

Serological Activity against Galactosyl- α (1-3)Galactose in Sera from Patients with Several Kinetoplastida Infections

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Using rabbit erythrocyte-derived neutral glycosphingolipids enriched for a defined ceramide pentasaccharide as antigens, we have detected elevated anti-galactosyl- α (1-3)galactose (anti-G α G) antibody values in patients with American cutaneous leishmaniasis (ACL), chronic Chagas' disease, and *Trypanosoma rangeli* infections compared with normal subjects or with patients suffering from any of 15 other infectious diseases. The specificity of the G α G antibodies was determined by inhibition enzyme-linked immunosorbent assays, which revealed that several α -galactosyl- but not β -galactosyl-bearing sugars blocked absorption of G α G antibodies to the specific antigen used. G α G antibodies were mainly distributed between immunoglobulin classes G and M in three Kinetoplastida infections studied, with a lower increase in reactivity detected in immunoglobulin A. Absorption of highly reactive G α G antibodies with purified murine laminin and nidogen, two basement membrane proteins, almost abolished G α G reactivity, suggesting the identity of anti-G α G with laminin and nidogen antibodies previously reported as elevated in Kinetoplastida infections. In ACL, G α G antibodies were detected in 71% of patients having skin lesions with a clinical evolution time of 0.5 month. This percentage increased with the time of evolution of skin lesions, reaching 93% in lesions older than 3 months, and tended to decrease inversely to the induration diameter in the skin leishmanin test. It is proposed that similar epitopes may exist on kinetoplast protozoa and that the determination of G α G antibodies may be a highly sensitive assay for the detection of humoral responses to Kinetoplastida infections.

The purification and chemical characterization of antigens derived from human infectious kinetoplast protozoa should provide valuable tools for analyzing the complex immune response to these organisms. If any of these antigens proved to be specific, it could also be used in immunological tests for diagnostic or taxonomic purposes. Recently, the production of antibodies recognizing the terminal galactosyl- α (1-3)galactose (G α G) residues in patients infected with *Trypanosoma* or *Leishmania* parasites has been reported elsewhere (27). The epitope involved is found on murine laminin (4, 26), nidogen (6), and some other basement membrane proteins (proteoglycan) (27).

The glycoconjugate nature of this epitope was demonstrated by its susceptibility toward α -galactosidase and treatment with periodate but resistance against reduction of disulfide bonds and treatment with other glycosidases, pronase, and alkali. Therefore, it appears very likely that this unusual cross-reactivity is due to carbohydrate structures shared by some murine basement membrane proteins and the parasites, which in the latter case may be present as a surface glycoprotein or glycolipid (27). This particular oligosaccharide, G α G, has been reported to be present in *Leishmania adleri* glycoconjugates (23). Furthermore, carbohydrate moieties similar to those on human blood type B antigens occur in several human pathogenic *Leishmania* species (8, 15).

In the present study, we examined the presence of antibodies to G α G in sera from patients with several Kinetoplastida infections or other inflammatory diseases by using the G α G-containing glycosphingolipids from rabbit erythrocyte plasma membrane as antigens. We attempted to evaluate the clinical significance of G α G antibodies in the diagnosis and prognosis of Kinetoplastida infections.

MATERIALS AND METHODS

Patients. Serum samples were obtained from patients with chronic Chagas' disease, several clinical stages of localized American cutaneous leishmaniasis (ACL), and *Trypanosoma rangeli* infections. A description of these patients has been published previously (4, 5, 7). A total of 169 healthy individuals and 69 patients with inflammatory diseases other than Kinetoplastida infections (5 with histoplasmosis, 10 with lepromatous leprosy, 2 with malaria, 8 with lupus erythematosus, 11 with paracoccidioidomycosis, 2 with toxoplasmosis, 6 with sporotrichosis or aspergillosis, 3 with chromomycosis, 4 with cutaneous mycoses caused by *Candida albicans*, 2 with cutaneous mycoses caused by *Trichophyton rubrum*, 3 with micetoma caused by *Nocardia brasiliensis* or with cryptococcosis, and 2 with amebiasis or coccidioidomycosis), preferably from the same geographical area, were also studied. All of the patients in this second group showed a negative leishmanin reaction and were serologically negative for Chagas' disease.

