CIRCULATING ANTIBODIES TO MOUSE LAMININ IN CHAGAS DISEASE, AMERICAN CUTANEOUS LEISHMANIASIS, AND NORMAL INDIVIDUALS RECOGNIZE TERMINAL GALACTOSYL(α1-3)-GALACTOSE EPITOPES

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Infections with protozoans of the family Trypanosomatidae cause several widespread human disorders including Chagas disease and various forms of leishmaniasis (1). Patients with Chagas disease develop antibodies reacting with blood vessels, endocardium, striated muscle, and peripheral nerves (2–6) and these antibodies are thought to be involved in the development of degenerative changes in various organs in these patients (7). Laminin, a large glycoprotein found exclusively in basement membranes (8) was able to bind most of the tissue-reacting antibodies present in these sera (6). Similar laminin-reacting antibodies have been detected in patients with American cutaneous and mucocutaneous leishmaniasis (9) and with poststreptococcal glomerulonephritis (10). However, these antibodies have been found to crossreact with various forms of the parasites (2, 3, 6, 11), as well as with animal erythrocytes (3, 11–13) and structurally distinct basement membrane proteins such as laminin and nidogen (13). A similar but unexpected crossreaction was observed between rat neurones and *Trypanosoma cruzi* using an mAb (14). These tissue-reacting antibodies may, however, not have a key role in invoking protective immunity against trypanosomal infections (15).

Since distinct proteins contained the epitope, it seemed likely that it was not these proteins themselves but rather a common posttranslational modification that was the actual reactant (13). Additionally, such epitopes could exist in sufficient amounts in human tissue to explain the occurrence of these antibodies and suggest a possible pathogenetic role of such antibodies in these disorders (11, 16). These antibodies show distinct reactions with epitopes present in rodent and other animal tissues (6, 11, 12, 16). Initial observations on binding to human tissues in situ and in vitro (2, 3) have been revised recently (12), and indicate

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that the tissue-reacting antibodies are generated due to a lack of immune tolerance for these epitopes in the human species.

In this study, we provide evidence for the carbohydrate nature of epitopes of mouse laminin that react with antibodies in the sera of leishmaniasis and chagasic patients, as well as in infected monkeys. The structure responsible for this interaction appears to be a terminal Galα1-3Gal group on *N*-linked oligosaccharides of mouse laminin. Surprisingly, an identical or very similar epitope is recognized by normal human laminin-binding antibodies, which apparently correspond to α-galactose-specific natural antibodies described in previous studies (17–19). Additional data indicate that these structures may also be present in some normal and pathological human tissues.

Materials and Methods

Serum Panels and Antisera. Sera with high-binding capacity for laminin were obtained by screening sera from patients with American cutaneous leishmaniasis and chronic Chagas disease as described previously (9, 13). Sera with similar high laminin binding were also obtained from rhesus monkeys infected by the ocular route with *T. cruzi* trypomastigotes (6) or inoculated intravenously with *Trypanosoma rhodesiense* trypomastigotes (20). Normal sera were collected in Germany from 8 female and 11 male volunteers (age range, 25–52 yr; average, 39 yr). The normal donors had normal values in several standard laboratory tests and lacked exposure to the parasitic diseases studied here.

Rabbit antisera against native laminin were described in previous studies (8, 21). Human blood group typing sera were obtained from Behringwerke AG, Marburg, Federal Republic of Germany. A polyvalent antiserum against human Ig was prepared in a sheep by repeated injections of Cohn fraction II serum protein mixed with CFA and was used in most binding studies as secondary antibody. In some studies we used specific goat antisera against human IgG, IgM, and IgA purchased from Medac, Hamburg, Federal Republic of Germany.

Sources of Antigens and Inhibitors. Laminin (8), nidogen, heparan sulfate proteoglycan (22), and protein BM-40 (23) were purified from the mouse Engelbreth-Holm-Swarm tumor. Laminin was also extracted with 10 mM EDTA containing buffer from human placenta (24) and purified by chromatography on Sepharose Cl-6B and DEAE-cellulose and was found to contain A, B, and M chains (25). Human plasma fibronectin was a kind gift of Dr. H. Richter, Max-Planck-Institut Für Biochemie, Martinsried, Federal Republic of Germany. Laminin B chains were prepared from reduced and alkylated bovine glomerular basement membranes (26) and were purified by ion exchange and molecular sieve chromatography. Conditioned meda of various human cell lines were obtained by incubating confluent cultures of the cells for 24 h in serum-free DMEM, provided by Dr. M. Aumailley, Max-Planck-Institut Für Biochemie. These materials were free of mycoplasma contaminations. Human saliva was used as a source of blood group substances. Glycosphingolipids from rabbit erythrocytes were extracted and separated into components with short and long oligosaccharide chains (27). Various mono- and oligosaccharides were obtained from Biocarb AB, Lund, Sweden, or Sigma Chemical Co., St. Louis, MO, and originated from natural sources or were synthetic products.

