Lytic anti-α-galactosyl antibodies from patients with chronic Chagas' disease recognize novel *O*-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma cruzi*

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Sera of patients with chronic Chagas' disease (American trypanosomiasis) contain elevated levels of anti-α-galactosyl antibodies that are lytic to *Trypanosoma cruzi*. The *T. cruzi* trypomastigote F2/3 antigen complex recognized by these antibodies runs as a broad smear on SDS/PAGE [Almeida, Krautz, Krettli and Travassos (1993) J. Clin. Lab. Anal. 7, 307–316]. Treatment of *T. cruzi* trypomastigote cells with bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) abolished most of their reactivity to chronic Chagas'-disease ((Chagasic, Ch) anti-α-galactosyl antibodies (anti-Gal). The F2/3 antigen complex, purified by solvent extraction and hydrophobic-interaction chromatography, contained 60% carbohydrate by weight and substantial amounts of Thr, Ser, Glx, Asx, Gly, Ala and Pro, but relatively few hydrophobic amino acids. The presence of *myo*-inositol, ethanolamine and 1-*O*-hexadecylglycerol suggested the presence of glycosyl-phosphatidylinositol membrane anchors. This was confirmed by PI-PLC treatment, which rendered the F2/3 molecules hydrophilic and reactive to anti-(cross-reacting determinant) antibodies. The majority of the GlcNAc content of the F2/3 antigens was found at the reducing termini of oligosaccharides in O-glycosidic linkage to Thr residues. These O-linked oligosaccharides could be released by β-elimination and by mild hydrazinolysis. The smallest released oligosaccharitol that was reactive with the Ch anti-Gal was Galα1-3Galβ1-4GlcNAcol (where GlcNAcol is *N*-acetyl-glucosaminitol). Several other Gal-containing oligosaccharitols were observed, most of which were branched and contained 4,6-di-*O*-substituted GlcNAcol at their reducing termini. About half of the total released oligosaccharitols could bind to immobilized Ch anti-Gal, but none of them bound to the anti-Gal isolated from normal human sera. These data suggest that the specificities of the Ch anti-Gal are quite different from the natural anti-Gal isolated from normal human sera. Therefore, these novel T. *cruzi* *O*-linked oligosaccharides are highly immunogenic under the conditions of natural infection and are the targets for lytic Ch anti-Gal.

INTRODUCTION

Chagas' disease (American trypanosomiasis) is caused by *Trypanosoma cruzi*, a flagellate of the Trypanosomatidae family, and affects several million individuals in South and Central America. The parasite proliferates in the triatomine insect vector midgut as the epimastigote form and appears in the insect hindgut and faeces as the metacyclic trypomastigote form. Infection in man is initiated by metacyclic trypomastigotes, which invade host cells and differentiate into amastigote forms that divide intracellularly. The amastigotes differentiate into non-dividing trypomastigotes immediately prior to host-cell rupture and are released into the bloodstream. Re-infection of host cells by trypomastigotes starts a cycle characterized by intermittent parasitaemia which is much reduced in the chronic phase. A favourable immune response to control the parasitaemia in Chagasic patients involves the production of specific antibodies that can (a) lyse or cause cytotoxic effects on trypomastigotes, and (b) reduce or abolish cell reinfection by the parasite. Thus active trypanosomiasis correlates directly with increased levels of antibodies lytic to trypomastigotes (Krettli and Brener, 1976). These antibodies are protective (Krettli and Brener, 1982), and a decrease in their serum titres has been used as a criterion of cure in patients submitted to chemotherapy (Galvão et al., 1993).

Recently, several contributions have appeared on the identification of the molecular targets for lytic antibodies on the surface of *T. cruzi* trypomastigotes, as reviewed by Travassos and Almeida (1993). We found that most of the lytic power in sera from patients with chronic Chagas' disease was due to anti-α-galactosyl antibodies (anti-Gal) which could be removed by absorption on immobilized Galα1-3Galβ1-4GlcNAc (Almeida et al., 1991). The natural anti-Gal antibodies of normal human serum (NHS anti-Gal) from healthy individuals are also prepared this way (Galili et al., 1988; Galili, 1993). However, Chagasic anti-Gal antibodies (Ch anti-Gal) show a much greater affinity for parasite surface antigens than do natural anti-Gal antibodies (Almeida et al., 1991). The cell-derived trypomastigotes produce glycoconjugates that migrate in the 60–200 kDa range on SDS/PAGE. These molecules, called the F2/3 antigens, specifically bind lytic Ch anti-Gal and can be used to diagnose active disease (Almeida et al., 1993).

Here we present evidence that the glycoconjugates from cell-derived trypomastigotes that are recognized by Ch anti-Gal are mucin-like glycosyl-phosphatidylinositol (GPI)-anchored molecules bearing several oligosaccharide chains. These oligo-

Abbreviations used: anti-CRD, anti-(cross-reacting determinant) antibody; anti-Gal, anti-α-galactosyl antibodies; anti-Gal[+], anti-Gal-binding fraction; anti-Gal[−], anti-Gal-non-binding fraction; Ch, chronic-chagasic-patient serum; Gal_f, galactofuranose; Gal_p, galactopyranose; GIPL, glycoinositolphospholipid; GlcNAcol, *N*-acetylglucosaminitol; GPI, glycosyl-phosphatidylinositol; GU, glucose unit; NHS, normal human serum; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoproteins; LIT, liver infusion/tryptose; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium; Bt-PLC, *Bacillus thuringiensis* PLC; QAE, quaternary aminoethyl.

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saccharides are *O*-glycosidically linked mainly to Thr residues in the peptide backbone via *N*-acetylglucosaminyl units.

MATERIALS AND METHODS

Parasites

Trypanosoma cruzi Y and G (clone D11) strains were provided by Dr. Nobuko Yoshida and Dr. Renato Mortara (Escola Paulista de Medicina, São Paulo, Brazil) respectively. Parasites were maintained by alternative passages in mice and *in vitro* growth in liver infusion/tryptose (LIT) medium (Camargo, 1964) containing 10% fetal-calf serum (FCS). LLC-MK_2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% FCS. The cell-derived trypomastigotes were collected 6 or 7 days later, following their release from infected cells (Schenkman et al., 1991).

Purification of anti-Gal

Polyclonal IgG NHS and Ch anti-Gal were purified by affinity chromatography on Synsorb 115 resin (ChemBiomed, Edmonton, Canada), containing the trisaccharide Galα1-3Galβ1-4GlcNAc, and subsequently on Protein A–Sepharose, as previously described (Almeida et al., 1993).