Leishmanin tests. Leishmanin was produced and tested as previously described (4). The diameter of the induration resulting from the intracutaneous injection of 0.1 ml of leishmanin (6.5×10^6 heat-killed parasites per ml) was measured after 24 and 48 h, with an induration diameter of >10 mm being considered a positive reaction.

***T. rangeli* identification and cultivation.** *T. rangeli* was detected in human subjects, either by direct microscopic examination of blood smears as previously described (2) or by blood culture in NNN medium and subsequent characterization by its nonagglutination with lectin II of *Aaptos papillata* (5).

Preparation of rabbit erythrocyte-derived neutral glycosphingolipids enriched for ceramide pentasaccharide. Glycosphingolipids were extracted from erythrocyte membranes (12), and the neutral glycosphingolipid fraction was isolated by DEAE-Sephadex chromatography (29).

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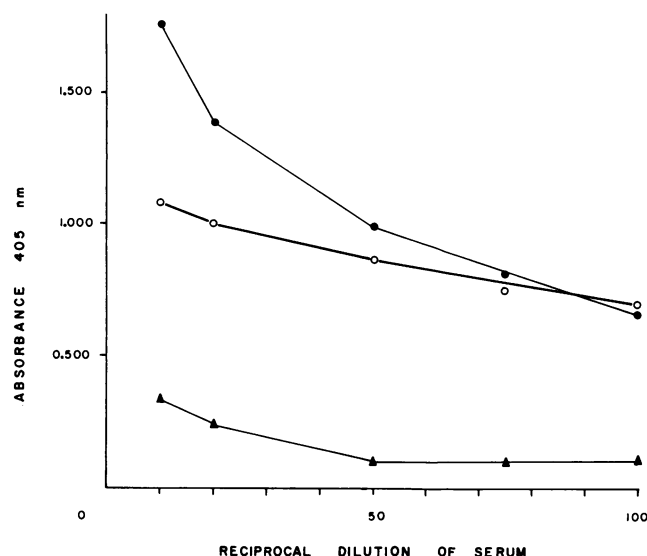


FIG. 1. Antibody activity against rabbit erythrocyte ceramide pentasaccharide in serial dilutions of the positive reference leishmanial (○), chagasic (●), and the negative reference normal human (▲) sera.

Enzyme-linked immunosorbent assay (ELISA). Anti-GαG antibodies were measured by using the neutral glycosphingolipids as antigens. Glycolipid (1 mg) was diluted with 1 ml of ethanol as stock solution. Polyvinyl chloride microtiter plate (Dynatech Laboratories, Inc.) wells were coated with 15 μl of a 100-fold dilution of stock solution in ethanol; this preparation contains 75 ng of carbohydrate. This glycolipid concentration was chosen for routine use, because it was sufficient for good discrimination between positive and negative sera. Wells were dried for 45 min under an electric fan. The plates were then blocked for 2 h with phosphate-buffered saline (PBS)–0.05% Tween 20–0.1% bovine serum albumin. Sera were diluted 1:50 in the aforementioned buffer, added, and incubated at 25°C for 2 h. The plates were washed three times, and 200 μl of horseradish peroxidase-labeled polyvalent goat conjugate antibody (Sigma Chemical Co.), diluted 1:3,500, was added to each well. After 15 h of incubation at 4°C, excess conjugate was removed, washed three times, and replaced with 200 μl of a 0.001% *o*-dianisidine–0.003% H₂O₂ solution in 0.1 M phosphate buffer (pH 6.0). After 1 h of incubation at 28°C, the reaction was fixed with 10 μl of 5 N HCl and the A₄₀₅ was read with a MicroELISA reader (Dynatech).