Modifications of Laminin and Other Antigenic Substances. Complete reduction and alkylation of laminin in 6 M guanidine-HCl followed a previously used procedure (21). For alkaline degradation, lyophilized laminin (3 mg/ml) was dissolved in 0.5 M NaOH, 0.1 M sodium borohydride and the solution was neutralized with glacial acetic acid after 2 d incubation at 37°C. Laminin dissolved in 0.5 M NaCl, 0.05 M Tris/HCl, pH 7.4, was also treated with pronase B (Sigma Chemical Co.) at an enzyme/substrate ratio of 1:50 for 2 d at 37°C to remove protein segments. Before periodate treatment, the pH of the laminin solution was lowered to 4.5, causing partial precipitation of the protein. After addition of periodic acid (0.02 M) and incubation for 24 h at 4°C the protein was redissolved by

dialysis against 0.5 M NaCl, 0.05 M Tris/HCl, pH 7.4, and the extent of degradation was measured by orcinol reaction (28).

Other samples of laminin were dialyzed against 0.5 M sodium acetate buffer, pH 5.0, and treated for 20 h at 37°C with either α-galactosidase (20–35 U/mg) or β-galactosidase (10–15 U/mg), both from *Aspergillus niger* (Sigma Chemical Co.), using 1 U of enzyme per 1.5 mg of laminin. These treatments did not cause degradation of the polypeptide chains of laminin, as judged from electrophoresis. Similar incubations were carried out with β-*N*-acetylglucosaminidase (10 U/ml; from *A. niger*) and α-*N*-acetylgalactosaminidase (2 U/ml, from *Charonia lampas*).

Chemical defucosylation (29) was achieved by heating antigens in 0.1 M TCA for 1 h at 100°C. Antigens were then precipitated at 4°C with 2 volumes of acetone, washed once with acetone, and dissolved in PBS, pH 7.2. Controls were heated without TCA and processed as above.

Immunological Assays. Laminin was labeled with ^{125}I to a specific activity of 8,000–12,000 cpm/ng by the chloramine T procedure and used at 2 ng/0.4 ml for direct binding analysis after standard protocols (30). For radioimmuno-inhibition assays, appropriate antiserum dilutions were incubated with nonlabeled inhibitors (24 h, 4°C) before the addition of labeled antigen and binding analysis (30).

Dot binding assays followed the procedure of Hawkes et al. (31), with slight modifications. Protein solutions (1 μl) were spotted onto nitrocellulose (Millipore, Molsheim, France), briefly washed with water, fixed with 0.1% glutaraldehyde/0.4% formaldehyde, again washed with water and blocked with 0.1% BSA, 0.05% Tween 20 in PBS (BTB)[1] for 1 h at 37°C. The strips were incubated with sera diluted 1:100–300 in BTB for 3 h at room temperature. Peroxidase conjugates of second antibodies (goat anti–monkey Ig from E.Y. Laboratories, Inc., San Mateo, CA; affinity-purified, polyvalent goat anti–human IgG from Tago Inc., Burlingame, CA) were used for incubations (1 h, room temperature) at dilutions of 1:500. The glycosphingolipid blotting assay followed a previous procedure (32), except that BTB was used as diluent and for blocking and washing.

Results

Laminin Binding of Normal and Patient Sera and Sera from Infected Monkeys in RIA. Sera from patients (n = 15–25) with American cutaneous and mucocutaneous leishmaniasis, with Chagas disease, as well as from monkeys infected either with *T. cruzi* or *T. rhodesiense* and from normal human subjects were examined for binding of ^{125}I-labeled mouse laminin by a double antibody RIA. All sera produced typical binding profiles with up to 50–60% of laminin bound at the lowest serum dilution (1:100) examined (Fig. 1, *a* and *b*). The titers of antisera were highest in the infected monkeys, followed by patient and normal sera. However, three individuals among the normal control group (n = 19) showed titers as high as those found in the patient group (Fig. 1 *a*). Binding of laminin was found to be mainly due to IgG rather than IgM or IgA antibodies, as illustrated for a normal serum (Fig. 1 *c*). A similar prevalence of IgG binding was previously demonstrated (9, 13) for patient sera by enzyme immunoassays.

