INCREASE IN ASIALOGLANGLIOSIDE- AND MONOSIALOGLANGLIOSIDE-REACTIVE ANTIBODIES IN CHRONIC CHAGAS’ DISEASE PATIENTS

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Abstract. Antibodies reactive with the core glycan of asialoganglioside (GA1), monosialoganglioside (GM1), and disialoganglioside (GD1a) were studied in human sera. In healthy individuals, GA1-, GM1-, and GD1a-reactive antibodies were mainly of the IgM class, but also of the IgA and IgG classes, and were present at low titers in the serum of 68%, 79%, and 91% of the individuals studied, respectively. Levels of anti-GA1 and anti-GM1 antibodies, mainly of the IgA and IgG classes, were significantly elevated (P < 0.001) in 62% and 72% of subjects, respectively, chronically infected with Trypanosoma cruzi, with no association found with the degree of myocardial damage. No significant increase in anti-GA1 and anti-GM1 antibodies was found in dilated cardiomyopathy patients. The level of anti-GD1a antibody was not significantly different between healthy controls and chronic chagasic or dilatary cardiomyopathy patients. Since the peripheral nervous system is very rich in gangliosides, it is possible that the increases in GA1- and GM1-specific antibodies that develop during chronic T. cruzi infection are involved in the pathology of peripheral neuropathy in Chagas’ disease.

Trypanosoma cruzi, the causative agent of Chagas’ disease is a parasitic protozoan that infects between 16 and 20 million people in South and Central America, with 90 million people at risk.¹

Chagas’ disease is characterized by chronic autoimmune pathology,² ³ including extensive neuronal degeneration. Thus, in the peripheral nervous system, there is massive degeneration of sympathetic,⁴ parasympathetic,⁵ and enteric neurons.⁶ ⁷ Lesions of Purkinje neurons in the cerebellar cortex have also been reported.⁸ With regard to the pathogenesis of chronic Chagas’ heart disease, it has been described as a cardiac neuromyopathy in which the sympathetic and parasympathetic systems are affected.² ⁵ ⁷ ⁹

Gangliosides are glycolipids containing one or more sialic acid residues, usually of the N-acetyl-neuraminic acid type. Owing to oligosaccharide heterogeneity, approximately 90 different gangliosides have been identified in bacteria, fungi, plants, and animals.¹⁰ Gangliosides usually occur in the outer leaflet of all eukaryotic cell membranes, and in neural tissues in the central and peripheral nervous system,¹¹ those where they are highly concentrated in the presynaptic region at the neuromuscular junction and in glial and axonal structures at the nodes of Ranvier.¹³

The antiganglioside antibodies are directed at the carbohydrate portion of the gangliosides. Recently, it was demonstrated that a monoclonal antibody raised against T. dionisi and T. vespertilions displays reactivity with cells from the cerebellum of embryonic, early postnatal, and adult mice¹⁴ and strongly cross-react with T. cruzi. The Dion 10.1b anti-T. dionisi monoclonal antibody specifically reacts with gangliosides, T. cruzi, and mouse cerebellar neurons.¹⁵ The T. cruzi and gangliosides cross-reactivity is interesting because the association of peripheral neuropathy with monoclonal gammopathy has been clearly demonstrated.¹⁶ Since peripheral nervous system involvement has been described in Chagas’ disease,¹⁷ we studied anti-ganglioside antibody levels in T. cruzi-infected patients and compared them with individuals having other chronic infections transmitted only in tropical environments or erythematous lupus and with healthy controls.