In each set of experiments, two positive reference serum samples and four negative reference human serum samples from apparently healthy donors were included as controls. Background levels of absorbance with PBS substituted for serum were subtracted from each serum sample. The class of antibodies reacting with GαG was determined in similar assays, with peroxidase-conjugated goat anti-human immunoglobulin G (IgG), IgM, and IgA obtained from the Institut Pasteur, Paris, France.

Absorption procedures. Some of the highly reactive GαG antibodies serum samples were retested by ELISA after being absorbed with either human type B or guinea pig erythrocytes (0 to 800 μl of packed cells in 2 ml of serum diluted 1:25 in PBS).

Enzymatic treatments of human erythrocytes. Washed erythrocytes (400 μl of packed cells) in citrate-buffered saline (pH 5.0) were incubated at 37°C for 10 h with 5 U of

α-L-fucosidase (Sigma). Cells were removed and washed three times in excess ice-cold PBS at 4°C before use. The efficacy of α-L-fucosidase was examined by its ability to hydrolyze methylumbelliferyl-α-L-fucoside as previously described (3). The same volume of cells was treated with 0.1% pronase (from *Streptomyces griseus* [Sigma]) in PBS for 3 h at 37°C and processed as described before for α-fucosidase. Under our experimental conditions, no more than 1 to 3% of cells were lysed.

α-Galactosyl specificity of GαG antibodies. For inhibition ELISAs, antiserum dilutions of 1:100 were incubated with carbohydrates (24 h, 4°C) before binding with specific glycolipids. Wells were then washed twice to remove free antibodies and tested for a decrease in bound GαG by ELISA as described before.

Statistical analysis. Each serum sample was assayed in triplicate, and the arithmetic mean of the A₄₀₅ readings was used for analysis. Statistical analysis of data was carried out by analysis of variance or Student's *t* test.

RESULTS

Standardization of ELISA for GαG antibodies. Optimal dilutions of sera and conjugate were chosen as those with the lowest number of false-positive and false-negative results. A serum dilution of 1:50 was found to be necessary, as antibodies from Kinetoplastida-infected patients had a relatively low affinity for the rabbit erythrocyte glycosphingolipid extract when compared with laminin and nidogen, when serum dilutions of >1,600 could be used (4, 6). Figure 1 shows the titration of the positive reference chagasic and leishmanial sera and the negative reference normal human sera. Positive reference sera gave a much stronger reaction than normal sera, and absorbance decreases with serial dilutions of the sera. An A₄₀₅ of 0.613 represents the upper limit of normality, defined as the mean plus 2.5 standard deviations of the control group.

Relatively important variations in the reaction with GαG were found in apparently healthy subjects, with significantly lower antibody values in subjects with blood type B compared with subjects with blood type A or O (Table 1).

Interestingly, in all apparently healthy subjects studied by ELISA, we detected low levels of GαG antibodies, a fact already noted for anti-α-galactosyl antibodies measured by a rosetting technique with rabbit erythrocytes and K562 cells (14). Thus, 13 and 20% of blood bank donors with blood type A and O had abnormal GαG levels, despite the fact that they displayed negative leishmanin skin test and Chagas' disease serology and had no blood-circulating trypanosoma (as established by direct examination of fresh blood films or blood culture in NNN medium). However, only 2% of subjects with blood type B showed significantly elevated GαG antibodies. The positivity for abnormal GαG antibody levels in the whole apparently healthy population studied was about 8

TABLE 1. Anti-GαG antibody levels in healthy controls as a function of blood group

Blood group (no. of subjects)	A ₄₀₅ ^a	Positivity (%)	P value ^b
A (30)	276 ± 223	13	<0.01
B (45)	131 ± 152	2	
O (29)	351 ± 274	20	<0.01

^a Mean ± standard deviation (10⁻³).

^b Determined in comparison with subjects with blood type B.

