

IMMUNOGENIC Gal α 1 $\rightarrow$ 3Gal CARBOHYDRATE EPITOPES ARE PRESENT ON PATHOGENIC AMERICAN *Trypanosoma* AND *Leishmania*¹

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Anti-Gal is a natural antibody present in unusually high concentrations in human sera. It constitutes as much as 1% of circulating IgG and displays a distinct specificity for the Gal α 1 $\rightarrow$ 3Gal carbohydrate epitope. In the present study, we have found in the sera of patients with Chagas' disease and *Leishmania* infection anti-Gal titers 10- and 16-fold higher than that of healthy or bacteria-infected individuals. This increase in anti-Gal titer seemed to be the result of a specific immune response toward parasitic Gal α 1 $\rightarrow$ 3Gal epitopes. Binding studies of affinity chromatography-purified anti-Gal antibodies to *Trypanosoma cruzi* and American *Leishmania* parasites indeed demonstrated the presence of Gal α 1 $\rightarrow$ 3Gal epitopes on these parasites. This finding was supported by the observed binding to the parasites of two additional Gal α 1 $\rightarrow$ 3Gal recognizing molecules: the mAb Gal-13, and the lectin, *Bandeiraea simplicifolia* I B₄. Furthermore, the binding of both anti-Gal antibody and of the *B. simplicifolia* I B₄ lectin could be inhibited by galactose, and not glucose. In addition, removal of the terminal α -galactosyl residues from the parasites by pretreatment with α -galactosidase, or the oxidation of the binding epitopes by periodate prevented the subsequent binding of both the antibody and the lectin. A crude leishmanial lipid extract readily bound these three reagents, suggesting that at least part of these epitopes are of a glycolipid nature. These Gal α 1 $\rightarrow$ 3Gal epitopes may thus serve as an antigenic source for the excess production of anti-Gal. In view of the naturally high level of anti-Gal in humans and its binding to *T. cruzi* and *Leishmania*, it is argued that these antibodies may contribute to the natural defense against the invasion of such parasites.

In our recent studies on antibody reactivities within the sera of patients with cutaneous and visceral leishmaniasis, and patients with Chagas' disease, we have demonstrated marked elevation in titers of antibodies which bind specifically to the carbohydrate epitope

Gal α 1 $\rightarrow$ 3Gal (1, 2). Furthermore, these antibodies were found to account for most of the anti-laminin antibody reactivity in these patients, because they interact with the Gal α 1 $\rightarrow$ 3Gal epitopes on the N-glycosylated carbohydrate chains of mouse laminin (3). Lower levels of such antibodies were found in sera of healthy individuals (3).

Separate studies on natural antibodies to Gal α 1 $\rightarrow$ 3Gal epitopes have shown that these antibodies constitute as much as 1% of circulating IgG in healthy individuals, when measured after isolation by affinity chromatography (4, 5). These antibodies were designated as anti-Gal (anti α -galactosyl IgG) antibodies (6). They are produced throughout life in humans as a result of constant antigenic stimulation probably by gastrointestinal bacteria which seem to express Gal α 1 $\rightarrow$ 3Gal epitopes on their cell wall LPS (7).

The observed elevation in antibodies with Gal α 1 $\rightarrow$ 3Gal reactivity in chagasic and leishmanic patients (1-3) suggested an increase in anti-Gal production as a result of a vigorous immune reaction to Gal α 1 $\rightarrow$ 3Gal epitopes on glycoconjugates of the pathogenic parasites, exceeding the regular immune response toward Gal α 1 $\rightarrow$ 3Gal epitopes on gastrointestinal bacteria. An alternative mechanism to be considered as the cause for the observed increase in this antibody reactivity could be a polyclonal activation of B lymphocytes as a result of the parasitic infection.

In our work, we analyzed these two hypothetical possibilities by determining the increase in reactivity of purified anti-Gal in chagasic and leishmanic patients, in comparison with the total IgG level, and by investigating the putative expression of Gal α 1 $\rightarrow$ 3Gal epitopes on *Trypanosoma cruzi*, *Leishmania braziliensis*, and *Leishmania mexicana* parasites. Our studies suggest that the severalfold increase in anti-Gal titer in these human Kinetoplastida infections is the result of specific immune reactivity toward Gal α 1 $\rightarrow$ 3Gal epitopes, many of which are residues on glycolipids of the cell membrane of the parasites.

MATERIALS AND METHODS

Anti-Gal in patients' sera. Sera were obtained from 31 patients with chronic Chagas' disease, 31 patients with active non-treated American cutaneous leishmaniasis (for clinical details, see Ref. 1), 23 patients with acute bacterial infections produced by β -hemolytic group A streptococcus, *Hemophilus influenza*, and *Staphylococcus aureus*, and 9 healthy individuals. Anti-Gal titers were determined by indirect hemagglutination assay with rabbit red cells, as previously described (6, 8). Rabbit red cells were incubated with twofold dilutions of various sera in V-shaped microtiter wells, for 30 min at

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³ Abbreviations used in this paper: Gal, galactose; anti-Gal, anti- α -galactosyl; BS lectin, *Bandeiraea simplicifolia* IB₄ isolectin; GlcNAc, N-acetylglucosamine.

24°C. The red cells were then washed three times with PBS by spinning the microtiter plates at 400 $\times$ *g* for 4 min, removal of supernatant and resuspension in PBS. The washed red cells were resuspended in rabbit anti-human IgG antibodies (Dako, Santa Barbara, CA) diluted 1/300, and the anti-Gal titer determined as the highest serum dilution yielding positive hemagglutination.