Study population and clinical samples. Cardiomyopathy patients. Serum samples were obtained from 142 individuals having some pathologic cardiac involvement. They were grouped according to their T. cruzi serology (quantitative complement fixation and indirect hemagglutination tests) into chagasic and nonchagasic patients. After obtaining their signed informed consent, the initial work-up of all patients consisted of noninvasive studies (clinical history, routine laboratory tests, chest radiographs, resting 12-lead electrocardiogram [EKG], stress test, M-mode echocardiogram, vectocardiogram, and 24-hr Holter monitoring) and invasive explorations (right and left catheterization plus cineventriculograms, coronary arteriograms, electrophysiologic evaluation of sino-atrial node function and AV conduction, and septal endomyocardial biopsies).¹⁸ ¹⁹ At completion of the study, the clinical and electrocardiographic characteristics and the results of the left cineventriculogram of all patients studied were used to include them in three groups with the following characteristics: group I was subdivided into groups Ia and Ib (group Ia = normal EKG and normal cineventriculogram; group Ib = normal EKG and abnormal left cineventriculogram); group II = abnormal EKG, but not signs of congestive failure; group III = abnormal EKG and congestive failure. (For additional clinical details, see the reports by Carrasco and others¹⁸ ¹⁹ and Avila and others²⁰ ²¹.) The protocol for human endomyocardial biopsies was reviewed and approved by the Ethical Committee at the Hospital Universitario (Merida, Merida State, Venezuela).

Control group. Control patients included 100 healthy individuals preferably of the same socioeconomic levels of cardiomyopathy patients. All of these subjects showed a negative leishmanin reaction and were serologically negative for Chagas’ disease.²¹ They were subjected between 1983 and 1990 to a work-up for evaluation of atypical chest pain; results of all noninvasive studies were normal. Patients with localized cutaneous and diffuse leishmaniasis, T. rangeli infection, visceral leishmaniasis, and 17 other infectious and inflammatory diseases previously described²⁰ ²¹ and briefly mentioned in Table 1 were also studied.
Glycolipids. Ganglioside nomenclature was according to Svennerholm.25

**Enzyme-linked immunosorbent assay.** The ELISA protocol was selected according to the recommendations of the workshop Measurement and Significance of Antibodies Against GM1 ganglioside.25 Five hundred nanograms of bovine brain asialo-ganglioside (GA1), monosialoganglioside (GM1), or disialoganglioside (GD1a) in 50 μl of ethanol were pipetted into polystyrene microtiter plate wells. Under standard experimental conditions, the anti-ganglioside test was performed as previously described.24

Anti-Gal(1-3)GalNac antibodies were measured using the Gal(1-3)GalNac-carboxyethylthioethyl (CETE)-bovine serum albumin (BSA) conjugate. Antigen was suspended in 0.01 M carbonate buffer, pH 9.6, at a concentration of 0.5 μg/ml. Other experimental procedures were as previously described.25

In each set of experiments, two positive reference serum samples and five negative reference human serum samples from healthy donors were included as controls. Background levels of absorbance with phosphate-buffered saline (PBS) instead of serum were subtracted from each serum sample. Optimal dilutions of sera and conjugate chosen were those with the lowest number of false-positive and false-negative results. The class of antibodies reactive with GA1, GM1, and GD1a was determined in similar assays with peroxidase-conjugated goat anti-human IgG, IgM, and IgA obtained from the Sigma Chemical Co. (St. Louis, MO).

**Absorption procedures.** From the 102 chagasic sera tested, we chose the five more reactive ganglioside antibody-reactive sera. They were retested by ELISA after being absorbed with human red blood cells, *T. cruzi* epimastigotes or trypomastigotes, *T. rangeli* culture forms, or *Leishmania mexicana* or *L. braziliensis* promastigotes grown as previously described.26 Cells were washed twice in PBS and mixed at 10^9 parasites/ml of serum diluted 1:800 in PBS.

Absorption was also carried out with ganglioside-containing liposomes (prepared from a lipid mixture containing dimyristoylphosphatidylcholine [0.5 μmol], cholesterol [0.5 μmol], dipalmitoylphosphatidic acid [0.5 μmol], and either GA1, GM1, or GD1a [0.05 μmol]). After removal of organic solvents by evaporation, the lipid suspension was ultrasonicated in PBS and centrifuged at 37,000 × g for 30 min to remove any lipid aggregates. Liposomes were then resuspended in 800 μl of human sera diluted 1:800 in PBS and incubated at 37°C for 4 hr.

