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Specificity of Human Antibodies Against Galα1-3Gal Carbohydrate Epitope and Distinction from Natural Antibodies Reacting with Galα1-2Gal or Galα1-4 Gal

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Antibodies against galactosyl-α1-3-galactose epitopes were characterized in normal and patient sera by radioimmunoassay binding to mouse laminin and oligosaccharide inhibition. Binding was strictly dependent on α-linked galactose in a terminal position. Reduced affinities were observed for digalactoses with α(1-2)-, α(1-6)- and α(1-4)-linkages and for the blood group B epitope, Galα1-3(Fucα1-2)Gal. Conformational models of various active and inactive oligosaccharides provided a clearer picture of the epitope requirements for the observed antibody specificity. Some antibody heterogeneity was detected by comparing individual sera and by hapten elution from a laminin adsorbent. New assays were developed with synthetic Galα1-3Gal-albumin conjugates and these were shown to be more sensitive than assays with mouse laminin. Two more ubiquitous human antibodies could be detected with Galα1-2Gal and Galα1-4Gal conjugates. They were distinct from Galα1-3Gal-specific antibodies as shown by carbohydrate inhibition. This demonstrates a considerable diversity in the recognition of α-linked galactose epitopes by natural antibodies.

Human beings acquire during their life-time a variety of natural antibodies specific for carbohydrate epitopes which are either phenotypically restricted or of more general occurrence. They include antibodies against a diverse number of blood group substances which in most cases have been defined with respect to their structural requirements [1, 2]. Ubiquitous antibodies with specificity for the Galα1-3Gal epitope located at the non-reducing termini of oligosaccharide chains were first identified by Galili *et al.* [3, 4]. This structure is part of the blood group B epitope [Galα1-3(Fucα1-2)Gal] but a subspectrum of Galα1-3Gal specific antibodies with low cross-reactivity has been shown to exist [5, 6] explaining why the human population responds to Galα1-3Gal irrespective of the B phenotype. Most interestingly, man and old world monkeys possess the natural antibodies

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but not new world monkeys and other mammalian species, in accordance with the presence of the Galα1-3Gal epitope in the latter species [7]. Yet, the antibodies react with human erythrocytes which are senescent or from patients with thalassemia [3, 4] or sickle cell anemia [8]. This could reflect the presence of the Galα1-3Gal epitope in a cryptic form [9].

The cryptic nature or absence of antigens explains the natural response to Galα1-3Gal structures in man and is possibly related to the lack or low activity of a specific α-galactosyl transferase found abundantly in other species [10]. As in the case of antibodies to blood group substances it was argued [1-3] that foreign substances (infectious agents, diet) are responsible for triggering antibodies to Galα1-3Gal. Evidence for this possibility was accidentally obtained in studies showing a strong antibody reaction to mouse laminin in patients with Chaga's disease and Leishmaniasis [11, 12]. Subsequently, it was found that these antibodies are specific for Galα1-3Gal epitopes [6]. Antibody titers in the patients were distinctly higher than in the normal population and elevated levels were also found in other infectious diseases [13, 14] and inner ear diseases [15]. It indicated that determining antibodies to the Galα1-3Gal epitope could have some clinical importance.

In the present study we have tried to achieve two goals. Firstly, to obtain a better immunochemical characterization of antibodies against the Galα1-3Gal epitope and, secondly to develop new antibody assays based on synthetic antigens. Additionally, we provide evidence for previously unrecognised natural human antibodies specific for terminal Galα1-2Gal and Galα1-4Gal epitopes.

Materials and Methods

Serum Panels

Normal sera were collected from eight female and 11 male volunteers (age range 25-52 years, average 39 years) and showed normal values in several standard laboratory tests. Sera from patients with American cutaneous Leishmaniasis and with sensorineural hearing loss were kindly provided by Drs. J. L. Avila, Caracas, and P. Berg, Tübingen. These antisera showed distinctly higher antibody titers against the Galα1-3Gal epitope compared to normal sera [6, 15]. A goat antiserum against human P blood group substance was purchased from Gödecke, Freiburg, FRG.

Antigens and Inhibitors

Laminin was purified from the mouse Engelbreth-Holm-Swarm tumor [16]. Porcine thyroglobulin and bovine fetuin were purchased from Sigma Chemical Co., St. Louis, MO, USA. Various mono- and oligosaccharides and arabinogalactan were obtained from BioCarb Chemicals, Lund, Sweden, or Sigma. Galα1-6Glc-BSA was from Sigma.

Synthetic Carbohydrate Conjugates

Coupling of disaccharide-glycosides to human or bovine serum albumin (Sigma) followed established procedures. Briefly, a solution of albumin (22 mg in 3.5 ml 0.1 M sodium

phosphate pH 9.5) was mixed with a solution of the disaccharide *p*-isothiocyanatophenylethylglycoside (prepared from the corresponding amine as described [17, 18]; 0.01 mmol) in water (1.0 ml). The pH was adjusted to 9.5 and the mixture stirred overnight at room temperature, followed by ultrafiltration with distilled water. The yield of coupled carbohydrate was determined by the anthrone reaction. The incorporation varied from 14-30 mol/mol.

