ENDOGENOUS AND SURFACE LABELING OF GLYCOCONJUGATES FROM THE THREE DIFFERENTIATION STAGES OF *TRYPANOSOMA CRUZI*

Bianca ZINGALES, Nuncio F. MARTIN, Rosa M. DE LEDERKREMER* and Walter COLLI

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, C.P. 20780, São Paulo, Brazil and **Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina*

Received 5 February 1982; revision received 8 April 1982

1. Introduction

We have been studying the glycoconjugates from epimastigote forms of *Trypanosoma cruzi* since 1975 [1–6]. A complex of four main carbohydrate-containing macromolecules (A–D) was isolated [1] and the plasma membrane localization was demonstrated [5]. One of the components (band D or lipopeptidophosphoglycan, LPPG) has been purified and a glycophosphoceramide structure was demonstrated for this compound [2–4].

The function of these cellular constituents as well as their presence in the different forms of the parasite are problems not yet elucidated. Due to the difficulties in producing an appreciable number of cells of the other two differentiation stages of *T. cruzi*, e.g., trypomastigotes and amastigotes which infect the vertebrate, it became important to develop sensitive methods to assess the fate of these macromolecules during differentiation of the parasite. Moreover, through the use of endogenous labeling of epimastigotes the influence of cycloheximide and tunicamycin on the synthesis of these glycoconjugates has been studied.

2. Experimental

Epimastigote forms of *T. cruzi* (Y strain) were cultivated in LIT medium [7]. After 2 days of growth (8×10^7 cells/ml) the epimastigotes were collected by centrifugation and washed twice with 150 mM NaCl containing 50 mM sodium phosphate (pH 7.2, PBS).

Address correspondence to W. Colli

Trypomastigotes from mouse blood or from tissue cultures were obtained as in [8]. Spleen and liver amastigotes were obtained as in [9] except that the enzyme treatment was omitted.

For the galactose oxidase-labeling experiments 2×10^8 *T. cruzi* cells were resuspended in 0.4 ml PBS containing 0.1 mg galactose oxidase (Worthington) and incubated for 40 min at 30°C. Then 0.021 mg NaB^3H_4 (New England Nuclear; spec. act. 282 mCi/mmol) were added and incubation proceeded for 10 min at room temperature. The reaction was stopped with 5 ml cold PBS and cells were washed twice in the same buffer. The final pellet was directly lysed with the electrophoresis sample buffer under reducing conditions and applied onto polyacrylamide gels.

To label the *T. cruzi* glycoconjugates by a non-enzymatic procedure 2×10^8 washed parasites were suspended in PBS (containing 0.1% BSA) and incubated at room temperature for 30 min with 50 μg/ml non-radioactive $NaBH_4$. Cells were then washed twice with excess buffer and further incubated in the dark for 20 min at room temperature with PBS containing 20 mM sodium periodate. Oxidation was stopped by diluting cells with cold buffer. After twice washing the cells with the same buffer they were resuspended in 0.2 ml PBS containing 0.0175 mg NaB^3H_4 and incubated at room temperature for 30 min. After reduction the cells were washed twice with PBS and the final pellet was lysed with electrophoresis sample buffer and applied onto polyacrylamide gels. The integrity of the parasites after the whole procedure was attested by microscope counting.

For [^{14}C]glucose incorporation studies epimastigote cells were preconditioned under agitation

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75 © 1982 Federation of European Biochemical Societies

(120 rev./min) for 4 h at 28°C in the medium of [10] in the absence of glucose (6×10^7 cells/ml). D-[U-^{14}C]-Glucose (New England Nuclear) was then added at 2 μmol/ml (spec. act. 4.8 μCi/μmol) and incubation was allowed to proceed for 16 h. Then, 2 mg/ml non-radioactive glucose was added and incubation continued for an additional 4 h. Phenol–water extraction of the glycoconjugate complex was as in [2]. The LPPG was isolated from the complex and purified as in [2]. In the kinetic experiments, as well as in those in which the effect of antibiotics was tested, the above procedure was employed except that the chase was omitted and the incubation time with radioactive glucose (spec. act. 138 μCi/μmol; 72 nmol/ml) was variable (see figure legends). Total incorporation of radioactivity was measured by precipitating aliquots of the incubation mixture with 10% trichloroacetic acid, filtration on nitrocellulose membranes, and counting in scintillation fluid in a Beckman LS-250 spectrometer (efficiency 74%).

