then added to the reaction tube. After 15 min the iodine was quenched with 100 μl of 1 mM sodium bisulfite and the iodinated protein was cleaned up on a Sephadex G-25 column (0.8 × 26.5 cm) pretreated with 1 mg mg^{-1} of BSA and equilibrated with TBS, 0.5% Triton X-100 buffer. The radioactive fractions that appeared in the void volume were pooled and were repurified on a melibiose-Sepharose column.

Binding assay of radiolabelled melibiose-binding protein The carbohydrate binding activity of ^{125}I-labelled melibiose-binding protein was ascertained by passing the sample through lactosyl-Sepharose (Galβ1 → 4Glc) and murine laminin-Sepharose (Galα1 → 3Gal) (1 ml each) equilibrated with TBS, 0.5% Triton X-100 buffer. After washing the columns with 10 bed volumes of the same buffer, the bound protein was eluted with either 0.1 M lactose (for lactosyl-Sepharose column) or 0.1 M melibiose (for laminin-Sepharose). The activity of the laminin-Sepharose column was checked by passing GS I (an αGal-binding lectin) through the column and 87% of the lectin was retarded by the column. As another positive control, an aliquot of the melibiose-binding protein was passed through a melibiose-Sepharose column and bound protein was eluted with 0.1 M melibiose.

Binding of 125I-labelled melibiose-binding protein to plastic-adsorbed laminin Immulon I, Removawells (Dynatech Laboratories, Chantilly, VA) were coated with murine and

human laminin (2.0 μg per 100 μl per well) as described [20]. After fixing and washing, the wells were blocked with 1% BSA/TBS for 1 h at 37°C. The blocking solution was aspirated and ^{125}I labelled melibiose-binding protein (800 cpm per 100 μl per well) was added. After 1 h incubation at 4°C, unbound protein was withdrawn and saved. The wells were washed three times with ice cold TBS (200 μl each time) and the washes were saved. Radioactivity of unbound protein, washes, and bound protein in the wells were assayed in a gamma counter. The wells were then allowed to react with galectin-1-peroxidase conjugate (0.20 ng per 100 μl per well) or conjugate 0.05 M lactose solution for 1 h at 4°C. After washing the wells three times with PBS, 0.01% thimerosal, 0.05% Tween 20, pH 7.3, the peroxidase activity was assayed with ABTS substrate [20].

Results and discussion

Isolation of melibiose-binding protein A melibiose-binding protein was isolated from human spleen by passing crude extracts sequentially through ME-Sepharose, lactose-Sepharose, mannose-Sepharose, and melibiose-Sepharose columns connected in series. The protein obtained from the melibiose column was rechromatographed on a fresh melibiose-Sepharose column. The final yield of melibiose-binding protein was 100 μg kg^{-1} of wet spleen. The melibiose-binding protein exhibited haemagglutinating activity at a concentration as low as 1 μg ml^{-1} when assayed with trypsinized glutaraldehyde-fixed rabbit erythrocytes. Haemagglutination was inhibited by melibiose (8 mM) but not by lactose (20 mM). Type A, B, and O human erythrocytes were not agglutinated by the protein. Although the protein was extracted and isolated in the presence of Triton X-100, Ca^{2+}, Mg^{2+}, and melibiose, studies were not carried out to investigate the requirement for these reagents. Nor were studies carried out to investigate the effects of chelating agents on the carbohydrate-binding activity of the melibiose-binding protein.

On SDS-PAGE, the purified melibiose-binding protein showed a major polypeptide of 58 kDa and two minor polypeptides of 26 kDa and 32 kDa (Fig. 1A, lane 2). Some very high molecular weight material was present near the gel origin. The lactose eluate from the lactose-Sepharose column revealed a polypeptide pattern distinctly different from the melibiose-binding protein (Fig. 1A, lane 3). The lactose-binding polypeptides at ~17 kDa probably correspond to those previously described for human spleen, B-lymphoblastoid cells and buffy coat cells [12, 13].

SDS-PAGE/fluorography of melibiose-binding protein isolated from [^{35}S]-methionine/[^{3}H]-leucine-labelled B142 lymphoblastoid cells also showed a major polypeptide at ~58 kDa and one at ~32 kDa (Fig. 1B). It has been found that the apparent molecular mass of the major polypeptide (58 kDa) of the melibiose-binding protein varies somewhat with changes in conditions of electrophoresis which suggests that this polypeptide may be glycosylated.