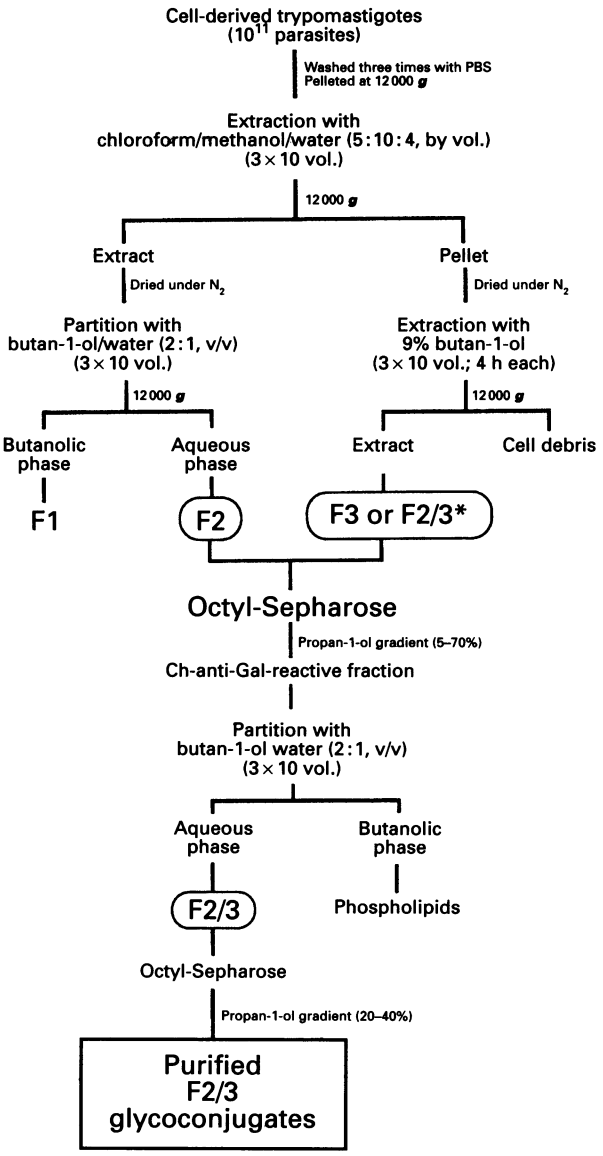
Preparation of αGal-containing GPI neutral glycan standards

Deaminated and NaB^3H_4-reduced GPI-anchor neutral glycans were prepared from *T. brucei* soluble-form variant surface glycoprotein (sVSG), variant 117 (MITat 1.4), and the individual glycoforms identified by Dionex high-performance anion-exchange chromatography as described previously (Ferguson et al., 1988; Ferguson, 1992).

Immunofluorescence of parasites and phospholipase C treatments

G strain (clone D11) trypomastigotes were treated with 500 μl of *Bacillus thurigiensis* phospholipase C (Bt-PLC) (500 μg/ml) (provided by Dr. T. L. Rosenberry, Case Western Reserve University, Cleveland, OH, USA), for 2 h at 37 °C in RPMI 1640 medium containing 10% FCS. Immunofluorescence experiments were carried out with purified Ch anti-Gal or NHS anti-Gal (20 μl; 5 μg/ml) using the biotin–streptavidin–fluorescein isothiocyanate system (GIBCO BRL; Life Technologies Inc.).

The extract containing the F2/3 complex (prior to octyl-Sepharose chromatography; see Scheme 1) was treated with 20 μl of Bt-PLC (500 μg/ml) in PBS for 4 h at 37 °C. The reaction was terminated by heating (100 °C, 3 min) and the products analysed by chemiluminescent Western blotting and immunocapture dot-blotting. Blotted products on poly(vinylidene difluoride) (PVDF) membranes (Millipore) were incubated with 5 μg/ml of either Ch anti-Gal or rabbit anti-CRD (cross-reacting determinant) antibodies (Cardoso de Almeida and Turner, 1983; Zamze et al., 1988). Further incubation steps and Western-blot development were carried out as previously described (Almeida et al., 1993). For immunocapture dot-blotting, the membranes were dotted with 2 μl of 10 μg/ml Ch anti-Gal, dried at 37 °C and blocked with 5% BSA in PBS for 1 h at room temperature. Bt-PLC-treated or control F2/3 glycoconjugates (2 μl) were then applied to each dot and allowed to dry. Membranes were washed with PBS containing 0.5% Tween 20, incubated with 5 μg/ml of anti-CRD and the reaction detected using the chemiluminescence method.



Scheme 1 Procedure for the purification of Ch-anti-Gal-reactive mucin-like glycoconjugates from cell-derived *T. cruzi* trypomastigotes

Starting from a wet-cell pellet, Ch-anti-Gal-reactive material was found in fractions F2 and F3; these fractions were combined. Starting from a freeze-dried cell pellet (*), Ch-anti-Gal-reactive material was found in a single fraction called F2/3. The combined F2 and F3 fractions and the F2/3 fraction were subsequently purified by two rounds of octyl-Sepharose chromatography.

Purification of F2/3 glycoconjugates from cell-derived trypomastigotes

T. cruzi trypomastigotes obtained from infected LLC-MK_2 cells were extracted with organic solvents as described by McConville and Blackwell (1991) (see Scheme 1). When wet cell pellets were used as starting material, Ch-anti-Gal-reactive material was recovered in fractions F2 and F3. SDS/PAGE and Western-blot analysis of these fractions showed that they contained Ch-anti-Gal-reactive material over the molecular-mass ranges 74–96 and 120–200 kDa for fractions F2 and F3 respectively (Almeida et al., 1993). Since both fractions were reactive with Ch anti-Gal, the F2 and F3 subfractions were combined (to form F2/3) and used in the subsequent purification steps. (Note: When freeze-

dried cells are used in the same protocol, all of the Ch-anti-Gal-reactive material appears in the F3 fraction.) The F2/3 glycoconjugates were freeze-dried and chromatographed on an octyl-Sepharose column (Pharmacia-LKB, Upsala, Sweden; 6 cm × 1.5 cm) pre-equilibrated with 5% propan-1-ol in 0.1 M ammonium acetate buffer, pH 7.2 (buffer A). The F2/3 sample in buffer A was applied to the column at a flow rate of 4 ml/h. The column was washed with 20 ml of buffer A and eluted with a propan-1-ol gradient (5–70%) over 100 ml at a flow rate of 12 ml/h. Fractions (0.8 ml) were assayed for Ch-anti-Gal-reactivity by chemiluminescence dot-blotting and for *myo*-inositol content by g.c.–m.s. The immunoreactive material from the octyl-Sepharose column was pooled, dried, and partitioned between water and butan-1-ol to remove any remaining phospholipids. The aqueous phase was freeze-dried, resuspended in 4 ml 20% propan-1-ol in ammonium acetate (0.1 M, pH 7.2) (buffer B) and re-applied to the octyl-Sepharose column (pre-equilibrated with buffer B). The column was washed with 20 ml of buffer B and eluted with a shallow propan-1-ol gradient (20–40%) over 40 ml at 12 ml/h. The fractions were assayed for Ch-anti-Gal- and 3C9-monoclonal-antibody-immunoreactivity by dot and Western blotting and for *myo*-inositol content by g.c.–m.s. (Figure 1). The 3C9 monoclonal antibody specifically recognizes sialic acid-containing epitopes in the Ssp-3 glycoconjugates of trypomastigotes (Andrews et al., 1987; Schenkman et al., 1991).

Amino acid and ethanolamine analyses

Amino acid and ethanolamine analyses were performed on samples containing approx. 30 pmol of *myo*-inositol using a Waters Pico-Tag system as previously described (Schneider et al., 1990; Ferguson, 1992).