Lipid oligosaccharides were isolated as in [11]. After incubation of epimastigotes for 90 min with [^{14}C]glucose the cells were centrifuged, washed with PBS and sonicated in water. Methanol (2 vol.), chloroform (3 vol.) and 10 mg carbohydrate complex as carrier were added and the tubes were centrifuged at low speed. The lower and upper phases were separated from the interface. The latter was extracted with chloroform/methanol (2:1) and then 3 times with chloroform/methanol/H_2O (10:10:3).

Electrophoretic analysis was performed in 8–15% linear polyacrylamide gradient slab gels in the presence of 0.1% SDS [12]. Gels were prepared for fluorography as in [13]. Tunicamycin was a kind gift from H. F. El-Dorry and its efficiency was tested by the inhibition of D-[^{3}H]mannose incorporation in HeLa cells. Cycloheximide was a product from Sigma (St Louis MO).

3. Results

With slight modifications, the method developed to label LPPG in vitro [6] was applied to living parasites. Due to the membrane location of LPPG and to the furanoic structure of its galactose residues this substance is strongly labeled when intact epimastigotes are subjected to mild periodate oxidation followed by reduction with NaB^3H_4 (fig.1). Bands A–C, but not LPPG, are strongly labeled when epimastigotes are

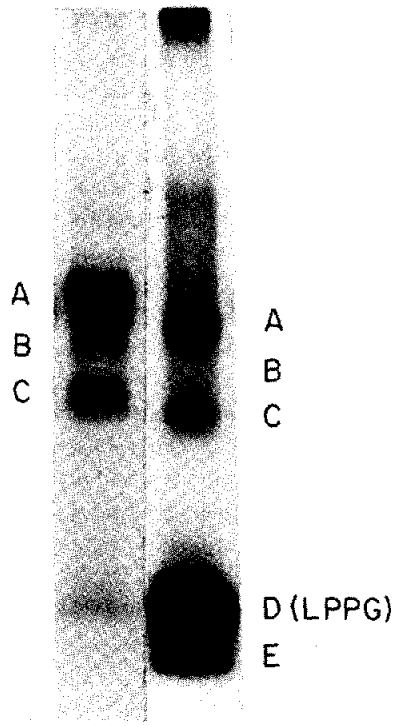

Fig.1. SDS–polyacrylamide gel electrophoresis of epimastigotes labeled with NaB^3H_4 after oxidation with galactose oxidase (left) or sodium metaperiodate (right).

reduced with NaB^3H_4 following oxidation via galactose oxidase (fig.1) [5,14].

When both intact spleen and liver-derived amastigotes and trypomastigotes (from tissue cultures or from blood of infected mice) were subjected to NaB^3H_4 reduction following chemical or enzymatic oxidation neither bands A–C nor LPPG could be detected. Equally negative results were obtained when lysates of these cells were subjected to the same procedures thus ruling out the possibility for the internal presence of these glycoconjugates in both differentiation stages of *T. cruzi*. These results have been further confirmed by direct extraction of 5×10^8 cells of each differentiation stage with phenol–water followed by SDS–polyacrylamide gel electrophoresis and PAS–Schiff staining [2]. Only epimastigotes displayed bands A–C and LPPG. Thus, it is imperative to conclude that 2 out of 3 differentiation forms of *T. cruzi* do not express these membrane-bound glycoconjugates

Fig.1 also shows that epimastigotes possess a compound (band E) which migrates ahead of LPPG and is strongly oxidized by mild periodate treatment. This

would suggest the presence of exocyclic glycols in, at least, part of its carbohydrate residues. Like LPPG, compound E is not reactive to galactose oxidase. Structural studies on this compound are underway.