Immunological Assays

Proteins were labelled with ^{125}I by the chloramine T procedure and used in fluid phase RIA binding and inhibition assays using 1 ng of ^{125}I-labelled antigen (10-15000 cpm/ng) per tube, following previously used procedures [6, 19]. For ELISA [20] plates were coated with disaccharide-albumin conjugates (1 µg/ml, 200 µl/well) dissolved in 0.05 M sodium carbonate pH 9.5-9.7. The plates were left overnight, then washed three times in 0.05% Tween 20, containing 0.15 M NaCl. Wells were incubated with human sera diluted in 0.05 M sodium phosphate pH 7.5, 0.15 M NaCl, 0.05% Tween 20 with 2 mg BSA/ml for 1 h, washed as before, then incubated for 1 h with alkaline phosphatase coupled to swine anti-human IgG (Orion, Esbo, Finland) diluted 1/1000 in the same phosphate buffer. The plates were again washed and bound reactants were then detected with *p*-nitrophenyl phosphate.

Conformational Models of Oligosaccharides

Conformational models of the oligosaccharides in solution were obtained by hard sphere exo-anomeric calculations (HSEA-calculations). Hydroxyl groups are represented by the oxygen atoms. Energy minimization was performed by simultaneous variation of torsion angles (multidimensional binary chop), using the HSEA potential described by Bock [21]. Ambiguity arose only for Galα1-6Galβ where the calculations resulted in two conformations of nearly equal HSEA-energy (<0.1 kcal/mol difference). The conformation with the lowest energy was chosen for the subsequent comparisons.

Results

Specificity and Diversity of Antibodies Reacting with Galα1-3Gal Structures

Previous studies have shown that RIA with mouse laminin is a useful method to detect antibodies with Galα1-3Gal specificity in normal and patient sera [6]. These reactions could be inhibited by high concentrations (0.2-30 mM) of galactose and methyl α-galactoside but not by other monosaccharides or methyl β-galactoside. Further analysis with some α-linked galactose-containing disaccharides indicated the importance of the α(1-3) bond.

Here we have extended these studies to several oligosaccharides (Table 1) in order to approach a more precise identification of the epitope. Galα1-3Gal was the most potent inhibitor even though as described before [6] it was less active on a molar and weight basis when compared to laminin. Other galactose-containing disaccharides showed distinctly lower activities which decreased in the order α(1-2)-, α(1-6)- to α(1-4)-linkage. Replacement of galactose at the non-reducing terminal but maintaining an α-glycosidic linkage

Table 1. Oligosaccharide inhibition of laminin-binding by individual sera from patients with Leishmaniasis (LS), with sensorineural hearing loss (SH) and from normal donors (NS).

	Concentrations of inhibitor (μM) required for 50% inhibition[a]					
Inhibitor	LS 1	LS 2	SH 1	SH 2	NS 1	NS 2
Laminin	$12x10^{-6}$	$20x10^{-6}$	$18x10^{-6}$	$18x10^{-6}$	$5x10^{-6}$	$7x10^{-6}$
Galα1-3Gal	0.9	0.6	0.1	0.3	2.0	8.0
Galα1-3Galβ1-3GlcNAc	2.5	0.6	0.5	0.5	7.3	22
Galα1-3(Fucα1-2)Gal	300	30	30	300	100	600
Galα1-2Gal	40	150	2.5	30	250	170
Glcα1-2Gal-phenyl	>>200[b]	>>200	>>200	>>200	>>200	>>200
Galα1-6Gal	180	300	8	300	300	100
Galα1-6Glc	380	>380	25	500	>500	500
Galα1-4Gal	>1500	>1500	2400	>2000	>1500	>1500
Glcα1-3Glc	>>1460	>>1460	>>1460	>>1460	>>1460	>>1460
Glcα1-6Glc	>>1170	>>1170	>>1170	>>1170	>>1170	>>1170
GalNAcα1-3(Fucα1-2)Gal	>>920	>>920	>>920	>>920	>>920	>>920
Forssman hapten[c]	>>100	>>100	>>100	>>100	>>100	>>100
Manα1-3Manβ1-4GlcNAc	>750	>750	>>750	>>750	>>750	>>750
Galβ1-3Galβ-methyl	>>1450	>>1450	>>1450	>>1450	>>1450	>>1450
GalNAcβ1-3Galα1-4Galβ1-4Glc	>>500	>>500	>>500	>>500	>>500	>>500

[a] determined by radioimmuno-inhibition assay.
[b] >> denotes less than 10% inhibition at the highest concentration tested.
[c] GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc.

produced an even more dramatic reduction in activity. Lack of activity was also found for Galβ1-3Gal and GalNAcβ1-3Gal structures. Among various blood group structures tested, the B epitope Galα1-3(Fucα1-2)Gal showed distinct inhibition even though reduced by a factor 60-1000 compared to Galα1-3Gal. No activity was found for the A epitope or Forssman hapten which both possess a terminal GalNAcα(1-3)- structure.