G.c.–m.s. analyses

G.c.–m.s. analyses were performed on a Hewlett–Packard 5890–5970 gc.–m.s. system. The *myo*-inositol contents were measured using deuterated *myo*-[^{2}H]inositol as internal standard, as described by Smith et al. (1987). Monosaccharide compositions and methylation-linkage analyses were performed as previously described (Ferguson, 1992).

^{1}H-n.m.r. spectroscopy

One-dimensional ^{1}H-n.m.r. spectra of oligosaccharides were obtained at 500 MHz as previously described (Greis et al., 1992), using a Bruker AM 500 spectrometer. Chemical shifts were referenced externally to acetone [chemical shift (δ) 2.225 p.p.m. at 300 K].

Galactose oxidase/NaB^{3}H$_4$ labelling

Purified F2/3 glycoconjugates (equivalent to 2.5 nmol of *myo*-inositol) were labelled with *Dactylium dendroides* galactose oxidase/NaB^{3}H$_4$ as previously described (McConville et al., 1990). The [^{3}H]Gal-labelled F2/3 glycoconjugates were re-purified from radioactive contaminants by octyl-Sepharose chromatography using the 20–40% propan-1-ol gradient as described above.

Reductive β-elimination of *O*-linked oligosaccharides

β-Elimination of [^{3}H]Gal-labelled and/or unlabelled F2/3 glycoconjugates was performed according to a modification of the protocol of Hanover et al. (1987). Briefly, F2/3 glycoconjugates were freeze-dried, resuspended in 250 μl of 0.1 M NaOH containing 1 M NaB^{2}H$_4$ and incubated for 24 h at 37 °C. The samples were neutralized with 250 μl of 1 M acetic acid and desalted in 0.6 ml of AG 50-X12(H$^+$) resin (Bio-Rad). Borate was removed by two evaporations with 250 μl of 5% acetic acid in methanol, two evaporations with 250 μl of methanol and two evaporations with 50 μl of toluene.

Anti-Gal–Sepharose-binding studies

Galactose-oxidase-labelled F2/3 oligosaccharides (10–20000 c.p.m.) were fractionated on Ch-anti-Gal–Sepharose and NHS-anti-Gal–Sepharose columns (0.2 ml of resin; 10 mg of IgG/ml). Samples (10 μl) were diluted in 0.1 M ammonium acetate buffer, pH 7.2 (90 μl), and applied and re-applied five times to each column. After washing with 2 ml of ammonium acetate buffer, the columns were eluted with 2 ml of 1 M propionic acid. An aliquot (50 μl) of each fraction was used for estimating the radioactivity, and the remaining material was freeze-dried. The Ch anti-Gal[+] and Ch anti-Gal[−] fractions were analysed by Bio-Gel P-4, as described below.

Radiolabelled GPI neutral glycans (800000 c.p.m.) from sVSG variant 117 were fractionated only on Ch-anti-Gal–Sepharose (0.8 ml; 10 mg/ml), since no reactivity with NHS anti-Gal was observed either with the intact sVSG molecules or the isolated glycans. Samples (40 μl) were diluted in 0.1 M ammonium acetate buffer, pH 7.2 (360 μl), and applied and re-applied five times to the column. After washing with 8 ml of ammonium acetate buffer, the column was eluted with 8 ml of 1 M propionic acid. An aliquot (50 μl) of each fraction was used for estimating the radioactivity, and the remaining material was freeze-dried. The Ch anti-Gal[+] fractions of the GPI neutral glycans were identified by Dionex high-performance anion-exchange chromatography, as described by Ferguson (1992).

Anion-exchange-resin-binding studies

Galactose-oxidase-labelled oligosaccharides (10000 c.p.m.) were applied on a column of 0.2 ml of AG 3-X4(OH-) resin over 0.1 ml of quaternary aminoethyl (QAE) -Sephadex A-25 (Pharmacia-LKB). The unbound (neutral) oligosaccharides were washed from the column with 3 ml of deionized water and the bound oligosaccharides eluted with 3 ml 1 M NaCl. Washing and elution fractions of 0.5 ml were collected and counted for radioactivity. Same protocol was used for analysing the samples treated with *Arthrobacter ureafaciens* sialidase (details of the enzymic treatment are given below).

Release of O-linked oligosaccharides by hydrazinolysis and NaB^{3}H$_4$ reduction

O-linked oligosaccharides were released from F2/3 glycoconjugates by mild hydrazinolysis (60 °C for 5 h; Patel et al., 1993) using an Oxford Glycosystems Module 1 apparatus. The released oligosaccharides were subsequently labelled by reduction with NaB^{3}H$_4$ as previously described (Schneider et al., 1993).

Bio-Gel P-4 gel filtration and exoglycosidase digestions

^{3}H-labelled glycans (10–15000 c.p.m.) dissolved in 0.2 ml of water were loaded on to a 1 m × 1.5 cm Bio-Gel P-4 (minus 400 mesh) column, maintained at 55 °C, and eluted with water at 0.2 ml/min. Fractions (1 ml) were collected, and the radiolabelled glycans detected by scintillation counting of aliquots. The elution positions of the F2/3 radioactive neutral oligosaccharides were

expressed in 'glucose units' (GU) as previously described (Yamashita et al., 1982).

Digestion with coffee-bean α-galactosidase (Boehringer) was performed in 30 μl of 100 mM sodium citrate/phosphate buffer, pH 6.0, containing 0.75 unit of enzyme, for 16 h at 37 °C. Bovine testis β-galactosidase (Boehringer) digestion was performed with 8.5 munits of freshly prepared enzyme in 30 μl of 100 mM sodium citrate/phosphate buffer, pH 4.5, for 16 h at 37 °C. The reactions with both galactosidases were terminated by heating (100 °C, 3 min) and the products desalted by passage through 0.2 ml of AG 50-X12(H^+) resin over 0.2 ml of AG 3-X4(OH^-) resin over 0.1 ml QAE-Sephadex A-25.

Digestion with Newcastle-disease-virus sialidase (Oxford Glycosystems) was carried out with 5 munits of enzyme in 25 μl of 50 mM sodium acetate, pH 5.5, for 18 h at 37 °C. *A. ureafaciens* sialidase (Oxford Glycosystems) digestion was performed with 40 munits of enzyme in 40 μl of 100 mM sodium acetate, pH 5.0, for 18 h at 37 °C.

The reactions were terminated by heating (100 °C, 3 min) and the products desalted in AG 50-X12(H^+) resin, drying, and two evaporations with 50 μl of toluene.

Acid hydrolysis and re-N-acetylation

Strong-acid hydrolysis of ^{3}H-labelled oligosaccharides (4–8000 c.p.m.) and unlabelled standards (10 μg) was carried out by heating the sample for 2 h at 100 °C in 200 μl of 2 M trifluoroacetic acid (McConville et al., 1990). Re-N-acetylation was performed as previously described (Ferguson, 1992).

T.l.c.

Labelled oligosaccharides (5000 c.p.m.) were chromatographed on aluminium-backed silica-gel 60 high-performance t.l.c. sheets (Merck) using butan-1-ol/acetone/water (6:5:4, by vol.) as the solvent system. Labelled glycans were detected by fluorography after the sheets had been sprayed with En^{3}Hance spray (du Pont-New England Nuclear). Unlabelled glycans were stained with orcinol/H_2SO_4 reagent.