Isolation of anti-Gal. Anti-Gal was isolated from various patient sera by affinity chromatography on a Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc adsorbent (Synsorb no. 115, Chembiomed, Edmonton, Alberta, Canada). The synthetic oligosaccharide Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc is linked to a silica matrix at a concentration of 0.8 μ mol/g of Synsorb matrix. Aliquots of 0.5 ml of heat-inactivated serum diluted 1/2 in PBS were applied on 0.5 g Synsorb no. 115 column and incubated for 10 min at 24°C. Subsequently the effluent was stored at 4°C for analyzing anti-Gal activity. After washing the column with PBS, the bound antibodies were eluted with 1 ml glycine-HCl buffer, pH 2.5, immediately neutralized, and then dialyzed against PBS. The eluates were concentrated in an Amicon chamber to a volume of 0.5 ml, which corresponds to the original volume of the serum used for anti-Gal isolation. The anti-Gal titer in the eluates was determined as described above.

Anti-Gal used for studying Gal α 1 $\rightarrow$ 3Gal epitopes on parasites' membrane was purified from 30 ml volumes of normal heat-inactivated AB sera on a 10-ml Synsorb no. 115 column by the same procedure. The eluted antibodies were brought to a concentration of 100 μ g/ml, which corresponds to the anti-Gal concentration in the blood of healthy individuals (6).

Determination of IgG concentration in various sera. IgG concentration was measured by the Mancini radial immunodiffusion assay (9) with rabbit anti-human IgG antibodies (Dako).

Parasites. *T. cruzi*, *L. mexicana*, and *L. braziliensis* strains studied were described before (10, 11). Several subspecies of *L. mexicana* (strain in parentheses) were studied: *L. m. pifanoi* (MP), *L. m. spp.* (NR, LR), *L. m. garhami* (JAP, HM, MHOM/JAP 78), *L. m. mexicana* (MHOM/BEI 21), *L. m. venezuelensis* (MHOM/PHM3), and *L. braziliensis*. *L. b. guayanensis* (MHOM/4147) and *L. b. braziliensis* (MHOM/M2903) were also used in this study. The subspecies of *T. cruzi* were: Marin-1, Bertolodo, F1, and Ma strains (belonging to zymodeme type 1) and Ya and Y strains (zymodeme type 2). For further details of *Leishmania* and *Trypanosoma* strains, see References 10 and 11, respectively. Most experiments were carried out with MP strain of *L. mexicana* and Marin-1 strain of *T. cruzi*.

Culture methods. *T. cruzi* epimastigote and American *Leishmania* promastigote strains were grown at 28°C in a semi-defined fetal calf serum-containing medium (12).

Glycolipids. *L. mexicana pifanoi* (MP strain) promastigotes (about 10¹⁰ cells resuspended in 5 ml cold 10 mM PMSF) were homogenized with a Teflon pestle in a Potter-Elvehjem homogenizer rotating at 1200 rpm. Parasitic lipids were successively extracted by adding 50 ml chloroform/methanol (2/1) to the cell suspension and stirring at 4°C for 24 h. Extracted cells were then sedimented by centrifugation at 1000 $\times$ *g* for 20 min at 4°C and the corresponding organic phase was separated. Extraction was repeated with the sediment with chloroform/methanol (1/2) solution. The two extracts were combined and evaporated to dryness. Residue thus obtained redissolved in chloroform/methanol 2/1 and washed several times with 0.15 M KCl until obtaining a clear saline phase. After this procedure (repeated 10 times), no proteins were detected within the organic phase, when 10 μ l of undiluted organic solution were subjected to Lowry analysis. This washing procedure removes compounds precipitating in the saline phase, and which seem to correspond to proteolipid contaminants. The organic phase was pipetted off and evaporated to dryness. The total lipids obtained by this procedure were subsequently monitored for ELISA immunoreactivity.

Glycosidase treatment of freshly isolated Kinetoplastida parasite culture forms. The incubation mixture contained the following components in 200 μ l of 50 mM sodium citrate buffer, pH 4.0: 2 $\times$ 10⁵ parasites and 10 U of green coffee bean α -galactosidase (Sigma Chemical Co., St. Louis, MO). After incubation at 37°C for 24 h, parasites were spun down at 3000 $\times$ *g* for 10 min, washed twice with PBS and then assayed for ELISA for binding anti-Gal.

Ligand analysis of Gal α 1 $\rightarrow$ 3Gal expression on parasites. The possible expression of Gal α 1 $\rightarrow$ 3Gal epitopes on the various parasites was assessed by the use of two antibodies and a lectin, all of which interact specifically with this epitope. 1) The natural anti-Gal antibody, as discussed above, binds with high specificity to Gal α 1 $\rightarrow$ 3Gal residues and not to Gal α 1 $\rightarrow$ 4Gal residues, β -galactosyl residues, or residues with carbohydrates other than galactose (4, 5); 2) the mAb Gal-13. This mouse IgG mAb was found to be highly specific for glycolipids with terminal Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc-R residues (13). Gal-13 does not bind, however, to these residues on N-glycosylated glycoproteins such as the Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc residues on mouse laminin or bovine thyroglobulin (U. Gallili,

unpublished observations). The narrow specificity of Gal-13 toward glycolipids stems from the fact that the myeloma cells producing this antibody, as well as other murine cells, express abundance of Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc residues on cell surface glycoproteins (see discussion below); 3) The BS lectin. This lectin was found to bind to Gal α 1 $\rightarrow$ 3Gal residues of glycoconjugates and with less affinity to the Gal α 1 $\rightarrow$ 3(Fuca1 $\rightarrow$ 2)Gal residues which represent the blood group B Ag (14–16).