Specificity of the binding reaction was demonstrated by passing patient and normal sera over columns containing immobilized laminin and observing that binding was reduced to background levels (Fig. 1 *b*). The residual binding was no longer dependent on serum concentration, indicating that it is probably due to nonspecific adsorption of labeled laminin to the test tubes. Adsorption of sera on nonsubstituted supporting material had no effect on laminin binding (Table

[1] *Abbreviations used in this paper:* BTB, bovine serum albumin in PBS/Tween.

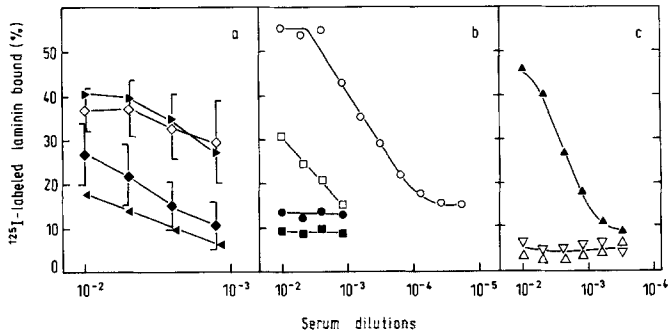

FIGURE 1. Comparison of RIA binding of mouse laminin by normal and leishmaniasis sera (*a*), the effect of laminin immunoadsorption on binding (*b*), and identification of Ig classes involved in binding by normal serum (*c*). The data in *a* show the average binding of serum panels from normal volunteers (◆, $n = 19$) and patients with American cutaneous leishmaniasis (◇, $n = 15$); vertical bars indicate the standard deviation. In addition, individual binding profiles of two normal sera which show the strongest (▶) and weakest (◀) binding activity are illustrated. Data in *b* show binding profiles before (*open symbols*) and after (*closed symbols*) immunoadsorption on laminin of an individual normal serum (□,■) and patient serum (○,●). Data in *c* were obtained with an individual normal serum after precipitation of immune complexes with antisera specific for either human IgG (▲), IgA (▽) or IgM (△).

TABLE I

Removal of Laminin-binding Activity from Patient (LS) and Normal (NS) Human Serum by Absorption with Erythrocytes

Absorption	Supernatant binding of ^{125}I-laminin	
	LS*	NS‡
	%	
Buffer control	37 ± 5	42 ± 1
Sepharose	38 ± 3	42 ± 2
Laminin-Sepharose	5 ± 1	13 ± 1
Human erythrocytes§	33 ± 4	40 ± 2
Sheep erythrocytes	5 ± 1	16 ± 1
Rabbit erythrocytes	4 ± 1	5 ± 1

Equal volumes of serum (diluted 1:10) and of 50% erythrocyte suspension incubated over night at 4°C. Supernatants were diluted 1:10 or 1:20 for binding tests.

* LS, leishmaniasis sera.

‡ NS, normal human sera.

§ No difference with A1, A2, B, or O blood group.

I). The binding of labeled laminin was also completely blocked by preincubation of sera with soluble, nonlabeled laminin (Fig. 2*a*) with a 50% reduction achieved at concentrations as low as 0.01–0.1 nM (0.01–0.1 μg/ml). These quantitative inhibition assays were subsequently used in the elucidation of the structure of epitopes involved in the reaction.

Nature of Laminin Epitopes Recognized by Patient and Monkey Antisera. A comparison of various murine basement membrane proteins by inhibition assays (Fig. 2*a*) demonstrated that other macromolecules, including nidogen and heparan sulfate proteoglycan, were also able to completely block laminin binding. Their activity was some 10–20% of laminin on a molar basis, indicating they