Chemiluminescence e.l.i.s.a.

To compare the reactivity with F2/3 of purified Ch as compared with NHS anti-Gal, a chemiluminescent e.l.i.s.a. was used as described previously (Almeida et al., 1993).

RESULTS

Purification and composition of Ch-anti-Gal-reactive glycoconjugates

The purification procedure used in the present study is shown in Scheme 1. The final octyl-Sepharose purification step is shown in Figure 1. The profile shows a broad peak of *myo*-inositol-containing material which contains two peaks of material that is immunoreactive both with polyclonal Ch anti-Gal and monoclonal 3C9 antibodies. Analysis of the peak fractions of Western blotting using Ch anti-Gal indicated that both peaks contained similar immunoreactive material of molecular mass 60–220 kDa (results not shown). Since the objective of this study was to identify the Ch anti-Gal epitope(s), the two immunoreactive peaks were pooled. Analysis of the combined material by Western blot showed that the same profile of molecules was reactive with Ch anti-Gal and 3C9 monoclonal antibody (Figure 1, insert). The combined material was analysed for amino acid, ethanolamine, carbohydrate and *myo*-inositol content (Table 1). The material was found to be rich in carbohydrate (Gal, Man, GlcNAc, sialic acid, Glc and Xyl) and certain amino acids (Thr,

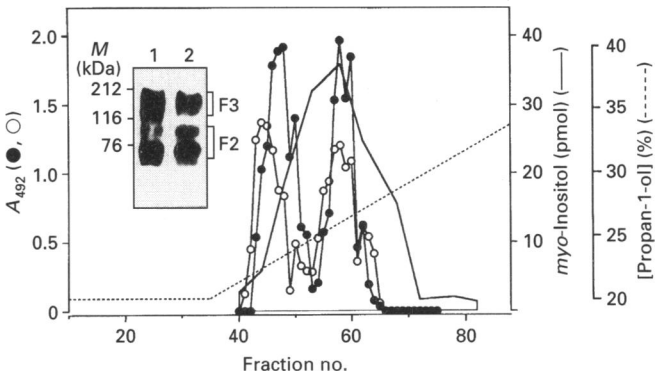


Figure 1 Fractionation of F2/3 glycoconjugates on octyl-Sepharose

The second octyl-Sepharose chromatography step (described in Scheme 1) was performed using a 20–40% propan-1-ol gradient. The peaks eluted at 23% and 26% propan-1-ol correspond to glycoconjugates recognized by Ch anti-Gal (●) and 3C9 monoclonal (○) antibodies. The one-peak central area (——, no symbol) represents the *myo*-inositol contents in pmol/fraction. In the insert immunoreactive peaks were pooled and analysed by chemiluminescence Western-blotting with Ch anti-Gal (lane 1) and 3C9 monoclonal antibody (lane 2) (*M* is molecular mass).

Table 1 Compositional analysis of F2/3 glycoconjugates from cell-derived trypomastigotes

Component	Amount (mol*†)	
	No treatment	Post β-elimination
Asx	5.3 (±0.4)	5.2
Glx	7.9 (±0.6)	7.2
Ser	5.5 (±0.8)	2.4
Gly	5.0 (±0.4)	9.8
Hys	0.5 (±0.1)	1.1
Arg	1.5 (±0.6)	1.7
Thr	11.9 (±1.0)	5.8
Ala	4.9 (±0.4)	5.7
Pro	5.5 (±0.4)	5.5
Tyr	0.4 (±0.1)	0.9
Val	1.8 (±0.2)	2.5
Met	0.0	0.0
Ile	1.1 (±0.1)	1.3
Leu	2.2 (±0.2)	2.9
Phe	0.9 (±0.1)	1.2
Lys	2.9 (±0.2)	3.1
Ethanolamine	0.8	1.1
Myo-Inositol	1.0	1.0
Galactose	15.0	16.7
Mannose	7.8	8.5
GlcNAc	5.1	1.4
GlcNAcol	0.0	4.1
Sialic acid	4.5	5.4
Glucose	5.7	6.1
Xylose	3.1	3.7
$C_{16:0}$ MAG‡	+	+

* The values were normalized with respect to *myo*-Inositol.

† Amino acid values for the untreated sample represent the mean values of three determinations (±S.D.); all other amino acid and carbohydrate values are means of two determinations.

‡ $C_{16:0}$ MAG (monoalkylglycerol) was not quantified.

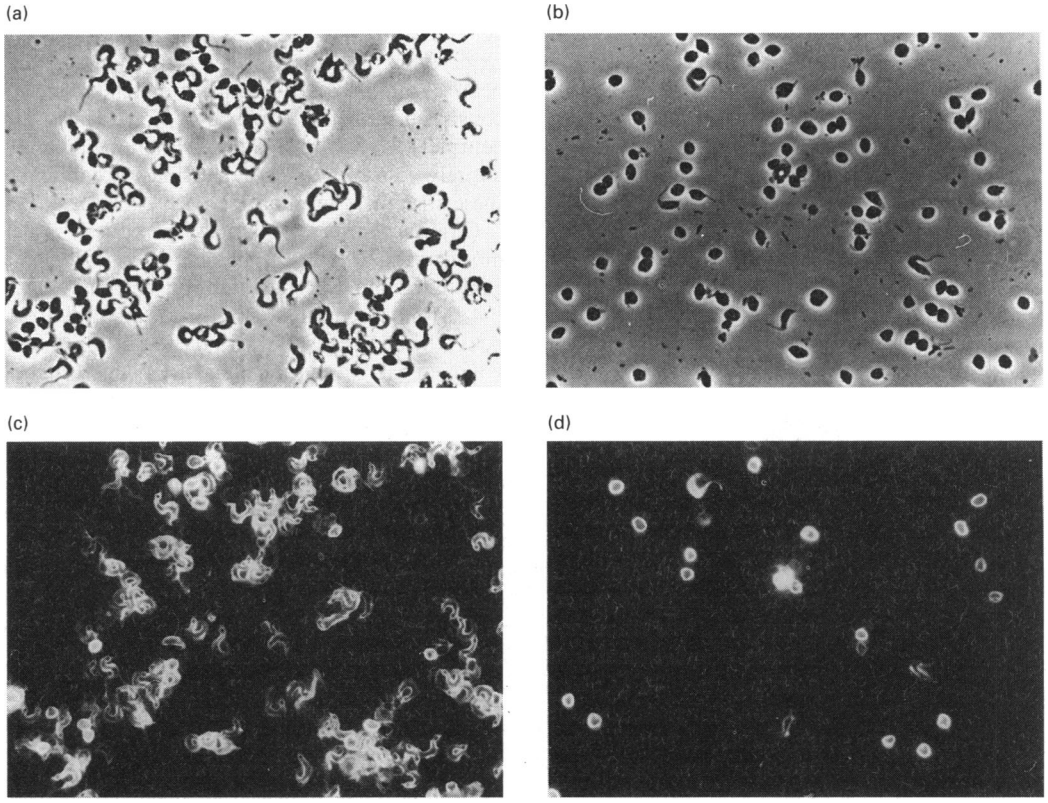


Figure 2 Effect of bacterial PI-PLC treatment on cell-derived trypomastigotes

Living-cell-derived trypomastigotes (**a**) were treated with bacterial PI-PLC (**b**). Parasites were fixed, stained with Ch anti-Gal antibodies and fluorescein isothiocyanate-labelled second antibody and analysed by fluorescence microscopy: (**c**) original trypomastigotes; (**d**) phospholipase-C treated cells. Only a small proportion of cells was still reactive with anti-Gal after PLC treatment.