Binding of BS lectin. The procedure for measuring binding of BS lectin to the parasites also was used for the ELISA experiments with the human polyclonal and mouse mAb. All binding assays were performed in PBS. Parasites (between 0 to 4 $\times$ 10⁵ cells) were placed in 100- μ l aliquots in polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) and dried overnight at 37°C. The wells were washed with PBS-0.05% Tween 20 and incubated for 2 h with 2% BSA to block non-specific binding of BS lectin and of avidin-peroxidase. Biotinylated BS lectin (30 μ g/ml) (Vector Laboratories, Burlingame, CA) either without or mixed with 0.5 M galactose in 50- μ l aliquots was incubated in the wells containing parasites for 1 h at 37°C. The assays were performed in triplicates. At the end of incubation, parasites were washed three times with PBS and incubated for 30 min at 37°C with 2.5 μ g avidin-peroxidase/ml. A further 3 $\times$ wash cycle with PBS was carried out. *O*-dianisidine was finally added and product formation was determined by measuring absorbance at 405 nm using a MicroELISA reader (Dynatech).

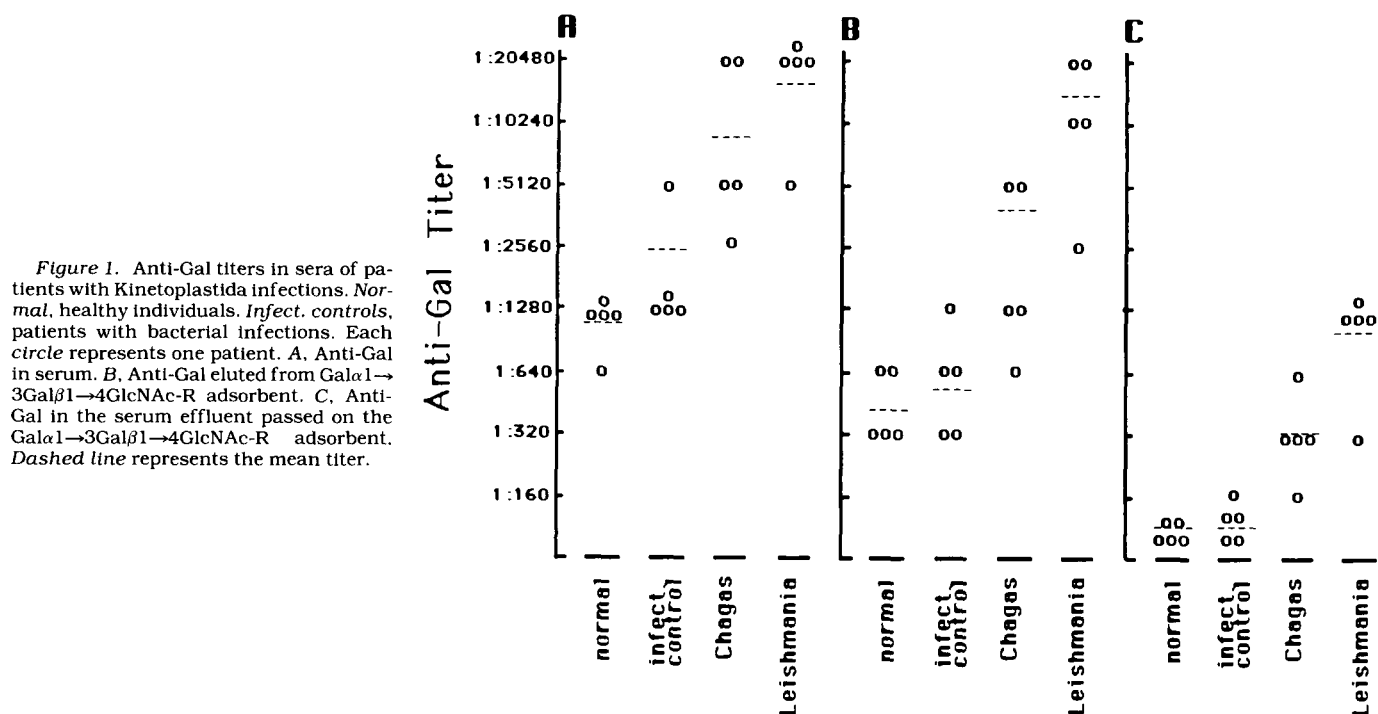
Binding of anti-Gal to *T. cruzi* and *Leishmania*. Experiments were carried out exactly as described for BS lectin, except that the anti-Gal binding was assayed at a dilution of 1/20 (5 μ g/ml, a concentration that corresponds to 5% of the physiologic concentration of anti-Gal) and peroxidase-labeled goat anti-human IgG 1/2,000 was used as a secondary antibody. Each plate included controls of the antibody for non-specific binding of anti-Gal to polystyrene plates and the non-specific binding of goat anti-human IgG to parasites and to polystyrene plates. The peroxidase-ELISA was carried out after cell fixation and blocking with 2% BSA. After one quick rinse with 50 mM sodium acetate buffer, pH 4.5, 10 mM sodium periodate in acetate buffer or just acetate buffer alone was added to each well. After 1 h at 25°C, the reaction was stopped by washing once with acetate buffer and adding a 1% glycerol solution in PBS (100 μ l) for 30 min. The plates were then washed three times with PBS and the assay was continued by incubating with anti-Gal antibody.