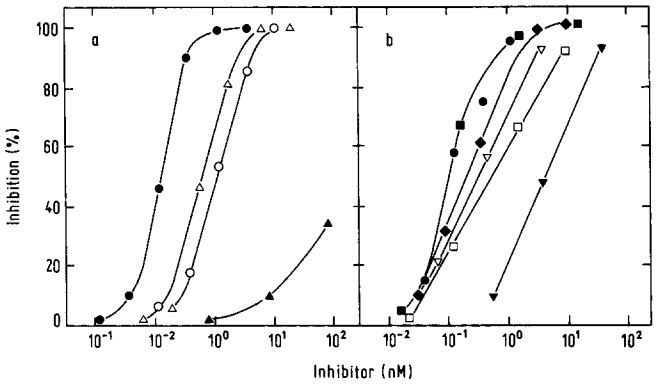

FIGURE 2. Crossreaction of laminin-binding leishmaniasis antibodies with other basement membrane proteins (*a*) and stability of epitopes on laminin against degradation (*b*). Antigenic activity was determined by radioimmuno-inhibition assay using ^{125}I-labeled laminin and two individual patient sera. Inhibitors used were laminin (●), heparan sulfate proteoglycan (△), nidogen (○), protein BM-40 (▲), reduced and alkylated laminin (■), laminin B chains-(◆), and laminin digests obtained after treatments with pronase (▽), alkali (□), or periodate (▼). All antigens originated from a mouse tumor except the B chains, which were from bovine glomerular basement membranes.

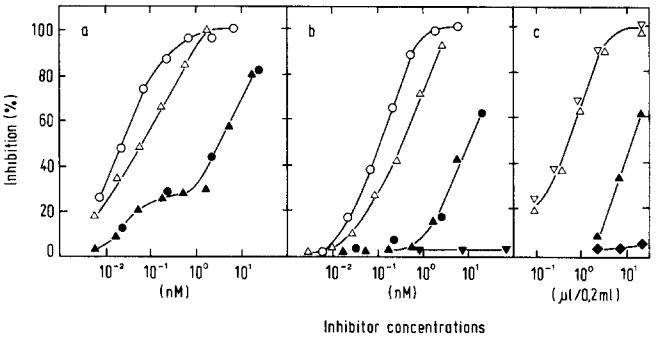

FIGURE 3. Comparison by radioimmuno-inhibition assay of mouse laminin before and after treatment with exoglycosidases (*a* and *b*) and cross-reactivity of human laminin (*a* and *b*) and conditioned cell culture medium (*c*). The test system consisted of ^{125}I-labeled mouse laminin and a normal serum (*a*) or leishmaniasis serum (*b* and *c*). Inhibitors of the reactions in *a* and *b* were mouse laminin (○), human placenta laminin (●), mouse laminin after treatment with β-galactosidase (△) or α-galactosidase (▲), and human plasma fibronectin (▼). Culture media in *c* were obtained from human embryonic fibroblasts (▲), fibrosarcoma HT 1080 cells (◆), astrocytoma cell line 251 MG (▽), and rhadbdomyosarcoma cell line A 204 (△).

contained a lower content of epitopes or had a lower affinity for the antibody. This interpretation was confirmed by inhibiting the reaction of the same antiserum with ^{125}I-labeled nidogen (13), which revealed laminin to be a 5–10-fold better competitor than nidogen and proteoglycan (data not shown). Another small protein, BM-40 which contains only one to two carbohydrate side chains (23), had a 1,000-fold lower inhibitory capacity than laminin (Fig. 2*a*). It is possible that the low activity observed with BM-40 is due to contaminants rather than the protein itself. However, a strong crossreaction was observed with laminin B chains obtained from bovine glomerular basement membranes (Fig. 2*b*).

Since these crossreactions with unrelated proteins indicated a possible glycoconjugate nature of epitopes (13), laminin was subjected to various chemical and enzymatic treatments. The digests were compared with intact laminin in inhibition assays (Figs. 2*b* and 3), demonstrating no loss of activity after complete reduction of disulfide bonds and only a three- to fivefold loss after treatments with pronase or alkali. Examination of the latter two digests by molecular sieve chromatography demonstrated all of the laminin had been degraded to small peptides. Conversely, periodate treatment, which reduced the carbohydrate

TABLE II

Inhibitory Activity of Mono- and Oligosaccharides for Laminin-binding by Leishmaniasis Sera (LS), Normal Human Sera (NS), and Serum from Monkey Infected with T. cruzi (MS)