Glx, Ser, Pro, Asx, Gly and Ala) and to contain *myo*-inositol and ethanolamine in a molar ratio of approx. 1:1. These latter components, together with lipid in the form of monoalkylglycerol (1-*O*-hexadecylglycerol), suggested the presence of GPI-anchored glycoconjugates.

The antigens recognized by Ch anti-Gal are GPI-anchored

Most cell-derived trypomastigotes react strongly with Ch anti-Gal, but not with NHS anti-Gal (Almeida et al., 1991; Souto-Padron et al., 1994). It has been previously noted that treatment of these cells with Bt-PLC drastically modified trypomastigote morphology, producing rounded cells (J. Ramalho-Pinto, personal communication). This observation was confirmed here (Figure 2). In addition, treatment with PI-PLC almost completely abolished binding of Ch anti-Gal to the parasites. Only a few (~ 20%) of the morphologically modified trypomastigotes still reacted with Ch anti-Gal. These results show that most of the antigens recognized by Ch anti-Gal on the trypomastigote cell surface are PI-PLC-sensitive and are therefore probably GPI-anchored.

Consistent with the above result, partially purified F2/3 glyconconjugates digested with PI-PLC were rendered hydrophilic and could no longer bind to the PVDF membrane (Figure 3a). In addition, the PI-PLC-digested antigens were shown to react with anti-CRD antibodies in an immunocapture assay on immobilized Ch anti-Gal (Figure 3b). Anti-CRD antibodies are specific for the GlcN1-6*myo*-inositol 1,2-cyclic phosphate epitope that is unique to PI-PLC-cleaved GPI membrane anchors (Zamze et al., 1988).

Taken together, these data demonstrate that the majority of the Ch anti-Gal reactive molecules were GPI-anchored glycoconjugates.

F2/F3 glycoconjugates contain GlcNAc-*O*-Thr/Ser linkages

A sample of purified F2/3 were subjected to reductive β-elimination and analysed for carbohydrate and amino acid content. The composition was similar to that of the starting material (Table 1), except that GlcNAc was substantially replaced by *N*-acetylglucosaminitol (GlcNAcol), and the Thr (mostly) and Ser contents were substantially reduced. Under the conditions used, only O-glycosidic linkages to Thr and/or Ser residues should be cleaved (Hanover et al., 1987). These data suggest that the majority of the GlcNAc of the F2/3 fraction is involved in O-glycosidic linkage to Thr and Ser residues in the polypeptide.

Galactose oxidase labelling and fractionation of the O-linked oligosaccharides

An aliquot of the purified F2/3 material was labelled with galactose oxidase/NaB^3H_4 and subjected to reductive β-elimination. Aliquots of this material were used for anti-Gal–Sepharose and anion-exchange-resin binding studies (Table 2). Over half of the labelled oligosaccharides bound to Ch-anti-Gal–Sepharose, whereas only 1% bound to NHS-anti-Gal–Sepharose. When the intact molecules before β-elimination were

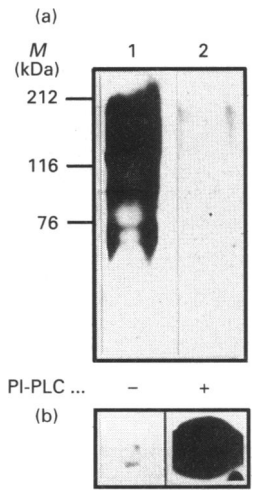


Figure 3 Treatment of the F2/3 complex with bacterial PI-PLC

(**a**) The crude F2/3 complex (prior to octyl-Sepharose chromatography) (lane 1) was treated with bacterial PI-PLC (lane 2) and analysed by SDS/PAGE and Western blotting with Ch anti-Gal. The lack of immunoreactive material in lane 2 was thought to be due to inability of the delipidated PI-PLC-treated material to bind to PVDF membrane. (**b**) Immunocapture dot-blot with immobilized Ch anti-Gal antibodies on PVDF membrane. The untreated (–) and PI-PLC-treated (+) crude F2/3 complex was incubated with the immobilized Ch anti-Gal antibodies and subsequently probed for reactivity with anti-CRD antibodies. *M* is molecular mass.

tested for reactivity with Ch-anti-Gal–Sepharose we observed that 90% of them bound to the antibody, suggesting that most of them contained α-Gal-terminating oligosaccharides (results not shown). Both the Ch-anti-Gal-binding fraction (Ch anti-Gal[+]) and the Ch-anti-Gal-non-binding fraction (Ch anti-Gal[–]) contained acidic material. Some of this material was rendered neutral by exhaustive neuraminidase treatment; however, substantial amounts of neuraminidase-resistant acidic material were observed in both fractions (see below).

The total β-eliminated material and the Ch anti-Gal[+] and Ch anti-Gal[–] subfractions were chromatographed on Bio-Gel P-4 (Figure 4). Both subfractions contained material eluting in the void volume and an array of overlapping peaks between 5 and 12 GU. A prominent 4.5 GU peak was found only in the Ch anti-Gal[+] fraction (Figure 4b). The Ch anti-Gal[+] void-volume material was treated with neuraminidase and re-chromatographed on Bio-Gel P-4. Some neutral species in the 5–12 GU range were observed, but the majority of the label remained in the void volume and no further 4.5 GU material was generated (results not shown). Treatment of the galactose oxidase/NaB^3H_4-labelled Ch anti-Gal[+] fraction with coffee-bean α-galactosidase produced labelled free Gal (Figure 4b), consistent with the presence of non-reducing αGal termini. In contrast, the Ch anti-Gal[–] fraction (Figure 4c) was almost entirely resistant to α-galactosidase treatment, consistent with these structures terminating with β-Gal residues.

Oligosaccharides obtained by reductive β-elimination were mixed with a trace of galactose oxidase/NaB^3H_4-labelled β-eliminated oligosaccharides, and chromatographed on Bio-Gel P-4. Monosaccharide composition analysis of the void-volume peak, the 5–12 GU region and the 4.5 GU showed that the void-volume peak was enriched in Man, which suggested the possible presence of N-linked oligosaccharides, and that the included peaks contained only Gal and GlcNAcol. The Glc and Xyl content of the starting material (Table 1) did not appear to be eluted from the Bio-Gel P-4 column.

In summary, these data showed that the β-eliminated F2/3 material contained the following: (1) a small (4.5 GU) Ch anti-Gal[+] oligosaccharitol that was not found in a sialylated form; (2) a group of oligosaccharitols in the 5–12 GU size range, some of which terminated in αGal and were Ch anti-Gal[+] and some of which (mostly from the Ch anti-Gal[–] fraction) were found in a sialylated form; (3) a group of Ch anti-Gal[+] and Ch anti-Gal[–] acidic structures that were neuraminidase-resistant. This latter fraction was not studied further.