Binding of mouse mAb Gal-13 to *T. cruzi* and American *Leishmania*. Experiments were carried out as described for BS lectin except that the ascitic solution of the mAb was used diluted 1/60 and as negative control pristane-produced ascitic fluid was used at the same dilution. Gal-13 was allowed to react with parasites for 2 h at 37°C and then 100 μ l biotinylated goat anti-mouse IgG diluted 1/300 added and incubated for another 2 h at 25°C.

Binding of antibodies and lectin to a crude leishmanial lipid extract. Lipids (1 mg) were diluted with 4.5 ml of ethanol as stock solution. The carbohydrate concentrations in this solution determined by phenol sulfuric acid reaction was 100 μ g/ml. Polyvinylchloride microtiter plate (Dynatech) wells were coated with 50 μ l of a 100-fold dilution of stock solution in ethanol, which was then evaporated to dryness. Other experimental procedures were exactly as described above.

RESULTS

Anti-Gal in sera of chagasic and leishmanic patients. When measured by hemagglutination assay with rabbit red cells, anti-Gal in healthy individuals usually yields a titer of 1/640 to 1/1280 (Fig. 1A). In sera passed through a Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc-R adsorbent (Synsorb no. 115), most (>85%) of the anti-Gal reactivity is retained on the column, as can be seen by a more than 10-fold decrease in the hemagglutinating reactivity in the effluent serum (Fig. 1C). The antibodies eluted from the adsorbent column by low pH displayed a titer of 1/320 to 1/640 in healthy individuals (Fig. 1B). A similar pattern of anti-Gal reactivity was observed in sera of five patients with various bacterial infections (infect. control group). Except for one patient with anti-Gal titer of 1/5120, all of the other four patients displayed normal activity of anti-Gal. However, in the serum of patients with Chagas' disease and *Leishmania* infection, the mean anti-Gal titer was 10-fold and 16-fold higher than that of healthy



individuals or infection controls (Fig. 1A). This marked elevation in hemagglutinating reactivity was due to an increase in anti-Gal reactivity because, as observed with sera from healthy individuals, passage on Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc-R adsorbent removed more than 90% of the hemagglutinating reactivity in the sera of the chagasic and leishmanic patients (Fig. 1C). The adsorbed anti-Gal of these patients, when eluted, displayed a 6- to 20-fold higher titers, respectively, as compared to that in normal individuals (Fig. 1B).

When IgG concentration was measured in the sera of the various individuals studied, all the patient groups exhibited only a two- to threefold increase in total IgG concentration when compared with that in healthy individuals (Table I). This suggested that the severalfold increase in anti-Gal reactivity in chagasic and leishmanic patients exceeded the increase in IgG concentration and thus may have resulted from a specific immune response against Gal α 1 $\rightarrow$ 3Gal epitopes in these patients. These data confirmed previous observations on elevation in

antibody reactivity to Gal α 1 $\rightarrow$ 3Gal epitopes in chagasic and leishmanic patients (1-3).

It should be noted that although the increase in anti-Gal titer in patients with Kinetoplastida infection was up to 20-fold (Fig. 1), the mean increase in the actual concentration of anti-Gal was only three- to fivefold (Table I). These quantitative studies suggest that the increase in anti-Gal reactivity in chagasic and leishmanic sera is comprised of an elevated synthesis of this antibody as well as the selective emerging of anti-Gal antibody clones with high affinity to the Gal α 1 $\rightarrow$ 3Gal epitope. These data on the quantitative and qualitative increases in anti-Gal level prompt us to study the possible expression of Gal α 1 $\rightarrow$ 3Gal epitopes on the parasites' membrane, which may account for the observed specific anti-Gal immune response.

Binding of anti-Gal antibodies to fixed *T. cruzi* and *American Leishmania*. Optimization of the binding procedures demonstrated that freshly isolated parasites treated with 0.01% sodium azide or 20 mM PMSF-10 mM EDTA and dried in microtiter wells showed a stronger significant specific anti-Gal binding compared to parasites fixed with 1% formaldehyde or glutaraldehyde. Treatment of well-bound parasites for 30 min at 37°C with 0.25% trypsin did not increase anti-Gal reactivity, but tended to maintain linearity of antibody binding as a function of the concentration of fixed parasites, at least in the range of 0 to 4×10^5 parasites/well (not shown). According to our previous results, we chose to resuspend freshly isolated parasites in 20 mM PMSF-10 mM EDTA and to trypsinize just before each experiment. Figure 2 shows the dose-dependent binding of anti-Gal to *T. cruzi* epimastigotes. Periodate treatment of the parasites greatly decreased the binding of anti-Gal (Fig. 2), further demonstrating the carbohydrate nature of the anti-Gal binding sites on the parasite. It should be noted that in experiments carried out with blood-borne *T. cruzi* trypomastigotes and with *American Leishmania* promasti-

TABLE I
IgG and anti-Gal concentrations in sera of various patients

	IgG Concentration (mg/ml)	Anti-Gal Concentration (μ g/ml)
Healthy individuals (n = 9)	7 - 11.0 ^a 8.7 $\pm$ 1.0 ^b	48 - 91 ^a 74 $\pm$ 2.8
Infection controls (n = 23)	10 - 40 26 $\pm$ 1.2	56 - 96 71 $\pm$ 2.6
Leishmanic patients (n = 31)	12 - 40 24 $\pm$ 1.1	302 - 490 380 $\pm$ 9.2
Chagasic patients (n = 31)	14 - 40 28 $\pm$ 1.6	72 - 419 213 $\pm$ 16.6

^a Range of antibody concentration.