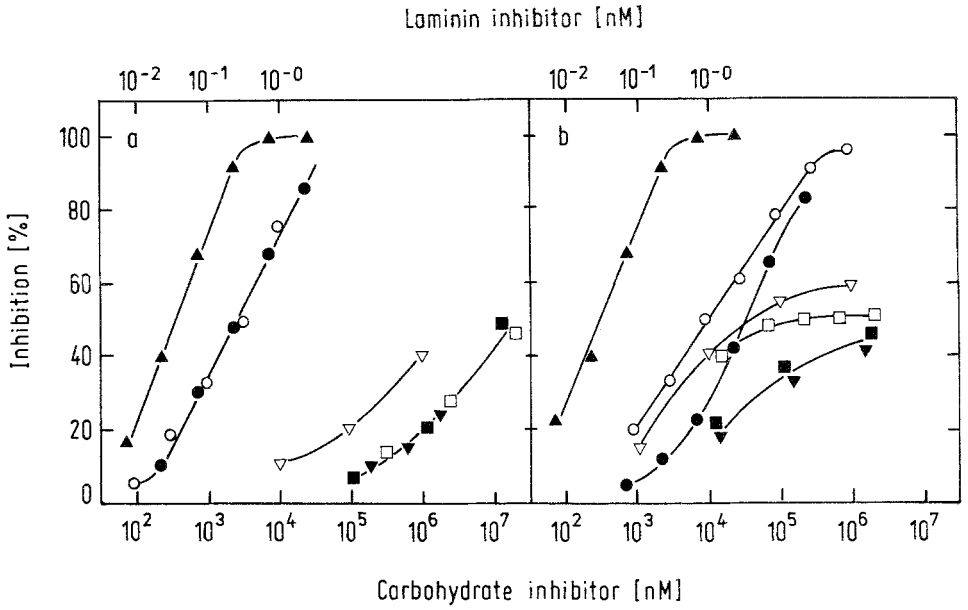


Figure 1. Inhibition of antibodies against the Galα1-3Gal epitope in patient (a) and normal serum (b) by various saccharide haptens. Activities were determined by radioimmuno-inhibition assays with mouse laminin as labelled antigen. Inhibitors used were laminin (▲), Galα1-3Gal (○), Galα1-3Galβ1-3GlcNAc (●), Galα1-6Gal (▽), Galα1-4Gal (▼), α-methyl galactose (□) and galactose(■).

The relative order of inhibiting activities of cross-reacting oligosaccharide structures compared to Galα1-3Gal appeared invariant in a variety of normal and patient sera (Table 1). More individual variations were, however, detected in the quantitative aspects of these comparisons. Further differences were noted in the nature of inhibition profiles for which two examples are shown in Fig. 1. Galα1-3Gal and Galα1-3Galβ1-3GlcNAc usually produced almost complete inhibitions with profiles close in steepness to that of laminin. Other, less active oligosaccharides and galactose showed usually less steep profiles and often resulted in a premature plateau region of the inhibition curve. This indicates individual variations in immunogenic stimuli and the activation of a diverse number of B cell clones.

It was also of interest, as discussed below, to compare several unrelated antigens in laminin RIA inhibition (Fig. 2). Porcine thyroglobulin showed an almost identical activity compared to mouse laminin. Bovine fetuin was also a distinct inhibitor although with 10-20 fold lower capacity. Arabinogalactan, which is a Galα1-3Gal polymer containing arabinose side chains, was also able to inhibit the reaction completely but with even lower potency. The latter fact may indicate that only terminal groups of the polymer have sufficient affinity for the antibodies.

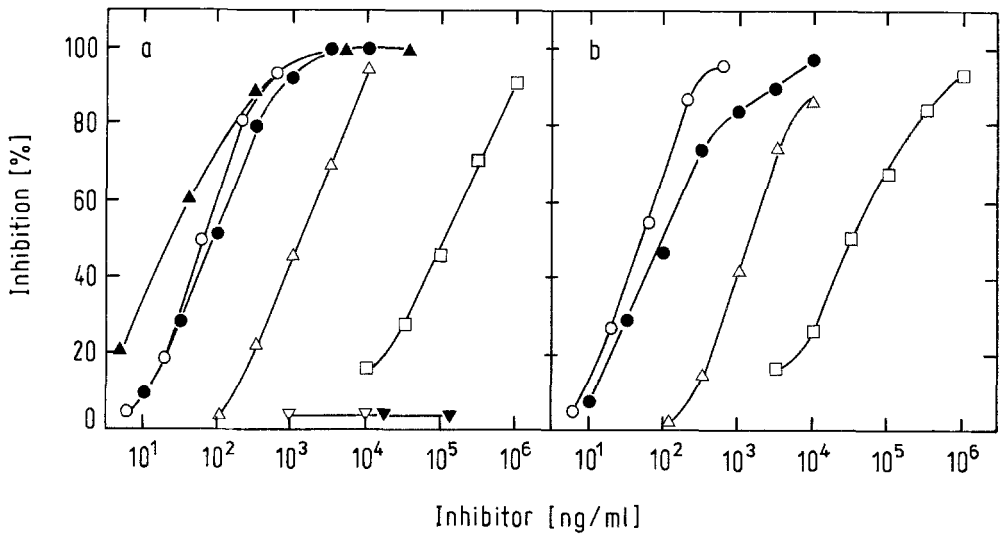


Figure 2. Comparison of various protein, synthetic saccharide conjugate and polysaccharide antigens with mouse laminin in radioimmuno-inhibition assays specific for the Galα1-3Gal epitope. The test system consisted of ^{125}I-labelled mouse laminin and a patient (a) or normal serum (b). Inhibitors used were mouse laminin (○), porcine thyroglobulin (●), bovine fetuin (△), arabinogalactan (□), Galα1-3Galβ-HSA (▲), Galα1-4Galβ-BSA (▽) and human serum albumin (HSA, ▼).