Release of O-linked oligosaccharides by mild hydrazinolysis

The released oligosaccharides were reduced with NaB^3H_4 and separated by Bio-Gel P-4 chromatography (results not shown). The profile was qualitatively similar to that observed with the galactose oxidase-labelled oligosaccharides shown in Figure 4(a). The hydrazinolysis/reduction procedure introduces one ^3H label per oligosaccharide and therefore gives a more accurate representation of the molar ratios of the individual oligosaccharides than does galactose-oxidase labelling. Using the hydrazinolysis/reduction-labelled oligosaccharides, it was found that 40% of them bound to the Ch anti-Gal affinity column and that the 4.5 GU peak represented 8% of the total oligosaccharides in molar terms.

Structure of the 4.5 GU oligosaccharide

The 4.5 GU oligosaccharitol, obtained by hydrazinolysis followed by NaB^3H_4 reduction and Bio-Gel P-4 purification, was subjected to strong acid hydrolysis and re-N-acetylation. The product co-chromatographed with authentic GlcNAcol on high-

Table 2 Fractionation of galactose oxidase-labelled β-eliminated oligosaccharides on anti-Gal-Sepharose and anion-exchange resin

Fraction	Proportion of total label (%)	Neutral fraction (%)	Acidic fraction (%)	
			Neuraminidase-sensitive	Neuraminidase-resistant
Total material	100	50	16	34
Ch anti-Gal[+]*	56	56	9	35
Ch anti-Gal[–]	44	44	26	30
NHS anti-Gal[+]	1	n.d.†	n.d.	n.d.
NHS anti-Gal[–]	99	n.d.	n.d.	n.d.

* [+] and [–] indicate bound and unbound fractions.
† n.d., not determined.

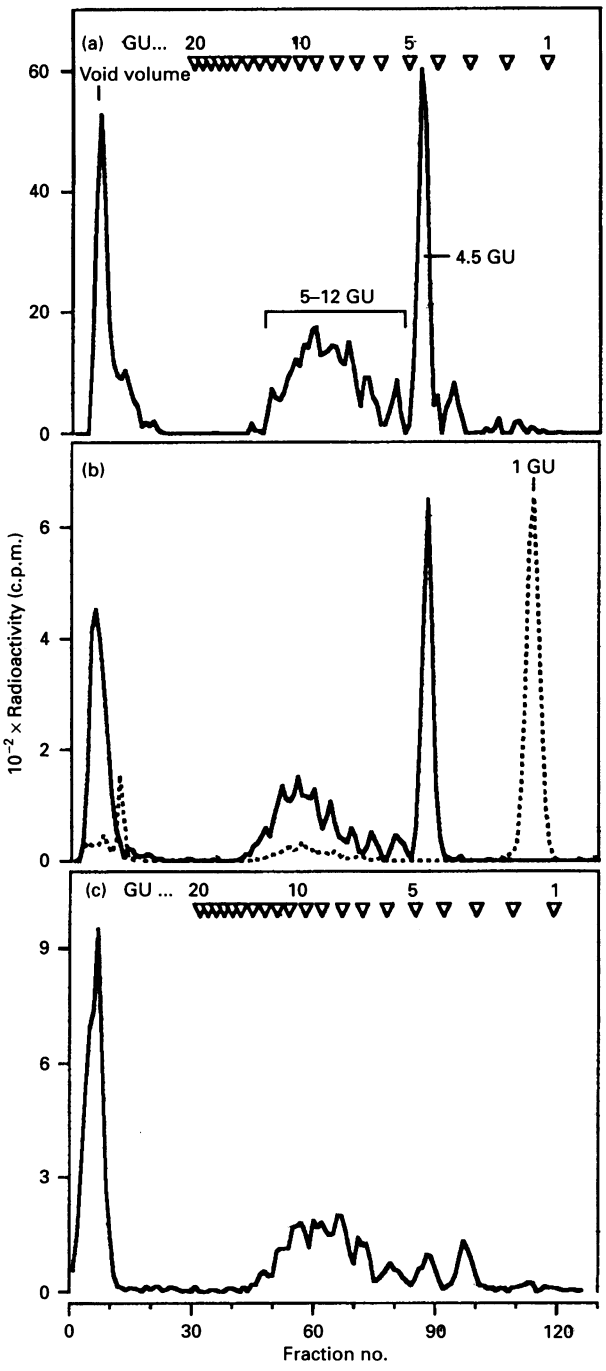


Figure 4 Bio-Gel P-4 chromatography of galactose oxidase/NaB^3H_4-labelled oligosaccharitols released from F2/3 by β-elimination

(**a**) Total oligosaccharitol fraction. (**b**) Oligosaccharitol fraction that bound to immobilized Ch anti-Gal antibodies (——); Ch anti-Gal binding fraction after treatment with coffee bean α-galactosidase (---). (**c**) Oligosaccharitol fraction that did not bind to immobilized Ch anti-Gal antibodies.

performance t.l.c. (results not shown), confirming the identity of the reduced terminus. Since GlcNAcol has a size of 2.5 GU on Bio-Gel P-4 (Yamashita et al., 1982), the size of the 4.5 GU peak suggested that it was a trisaccharide. Treatment with coffee-bean α-galactosidase generated a product that co-chromatographed with authentic Galβ1-4GlcNAcol on high-performance t.l.c.

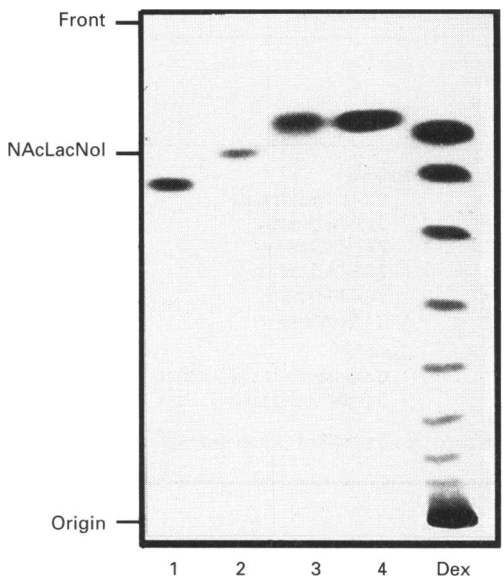


Figure 5 Sequencing of the 4.5 GU oligosaccharide by high-performance t.l.c.

Lane 1, untreated NaB^3H_4-labelled 4.5 GU oligosaccharide; lanes 2 and 3, labelled 4.5 GU oligosaccharide digested with coffee-bean α-galactosidase and freshly prepared bovine testis β-galactosidase respectively; lane 4, [^{3}H]GlcNAcol standard; Dex, NaB^3H_4-reduced dextran hydrolysate; NAcLacNol, unlabelled *N*-acetyl-lactosaminitol standard.

Further digestion of this product with freshly prepared bovine testis β-galactosidase generated a compound that co-migrated with authentic GlcNAcol (Figure 5).