TABLE 2. Anti-GαG antibody levels in healthy controls and in study group of patients

Study group (no. of patients)	A ₄₀₅ ^a	Positivity (%)	P value ^b
Controls (169)	235 ± 151	8	
Cutaneous ACL (91)	1,594 ± 1,208	67	<0.01
Mucocutaneous leishmaniasis (6)	1,975 ± 657	87	<0.01
Recently closed ACL ulcer ^c (54)	1,493 ± 993	80	<0.01
Chronic Chagas' disease (26)	972 ± 812	50	<0.05
<i>T. rangeli</i> infection (31)	946 ± 582	74	<0.05
Inflammatory diseases (69)	293 ± 126	4	>0.1

^a Mean ± standard deviation (10⁻³).^b Determined in comparison with GαG antibody values found in control subjects.^c These patients were studied after ACL ulcer had been clinically cured for 2 to 3 months.

± 0.5%. The significance of this finding is, at present, unclear (Table 2).

Detection of GαG antibodies in Kinetoplastida-infected human subjects. Table 2 shows that elevated antibody activity to GαG has so far been found consistently only in patients with Kinetoplastida infections when compared with apparently healthy subjects or individuals with any of 15 other inflammatory diseases. Table 2 also shows that significantly elevated GαG antibody levels were found in 50% of chronic chagasic patients, 67% of patients with ACL, and 74% of *T. rangeli*-infected patients and that antibody values were higher in cutaneous or mucocutaneous leishmaniasis when compared with more chronic Kinetoplastida infections, such as chronic Chagas' disease or *T. rangeli* infection.

There was a wide variation of antibody levels in individual serum samples from patients with similar kinetoplast infection, as shown by the relatively high standard deviations found in these patients.

To investigate whether results in ACL patients were associated with the time of evolution of the disease, reactivity with leishmanin, or age of patients, the cutaneous leishmaniasis group was subdivided into several groups (Fig. 2 to 4). Except when leishmanial patients older than 61 years were compared with younger patients and when subjects having skin lesions that had evolved over 3.1 to 4.5 months were compared with subjects having lesions with a shorter evolution time, there was no significant difference between the antibody levels in all other groups studied ($P > 0.05$). Regarding positivity, the percentage of ACL patients having abnormal GαG antibody levels tended to decrease in patients older than 61 years ($P < 0.1$). We found that 71% of patients with skin lesions with 0.5 month of clinical evolution already had elevated GαG antibody levels; this percentage increased to about 93% after 3 months of clinical evolution. On the other hand, the positivity for elevated GαG antibody levels

was 100% in ACL patients having lesions with a diameter of 15 to 18 mm in the leishmanin skin test response, this value decreased to about 65% in ACL patients with higher leishmanin reactivity ($P < 0.1$).

The GαG antibody levels and percentage of positivity in recently closed ACL ulcer patients did not differ significantly from patients with active ACL skin ulcers (Table 2).

Table 3 shows the immunoglobulin class distribution found in the healthy human population. GαG antibodies distributed evenly between IgA, IgG, and IgM. However, the significantly elevated GαG antibody levels found in all the three Kinetoplastida infections studied were mainly IgG, with lower reactivity detected in IgM and very little reactivity found in IgA.

Specificity of anti-GαG antibodies. As correlation studies showed that GαG antibodies values fit well with laminin ($r = 0.70$, $P < 0.05$) and nidogen ($r = 0.75$, $P < 0.05$) antibody values measured in same leishmanial, chagasic, and normal sera, further evidence for this relationship was searched by immunoadsorption studies with human chagasic or leishmanial sera. Under these experimental conditions, almost complete inhibition by both basement membrane proteins was found (Fig. 5). Similar results were found when Kinetoplastida-infected human sera were absorbed on guinea pig erythrocytes (results not shown). Notably, absorption with washed human blood type B erythrocytes did not decrease anti-GαG, antilaminin, and antinidogen reactivities of the abovementioned sera. Table 4 shows that the α-galactosyl-containing carbohydrates, GαG, melibiose, α-methylgalactoside, and stachyose significantly inhibited the GαG reactivity at low concentrations (0.4 to 40 mM). In contrast, β-galactosyl-containing disaccharides affect the binding only at 100 to 200 mM.