Saccharides	Concentrations of inhibitor (μM) required for 50% inhibition*				
	LS 1	LS 15	NS 21	NS 25	MS
Mouse laminin	40×10^{-6}	32×10^{-6}	14×10^{-6}	30×10^{-6}	700×10^{-6}
Gal α1-3 Gal	0.5	0.6	5.4	8.9	7.0
Gal α1-6 Gal	81	2,400	1,400	40	>1,000
Gal α1-4 Gal	>1,500	>1,500	>1,500	>1,500	>1,500
Galα1-3(Fucα1-2)Gal	300	30	78	630	>700
α-methyl galactose	400	5×10^3	400	200	$>20 \times 10^3$
β-methyl galactose	$>20 \times 10^3$	$>20 \times 10^3$	20×10^3	$>20 \times 10^3$	$>20 \times 10^3$
Galactose	11×10^3	10×10^3	33×10^3	1.5×10^3	$>30 \times 10^3$
Glucose	$>55 \times 10^3$	$>55 \times 10^3$	$>55 \times 10^3$	$>55 \times 10^3$	$>55 \times 10^3$
Mannose	$>55 \times 10^3$	$>55 \times 10^3$	$>55 \times 10^3$	$>55 \times 10^3$	$>55 \times 10^3$
Gal NAc	$>45 \times 10^3$	$>45 \times 10^3$	40×10^3	$>45 \times 10^3$	$>45 \times 10^3$
Glc NAc	$>45 \times 10^3$	$>45 \times 10^3$	$>45 \times 10^3$	$>45 \times 10^3$	$>45 \times 10^3$

* Determined by radioimmuno-inhibition assays.

content of laminin by 70%, reduced its inhibitory activity to 2% of control (Fig. 2*b*). Inhibitory activity of the pronase and alkali digests of laminin were completely destroyed by periodate treatment. In contrast to these results, only the periodate-treated laminin was able to inhibit rabbit antisera raised against native laminin (data not shown), which are known to react with peptide epitopes (21).

The possible involvement of terminal sugar residues present at the nonreducing end of oligosaccharides in laminin was evaluated using specific exoglycosidases. Treatment with α-galactosidase caused a 100-fold loss of inhibitory capacity of laminin, while only a two- to threefold decrease was observed with β-galactosidase (Fig. 3*b*). The latter effect can be presumably attributed to a small contamination of α-galactosidase usually present in such enzyme preparations. No substantial effects on antigenicity were observed after treatments with β-*N*-acetylglucosaminidase and α-*N*-acetylgalactosaminidase in dot binding assays (data not shown). This indicates that terminal α-galactose residues are part of the carbohydrate epitopes on mouse laminin recognized by patient and monkey antibodies.

Reaction of Antibodies with Defined Carbohydrate Structures. The presence of terminal α-galactose groups on laminin was recognized in previous studies (33–35) but their precise linkage to other galactose residues has so far not been established (35). We have therefore used various mono- and oligosaccharides to study the specificity of epitope recognition by inhibition assays (Table II). Among the various components tested Galα1-3Gal and Galα1-3Galβ 1-3GlcNAc showed the strongest activity in completely blocking the binding of laminin at concentrations of 10–100 μM. A 10^5-fold molar excess of Galα1-3Gal compared with laminin is required to produce 50% inhibition (Table II), which corresponds to a 300-fold difference compared on a carbohydrate weight basis. Other galactose disaccharides that differ in α-glycosidic linkage (α1–4 and α1–6) were 100–1,000-fold less competent inhibitors than Galα1-3Gal. A lower activity was also