Since *T. cruzi* is able to strongly adhere macromolecules from the culture medium [14] it became important to definitively demonstrate the endogenous nature of the glycoconjugates in the epimastigote form. After incubation of 6×10^7 epimastigotes/ml with [^{14}C]glucose (10 μCi/ml) for 16 h, followed by a 4 h chase with excess non-radioactive glucose, an important amount of radioactivity was incorporated into 10% trichloroacetic acid-precipitable material (5.5×10^6 cpm for 3×10^8 cells). The glycoconjugate complex, extracted with phenol–water and precipitated with ethanol from the aqueous phase [2] accounted for ~82% of the counts. Fluorography showed most of the label in bands A–C (fig.2). LPPG which was less intensely labeled could be extracted with chloroform/methanol/water (10:10:3) [2]. The extracted material showed the same mobility as a sample of the purified glycophosphoceramide (not shown). The compound which appears strongly labeled in fig.2 (arrow) coincides with a faint band that is occasionally seen in PAS–Schiff-stained gels. The possibility that this compound lacks galactose must be entertained since it could not be reduced by NaB^3H_4 after oxidation with either galactose oxidase or metaperiodate. Whether the amount of this substance depends on the growth stage of the parasite remains to be elucidated. We do not yet know why LPPG incorporates ^{14}C from glucose at a slower rate than bands A–C. The conditions used for isotope incorporation might not have been the best due to the extreme complexity of this molecule [3,4,6].

The effect of tunicamycin and cycloheximide on ^{14}C incorporation into trichloroacetic acid-precipitable material from epimastigotes was followed for 90 min (fig.3). While cycloheximide (20 μg/ml) inhibited incorporation by 50%, tunicamycin had no effect over 0.15–5 μg/ml. Incubation with tunicamycin (2.5 μg/ml) for 2 h before addition of [^{14}C]glucose

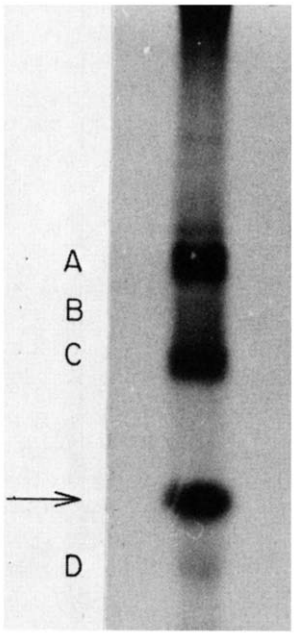

Fig.2. Fluorography of SDS–polyacrylamide gel electrophoresis of epimastigotes endogenously labeled with [^{14}C]glucose.

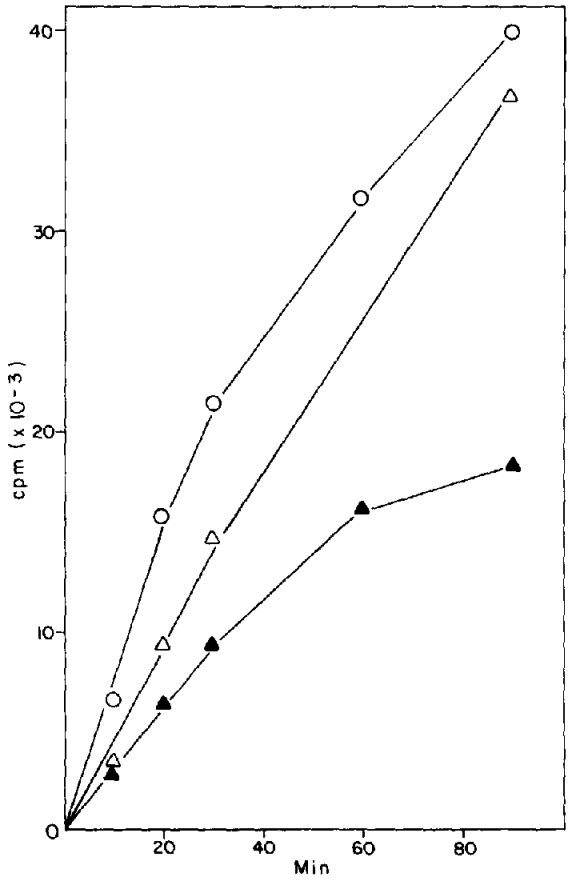

Fig.3. Kinetics of ^{14}C incorporation from [^{14}C]glucose into *T. cruzi* epimastigotes: control (△); 20 μg cycloheximide/ml (▲); 1 μg tunicamycin/ml (○). Each experimental point contained 8×10^6 cells.