Unmasking the binding site on human type B erythrocytes. We were unable to demonstrate GαG antibodies from patient

TABLE 3. Distribution of GαG antibodies among serum immunoglobulin classes in Kinetoplastida-infected patients and in control subjects

Study group (no. of patients)	Absorbance ^a			
	Total immunoglobulins	IgA	IgG	IgM
Controls (6)	120 ± 77	58 ± 30	82 ± 39	73 ± 20
ACL ulcer (11)	1,981 ± 826 ^b	251 ± 102 ^d	2,984 ± 1,239 ^b	1,176 ± 820 ^c
Chagas' disease (8)	1,611 ± 959 ^c	447 ± 171 ^c	2,499 ± 1,798 ^c	935 ± 611 ^c
<i>T. rangeli</i> infection (6)	962 ± 381 ^d	367 ± 108 ^c	1,459 ± 627 ^d	259 ± 97 ^d

^a Mean ± standard deviation (10⁻³). Peroxidase-conjugated goat anti-human immunoglobulin sera were used at the following concentrations: total immunoglobulin, 1:3,500; IgA, 1:500; IgG, 1:1,500; IgM, 1:2,000.^b $P < 0.001$ compared with control group.^c $P < 0.005$ compared with control group.^d $P < 0.010$ compared with control group.

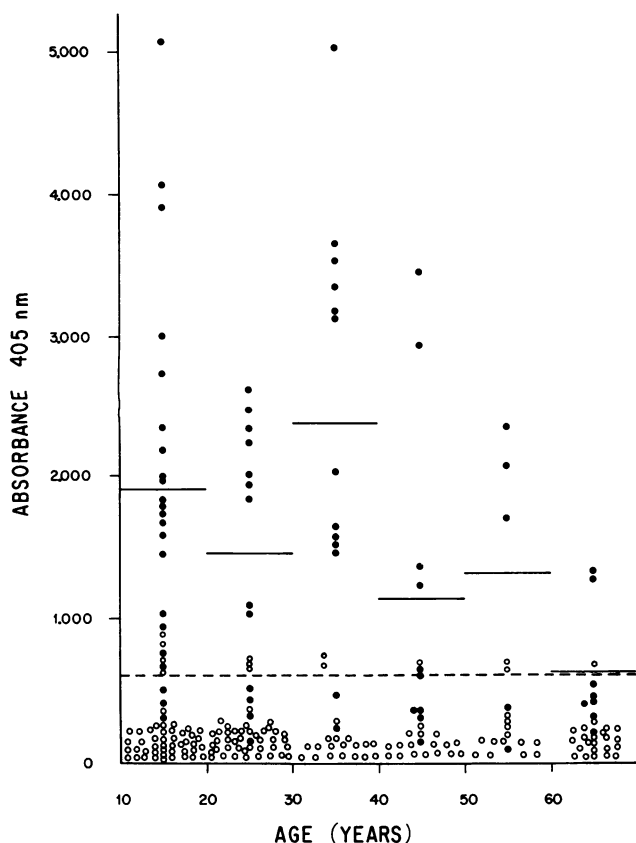


FIG. 2. Anti-G α G antibodies in localized cutaneous leishmaniasis patients (●) and in control subjects (○) as a function of age. ----, Upper limit of normal as defined in the text. Each point represents the value obtained from an individual by an ELISA using polyvalent goat anti-human immunoglobulins. The horizontal bars represent the arithmetic mean values.

serum samples to human type B erythrocytes, which are known to contain terminal α -linked galactose moieties close to that of rabbit ceramide pentasaccharide (25).