^b Mean of antibody concentration $\pm$ SE.

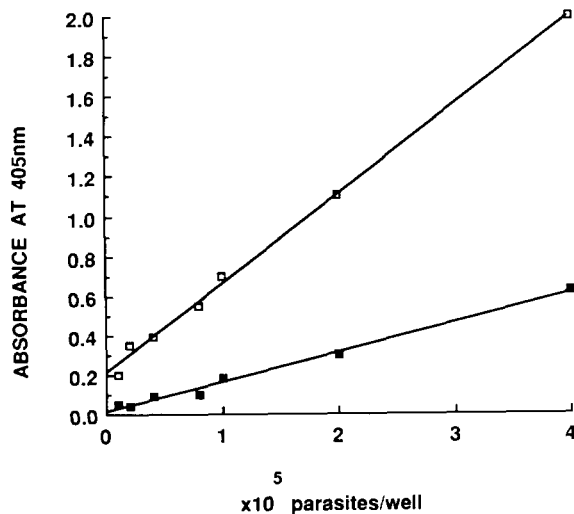


Figure 2. Binding of purified human anti-Gal to *T. cruzi* epimastigotes. Several parasite concentrations (0 to 4×10^5 parasites/well) were exposed to freshly purified human anti-Gal antibody (10 μ g/ml) for 1 h at 37°C. □, Anti-Gal binding to *T. cruzi* epimastigotes; ■, anti-Gal binding to 10 mM periodate-treated *T. cruzi* epimastigotes.

gotes, similar anti-Gal binding was observed as with *T. cruzi* epimastigotes. The binding characteristics of human anti-Gal to fixed *Leishmania* promastigote and *T. cruzi* epimastigote and trypomastigotes is presented in Table II. Incubation of anti-Gal in the presence of 0.5 M galactose decreased binding to both parasites by 70%, whereas 0.5 M glucose did not affect anti-Gal reactivity, demonstrating the specific blocking effect of galactose on this natural antibody. Accordingly, treatment of freshly isolated parasites with green coffee bean α -galactosidase markedly decreased anti-Gal binding to *T. cruzi* and American *Leishmania* (Table II). It is of interest to note that anti-Gal binding to *T. cruzi* epimastigote and trypomastigote forms was quite similar. Furthermore, when human anti-Gal binding was studied by immunofluorescence a stronger diffuse staining was found on trypomastigotes and amastigotes than on epimastigotes (J. L. Avila and N. Salma, manuscript in preparation). Anti-Gal staining of the different *Leishmania* parasitic forms demonstrated a diffused distribution of the antibody in promastigotes and a patchy distribution in the amastigotes. Proteolytic treatment of the parasites with either trypsin or pronase did not alter anti-Gal binding to the parasites. The activity of the proteolytic enzymes in these studies was confirmed before the experiments by demonstration of hydrolysis of N-benzoyl-L-arginine

ethyl ester at pH 8.0. Notably all *T. cruzi* and American *Leishmania* strains studied showed anti-Gal binding, some strains being more reactive than others. No relation with taxonomic classification was found in the case of American *Leishmania*, or with zymodeme in the case of *T. cruzi* strains (Table III).

Binding of mAb Gal-13. The mAb Gal-13, used as the second antibody in this study, was previously shown to bind specifically to Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc residues on glycolipids (13). Figure 3 shows the binding of Gal-13 to 20 mM PMSF-10 mM EDTA-fixed *T. cruzi* epimastigotes and American *Leishmania* promastigotes as a function of the concentration of parasites. The strong binding of Gal-13 to the parasite surface suggested that a substantial proportion of the Gal α 1 $\rightarrow$ 3Gal epitopes on these parasites are expressed as residues on glycolipids, because this mAb does not bind to Gal α 1 $\rightarrow$ 3Gal residues on N-glycosylated glycoproteins. Two negative mAb controls were studied: anti-desmin mAb (Amersham, Ltd. catalog no. BRN 1101, U.K.) and Synsorb no. 115 adsorbed Gal-13 antibody. Neither of the two antibody preparations were found to bind to the parasites, as indicated by background absorbance levels (not shown).

Binding of BS lectin. To further corroborate the presence of terminal α -galactosyl-bearing glycoconjugates in *T. cruzi* and American *Leishmania* indicated by studies with anti-Gal and Gal-13 antibodies, we used BS lectin, which binds with high affinity to terminal Gal α 1 $\rightarrow$ 3Gal residues (14, 15). Table IV and Figure 4 shows BS lectin binding to *T. cruzi* epimastigotes and trypomastigotes and American *L. mexicana pifanoi* (MP strain) promastigotes. It can be seen that a better binding was obtained with parasites fixed either with 20 mM PMSF-10 mM EDTA or with 1% formaldehyde when compared with 1% glutaraldehyde. Also shown in Table IV is the specific blocking effect of 0.5 M galactose, but not of 0.5 M glucose. Periodate treatment of fixed parasites strongly blocked lectin binding, confirming the carbohydrate nature of the lectin binding sites on *T. cruzi* epimastigotes and promastigotes and *Leishmania mexicana* promastigotes.