**Extraction of *T. cruzi* and human red blood cell lipids.** To a freshly collected 2-ml cell pellet (2 × 10^11 cells) of *T. cruzi* trypomastigotes (grown in Vero cell tissue culture26), epimastigotes or human red blood cells, a mixture of 10 ml of chloroform:methanol (1:1 [v/v]) was added. Gangliosides were extracted as described by Ladisch and Gillard27 and chromatographed by high-performance, thin-layer chromatography (HPTLC) using chloroform:methanol:0.2% CaCl₂ (50:40:10) as the solvent. Staining was carried out with resorcinal. To obtain information on the structure of these lipids, samples were analyzed for sugars and long chain bases by gas chromatography as described by Taki and others.28

**Reagents.** Asialo-ganglioside-GM1, monosialoganglioside GM1, and disialoganglioside GD1a from bovine brain, Gal(1-3)GalNac-CETE-BSA, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diaminonium salt, octyl-Sepharose, galactose, galactosamine, stachyose, melibiose, mannose, methyl-α-D- and β-D-galactopyranoside, galactosyl(1-3)galactose, glucose, glucosamine, lactose, N-acetylgalactosamine, BSA, pronase, *Vibrio cholerae* neuraminidase (type II), and precoated silica gel plates were obtained from the Sigma Chemical Co. Fetal calf serum and minimal essential medium were obtained from Gibco-BRL (Gaithersburg, MD). All other chemicals were of reagent grade.

**Statistical analysis.** The statistical package Statgraph, version 6.0 (Statistical Graphics Corp., Rockville, MD) was used for all analyses. All tests were two-tailed. A P value < 0.05 was considered to denote statistical significance.

**RESULTS**

**Standardization of ELISA for ganglioside-reactive antibodies.** Using either bovine GA1, GM1, or GD1a as antigens, positive reference sera gave a much stronger reaction than normal sera, and optical density (OD) decreased with serial dilutions of the sera.
We chose a 1:1,600 dilution for anti-GA1 antibody or a 1:800 dilution for anti-GM1 or -GD1a antibodies because they were optimal for a clearer separation between positive chagasic and negative healthy control sera. Using these experimental conditions, 68%, 79%, and 91% of healthy human sera showed immunoreactivity for bovine GA1, GM1, or GD1a, respectively. Higher antigen concentrations (500–5,000 ng/well) did not increase this negative percentage, which suggests that in healthy subjects, antibodies have poor affinity for three antigens tested. For anti-GA1, -GM1, and GD1a antibodies, the OD values at 405 nm (OD\textsubscript{405}) of 0.750, 0.590, and 0.290, respectively, represent the upper limit of normality, defined as the mean plus 2 SD of the control group. The coefficients of intra-assay and inter-assay variation estimated using a serum sample containing anti-GA1, -GM1, and -GD1a antibodies were 4%, 10%, and 6%, respectively, after more than 50 separate measurements. Similar antibody levels were found in individuals with A, B, AB, and O blood types.

In chronic chagasic patients a significant correlation was found between anti-GA1 and -GM1 antibodies (R\textsuperscript{2} = 49%; r = 0.70, P < 0.0001), suggesting that we could be measuring the same antibody using two different but closely related antigens.

**Detection of GA1- and GM1-reactive antibodies in Trypanosoma infected human subjects.** Table 1 shows that while their levels were significantly elevated (P < 0.001) GA1- and GM1-reactive antibodies were found in 62% and 72%, respectively, of individuals chronically infected with *T. cruzi*, other acute or chronic infections produced by Trypanosomatidae family members did not induce significantly elevated ganglioside antibody levels. The GA1- and GM1-reactive antibodies increased in only 6% and 8%, respectively, of dilated cardiomyopathy patients (Table 1), but in 53% and 64%, respectively, of patients with erythematous lupus as reported by Endo and others.\textsuperscript{20}

To investigate whether the results in chronic Chagas’ disease patients were associated with the degree of myocardial damage, patients were subdivided into several groups as described. Among chagasic groups there was no significant difference in GA1-, GM1-, or GD1a-reactive antibody levels or in the percentage of patients having elevated immunoreactivity, although anti-GA1 and -GM1 antibody levels were significantly higher when any of the chagasic groups were compared with healthy human subjects (P < 0.001).