Analysis of Antibodies with Defined Digalactose-Albumin Conjugates

Assays used here and previously for the detection of antibodies with Galα1-3Gal specificity [3, 4, 6, 12, 22] were based on proteins possessing the epitope on *N*-linked oligosaccharides or on cellular glycolipid antigens. In order to make the assay independent of the carrier moieties and to allow a more precise comparison of different disaccharide epitopes we have now coupled Galα1-3Gal, Galα1-2Gal, Galα1-4Gal and Galα1-6Glc to either human (HSA) or bovine (BSA) serum albumin, resulting in conjugates with 14-30 epitopes per molecule. The hapten density estimated for mouse laminin is 50-60 [6]. As expected, Galα1-3Galβ-HSA was at least as efficient an inhibitor as mouse laminin in RIA inhibition using laminin as tracer while Galα1-4Galβ-BSA and HSA were inactive inhibitors (Fig. 2a). This indicated that the synthetic antigen could completely replace the natural antigens used before. This was also demonstrated by using labelled Galα1-3Galβ-HSA with human serum in RIA inhibition (Fig. 3). Low concentrations of Galα1-3Galβ-HSA could completely inhibit the reaction while other digalactose-albumin conjugates were rather inefficient. Laminin was also inhibitory but at 10-30 fold higher concentrations when compared to Galα1-3Galβ-HSA on a weight basis. The disaccharide Galα1-3Gal could completely block the reaction followed in activity by the B epitope Galα1-3(Fucα1-2)Gal. Other α-linked digalactose structures and methyl α-galactoside were poor inhibitors (Fig. 3).

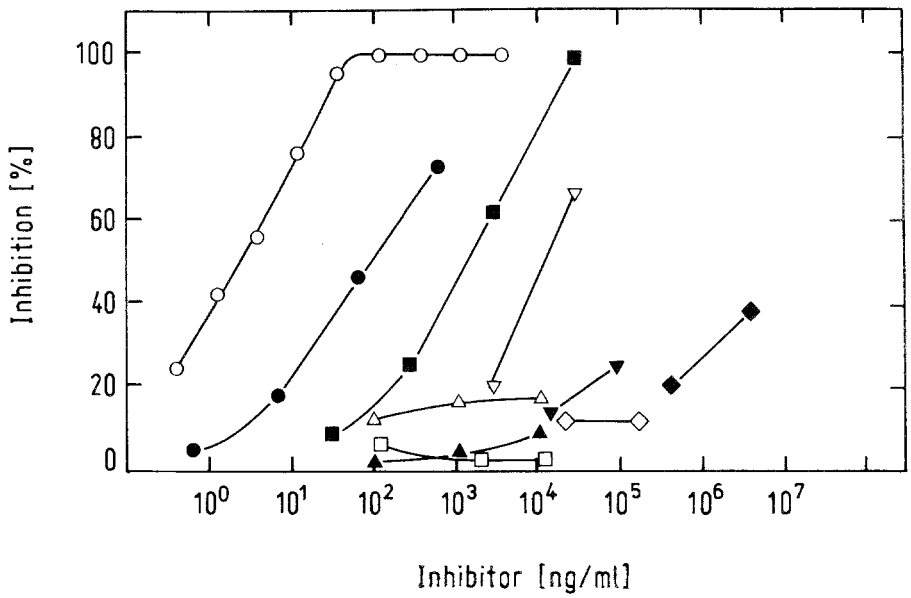


Figure 3. Inhibition of the RIA reaction between synthetic Galα1-3Galβ-HSA conjugate and human antibodies by various carbohydrate antigens. Inhibitors of the reaction were Galα1-3Galβ-HSA (○), laminin (●), Galα1-6Glc-BSA (△), Galα1-4Gal-BSA (▲), HSA (□), Galα1-3Gal (■), Galα1-3(Fucα1-2)Gal (▽), Galα1-6Gal (▼), Galα1-4Gal (◇) and α-methyl galactose (◆), The test system consisted of ^{125}I-labelled conjugate and a Leishmaniasis serum.

Studies with several patients' sera showed with labelled Galα1-3Galβ-HSA typical RIA binding profiles and up to 100% binding at high serum concentrations (Fig. 4a). The detection of Galα1-3Gal-specific antibodies was more sensitive with the synthetic conjugate compared to mouse laminin which normally produced binding profiles with 40-50% maximal binding [6, 15]. Distinct although weaker binding of Galα1-3Galβ-HSA was also observed for all 19 normal sera examined with only two individuals having titers close to those observed in the patients (Figs. 4a-c, 5). A similar sensitivity in antibody detection was observed in ELISA with Galα1-3Galβ-HSA when compared to RIA with an equivalent discrimination between patient and normal sera. (Fig. 4e).

A comparison of conjugates prepared from Galα1-2Gal, Galα1-4Gal or Galα1-6Glc with those of Galα1-3Gal showed usually weaker binding profiles (Fig. 4a-d) and lower maximal binding (at 1:50 serum dilution, Fig. 5) for both patient and normal sera. No clear distinction could be observed between Leishmaniasis patients and normal individuals particularly in studies with the Galα1-2Galβ-HSA conjugate for which normal sera even exhibited stronger binding than to Galα1-3Galβ-HSA. The latter observation could be confirmed by ELISA studies (data not shown). No binding of Galα1-4Galβ-BSA was, however, observed with a goat antiserum against human blood group substance P.