Because blood group B substance galactosyl α 1-3(fucosyl α 1-2)galactosyl β 1-4 *N*-acetyl glucosamine contains an α 1-2fucosyl unit linked to the penultimate galactose, experiments were designed to test whether enzymatic removal of fucose would allow G α G antibodies to bind human type B erythrocytes. When these cells were pretreated with increasing concentrations of α -fucosidase, they bound G α G antibodies in a dose-dependent manner, as in absorption experiments (results not shown). Same results were obtained after pronase treatment of human type B erythrocytes.

DISCUSSION

Serological activity has been demonstrated against the G α G terminal residue present in ceramide pentasaccharide isolated from rabbit erythrocytes in sera from humans infected with *Trypanosoma cruzi*, *T. rangeli*, and American *Leishmania* spp.

We found that immunoadsorption of positive sera with purified murine laminin or nidogen almost abolished completely G α G antibody reactivity. This confirmed our previous results that these antibodies cross-react with murine laminin, nidogen, and some other basement membrane proteins (27) (the disaccharide G α G being an essential structure

of the major epitope). Similarly, treatment of purified murine laminin or nidogen with α -galactosidase almost completely abolished antigenicity towards chagasic or leishmanial sera (27). Previous results suggested that some analogy might exist between G α G antibodies found in Kinetoplastida-infected human patients and anti- α -galactosyl antibodies reported in normal subjects (14). However, in contrast with this latter antigenic system, which is mainly restricted to IgG antibodies in healthy subjects, we found that in Kinetoplastida-infected subjects, G α G antibodies were mainly distributed between IgG and IgM, with little reactivity in IgA antibodies. The immunoglobulin class distribution pattern of G α G antibodies in patients infected with three different kinetoplast protozoa is similar to that reported for laminin and nidogen antibodies (4, 6). It should be emphasized however, that in general, antibody reactivity against ceramide pentasaccharide was lower than against laminin or nidogen, as G α G antibodies were optimally detected with a serum dilution of 1:50 compared with 1:1,600 for laminin and nidogen (4, 6). This difference of affinity for the epitope could be explained by differences in the local density or in the steric configuration of the oligosaccharide, as recently demonstrated by the reaction of glycosphingolipids with several lectins (21).

The advantage of using the specific epitope of murine laminin reacting with Kinetoplastida-infected human serum present in G α G-ceramide and not the whole protein molecule lies in the fact that antibodies against this glycoprotein have also been described in acute poststreptococcal glomer-

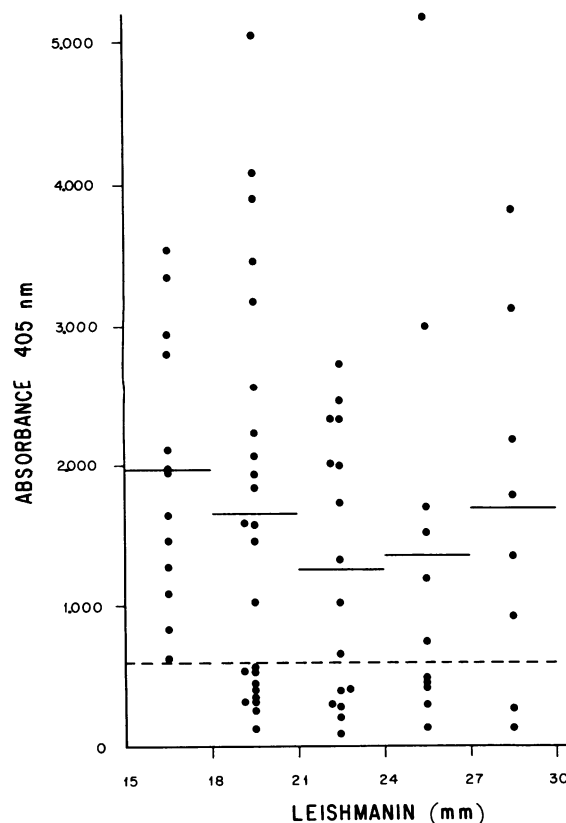


FIG. 3. Anti-G α G antibodies in localized cutaneous leishmaniasis patients as a function of leishmanin reactivity. For explanation of figure, see the legend to Fig. 2.