Interestingly, no significant difference in GD1a antibody levels was found between healthy human subjects (0.170 ± 0.060 [mean ± SD]) and chronic chagasic patients (0.187 ± 0.073).

**Reactivity of T. cruzi and human and bovine GA1, GM1, and GD1a.** When chronic chagasic or healthy control serum were checked for immunoreactivity using either *T. cruzi*, bovine brain, or human red blood cell GA1, GM1, or GD1a as antigens, it was found that *T. cruzi* GA1 and GM1 were as immunoreactive as respective human or bovine antigens. On the other hand, *T. cruzi* GD1a is more immunoreactive than bovine brain or human red blood cell GD1a.

**Detection of Gal(β1-3)GalNac-linked BSA-reactive antibodies in Trypanosoma-infected and healthy human subjects.** When the GA1 and GM1 terminal disaccharide sequence Gal(β1-3)GalNac was linked to BSA and used as antigen, all healthy human sera studied had Gal(β1-3)GalNac-BSA antibodies at OD\textsubscript{405} as assessed by microELISAs, with the mean ± SD value of the entire healthy group being 0.872 ± 0.364 (range = 0.162–1.561).

No significant difference in Gal(β1-3)GalNac-BSA antibody levels was found between healthy individuals, chronic chagasic patients (with only 25% having abnormal immunoreactivity), and in other acute or chronic infections produced by other Trypanosomatidae family members.

**Evidence that anti-GM1 and -GA1 are different from Gal(a1-3)Gal antibodies.** To investigate the possible association with Gal(a1-3)Gal antibodies, red blood cell and murine laminin and nidogen binding experiments were performed. Table 2 shows that as expected, human red blood cells did not bind GA1- or GM1-reactive antibodies. Proteolytic treatment of human red blood cells with 0.1% pronase for 60 min at 37°C did not result in binding of both antibodies. Also, absorption with 0–6 μg/ml of murine laminin or nidogen (Table 2) did not decrease serum GA1- and GM1-reactivity. Previous results are evidence that Gal(a1-3)gal antibody levels, which we have found elevated in chronic Chagas’ disease,\textsuperscript{24} were not being measured.

**Specificity of human antibody against GA1 and GM1.** Chronic chagasic sera anti-GA1 reactivity absorbed strongly on GA1-bearing liposomes but very weakly on GM1. The contrary was true for GM1-reactive antibodies (Table 2).

**Immunoglobulin class distribution of GA1- and GM1-reactive antibodies.** Table 3 shows that in healthy human populations, anti-GA1 reactivity was mainly of the IgM class, but also of the IgA and IgG classes, while in *T. cruzi*-infected patients, anti-GA1 reactivity increased significantly only in the IgA and IgG classes. Similar results were found for GM1-reactive antibodies. The immunoglobulin nature of the antibodies obtained after the absorption with GA1- and GM1-containing liposomes was further confirmed by the single precipitin line obtained in Ouchterlony immunodiffusion assay against specific rabbit anti-human immunoglobulin serum.

**Absorption of GA1 and GM1-reactive antibodies to Kinetoplastidae family culture forms.** When purified GA1-

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*Chagasic GA1- and GM1-reactive antibodies were purified on a asialolectose-GM1 affinity chromatography column as previously described.\textsuperscript{18} RBC = red blood cells.
and GM1-reactive antibodies were incubated at 37°C with living *T. cruzi* trypomastigotes and epimastigotes as well as other Kinetoplastidae family parasites (*T. rangeli, L. mexicana, L. braziliensis,* and *L. donovani*) a strong absorption of these antibodies was found (Table 2).