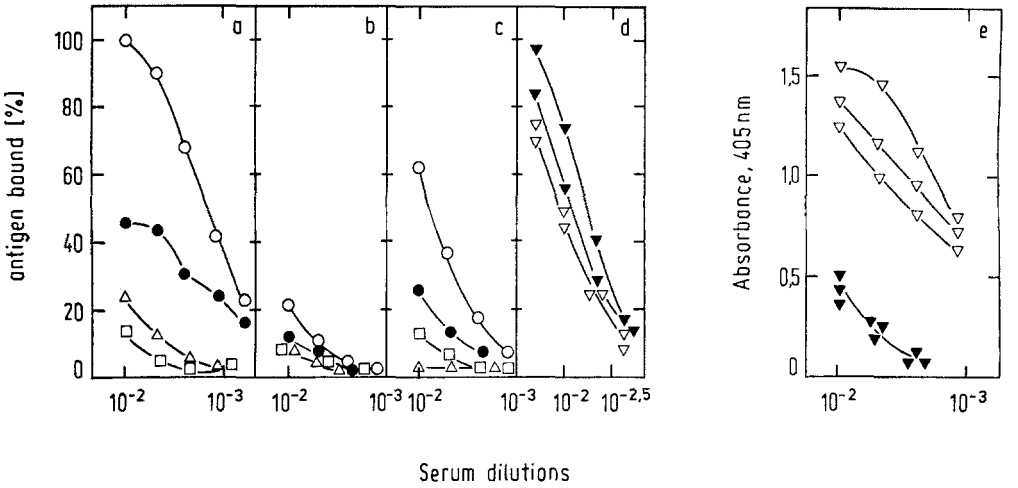


Figure 4. Binding curves of human antibodies with various digalactose albumin conjugates in RIA (a-d) and ELISA (e). RIA curves in (a-c) were obtained with ^{125}I-labelled Galα1-3Galβ-HSA (○), mouse laminin (●), Galα1-4Galβ-BSA (△) and Galα1-6Glcβ-BSA (□) using a Leishmaniasis serum (a), a typical normal serum (b) and a stronger reacting normal serum (c). Binding of ^{125}I-labelled Galα1-2Galβ-HSA (d) is shown for two stronger reacting normal (▼) and Leishmaniasis (▽) sera. ELISA reactions with Galα1-3Galβ-HSA (e) is illustrated for three Leishmaniasis (▽) and normal sera (▼).

Specificity of Natural Antibodies Against Galα1-2Gal and Galα1-4Gal Structures

The obvious lack of correlation of antibody binding to Galα1-3Galβ-HSA when compared to Galα1-2Gal or Galα1-4Gal conjugates in patient and/or normal sera (Fig. 5) indicated that the reaction with the two latter antigens is due to the presence of additional natural antibodies with different carbohydrate specificities. This could be demonstrated by RIA inhibition assays (Table 2). The reaction with Galα1-2Galβ-HSA could be inhibited in a sensitive fashion by the same but not by the related conjugates. Similarly, the Galα1-2Gal disaccharide was a distinctly better inhibitor than the other α-linked digalactose components. A comparison of Galα1-2Gal disaccharide with the corresponding conjugate showed a 10^4-10^5 fold higher inhibitory capacity for the latter on a molar basis. When calculated on a weight basis including a correction for the hapten content of the conjugate the difference is reduced to a factor 10-100. Similar differences were noted before [6] and here (Table 1, Fig. 3) for antibodies specific for the Galα1-3Gal epitope.

The binding of Galα1-4Gal conjugate by patient and normal sera was also apparently specific for this carbohydrate structure as shown by inhibition assays with related digalactose α-linked to albumin and disaccharides (Table 2). The difference in inhibiting capacity

Table 2. Inhibition of Galα1-2Gal-HSA (A) and Galα1-4Gal-BSA (B) binding by patient (LS) and normal sera (NS) with synthetic conjugates and disaccharides.

Inhibitor	50% Inhibitory level (μM)[a]			
	A		B	
	LS 64	NS 17	LS 6	NS 29
Galα1-2Gal-HSA	0.0008	0.00015	n.t.[b]	n.t.
Galα1-4Gal-BSA	>0.15	>0.15	0.00001	0.00002
Galα1-3Gal-HSA	>0.5	>0.5	0.004	0.05
Galα1-6Glc-BSA	>0.15	>0.15	>0.015	>0.015
Galα1-2Gal	50	1.0	>500	>500
Galα1-4Gal	>150	>150	10	10
Galα1-3Gal	>88	>88	50	>88
Galα1-6Gal	>91	>91	>91	>91

[a] Determined by RIA inhibition assay.
[b] n.t. = not tested.

between Galα1-4Gal conjugate and disaccharide was, however, 10-fold higher than for the other digalactose epitopes. The reaction of human sera with Galα1-6Glc conjugate was usually too low (Fig. 5) to allow specificity analysis by inhibition assays. Two patients sera with higher titers could, however, be examined, following protocols similar to those of Table 2. Both reactions could be inhibited within a factor of 10 by all α-linked digalactose disaccharides except for Galα1-4Gal (data not shown). It indicates that this binding reaction measures a subfraction of antibodies specific for the Galα1-3Gal epitope in agreement with previous observations [3, 4] that the same antibodies can be bound to an immunoadsorbent containing the Galα1-6Glc disaccharide.