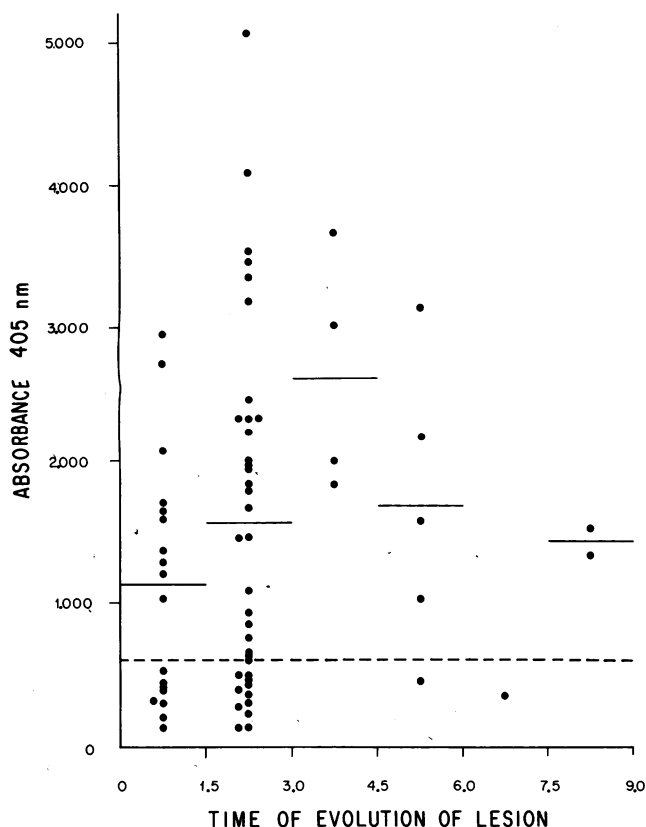


FIG. 4. Anti-G α G antibodies in localized cutaneous leishmaniasis patients as a function of time of evolution of lesion(s). For explanation of figure, see the legend to Fig. 2.

ulonephritis (17) and preeclampsia (11). These latter antibodies could be reactive with other epitopes present on laminin; if so, elevated G α G antibodies could be specific for Kinetoplastida infections.

The α 1-3galactose moiety in human tissues has been identified by structural analysis on blood group B substance (28) and teratocarcinoma cells (19). The blood group B

substance is fucosylated at the penultimate galactose residue and is not recognized by G α G antibodies characteristic for Kinetoplastida-infected patients. However, natural anti-B antibodies from normal blood group A and O donors have been reported to cross-react with G α G (13). This observation may be related to the higher positivity of normal A and O donors against the G α G-containing glycosphingolipid (Table 1) in our experiments. The α -galactose in the teratocarcinoma cells is linked to *N*-acetylgalactosamine (19) and may occur on further glycoconjugates, notably in nervous tissue (10). It remains to be determined whether G α G-antibodies, particularly from Kinetoplastida-infected patients, recognize this apparently nervous tissue-specific moiety. Further indirect evidence for G α G is derived from lectin-binding studies on human tissues. Thus, G α G is expressed on human peripheral nerve (16), parietal cells of gastric mucosa (18), fibroblasts and several cancer cells (18, 19), and laminin (27) (although this latter protein has lower terminal G α G residues than the murine molecule). Thus, regarding the pathogenetic significance of these antibodies, the possibility of some immunological reactions between cellular surface G α G-bearing residues and elevated levels of circulating G α G antibodies seems possible.