**Biochemical identification of gangliosides present in *T. cruzi* trypomastigotes.** The HPTLC using chloroform:methanol:0.2% CaCl₂ (50:40:10) as the solvent and staining with resorcinol revealed the presence in *T. cruzi* trypomastigote lipid extracts of GD1a (RF = 0.25), GM1 (RF = 0.36), GA1 (RF = 0.47), and GM3 (RF = 0.49) among other gangliosides. Acid hydrolysis of these lipids revealed a spot with the same mobility as dihydrophosphoglucomine and molar ratios of sugar components of Gal:Glc:GalNac:NeuAc of 2:1:1; 2:1:1:1; 2:1:1:1; and 1:1:0:1 for GA1, GD1a, GM1, and GM3, respectively.

**DISCUSSION**

This study describes a microELISA technique using purified bovine brain asialoganglioside-GM1, monosialoganglioside-GM1, and disialoganglioside-GD1a as antigens for determination of anti-GA1, -GM1 and -GD1a antibody levels in human serum. These antibodies are different from each other based on specific absorption with ganglioside-bearing liposomes.

This ELISA method indicated that 68%, 79%, and 91% of healthy individuals had some anti-GA1, -GM1, and -GD1a reactivity, respectively, similar to results recently reported. When the terminal Gal[β1-3]GalNac sequence linked to BSA was used as antigen, antibody reactivity was comparable between healthy humans and chronic chagasic patients, suggesting the importance of the Gal[β1-3]GalNac[β1-4]Gal[β1-4]Glc epitope for antigen-chagasic antibody binding determined using GA1.

It is important to point out that increased anti-GM1 and anti-GA1 antibodies found in Chagas’ disease are not a consequence of the chronic infection by itself because other long-lasting diseases such as lepromatous leprosy, diffuse cutaneous leishmaniasis, or kala-azar did not induce an increase in levels of anti-GM1 and -GA1 antibodies. They are also not a consequence of nonspecific polyvalent B cell activation leading to the production of unspecific anti-ganglioside antibodies because anti-glucosylceramide, anti-Gal[β1-3]GalNac, and anti-GD1a reactivities were similar between healthy controls and chronic chagasic patients.

The fact that *T. cruzi* GA1 and GM1 were more immunoreactive with chagasic serum than with control serum suggests that parasitic GA1 and GM1 could be highly immunogenic for some *T. cruzi*-infected subjects. There are several explanations for a specific GA1 and GM1 immunogenicity. 1) The GA1 and GM1 at the cell surface of *T. cruzi* could be highly exposed when compared with host cells, which might convert nonimmunogenic GA1 and GM1 to an immunogenic status. 2) Part of the GA1 and GM1 in *T. cruzi* may exist in the lactone form, which has a very different conformation than GA1 and GM1 or both gangliosides when associated with membrane proteins, may expose a lactone-like conformation. These two possibilities have already been demonstrated in B16 melanoma cells using GM3 as antigen. 3) Other protozoal antigens might act as immunologic adjuvants. 4) Differences in ganglioside fatty acid composition between *T. cruzi* and host GA1 and GM1 may increase their immunogenicity as demonstrated for murine lymphoma. Alternatively, this terminal sequence could exist on some parasitic glycoproteins also acting as an antigen, a possibility already demonstrated by the reaction of a human monoclonal antibody directed against GA1 with a high molecular weight neural-specific glycoprotein.

As for the pathologic relevance of elevated anti-GA1 and anti-GM1 antibodies, such autoantibodies can cause the peripheral neuropathy seen in Chagas’ disease. It should be noted that 10–15% of patients with peripheral neuropathies have elevated GA1 and GM1 antibody levels, and that peripheral neuropathy has been induced by passive transfer of monoclonal antibodies to GA1 and GM1, while therapeutic reduction of GA1 and GM1 antibody concentrations is associated with clinical improvement. It is possible that the increases in GA1- and GM1-specific antibodies that develop during chronic *T. cruzi* infection are involved in the pathogenesis of peripheral neuropathy seen in Chagas’ disease. Gangliosides have been successfully used in the treatment of cardiac symptoms of chronic chagasic patients and mice.

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