Table 1
Incorporation of ^{14}C (cpm × 10^{-4})[a] into different fractions from epimastigote cells incubated with [^{14}C]glucose[b]

Additions	Solvent A, phase:		Solvent B
	Upper	Lower	
None	49.4	9.9	3.4
Tunicamycin (0.15 μg/ml)	74.8	3.6	2.6
Cycloheximide (20 μg/ml)	117.8	15.1	5.2

[a] From 8 × 10^7 cells
[b] Incubation for 90 min at 28°C as in section 2

Solvent (A): chloroform/methanol/water (3:2:1)

Solvent (B): chloroform/methanol/water (10:10:3)

gave a similar incorporation than when the radioactive sugar was added concomitantly with the antibiotic.

Epimastigote cells incubated for 90 min with [^{14}C]glucose were processed as recommended [11] for the separation of the polyprenol oligosaccharide pyrophosphate. The radioactivities of the different fractions are shown in table 1. Inhibition by tunicamycin is now observed in the lower phase of the chloroform/methanol/water (3:2:1) extract and in the chloroform/methanol/water (10:10:3) extract of the interface. Although LPPG can also be extracted by the latter solvent, it contains no *N*-asparagine linkages in its structure, being a glycophosphoceramide. Therefore the inhibition observed with tunicamycin suggests the presence in *T. cruzi* epimastigotes of the lipid oligosaccharide pyrophosphate.

4. Discussion

The glycoconjugates extracted with phenol–water [1,2] are major components of the cell surface membrane of epimastigote forms of *T. cruzi* [5]. That these macromolecules incorporate 82% of the total trichloroacetic acid-precipitable radioactivity upon [^{14}C]glucose incorporation would confirm that they are the main sugar constituents of the cell. However, their function is not yet known. In vivo labeling of these macromolecules should facilitate these studies and help to follow the influence of different factors on their biosynthesis. However, the culture medium for *T. cruzi* growth is very complex and the possibility that certain components could adhere to the cells has been considered (cf. [14]). Labeling of the glycoconjugates by incubation with [^{14}C]glucose confirmed their endogenous nature. We tried to inhibit the incorporation of radioactivity with tunicamycin, an antibiotic that blocks the production of polyprenol *N*-acetylglucosamine pyrophosphate [15], the intermediate in the biosynthesis of the N-linked oligosaccharides in glycoproteins. No inhibition was evident for 90 min with different concentrations of tunicamycin even when using 50-times the concentration that produced considerable inhibition in *T. brucei* [16]. In fact, a slight stimulation was observed (fig.3). The same incorporation was obtained when cells were incubated with tunicamycin for 2 h before addition of [^{14}C]glucose and then left for another 2 h. This result excludes the possibility that the pool of precursors pre-existing in the cell would preclude inhibition by tunicamycin. However, cycloheximide caused inhibition of sugar incorporation. Fluorography of the glycoconjugates extracted with phenol–water from cells incubated with tunicamycin showed no difference when compared with the control, while all bands were less intense upon incubation with cycloheximide (not shown). We know that one of the components of the complex (LPPG) has a glycophosphoceramide structure. The biosynthesis of LPPG has yet not been studied, but most probably polyprenol-*N*-acetylglucosamine pyrophosphate is not an intermediate in its biosynthesis. The structure of the other 3 components (bands A–C) is unknown, but although they have as a mixture an important amount of protein (unpublished) they would not be rich in *N*-glycosidic linkages.