Binding to parasites' glycolipids. The binding of Gal-13 antibodies to the parasites and the lack of inhibitory effect of proteolysis of parasites on BS lectin binding suggested that a substantial proportion of the epitopes binding these reagents is part of the glycolipid moiety of the parasitic membrane. To study the occurrence of glycolipids with Gal α 1 $\rightarrow$ 3Gal residues in the parasites' membrane, crude total lipids fraction was assessed for

TABLE II
Human anti-Gal antibody binding to American *Leishmania* promastigote and *T. cruzi* epimastigotes and trypomastigotes after various experimental procedures

Experimental Procedures	% of Control*		
	<i>Leishmania</i> ^a	<i>T. cruzi</i> [†]	
		Epimastigotes	Trypomastigotes
None	100	100	100
10 mM periodate	18 $\pm$ 3	21 $\pm$ 5	34 $\pm$ 6
0.5 M galactose	33 $\pm$ 4	29 $\pm$ 6	21 $\pm$ 4
0.5 M glucose	90 $\pm$ 3	90 $\pm$ 4	93 $\pm$ 6
0.1% pronase (1 h, 37°C)	94 $\pm$ 4	92 $\pm$ 5	90 $\pm$ 6
α -Galactosidase (10 U, 24 h, 37°C)	30 $\pm$ 3	29 $\pm$ 4	23 $\pm$ 3
0.25% trypsin (1 h, 37°C)	87 $\pm$ 6	91 $\pm$ 8	89 $\pm$ 10

^a Mean $\pm$ SD of three separate experiments.

[†] Data were obtained with MP, JAP, and MHOM/4147 strains of American *Leishmania* and Marin-1 F, and Y strains of *T. cruzi*.

TABLE III

Comparison of binding of human anti-Gal to several strains of *Leishmania mexicana*, *Leishmania braziliensis* promastigotes and *T. cruzi* promastigote and trypomastigote forms^a

Parasites (Strain)	Absorbance at 405 nm ^b
<i>L. mexicana</i>	
L.m. pifanoi (MP)	1.120 $\pm$ 0.080
L.m. spp. (NR)	1.209 $\pm$ 0.093
L.m. spp. (LR)	1.003 $\pm$ 0.126
L.m. garhami (JAP)	1.286 $\pm$ 0.153
L.m. garhami (HM)	1.325 $\pm$ 0.108
L.m. garhami (MHOM/JAP78)	1.096 $\pm$ 0.103
L.m. mexicana (MHOM/BEL21)	1.184 $\pm$ 0.096
L.m. venezuelensis (MHOM/PHM3)	1.228 $\pm$ 0.065
<i>L. braziliensis</i>	
L.b. guyanensis (MHOM/4147)	1.192 $\pm$ 0.253
L.b. braziliensis (MHOM/M2903)	1.287 $\pm$ 0.090
<i>T. cruzi</i>	
Marin-1	1.143 $\pm$ 0.056 (1.286 $\pm$ 0.083) ^c
Bertoldo	1.149 $\pm$ 0.083 (1.394 $\pm$ 0.051)
F1	1.293 $\pm$ 0.075 (1.094 $\pm$ 0.126)
Ma	1.147 $\pm$ 0.082 (0.956 $\pm$ 0.123)
Ya	1.314 $\pm$ 0.098 (1.084 $\pm$ 0.106)
Y	1.126 $\pm$ 0.102 (0.958 $\pm$ 0.082)

^a Freshly PMSF-EDTA-fixed parasites (2×10^5 /well) were incubated with 10 μ g/ml human anti-Gal antibody for 4 h at 37°C.

^b Mean of three different experiments.

^c Values in parentheses correspond to trypomastigote forms.

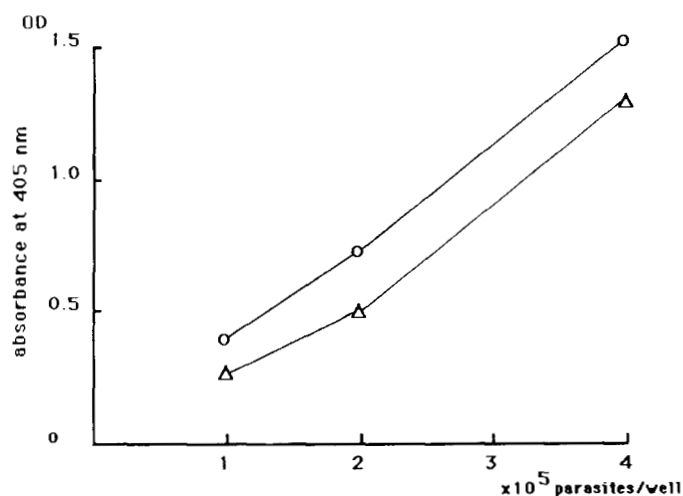


Figure 3. Binding of the mAb Gal-13 to fixed *T. cruzi* epimastigotes and American *Leishmania* promastigotes as a function of parasite concentration. (O), *T. cruzi* epimastigotes; (Δ), *Leishmania* promastigotes.

interaction with the three Gal α 1 $\rightarrow$ 3Gal binding molecules.

Figure 5 shows the dose-dependent binding of purified human anti-Gal, of mouse mAb Gal-13, and of BS lectin to increasing amounts of *Leishmania* crude lipids. These

data indicate that at least part of the Gal α 1 $\rightarrow$ 3Gal epitopes on the parasite are on glycolipid molecules.