The anomeric region of the ^{1}H-n.m.r. spectrum of the 4.5 GU oligosaccharide (results not shown) suggested the presence of a β-Gal residue (H-1 at 4.58 p.p.m., J 1,2 = 8.7 Hz), an α-Gal residue (H-1 at 5.16 p.p.m., J 1,2 = 3.6 Hz) and *N*-acetamido protons at 2.1 p.p.m. Methylation analysis showed derivatives consistent with non-reducing terminal galactopyranose (Gal*p*), 3-O-substituted Gal*p* and 4-*O*-substituted GlcNAcol (Table 3). Taken together, these data define the 4.5 GU oligosaccharide as Galα1-3Galβ1-4GlcNAcol.

Partial characterization of the 5–12 GU oligosaccharides

Methylation analysis of the 5–12 GU region revealed a mixture of derivatives (Table 3). The most notable feature is the presence of 4-O-substituted GlcNAcol and 4,6-di-O-substituted GlcNAcol in a ratio of about 1:3. This suggests that the majority of these structures are branched at the reducing terminus. The oligosaccharitols appeared, both from the compositional data and from the methylation analysis, to contain only Gal residues in addition to their GlcNAcol reducing termini. The presence of some 2,6-di-O-substituted Gal suggested that some of the structures were also branched elsewhere in the chain.

Comparison of the Ch anti-Gal and NHS anti-Gal reactivity with F2/3 glycoconjugates

The trypanolytic activity of Ch anti-Gal was tested before and after absorption of the antibodies on immobilized F2/3 glycoconjugates obtained from cell-derived trypomastigotes. More than 90% of the lytic activity of Ch anti-Gal was absorbed (results not shown). Binding studies with immobilized F2/3 in

Table 3 Partially methylated alditol acetates (PMAAs) derived from F2/3 neutral oligosaccharides

PMAA derivative	Origin	Oligosaccharide fraction...	Molar Ratio* 5–12 GU	Molar Ratio* 4.5 GU
Galactitol				
2,3,4,6-Tetra-*O*-methyl	Terminal Gal*p*		4.7	1.2
3,4,6-Tri-*O*-methyl	2-*O*-Substituted Gal*p*		0.8	–
2,4,6-Tri-*O*-methyl	3-*O*-Substituted Gal*p*		1.9	0.9
2,3,6-Tri-*O*-methyl	4-*O*-Substituted Gal*p*		1.4	–
2,3,4-Tri-*O*-methyl	6-*O*-Substituted Gal*p*		0.7	–
3,4-Di-*O*-methyl	2,6-Di-*O*-substituted Gal*p*		1.0	–
GlcNAcol				
3,6-di-*O*-Methyl-1,5-di-*O*-acetyl	4-*O*-Substituted GlcNAcol		1.1	0.8
3-*O*-Methyl-di-*O*-acetyl	4,6-Di-*O*-substituted GlcNAcol		3.2	–

* The molar ratio of the 3-*O*-substituted Gal*p* derivative was corrected using an empirically derived molar response factor from the analysis of a standard (lacto-*N*-tetraose) containing this derivative.

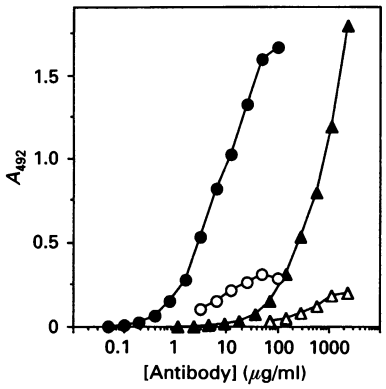


Figure 6 Reactivity of the F/3 complex with Ch anti-Gal and NHS anti-Gal antibodies

Microtitre plates coated with F2/3 were probed with a range of Ch anti-Gal (●) and NHS anti-Gal (▲) antibody concentrations. The intensity of the reactions was determined by chemiluminescent e.l.i.s.a. The open symbols represent the same experiment following prior treatment of the coated plates with coffee-bean α-galactosidase.

chemiluminescence e.l.i.s.a. showed that the affinity of Ch anti-Gal for these glycoconjugates was about 100-fold that of NHS anti-Gal (Figure 6). As expected, treatment of the F2/3 glycoconjugates with α-galactosidase abolished almost all of the immunoreactivity. These data show that, although both antibodies were immunopurified on the same Synsorb 115 column, the Ch anti-Gal antibodies had quite different specificity compared with NHS anti-Gal antibodies with respect to the *T. cruzi* antigens.

Specificities of the Ch anti-Gal antibodies

Both Ch anti-Gal and NHS anti-Gal were purified on Synsorb 115 and both, therefore, bind to the epitope Galα1-3Galβ1-4GlcNAc1-*O*-R. However, only Ch anti-Gal could bind the corresponding reduced oligosaccharitol, Galα1-3Galβ1-4GlcNAcol. Similarly, only Ch anti-Gal bound several αGal-containing structures from the GPI anchor of *T. brucei* VSG used in this study and the glycoinositol phospholipids of *Leishmania major* (Almeida et al., 1993). These data are summarized in Table 4.

DISCUSSION

Anti-Gal antibodies obtained from patients with chronic Chagas' disease bind to 72 kDa glycoproteins in metacyclic trypomastigotes obtained from axenic cultures and cause complement-mediated lysis (Almeida et al., 1991; Travassos et al., 1993). In contrast, the lysis of cell-derived trypomastigotes by the

Table 4 Binding of Ch and NHS anti-Gal to different αGal-containing epitopes

Epitope	Origin	Anti-Gal binding Ch	Anti-Gal binding NHS
Gal*p*α1,3Gal*p*β1,4GlcNAcβ1-*O*-R	Synsorb 115; CPH‡	+	+
Gal*p*α1,3Gal*p*β1,4GalNAcol	F2/3 (Ssp-3) (*T. cruzi*)	+	–
Gal*p*α1,2Gal*p*α1,6Gal*p*α1,3[Man_3AHM]*	VSG 117 (*T. brucei*)	+	–
Gal*p*α1,2Gal*p*α1,6(Gal*p*α1,2)Gal*p*α1,3[Man_3AHM]	VSG 117 (*T. brucei*)	+	–
Gal*p*α1,6(Gal*p*α1,2)Gal*p*α1,3[Man_3AHM]	VSG 117 (*T. brucei*)	+	–
Gal*p*α1,3Gal*f*β1,3Man_2GlcNH_2-PI†	GIPL-2 (*L. major*)	+	–
Gal*p*α1,6Gal*p*α1,3Gal*f*β1,3Man_2GlcNH_2-PI	GIPL-3 (*L. major*)	+	–

* AHM, Anhydromannitol.
† $GlcNH_2$-PI, glucosamine-phosphatidylinositol.
‡ CPH, ceramide pentahexoside from rabbit erythrocytes.

same antibodies proceeds via the intense agglutination of the parasites and is complement-independent. The targets for antibody binding on cell-derived trypomastigotes were identified by Western blotting as a polydisperse complex of molecules migrating as a broad smear between 60 and 200 kDa on SDS/PAGE. According to the extraction conditions used, these molecules can be fractionated into low- and high-apparent-molecular-mass groups called F2 and F3 (Almeida et al., 1993) or they can be combined into a single fraction called F2/3 (Scheme 1). These fractions have been used successfully in immunoassays to diagnose active Chagas' disease (Almeida et al., 1993; Travassos and Almeida, 1993). The cell-derived trypomastigote forms mostly resemble the bloodstream-form trypomastigotes found *in vivo*. This study was undertaken to characterize the target antigens in cell-derived trypomastigotes and the epitopes recognized by the Chagasic anti-Gal antibodies.