Discussion

Human natural antibodies against the Galα1-3Gal epitope were discovered some five years ago [3, 4] and are likely to be triggered by exogeneous stimuli of yet unknown nature. They have been implicated in the recognition and removal of altered human erythrocytes [3, 4, 8] and possibly in recognition of cellular alterations due to parasitism or tumor development [6]. These antibodies are restricted to humans and old world monkeys [7] indicating some

Table 3. Relative contributions of particular substructures of the Galα1-3Gal epitope to antibody binding.

	Structure	Loss of binding capacity (factor)
1	Terminal galactose	>>3,000-10,000
2	α- or β-anomeric linkage	>>3,000-10,000
3	Position of α-linkage	40-1,000
4	Second galactose	2-5[a], 60-1,000[b]
5	Further linkage of second galactose	1-5

[a] By comparison with Galα1-6Gal and Galα1-6Glc.
[b] By comparison with Galα1-3(Fucα1-2)Gal.

evolutionary implications that are also not yet understood. Elevated antibody levels have been reported for several unrelated diseases [6, 11-15]. The better characterization of the origin and specificity of these antibodies seems therefore important for the understanding of their role under normal and pathological circumstances.

In the present study we have achieved a better definition of antibody specificity by using a variety of oligosaccharide haptens. Basic conclusions from these studies (Table 3) are that the change from non-reducing terminal galactose to other common monosaccharides (glucose, mannose, *N*-acetylgalactosamine) and the change from α(1-3)- to β(1-3)-linkage abolishes binding to the antibodies. A lower, but still a distinct reduction in affinity was observed by varying the position of α-linkage between two galactose residues, decreasing in the order α(1-3), α(1-2), α(1-6) to the almost inactive α(1-4) bond.

The importance of the second galactose in the Galα1-3Gal structure is less defined. A change from the cross-reacting Galα1-6Gal structure to Galα1-6Glc produces only marginal effects. Substitution of fucose on the second galactose which generates the blood group B epitope causes considerably stronger losses in affinity, presumably due to steric interferences. The contribution of other sugar residues to affinity besides Galα1-3Gal seems negligible since a change to Galα1-3Galβ1-3GlcNAc which is the natural structure in *N*-linked laminin oligosaccharides [22, 23] does not increase activity. That terminal rather than internal Galα1-3Gal structures possess activity was previously concluded from studies with natural antigens which is supported here by showing a low activity of polymeric Galα1-3Gal as present in arabinogalactan. Studies with oligosaccharides possessing internal Galα1-3Gal are, however, still required to substantiate this point.

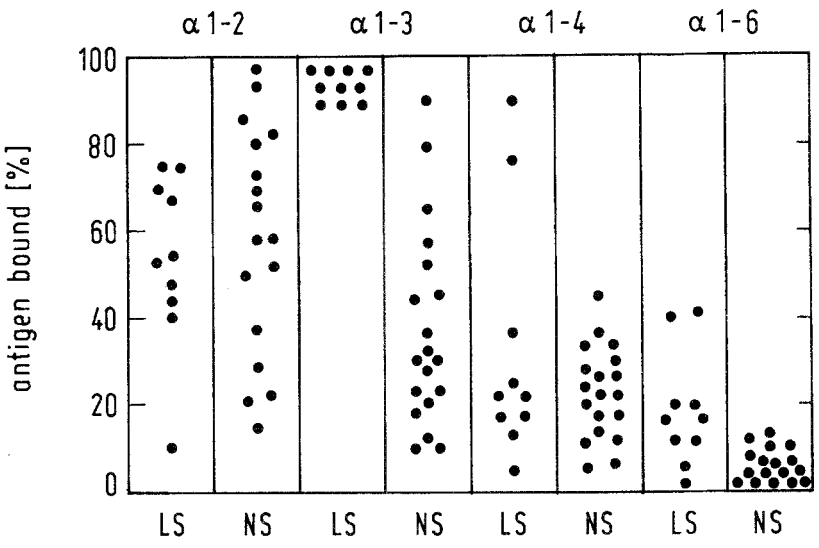
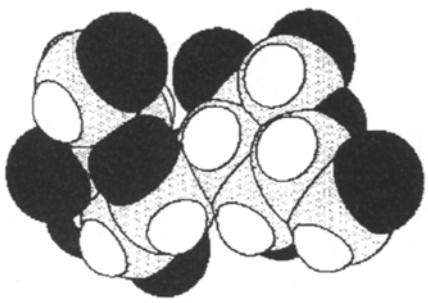


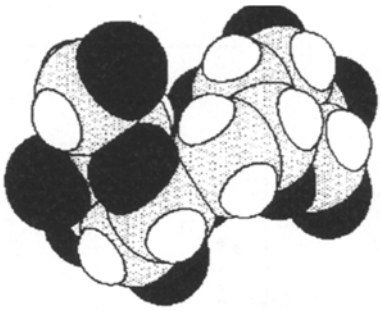
Figure 5. RIA binding of Galα1-2Gal, Galα1-3Gal, Galα1-4Gal and Galα1-6Glc albumin-conjugates to individual Leishmaniasis (LS, n=10) and normal (NS, n=19) sera all used at 1:50 dilution.