The fact that no inhibition of incorporation of ^{14}C into these glycoconjugates was observed, upon addition of tunicamycin, suggests that most of the linkages in A–C are *O*-glycosidic. A few short *N*-glycosidic chains would not noticeably alter the electrophoretic behavior. Tunicamycin, however, inhibited incorporation of radioactivity in the lower phase of the chloroform/methanol/water (3:2:1) extract and in the chloroform/methanol/water (10:10:3) fraction (table 1). This inhibition would indicate the existence of N-linked oligosaccharides, but as a minor feature, in the pool of the glycoproteins of *T. cruzi* epimastigotes. The data obtained using cycloheximide in the labeling experiments support this hypothesis. Although total incorporation of ^{14}C was clearly inhibited by the drug, the radioactivity in the chloroform/methanol/water extract considerably increased, probably

due to the accumulation of the lipid oligosaccharide precursor. The inhibition of radioactivity observed in the lower phase of the chloroform/methanol/water (3:2:1) extract in the tunicamycin experiments suggests the existence of N-linked oligosaccharide intermediates with shorter chains than those found in other glycoproteins [17]. Oligosaccharide transferred to proteins in *C. fasciculata* contained 2 *N*-acetylglucosamine and only 7 mannose residues [18].

That only epimastigotes, as opposed to trypomastigotes and amastigotes, contain the glycoconjugates A–D suggests that they might play an important function in the plasma membrane of the epimastigote cell. Such function could be structural. However, one cannot rule out the possibility of their involvement in phenomena which are specific for the epimastigotes, e.g., their lability to the alternative pathway of the complement [19] and their capacity to divide in particular environments such as the midgut of the insect vector.

Further studies on the structure and function of the glycoconjugates of *T. cruzi* epimastigotes are in progress.

Acknowledgements

This investigation received financial support from Secretaria de Estado de Ciencia y Tecnologia (SECYT) to R. M. L.; project PNUD/UNESCO RLA 78/024 to W. C. and R. M. L.; CNPq/FINEP and UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases to W. C.; and FAPESP to B. Z. The authors are indebted to M. F. Gonçalves and C. Carniol for help during the experimental work. R. M. L. is a research member of the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

References

[1] Alves, M. J. M. and Colli, W. (1975) FEBS Lett. 52, 188–190.
[2] Lederkremer, R. M., Alves, M. J. M., Fonseca, G. C. and Colli, W. (1976) Biochim. Biophys. Acta 444, 85–96.
[3] Lederkremer, R. M., Tanaka, C. T., Alves, M. J. M. and Colli, W. (1977) Eur. J. Biochem. 74, 263–267.
[4] Lederkremer, R. M., Casal, O. L., Tanaka, C. T. and Colli, W. (1978) Biochem. Biophys. Res. Commun. 85, 1268–1274.
[5] Alves, M. J. M., da Silveira, J. F., De Paiva, C. H. R., Tanaka, C. T. and Colli, W. (1979) FEBS Lett. 99, 81–85.
[6] Lederkremer, R. M., Casal, O. L., Alves, M. J. M. and Colli, W. (1980) FEBS Lett. 116, 25–29.
[7] Fernandes, J. F. and Castellani, O. (1966) Exp. Parasitol. 18, 195–202.
[8] Andrews, N. W. and Colli, W. (1982) J. Protozool. in press.
[9] Pereira, M. E. A., Loures, M. A., Villalta, F. and Andrade, A. F. B. (1980) J. Exp. Med. 152, 1375–1392.
[10] Boné, G. J. and Parent, G. (1963) J. Gen. Microbiol. 31, 261–266.
[11] Parodi, A. J. (1979) J. Biol. Chem. 254, 10051–10060.
[12] Laemli, U. K. (1970) Nature 227, 680–685.
[13] Chamberlain, J. P. (1979) Anal. Biochem. 98, 132–135.
[14] Colli, W., Andrews, N. W. and Zingales, B. (1981) in: International Cell Biology (Schweiger, H. D. ed) pp. 401–410, Springer Verlag, Heidelberg, New York.
[15] Lehle, L. and Tanner, W. (1976) FEBS Lett. 71, 167–170.
[16] Strickler, J. E. and Patton, C. L. (1980) Proc. Natl. Acad. Sci. USA 77, 1529–1533.
[17] Parodi, A. J. and Leloir, L. F. (1979) Biochim. Biophys. Acta 559, 1–37.
[18] Parodi, A. J., Quesada Allue, L. A. and Cazzulo, J. J. (1981) Proc. Natl. Acad. Sci. USA 78, 6201–6205.
[19] Nogueira, N., Bianco, C. and Cohn, Z. (1975) J. Exp. Med. 142, 224–229.