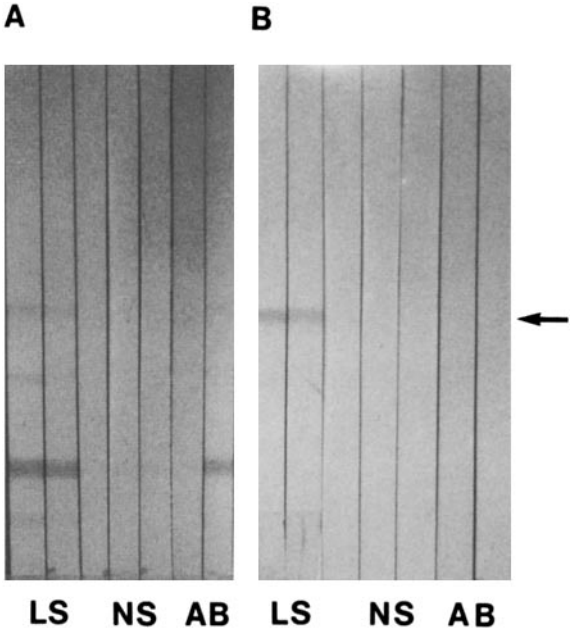

FIGURE 4. Detection of α-galactose epitopes on glycosphingolipids prepared from rabbit erythrocytes. The lipids were enriched by Folch's partition for components with long (*A*) and short (*B*) carbohydrate chains (27). After separation by TLC and blotting to nitrocellulose they were reacted against two different leishmaniasis patient sera (*LS*); three different normal human sera (*NS*) all diluted 1:100, and against blood group typing sera specific for A and B blood group substances (dilution 1:10). The arrow denotes the position of a pentasaccharide ceramide described by Stellner et al. (37). The slow migrating band in *A*, which also reacts with anti-B antisera, may correspond to a ceramide decasaccharide described by Hanfland et al. (38). Reaction patterns similar to LS sera are observed at higher concentrations of normal sera. Migration is from bottom to top.

found after introducing substitution on the second galactose, such as in Galα1-3(Fucα1-2)Gal (Table II).

The antibody reaction was also inhibited by monosaccharides with galactose being distinctly more active (50% inhibition at ~10 mM) than glucose, mannose, and *N*-acetylhexosamines. A distinct increase of galactose inhibitory activity was observed after methylation in α- but not in β-glycosidic linkage (Table II).

The specific recognition of carbohydrate structures by the patient antibodies suggests that the adsorption of tissue-reacting antibodies by erythrocytes (3, 11–13) is due to similar surface epitopes. By using RIAs we found that the adsorption of sera with rabbit and sheep but not with human erythrocytes reduced laminin binding of patient sera to background values (Table I). Reactive components were detected in two fractions enriched for short or long carbohydrate chains of purified neutral glycosphingolipids prepared from rabbit erythrocyte plasma membranes. These components were separated by TLC and, after blotting, reacted with patient and monkey antisera. The only stained band observed in the short chain fraction (Fig. 4, *B*) corresponded to a glycosphingolipid with five monosaccharide units, as judged from its mobility. It very likely corresponds to Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-ceramide identified and characterized in previous studies (36, 37). The mobility of the reacting, slow-migrating band of the long chain fraction (Fig. 4*A*) is compatible with an I and B blood group, active branched ceramide decasaccharide with two terminal α-galactose residues (38).

Specificity of Laminin-binding Antibodies from Normal Human Sera. Natural antibodies with Galα1-3Gal specificity have been previously described (17, 18) to be ubiquitous in normal human sera implying that the normal laminin-binding antibodies (Fig. 1) are identical. The specificity of the reaction of normal sera

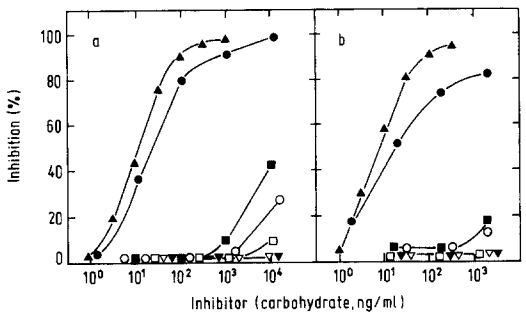

FIGURE 5. Comparison of the α-galactose epitope on mouse laminin with human blood group substances by radioimmunoinhibition assay. The assay consisted of ^{125}I-labeled mouse laminin and serum from a monkey infected with *T. rhodesiense* (*a*) or normal human serum (*b*). Inhibitors used in the assay were laminin (▲), saliva blood group substances A (□), B (○), 0 (▽) before and after (A, ■; B, ●; 0, ▼) removal of fucose by TCA hydrolysis. Inhibitor concentrations are expressed as carbohydrate equivalents, which were determined by an orcinol assay.

with laminin was therefore determined by inhibition assays and showed no substantial difference to the patterns found with patient and monkey antibodies. The natural antibodies exhibited a similar crossreaction with various basement membrane proteins that could be blocked most efficiently with Galα1-3Gal disaccharide (Table II), and the epitopes on laminin were found to be sensitive to treatment with periodate and α-galactosidase (Fig. 3*a*). Some differences in the fine specificity of natural and patient antibodies may exist as illustrated in Fig. 3, *a* and *b*, and Table II. The structural basis of such differences needs further exploration.