DISCUSSION

Our study confirms the previous observations on the increased production of antibodies with Gal α 1 $\rightarrow$ 3Gal specificity (i.e., anti-Gal) in human Kinetoplastida infections (1-3). This increase in anti-Gal titer in chagasic and leishmanic patients seems to be the result of a specific immune response to Gal α 1 $\rightarrow$ 3Gal epitopes, rather than of a polyclonal activation of B lymphocytes for two reasons: 1) control patients with bacterial infections who also demonstrated hypergammaglobulinemia showed no increase in anti-Gal titers; and 2) whereas the mean increase in IgG concentration in the patients with chronic parasite infections was threefold the normal levels, the mean elevation in anti-Gal titer exceeded the increase in IgG concentration and was 10- to 20-fold the normal titer. This increase in anti-Gal reactivity was contributed by the elevation in the actual concentration of anti-Gal in the serum as well as by the increase in the proportion of high-affinity clones of IgG antibodies directed against Gal α 1 $\rightarrow$ 3Gal epitopes. These characteristics of a specific immune response toward a given antigenic epitope suggested that putative Gal α 1 $\rightarrow$ 3Gal epitopes on the parasites' membrane may serve as an antigenic source for the excess production of anti-Gal.

The present study also demonstrates Gal α 1 $\rightarrow$ 3Gal epitopes on both American *Leishmania* promastigotes and *T. cruzi* blood and culture forms. The expression of these epitopes could be readily studied by measuring the binding of human polyclonal anti-Gal antibody and mouse Gal-13 mAb to the parasites. The binding of BS lectin to the parasites further supported these observations. Our studies were carried out on dried parasites fixed to microtiter wells. Nevertheless, the expression of these epitopes on live parasites could be readily demonstrated by the agglutination of live *T. cruzi* by either anti-Gal or BS lectin (not shown). These findings support the observations of Milani and Travassos (17) who reported recently on the complement-mediated anti-Gal lysis of *T. cruzi* trypomastigotes.

The occurrence of α -galactosyl epitopes on *T. cruzi* membrane was also previously suggested by Villalta and Kierszenbaum (18). These investigators observed that α -galactosidase treatment of *T. cruzi* enhanced their in vitro ability to associate with mouse peritoneal macrophages or rat heart myoblasts. Further, this modification

TABLE IV

Binding of BS lectin to American *Leishmania* promastigotes and *T. cruzi* epimastigotes and trypomastigotes under different experimental conditions

Experimental Conditions	<i>Leishmania</i> ^a	% of Control ^a	
		<i>T. cruzi</i> ^b	
		Epimastigotes	Trypomastigotes
20 mM PMSF-10 mM EDTA (control)	100	100	100
1% formaldehyde	94 $\pm$ 4	90 $\pm$ 2	93 $\pm$ 6
1% glutaraldehyde	65 $\pm$ 6	60 $\pm$ 9	74 $\pm$ 8
0.01% sodium azide	83 $\pm$ 5	78 $\pm$ 7	69 $\pm$ 6
0.5 M galactose ^c	18 $\pm$ 3	21 $\pm$ 4	19 $\pm$ 4
0.5 M glucose ^c	94 $\pm$ 5	91 $\pm$ 7	87 $\pm$ 6
10 mM periodate ^c	23 $\pm$ 3	20 $\pm$ 6	16 $\pm$ 3

^a Mean $\pm$ SD of six different experiments.

^b Data obtained with MP, HM, LR, JAP, MHOM/4147, and MHOM/M2903 strains of American *Leishmania* and Marin-1, Bertoldo, F1, Ma, Ya, and Y strains of *T. cruzi*.

^c Experiments were carried out in PMSF-EDTA-fixed parasites.

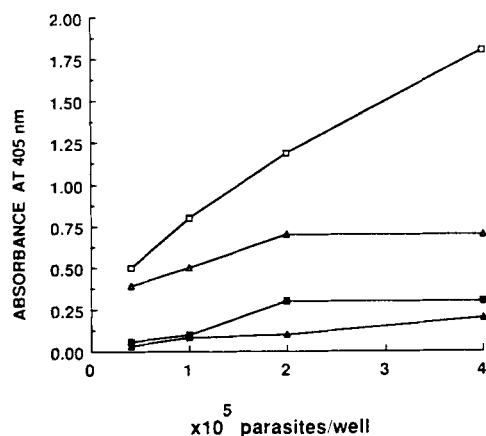


Figure 4. Binding of BS lectin to fixed *T. cruzi* epimastigotes and American *Leishmania* promastigotes as a function of parasite concentration. (□), *T. cruzi*, (Δ) American *Leishmania*; (■, ▲), binding in the presence of 0.5 M galactose.

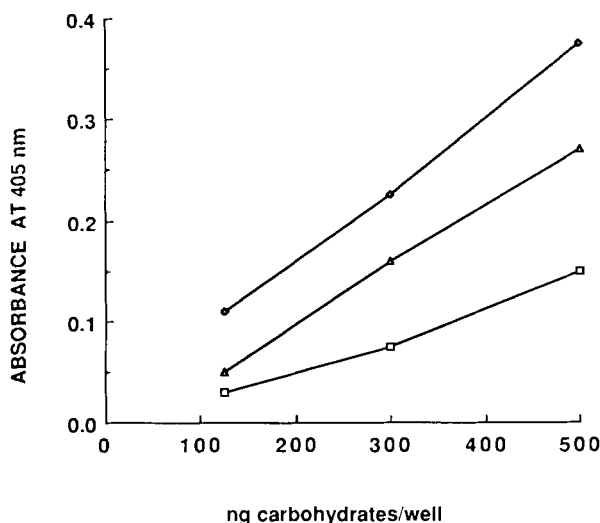


Figure 5. Binding of (□), BS lectin; (Δ), Gal-13; and (◇) human anti-Gal to American *Leishmania* lipid fraction, as a function of carbohydrate concentration.

was found to be reversible in nature, suggesting a potential mechanism of modulating these epitopes (18).