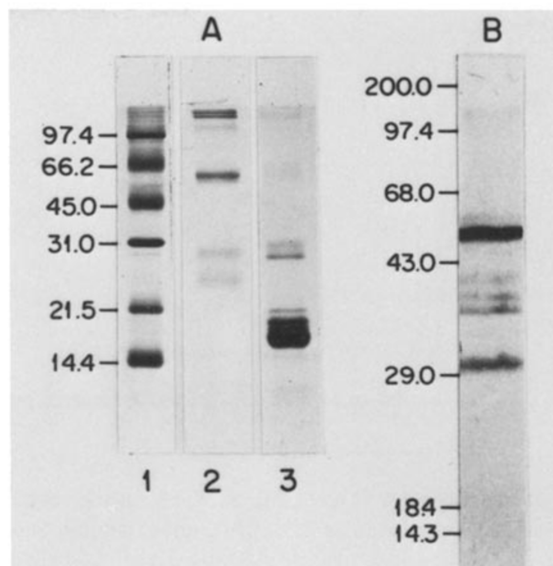

Figure 1. SDS-PAGE of melibiose-binding and lactose-binding proteins isolated from spleen (A); and melibiose-binding protein from B lymphoblastoid cells (B). The samples for electrophoresis were reduced in 0.6 M Tris, 0.018 M EDTA, 0.030 M dithiothreitol, pH 8.4, and alkylated with 0.050 M iodoacetamide under N_2 at room temperature. The samples were run on a 12.5% acrylamide slab gel. Lane 1: MW standards; lane 2: 5 μg melibiose-binding protein; lane 3: 10 μg lactose-binding protein; B: ^{35}S/^{3}H-labelled lymphoblastoid cell melibiose-binding protein. Lanes 1–3 stained with Coomassie blue; B developed by fluorography.

Binding characteristics of melibiose-binding protein The results of haemagglutination inhibition assays suggested that the melibiose-binding protein had a distinct preference for α-galactoside residues rather than for β-galactoside residues. To investigate this further, the melibiose-binding protein was radiolabelled with ^{125}I and it was passed through columns of murine laminin-Sepharose, lactose-Sepharose, and melibiose-Sepharose. None of the protein bound to lactose nor to murine laminin, while about 20% re-bound to melibiose (Fig. 2). This confirmed the specificity of melibiose relative to lactose. The data also suggest that the protein has a binding preference for Galα1 $\rightarrow$ 6 residues rather than Galα1 $\rightarrow$ 3 residues. The presence of non-reducing terminal Galα1$\rightarrow$3Gal residues in murine laminin, as well as Galβ1 $\rightarrow$ 4GlcNAc residues, has been reported [21]. GSI lectin acid did bind to murine laminin-Sepharose indicating the availability of αGal residues (not shown). The data also suggest that the conditions of iodination may partially destroy the carbohydrate-binding activity of the melibiose-binding protein.

To confirm the failure of melibiose-binding protein to bind to laminin, binding of the protein to plastic-adsorbed murine and human laminin was assayed. The protein did not bind to either of these components. In contrast, both plastic-adsorbed laminins bound galectin-1 isolated from human spleen (data not shown). These observations showed that the laminin oligosaccharides were available for binding but that the melibiose-binding protein had no specificity for them.

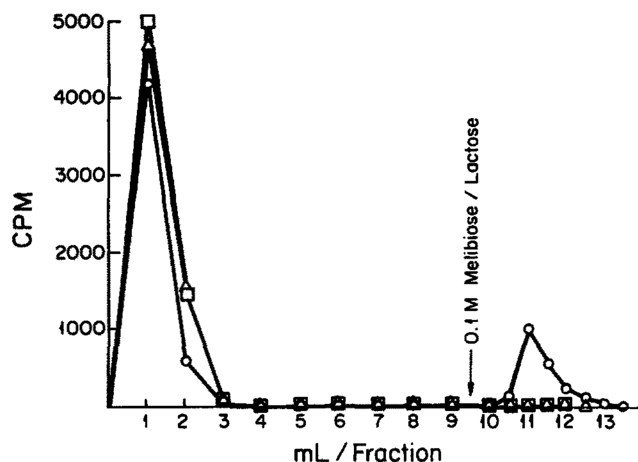

Figure 2. Binding assay of melibiose-protein by affinity chromatography on lactosyl-Sepharose (△–△), murine laminin-Sepharose (□–□), and melibiose-Sepharose (○–○). The ^{125}I-labelled melibiose-binding protein (7000 cpm) was loaded on each column (1 ml bed volume) equilibrated with TBS, 0.5% Triton X-100. The columns were washed with 9 ml of buffer and 1 ml fractions were collected. The bound protein was eluted from each column with a mixture of 0.1 M lactose, 0.1 M melibiose and 0.5 ml fractions were collected.