Search for the Presence of Galα1-3Gal Epitopes on Human Antigens. Laminin was purified from human placenta but was 100–200-fold less active in inhibition assays with normal and patient sera than mouse laminin. Its low activity was identical to that of mouse laminin treated with α-galactosidase (Fig. 3, *a* and *b*). The residual activity may represent distinct epitopes since no activity was detected with another human glycoprotein, fibronectin. Presumably, such activity could be due to small amounts of α-galactose or reflect a weak crossreaction with related carbohydrate structures present on human laminin. A comparably higher inhibitor activity, equivalent to 50 μg/ml of mouse laminin, was found in conditioned medium of some human tumor cell lines (Fig. 3*c*) and some activity was also produced by cultured embryonic fibroblasts. Since these media contain a maximum of 1–2 μg/ml of laminin as determined by a specific RIA for human placenta laminin, it indicates that the epitope may also be present on other secreted products.

The terminal immunodominant structure of human B blood group substance (39), Galα1-3(Fucα1-2)Gal, shows some similarity to the epitope characterized here but has a low affinity for the antibodies, as shown in erythrocyte adsorption tests (Table I) and inhibition assays with salivary blood group substances (Fig. 5). Defucosylation of the B substance by TCA hydrolysis (29) generated a highly active inhibitor of laminin binding to normal and patient antibodies. Little or no activity was found in defucosylated A and O substance (Fig. 5). This indicates the potential for an efficient generation of terminal Galα1-3Gal structures in various components by a portion of the human population.

A comparison of Galα1-3Gal with Galα1-3(Fucα1-2)Gal inhibitors that correspond to the terminal structures of defucosylated and nonmodified human B blood group substance, respectively, demonstrated a 10–600-fold difference in

inhibitory activity depending on the human serum used (Table II). All the sera examined contained anti-A and -B antibodies, indicating that the individuals lack A and B blood group substances. Therefore, the variation in inhibitory potency of the B epitope seems not to be related to recent observations (19) that antibodies from B^+ persons exhibit a narrower recognition of the Galα1-3Gal epitope than those lacking the B phenotype.

Discussion

The data presented here demonstrate that laminin-binding antibodies identified previously in patients and monkeys infected with Trypanosoma (6, 9) and Leishmania parasites (9) recognize oligosaccharide epitopes present on mouse laminin. This was shown most definitely in inhibition studies with oligosaccharides, which indicated that terminal Galα1-3Gal residues are likely to be the structures involved in the reaction. The carbohydrate nature of epitopes was additionally shown by their destruction after treating laminin with α-galactosidase and periodate but not by alkali, pronase, or the reduction of disulfide bonds. The antibodies also crossreacted with rabbit erythrocytes involving a glycosphingolipid with a terminal Galα1-3Gal structure (36, 37) and with an identical sequence produced from human blood group B substance by defucosylation.

A comparison of various mono- and oligosaccharides as inhibitors (Table II) showed that terminal galactose in α(1-3) linkage to a second galactose contributes most significantly to the antigenic specificity of the epitope. The penultimate galactose is apparently also recognized by the antibodies, since the substitution of an additional fucose residue, such as that occurring in B blood group substance, substantially reduces its affinity. Whether the disaccharide Galα1-3Gal composes the entire epitope is unknown. However, there is a strikingly lower inhibition by the disaccharide compared with laminin, although similar differences between macromolecular blood group substances and small carbohydrate haptens have been noted before (39). Presumably, polymeric carbohydrate antigens can interact simultaneously with both antibody binding sites increasing their affinity and indicating that mouse laminin possesses several α-galactose epitopes.

The presence of some 50–60 residues of α-galactose on mouse laminin was indicated in binding studies with *Griffonia simplicifolia* I-B_4 lectin (33, 34, 40) and by degradation with α-galactosidase (33, 35). Several α-galactose containing *N*-linked oligosaccharides have been isolated from laminin and found to contain terminal Galα1-?Gal structures (35). The same structures are also likely to be present on other murine basement membrane proteins and on bovine laminin B chains (Fig. 2). However, terminal α-galactose residues are not restricted to basement membrane proteins and are found, for example, on erythrocytes (41) and thyroglobulin (40) in several species.

Natural antibodies with Galα1-3Gal specificity are present in all adult human sera and have been previously detected (17, 18) by affinity chromatography on Galα1-6Glc, by rabbit erythrocyte agglutination and rosette tests. As shown here, these antibodies react in RIAs with mouse laminin but with 10–100-fold lower titers than those in patient and monkey sera. These natural antibodies, however, closely correspond with those arising after parasitic infections in terms of their disaccharide specificity and reaction with rabbit glycosphingolipids (reference

18; Fig. 4). Other antibody reactions reported for normal human sera and mouse laminin (10, 42) are therefore very likely due to the same antibodies.