The binding of mAb Gal-13 suggested that at least part of the Gal α 1 $\rightarrow$ 3Gal epitopes are of glycolipid nature, because this antibody displays a highly restricted specificity to Gal α 1 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc residues which are not on N-glycosylated glycoproteins (13) (U. Galili, unpublished observations). Indeed, lipid extracts from *Leishmania* contained glycolipids with Gal α 1 $\rightarrow$ 3Gal residues, as indicated by the binding of the three reagents to the lipid extracts. The exact nature of these glycolipids is currently under study. It should be stressed that these findings do not exclude the possibility that some of the Gal α 1 $\rightarrow$ 3Gal epitopes are on lipid-linked proteoglycan molecules of the parasites or lipophosphoglycans. Lipophosphoglycans have recently been shown to be major components on *Leishmania* membranes (19) and may theoretically serve as powerful immunogens.

Glycolipids from membranes of *Trypanosoma* and *Leishmania* parasites were examined in a number of studies (19–24), none of which identified Gal α 1 $\rightarrow$ 3Gal residues. The report on *Leishmania adleri*, where a polysaccharide showing galactose as the predominant sugar with branched 1-3 galactan associated with α -D-

mannose (25), may be related to such glycolipid structures, suggested in the present study to interact with anti-Gal. It should be stressed that our studies also do not exclude the possible expression of the Gal α 1 $\rightarrow$ 3Gal epitopes on membrane proteins. The inability of proteolytic enzymes to remove these epitopes may be because such molecules on the surface of the parasites are not accessible to proteases. In addition, we currently have no information on the interaction of the Gal-13 mAb with Gal α 1 $\rightarrow$ 3Gal residues on O-glycosylated glycoproteins, some of which may occur on the parasites' membrane. A detailed analysis of the carbohydrate moiety of glycoproteins isolated from these parasites may elucidate this question.

Our studies suggest that different life cycle forms of both *Leishmania* and *T. cruzi* may induce increased production of anti-Gal antibodies in infected patients. Subsequent to s.c. inoculation, promastigotes and metacyclic trypomastigotes are rapidly phagocytized and transform into intracellular amastigotes. Later on, these forms transform, in the case of *T. cruzi* into extracellular trypomastigotes, which are again phagocytized to complete the mammalian cycle. The observed interaction of anti-Gal with the different parasitic forms may thus imply that amastigotes and trypomastigotes in Chagas' disease and amastigotes in leishmanial infection are able to rapidly trigger specific antigenic stimulation. The observation that about 75% of Kinetoplastidae-infected human subjects show elevated levels of anti-Gal antibodies as early as 2 wk after clinical detection (1) suggests that the Gal α 1 $\rightarrow$ 3Gal epitopes on the parasites are highly immunogenic. Their expression in a form linked to lipids may contribute to this immunogenicity because of the low rate of degradation of glycolipid antigens.

The expression of Gal α 1 $\rightarrow$ 3Gal epitopes in mammals displays a unique evolutionary distribution. They are on red cells and nucleated cells of non-primate mammals, prosimians and New World monkeys. However, in Old World monkeys, apes, and humans, no such epitopes are detected (8, 26). This difference is because of diminished activity of the enzyme α 1 $\rightarrow$ 3 galactosyltransferase which links terminal α -galactosyl to lactosamine residues (26). The loss of immune tolerance to Gal α 1 $\rightarrow$ 3Gal residues in the latter group of primates resulted in the immune reaction against Gal α 1 $\rightarrow$ 3Gal epitopes on gastrointestinal bacteria cell wall components and production of anti-Gal (7). In man, the immune response to this epitope seems to be intensified upon exposure to Gal α 1 $\rightarrow$ 3Gal carbohydrate structure on the parasites.

There have been an increasing number of reports in recent years which suggest a possible role for human natural antibodies in defense against *Leishmania* and *T. cruzi* infections (27–29). Such a role may also be attributed to anti-Gal (17). In view of the naturally high level of anti-Gal in humans and its binding to *T. cruzi* and *Leishmania* as reported here, it could be speculated that anti-Gal may contribute to the natural defense against the invasion of such parasites. This assumption is supported by the observations of Milani et al. (30) who have recently reported that pre-incubation of *T. cruzi* trypomastigotes with purified anti-Gal antibodies abolished the capacity of the parasite to cause parasitemia in inoculated mice. Thus it may be possible that in individuals contracting Chagas' disease, the anti-Gal titer is

transiently low at the time of infection; or the diversity of anti-Gal clones (5) is limited to the extent that the parasite succeeds in evading the antibody and penetrates cells before being destroyed by anti-Gal. Within the infected cells, the parasite can proliferate and possibly continue to produce Gal α 1 $\rightarrow$ 3Gal glycoconjugates which keep stimulating the immune system for excessive anti-Gal production. The possible de novo expression of Gal α 1 $\rightarrow$ 3Gal glycoconjugates on infected tissues would also result in anti-Gal binding to these carbohydrate structures. As previously suggested (3), this interaction may underlie the massive inflammatory reactions observed in infected tissues of chagasic patients. Analysis of the occurrence of Gal α 1 $\rightarrow$ 3Gal epitopes on pathologic tissues of patients with these parasitic infections will help to elucidate the contribution of anti-Gal to such inflammatory processes.

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