Western blot analyses Western blots of melibiose-binding protein that were probed with anti-[buffy coat] serum revealed polypeptides of apparent 58 kDa and 32 kDa molecular mass. This result shows that the melibiose-binding protein is present in peripheral leucocytes as well as in spleen (Fig. 3, lane 1). Anti-[human core-specific lectin] serum reacted very weakly with the 58 kDa polypeptide of melibiose-binding protein (Fig. 3, lane 2). This result suggests that a very small amount of the core-specific lectin may co-isolate with melibiose-binding protein, or they may be weakly cross-reactive. The 58, 32 and 26 kDa polypeptides did not react with anti-[rat lung lectin 29] serum (Fig. 3, lane 3). However, reactivity with this antiserum was observed at ~35 kDa even though staining of gels with Coomassie blue did not detect a polypeptide at this region. Western blot analysis of spleen extract with anti-[58 kDa polypeptide] serum detected a major polypeptide at 58 kDa (Fig. 3, lane 4), as expected. The lactose eluate from the lactose-Sepharose column showed some reactivity with anti-[58 kDa polypeptide] serum (Fig. 3, lane 5). This reactivity was weaker when compared with that for a comparable amount of melibiose-binding protein (Fig. 3, lane 6).

None of the polypeptides of the melibiose-binding protein reacted with anti-[human splenic galectin-1] serum (Fig. 4, lane A2), anti-[C-reactive protein] serum (Fig. 4, lane B2), anti-[amyloid P component] serum (Fig. 4, lane C2), and anti-[epidermal keratin] serum (not shown).

The results of Western blot analyses show that some melibiose-binding protein, or a cross-reactive component, may bind to lactose-Sepharose. However, C-reactive protein and amyloid P component, which have a propensity to bind to Sepharose-based adsorbents, were absent. It should be noted that in these studies the quantity of melibiose-binding protein

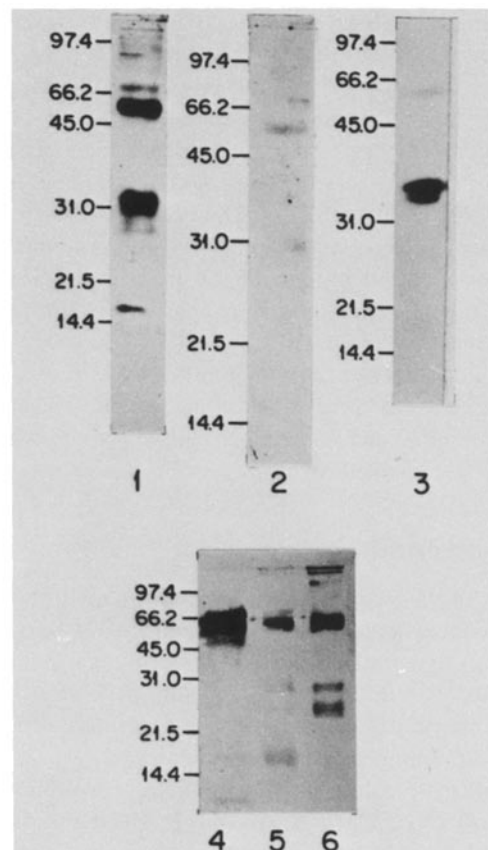

Figure 3. Western blot analyses of melibiose-binding protein. Melibiose-binding protein (5 μg), lanes 1–3, 6; spleen extract, lane 4; and lactose-binding protein (5 μg), lane 5; proteins were reduced, alkylated, electrophoresed on 12.5% acrylamide slab gels, and blotted to nitrocellulose. The blots were probed with anti-[buffy coat] serum (1:100), lane 1; anti-[human core-specific lectin] serum (1:100), lane 2; anti-[rat lung lectin 29] serum (1:100), lane 3; and anti-[58 kDa polypeptide] serum (1:1000), lanes 4–6, as described [13].