For stereochemical considerations of the hapten structure we also calculated conformations of the oligosaccharides tested for inhibition of laminin binding (Table 1, Fig. 6). The aim was to correlate conformations to inhibition data in order to gain information about within what limits the three-dimensional shape of a hapten is allowed to vary and still give appreciable binding to the antibodies. Two comments have to be made to this approach. Carbohydrates are molecules with a high degree of flexibility, which might result in a conformation for an oligosaccharide bound in a combining site, that differs from that in solution. In the following reasoning, however, the conformations calculated by HSEA will be used. It should also be stressed that polyclonal antisera were used in the inhibition study. Structures with significant but weak inhibition may bind to different subpopulations of the antibodies, which to some extent can be indicated by different inhibition profiles. The resulting three-dimensional map should therefore not be interpreted as representing an individual antibody binding site, but rather the binding ability of the sera as a whole, when screened for a given epitope.

An initial study was made to test the validity of the approach. Prior to the experimental testing, a theoretical estimation was made for the probability of appreciable inhibition by Galα1-2Gal. Data on laminin-inhibition for Leishmaniasis and normal sera was taken from a previous work [6], where Galα1-3Gal was shown to give good, Galα1-3(Fucα1-2)Gal and Galα1-6Gal weak, and Galα1-4Gal negligible inhibition. As the non-reducing terminal galactose had been shown to be essential for the binding to the antibodies, the different structures were compared with the non-reducing pyranose rings in identical orientation and



Gal α1-3Galß



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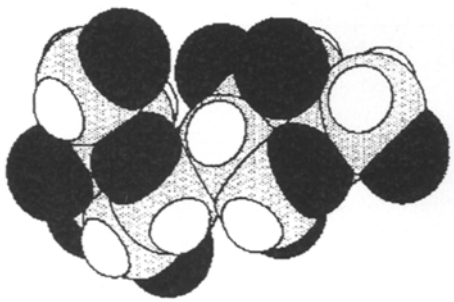

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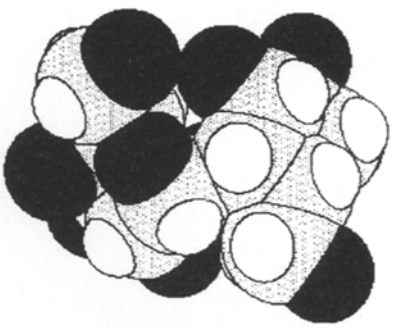
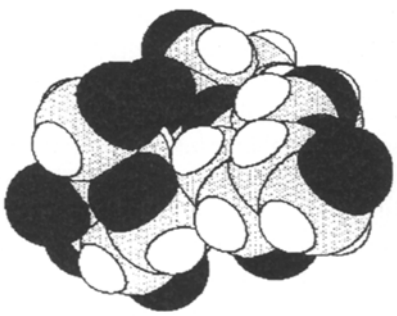
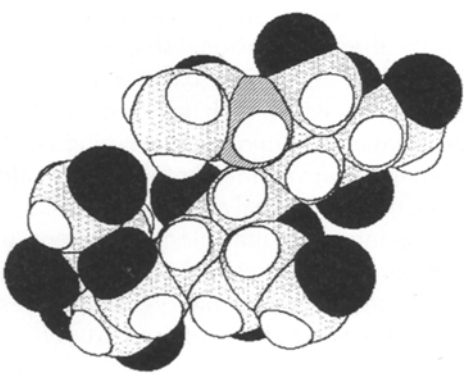


Galα1-4Galß



Galα1-3(Fucα1-2)Galß



Galα1-3Galß1-3GlcNAcß

Figure 6. Space filling models, based on HSEA calculations, of oligosaccharides used for antibody inhibition. Hydroxyl groups are represented by the oxygen atoms. Carbon atoms are light grey, oxygen dark, nitrogen grey, and hydrogen white. The non-reducing terminal is always to the left. a) Galα1-3Galβ, b) Galα1-6Galβ, c) Galα1-2Galα, d) Galα1-4Galβ, e) Galα1-3(Fucα1-2)Galβ, f) Galα1-3Galβ1-3GlcNAcβ.