Almost all of the Ch-anti-Gal-binding sites on the trypomastigote surface could be removed by *B. thuringiensis* PI-PLC. Treatment with PI-PLC also caused most of the cells to round up; however, the significance of this is unclear. Solvent extraction of trypomastigotes, followed by hydrophobic-interaction chromatography, revealed a group of *myo*-inositol-containing molecules and subsets of these, in the form of two peaks that bound Ch anti-Gal. They were also reactive with the monoclonal antibody 3C9 (Andrews et al., 1987; Schenkman et al., 1991), but the two antibodies did not compete with each other in e.l.i.s.a. assays (results not shown), demonstrating that their respective epitopes are different. They could, however, be attached to the same polypeptide backbone(s). Presumably the molecules in the two peaks differ in their hydrophobic lipid moieties (hence their separation on octyl-Sepharose), but contain similar glycoprotein moieties (giving rise to co-migration and antibody staining on Western blots). The two peaks were pooled and the F2/3 antigenic complex was rendered hydrophilic and reactive with anti-CRD antibodies by treatment with PI-PLC. These data define the Ch anti-Gal target molecules as a complex of GPI-anchored glycoconjugates.

Judging from compositional analyses, the F2/3 complex is similar to the 35/50 kDa antigen of *T. cruzi* metacyclic trypomastigotes (Schenkman et al., 1993) and the previously described 38/43 kDa glycoproteins of *T. cruzi* epimastigotes (Previato et al., 1985, 1994). All of these groups of molecules appear to be mucin-like in that they contain large amounts of Thr, Ser, Asx, Glx, Gly and Pro, together with O-linked oligosaccharides rich in GlcNAc, Gal and sialic acid. Both mucin-like antigens, the 35/50 kDa component of metacyclic forms and the F2/3 complex of trypomastigotes (equivalent to the glycoconjugate family carrying the 3C9-specific Ssp3 epitope) are GPI-anchored molecules, being also the major sialic acid acceptors in trans-sialidase reactions (Schenkman et al., 1991, 1993). There are clear structural differences in the molecules produced by metacyclics and epimastigotes versus trypomastigotes, as judged by their behaviour on SDS/PAGE. Some differences in the oligosaccharide side chains can be inferred from antibody reactivities. For example, the trypomastigote F2/3 complex reacts with Ch anti-Gal and the 3C9 monoclonal antibody, whereas the metacyclic 35/50 kDa antigen does not react with either. How many different underlying polypeptide chains are involved in these families of molecules is unknown.

In mammalian mucins the carbohydrate–protein linkage tends to be GalNAcα1-*O*-Thr (or Ser). However, GalNAc has not been described in *T. cruzi*, and the carbohydrate-protein linkage sugar for the epimastigote 38/43 kDa glycoproteins has been recently identified as GlcNAc (Previato et al., 1994). The results reported here for the trypomastigote F2/3 complex confirm this observation and further suggest that the linkage is mostly GlcNAc1-*O*-Thr. The attachment of GlcNAc to Ser residues in cytoplasmic and nuclear proteins is well documented in mammalian cells (Holt and Hart, 1986; Hanover et al., 1987; Haltiwanger et al., 1992) and in protozoan parasites (Handman et al., 1993). The attachment of larger oligosaccharides to cell surface proteins via O-linked GlcNAc, however, is unusual. An O-linked βGal-GlcNAc disaccharide has been reported in the malarial parasite *Plasmodium falciparum*, but the cellular location of the glycoprotein(s) bearing this structure is unknown (Dieckmann-Schuppert et al., 1993).

The O-GlcNAc linked oligosaccharides released from F2/3 by reductive β-elimination were a complex mixture of structures. The smallest (4.5 GU) structure was the trisacharide Galα1-3Galβ1-4GlcNAcol. The larger (5–12 GU) structures contained both 4-substituted GlcNAcol and 4,6-disubstituted GlcNAcol termini, together with non-reducing terminal Gal residues and 2-, 3-, 4- and 6-substituted and 2,6-disubstituted Gal residues. Unlike the trisaccharide, some of these structures can exist in a sialylated form. Owing to the limiting quantities of material purified from cell-derived trypomastigotes, these more complex (mostly branched) structures were not solved. However, the same proportion of these oligosaccharides were sensitive to Newcastle-disease-virus neuraminidase as well as to *A. ureafaciens* neuraminidase. The former enzyme is specific for α2-3-linked sialic acid, whereas the latter will remove α2-3-, α2-6- and α2-8-linked sialic acid. Thus all of the neuraminidase sensitive sialic acid residues in the F2/3 complex appear to be α2-3-linked. This would be consistent with their addition to the F2/3 oligosaccharides by *T. cruzi* trans-sialidase, an enzyme that catalyses the transfer of sialic acid in α2-3 linkage from host glycoconjugates to the parasite surface (Schenkman et al., 1991). In addition to the structures described above, a peak of galactose oxidase-labelled carbohydrate enriched in Man, and resistant to neuraminidase, was found in the void volume of the Bio-Gel P-4 chromatogram. This material was not studied further, but, judging from the Man content, it might represent N-linked oligosaccharides still linked to polypeptide and/or released neuraminidase-resistant acidic oligosaccharides. The specificities of the polyclonal human Chagasic anti-Gal antibodies were examined in this study. The specificity of these antibodies for *T. cruzi* trypomastigote structures was evident from e.l.i.s.a. studies using immobilized F2/3 antigens. NHS anti-Gal bound poorly to these antigens, whereas Ch anti-Gal binding to F2/3 was about 100-fold higher. In both cases binding was abrogated by treating the antigens with coffee-bean α-galactosidase. Both anti-Gal preparations were purified on Synsorb 115 resin, which contains Galα1-3Galβ1-4GlcNAc1-*O*-R. However, only the Ch anti-Gal antibodies were able to bind the reduced Galα1-3Galβ1-4GlcNAcol derivative. In addition, Ch anti-Gal antibodies could bind a range of αGal-terminating (non-*T. cruzi*) structures, whereas NHS anti-Gal did not. Thus it would appear that Ch anti-Gal can bind to structures terminating in Galα1-2Gal, Galα1-3Gal, Galα1-6Gal and Galα1-3Gal*f* (where Gal*f* is galactofuranose). Similar conclusions have been reported by Avila et al. (1989, 1991, 1992). This suggests that Ch anti-Gal has little specificity for the subterminal units or longer oligosaccharides linked to the terminal non-reducing αGal residues. Presumably the novel and highly heterogeneous *T. cruzi* O-linked structures are highly immunogenic and induce antibodies that are specific for the terminal αGal components. An increased binding of Ch anti-Gal to these structures in comparison with the natural anti-Gal results in intense agglutination and lysis of cell-derived trypomastigotes, a defense mechanism that is probably effective *in vivo*.

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