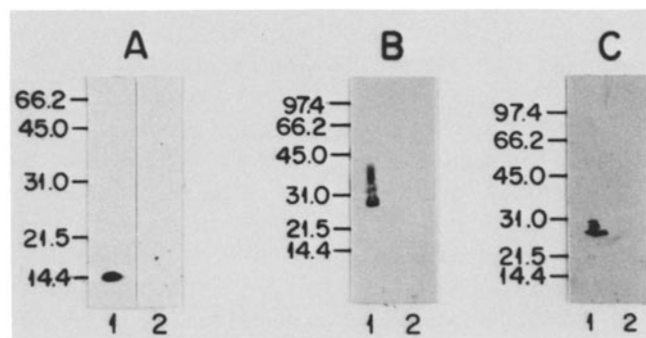

Figure 4. Western blot analyses of melibiose-binding protein. Melibiose-binding protein (5 μg), splenic galectin-1 (25 ng), and post-surgical human serum were electrophoresed, blotted, and probed as for Fig. 3. Lane A1: galectin-1; lanes B1 and C1: serum; lanes A2, B2 and C2: melibiose-binding protein. A: anti-[human splenic galectin-1] serum (1:1000); B: anti-[C-reactive protein] serum (1:1000); C: anti-[amyloid P component] serum (1: 1000).

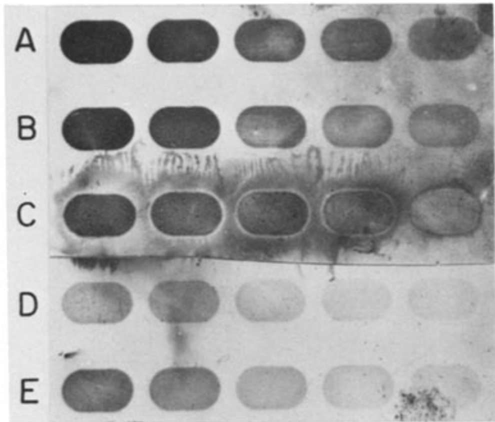

Figure 5. Dot blot assay of melibiose-binding protein. The blots were probed with anti-[human IgG] serum (1:1000), rows A–C, and with anti-[58 kDa polypeptide] serum (1:1000), rows D, E as described in Methods. Rows A, D: melibiose-binding protein (200–12.5 ng); row B: human IgG (200–12.5 ng); rows C, E: human serum (1:1–1:16).

loaded on the gels for subsequent blotting was about 500 times greater than the amount typically required for antisera probing of blots (5 μg vs 10 ng).

Dot blot assay Dot blot assays were carried out to further explore the identity of the melibiose-binding protein. The molecular mass of the major and minor polypeptide bands, the possible glycosylation of the 58 kDa polypeptide, and the weak Coomassie blue staining of the lower molecular mass bands suggested that the major melibiose-binding protein might be an immunoglobulin. To test this possibility, dot blots of melibiose-binding protein were probed with anti-[human IgG] serum. Likewise, human IgG and human serum dot blots were probed with anti-[58 kDa polypeptide] serum. The results are shown in Fig. 5. In this assay, anti-[human IgG] serum reacted with melibiose-binding protein, human IgG, and human serum, row A, B, C, respectively. Anti-[58 kDa polypeptide] serum reacted with melibiose-binding protein and human serum, row D and E, respectively.

The data presented here, taken collectively, indicate that the major melibiose-binding protein present in human spleen, human buffy coat cells, and Epstein-Barr virus-transformed human B lymphocytes is a Galα1 $\rightarrow$ 6 specific IgG. The presence of naturally occurring anti-α-Gal antibodies in human sera is well known [22]. Perhaps the most important subclass of the anti-α-Gal antibodies are those with Galα1 $\rightarrow$ 3Gal binding specificity. These are thought to play a role in red blood cell senescence [23] and they are important mediators of xenograft rejection [24, 25]. These antibodies are different from the melibiose-binding IgG reported here in that the anti-Galα1 $\rightarrow$ 3Gal bind to murine laminin [26]. However, the function and physiological relevance of anti-Galα1 $\rightarrow$ 6 IgG is currently unknown and is open to speculation. It is interesting to note that Joubert-Caron *et al.* [27] recently reported the detection of β-galactoside-binding IgG in human serum.

Our studies emphasize the importance of identifying presumed endogenous lectins as being distinct from immunoglobulins. This is especially important when the tissue sources of lectin may be immunoglobulin-producing cells; or the sources are excised from animals and humans and, hence, may contain serum IgG or lymphoid infiltrates.

Acknowledgements

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