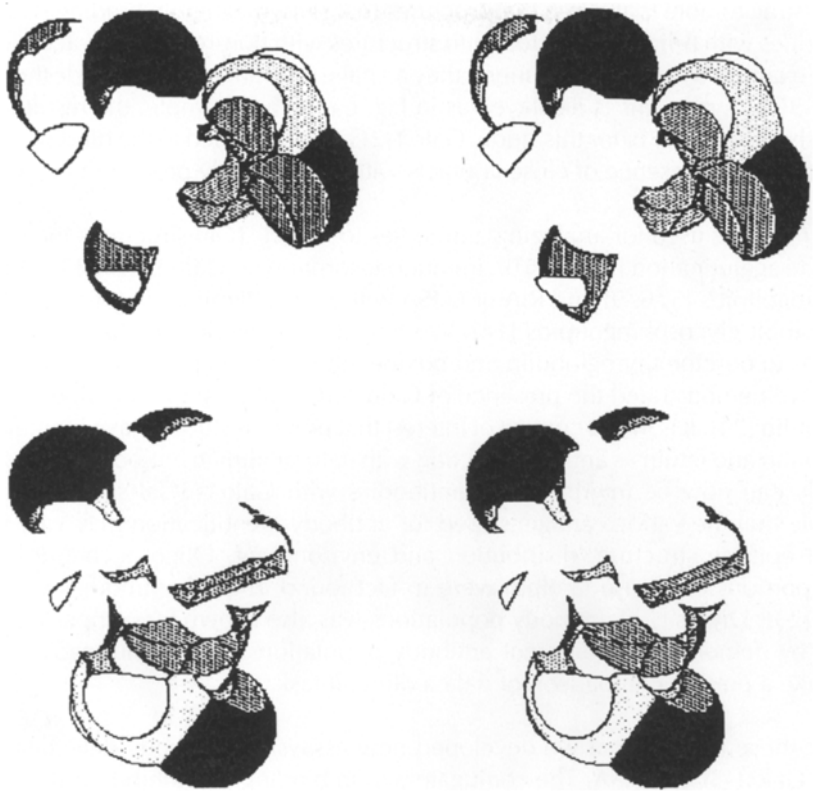


Figure 7. a) Stereo view showing areas of Galα1-2Galα not overlapping Galα1-3Galβ, Galα1-6Galβ, or Galα1-3(Fucα1-2)Galβ when all oligosaccharides are located and orientated with reference to the non-reducing terminal galactose. Surfaces inside the molecule are shown in different shades only to simplify the perception of the three-dimensional structure.
b) Stereo view showing areas of Galα1-4Galβ not overlapping Galα1-3Galβ, Galα1-6Galβ, Galα1-3(Fucα1-2)Galβ or Galα1-2Galα when all oligosaccharides are located and orientated with reference to the non-reducing terminal galactose.

location. The calculated structures for some of the oligosaccharides studied are shown in Fig. 6, represented as space-filling models using standard van der Waals radii for the atoms. For Galα1-2Gal the α-anomer was chosen, as this resulted in closest resemblance to Galα1-3Gal. In all other oligosaccharides the subterminal galactose is shown as the β-anomer. Regions of Galα1-2Gal which did not overlap any of the three oligosaccharides with previously shown binding activity are displayed in Fig. 7a. The residual regions were small, located far from the non-reducing terminal, and for the most part surrounded on two sides by regions of oligosaccharides that bind. This suggests a low probability for hard contacts with the antibody protein. It was concluded that Galα1-2Gal probably also would bind to the antibodies. The inhibition tests in the present study showed this to be correct. (Table 1).

It is interesting to note that when the structures that show negligible binding (Galα1-4Gal, disaccharides with β-linked galactose and structures with non-reducing *N*-acetylgalactosamine) are examined in the same manner, they all have residual regions outside the lower part of Galα1-3Gal, when this is displayed as in Fig. 6a. As an example, the result for Galα1-4Galβ is shown in Fig. 7b (for this study, Galα1-2Galα was added to the binding structures). This indicates the presence of close contacts with the antibody protein in this region.

Previous methods used for analyzing antibodies to Galα1-3Gal structures included rabbit erythrocyte agglutination [3, 4, 9, 10], immunoadsorption on Galα1-3Glc [3, 4], blotting of glycosphingolipids [5, 6, 9] and RIA or ELISA with mouse laminin and nidogen [6, 12-15, 22] and rabbit glycosphingolipids [14]. We have here extended the list of cross-reacting substances to porcine thyroglobulin and bovine fetuin. This is no surprise since chemical studies have demonstrated the presence of Galα1-3Gal at least in animal, but not human thyroglobulin [24]. It is in this context of interest that previous studies have identified animal thyroglobulin and fetuin as antigens reacting with natural human antibodies [25, 26] which very likely can now be interpreted as antibodies with Galα1-3Gal specificity. It is also imaginable that the various antigens used for antibody identification may vary in various details of epitope structure, distribution and environment. Oligosaccharides bound to different portions of mouse laminin were in fact found to bind antibodies with different affinities [23]. Diversity of antibody populations was also shown by comparing individual sera and by demonstrating different antibody populations with immunoadsorption. This could make a precise comparison of data a difficult task.

It was for those reasons that we developed new assays for antibody detection based on synthetic Galα1-3Galβ-HSA. The conjugate was in binding and inhibition studies equivalent or even better than mouse laminin used previously [6, 15]. It showed also as expected a strict Galα1-3Gal specificity with only marginal cross reactions with other α-linked digalactose structures. The hapten carrier used will also exclude the detection of autoantibodies which in patient studies, for example with mouse laminin, may require more laborious assessments of antibody specificities. The ELISA with Galα1-3Galβ-HSA may be as shown here of particular clinical utility for assays of antibodies or circulating antigen.

In the context of specificity examinations we made also the surprising observations of a distinct binding of normal and Leishmaniasis sera to albumin conjugates of Galα1-2Gal and Galα1-4Gal structures. Since inhibition studies did not reveal significant antibody cross-reactions between both carbohydrates as well as with Galα1-3Gal we interprete these observations as the demonstration of natural antibodies against two new α-linked digalactose epitopes. Terminal Galα1-2Gal has been recently shown to be a structural unit of a phosphatidyl inositol "anchor" for inserting variable surface glycoproteins of trypanosomal parasites into their cell surface [27]. Increased antibody responses to this structure could therefore be common in some parasitic infections but are missing in Leishmaniasis patients. The Galα1-4Gal structure has been identified as the human blood group substance P but in an internal position of a glycosphingolipid chain [28]. Yet, a P-specific antiserum failed to react with our synthetic conjugate. A more comprehensive analysis still seems to be required for the antibodies reacting with the albumin conjugates of Galα1-2Gal and Galα1-4Gal to understand their specificity, significance and relationship to other carbohydrate-specific antibodies.

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