A further anti-carbohydrate antibody directed against galactosyl- β 1-3-*N*-acetylgalactosamine (Thomsen-Friedenreich antigen) has recently been described in Chagas' disease (24). The relationship of these antibodies with the pathogenesis of this disease has not been determined. It is remarkable that in ACL, the positivity for G α G antibodies increased with the time of evolution of lesions, reaching 93% in lesions older than 3 months, tending to decrease inversely to the induration diameter of skin leishmanin test. The immunological factors controlling this reactivity remain to be established. They could be related to the parasitic load of some infected subjects, as found for the phenolic glycolipid I from *Mycobacterium leprae*-infected patients, when lepromatous subjects with heavy bacterial load had significantly elevated anti-phenolic glycolipid I levels (20). It is interesting that patients having recently closed ACL ulcers maintained high anti-G α G levels and a high percentage of positivity, even after 2 to 3 months of clinical healing. Possibly, immunogenic residues remaining at the lesion site maintained these elevated circulating anti-G α G levels, so that persistent ab-

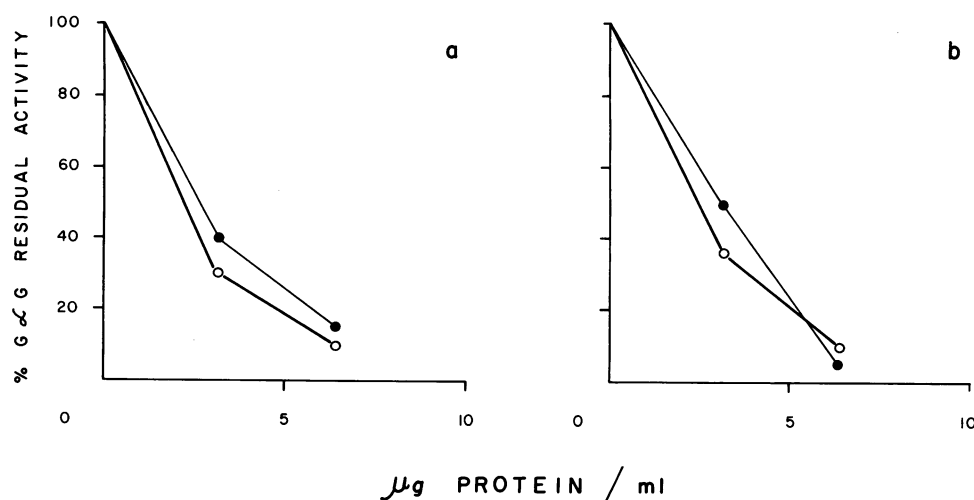


FIG. 5. Absorption of G α G antibodies present in chagasic (a) and leishmanial (b) serum by preincubation for 1 h at 37°C with several concentrations of murine laminin (○) and nidogen (●).

TABLE 4. Inhibition of the interaction between GαG antibodies from ACL patients and rabbit erythrocyte neutral glycosphingolipids enriched for ceramide pentasaccharide by various sugars

Sugar	Sugar concn (mM) inhibiting 50% antibody binding
None (control)	—
D-Galactose	20
Galactosyl-α(1-3)galactoside	0.4
Galactosyl-α(1-6)galactoside	40
Stachyose ^a	40
α-Methylgalactoside	20
β-Methylgalactoside	100
Galactosyl-β(1-6)glucoside	200
Galactosyl-β(1-6)galactoside	200

^a α-Galactosyl-α-galactosyl-α-glucosyl-β-fructose.

normal anti-GαG levels should alert the clinician to a silent leishmanial residual infection, as mentioned in several papers, by the demonstration of living organisms in humans years after complete clinical cure (1, 22, 30), as well as in apparently cured *Leishmania*-infected treated animals (9).

Since GαG antibodies were detectable in 71% of ACL patients after 0.5 month of clinical evolution as well as in acutely infected chagasic patients (26), it will be of special interest to apply such tests to detect the very early stages of Kinetoplastida infection. Our results suggest that the ELISA using GαG-containing glycolipid is a highly sensitive assay for detection of humoral responses to Kinetoplastida infection.

Early detection and treatment of these infections have the potential benefits of reducing the incidence of skin, mucous, or cardiac lesions due to cutaneous and mucocutaneous leishmaniasis and Chagas' disease, respectively, and also of reducing the transmission of these diseases in the community.

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