The identification of the carbohydrate nature of crossreacting laminin epitopes also leaves little doubt that the antibodies arising after parasitic infections are triggered by Galα1-3Gal or similar epitopes present on glycolipids or glycoproteins of Trypanosoma and Leishmania parasites, since tissue-reacting antibodies can be adsorbed with parasites (reviewed in reference 11) and such antibodies purified by laminin immunoadsorption react strongly with various forms of *T. cruzi* (6). It appears unlikely that the same stimuli are responsible for generating the natural antibodies, particularly in Caucasian populations that are normally not exposed to these parasites. As discussed elsewhere, for the generation of natural antibodies against blood group substances (17, 39, 43) they may be stimulated by antigens of the intestinal flora, diet, or by other infections. Anti–basement membrane antibodies that react with mouse laminin in individuals with poststreptococcal glomerulonephritis (10), preeclampsia (44), and mercury exposure (45) could be related to the carbohydrate-specific antibodies defined here and may have some bearing on the etiology of these conditions.

The ubiquitous occurence of the antibody response to Galα1-3Gal structures, augmented in certain diseases, indicates that this is a lack of immune tolerance to these epitopes in the human species (41). Indeed, most data show the lack of terminal α-galactose structures in human tissues as studied by specific lectin binding of tissues (46), glomerular basement membrane proteins (47), and thyroglobulin (40). Our antibody binding data agree with these observations by showing their absence on human fibronectin and a low reactivity and presumed content on human placenta laminin. This could be due to the absence or low activity of an α-galactosyl transferase as present in rabbit tissue, producing an α 1-3-linkage to another galactose residue. Two enzymes produce this structure by using either Fucα1-2Gal (resulting in B group structure) or a terminal galactose as substrate (48). The latter transferase, which would generate the Galα1-3Gal epitope, has not been detected in human tissues, although its possible existence was indicated in glial cells of a Tay-Sachs embryo (49).

Some other data, however, indicate that terminal α-galactose groups, and possibly Galα1-3Gal epitopes, may exist in some normal or pathological human tissues. This was demonstrated by lectin binding to some extracellular structures of peripheral nerves (50), which is of particular interest in view of mAb reactions with neurones and *T. cruzi* (14) and the occurence of nerve injury as a late complication in Chagas disease (7). Such epitopes apparently also appear on senescent, thalassemic (17, 18) and sickle cell erythrocytes (51), and various human tumors (52, 53). Our data extend these observations by demonstrating the epitope on various other tumor cell products and tissues.

Given the existence of antibodies specific for the Galα1-3Gal epitope during normal life (17) and modulation of their levels by infections, a variety of situations may arise that generate the epitope at certain stages of aging or as a consequence of pathological alterations. These may then be exposed to specific antibodies raised before by foreign antigenic stimuli. Such situations could include the removal of altered erythrocytes considered to be a physiological process (17, 18, 51). In some parasitic diseases it may include altered cells (macrophages, muscle

cells) which, due to the intracellular residence of parasites, may change their surface structure by inserting parasite-derived epitopes (1) or changing host components by new posttranslational modifications. The autoimmunity observed in Chagas disease may therefore not be to cross reacting structures (7, 11, 16) present per se, but to a modified structure arising in the infected individuals. Comparable expressions of new carbohydrate epitopes have been observed in cancer cells (54), including antibodies to α-galactose epitopes in patients with malignant ovarian germ cell tumors (55). Such modifications of the normal repertoire of glycosylation reactions may be common sequelae of a variety of degenerative conditions.

Summary

Sera from patients with American cutaneous leishmaniasis and Chagas disease and from monkeys infected with either *Trypanosoma cruzi* or *Trypanosoma rhodesiense* show, in RIAs, strong binding to mouse laminin. A distinct although weaker binding activity is also detected in normal human sera. The antibodies recognize a common carbohydrate epitope present on mouse laminin, which was assigned to a terminal galactosyl(α1-3)-galactose group. Distinct crossreactions were observed with some other basement membrane proteins, rabbit glycosphingolipids, defucosylated human B blood group substance and components produced by some human tumor cells. Only little activity was, however, found on laminin obtained from human placenta. The data indicate that the antibodies arising in infectious diseases are stimulated by similar carbohydrate epitopes present on the surface of parasites. Tissue-specific occurrence of such epitopes may exist and explain the involvement of distinct tissues in autoimmune disorders.

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