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Original Article

Chemiluminescent detection of glycocode alterations in hepatic granulomatous lesions of experimental schistosomiasis

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Abstract: This work describes the glycophenotype evaluation of mice liver granulomatous lesion caused by infection of *Schistosoma mansoni* by using lectins labeled to acridinium ester (AE). The employed lectins were Concanavalin A (Con A), wheat germ agglutinin (WGA) and *Sambucus nigra* agglutinin (SNA) that specifically recognize α-D-glucose/mannose, N-acetyl-D-glucosamine and α-NeuNAc-[2→6]-Gal/GalNAc. The chemiluminescence expressed in relative light unit (RLU) obtained from the hepatic granuloma tissues (0.25 cm^2) treated with the lectins-AE was compared with control tissues. Con A-AE infected tissues showed higher statistically significant values (1,501,182 ± 163,450 RLU) compared with the control tissue (575,280 ± 97,216 RLU). WGA-AE results also showed higher values (189,654 ± 20,686 RLU) than that found for the controls (82,878 ± 24,411). SNA-AE results did not present statistical difference between granulomatous tissues (198,990 ± 15,131) and controls (167,290 ± 25,194). There is a significant increase in glucose/mannose residues and N-acetyl-D-glucosamine in hepatic granuloma caused by *S. mansoni*, while the sialic acid remains virtually unchanged. The understanding of schistosome glycophenotype is relevant for the development of new diagnostic methods for schistosomiasis, design of new drug targets and preparation of glycan-based vaccines.

Keywords: Carbohydrates, glycophenotype, chemiluminescence, lectins, *schistosomiasis mansoni*, acridinium ester

Introduction

The relevance of glycoconjugates in many biological processes has been exhaustively demonstrated [1]. They have a key role in many life phenomenologies acting as signals in biological translations. It is not different in host-parasite interactions. Schistosomes are complex multicellular organisms, which synthesize a diverse variety of O- and N-linked (Lewis^x antigen, LacdiNAc and fucosylated LacdiNAc) antigen glycoproteins, glycosylphosphatidylinositol anchors and glycolipids [2]. These glycoconjugates are involved in the increase of *S. mansoni* survival within the host [3]. They varied according to the various stages of Schistosoma [4, 5]. They may be valuable for diagnosis and treatment proposals. Fucosylated oligosaccharides glycan fragments of the cercarial glycocalyx of the parasite stage, for instance, are potential tools for diagnostic procedures based on antibodies raised against the glycan in the serum of humans infected [6].

Although it is known that the cercariae and adult worms of *S. mansoni* have their glycocalyx rich in fucose residues the carbohydrate alterations in the granulomatous lesions/liver complex in schistosomiasis deserve better investigations, once glycans of schistosome eggs have been identified as initiators and/or modulators of granuloma formation [7].

Lectins have been widely used to decipher tissue glycocode. In our lab, lectins conjugated to

peroxidase have been used in histochemical studies of the murine granulomatous lesions/liver complex caused by schistosomiasis [8, 9].

Here, lectins conjugated to acridinium ester (AE) are proposed to replace those labeled with peroxidase. Therefore, chemiluminescence can be employed instead enzymatic methods to investigate the tissues glyconjugates analyses based on staining procedures. In our lab, a set of contributions demonstrated that the use of lectins labeled with acridinium ester as probes is capable to reveal specifically and quantitatively the respective glyconjugates [10-15]. Furthermore, these works showed that there is a correlation between the light emitted by these glyconjugates/lectins-acridinium ester complexes and the surface area of the tissues. In this work the expression of N-acetyl-D-glucosamine, α-D-glucose/mannose and α-NeuNAc-[2→6]-Gal/GalNAc residues on hepatic granulomas system in murine experimental schistosomiasis were investigated by using, respectively, wheat germ agglutinin (WGA), Concanavalin A (Con A) and *Sambucus nigra* agglutinin (SNA) conjugated with AE (WGA-AE, Con A-AE and SNA-AE).

Materials and methods

Reagents

N-hydroxysuccinimide-activated dimethyl acridinium ester (DMAE-NHS)/1966-1-53-2/Organic Lab) was kindly supplied by Dr. H. H. Weetall. Con A, WGA, SNA, N, N-dimethylformamide, methyl-α-D-mannoside, N-acetyl-D-glucosamine and Sephadex G-25 were supplied from Sigma-Aldrich (St. Louis, MO, USA). Chemiluminescent detection was performed using Siemens Reagent TSH 500T (Siemens Medical Solutions Diagnostics, Malvern, PA, USA) composed of 0.5% H_2O_2 in 0.1 N HNO_3 and 0.25 M NaOH. Xylene and ethanol were obtained from Merck (Darmstadt, Germany).

Experimental model

Female mice (*Mus musculus*) with six weeks old and weighting 35-45 g were used in this experiment. The animals (n=20) were housed in cages (20×30×13 cm) containing sterile wood shaving bed; standard diet (LABINA®, Purina do Brasil S.A., Recife-PE, Brazil) and water were available *ad libitum*. Room temperature was kept at 25 ± 2°C and 12:12 h light/dark cycle. Ten of those mice were used as control group and the others ten mice were individually infected by *Schistosoma mansoni* (Belo Horizonte-BH strain): 50 cercariae on its abdominal region under artificial light for at least 2 h. The mice were intramuscularly anesthetized with ketamine: xylazine (115 mg: 10 mg per kg, respectively). Control group was submitted to the same stress processes than experimental group. Past 60 days of infection, the 20 mice were euthanized by an overdose of anesthetic and their livers were removed with surgical scissors. The experimental protocols were in accordance with the requirements of the Animal Experiments Ethics Committee of the Federal University of Pernambuco, Brazil (nº 23076.017619/2015-60).

Lectins conjugation with Acridinium-Ester (AE) and chemiluminescent measurement

Lectins Con A, WGA and SNA were conjugated to acridinium ester (DMAE-NHS) according to procedure previously described [15]. Briefly: lectins (2 mg of protein/mL) were incubated with 10 µL of acridinium ester solution (0.2 mg diluted in 400 µL of N, N-dimethylformamide) for 1 h at 25°C under rotary stirring. The conjugate (lectin-AE) were applied to a column of Sephadex G-25 (10×1 cm), previously equilibrated with 10 mM phosphate buffer, containing 0.15 M de NaCl, pH 7.2, from known on called PBS, and eluted with this buffer. Aliquots (1 mL) were collected and their protein content was spectrophotometrically determined at 280 nm. Chemiluminescence of the same aliquots was also assayed with solutions of 0.5% H_2O_2 prepared in 0.1 N HNO_3 (50 µL) and 0.25 M NaOH (50 µL) using a luminometer Modulus Single Tube 9200-001 (Turner BioSystems, USA). The light emission intensity was determined as relative light units (RLU) with a counting time of 5 seconds per sample. Fractions corresponding to protein and chemiluminescence peaks were pooled and used throughout this work.

Lectin histochemiluminescence

The livers were washed several times with PBS and preserved in 10% formalin in PBS for histopathological study. The selected fragments underwent dehydration steps (diving in ethanol) and diaphanization (diving in xylene), followed

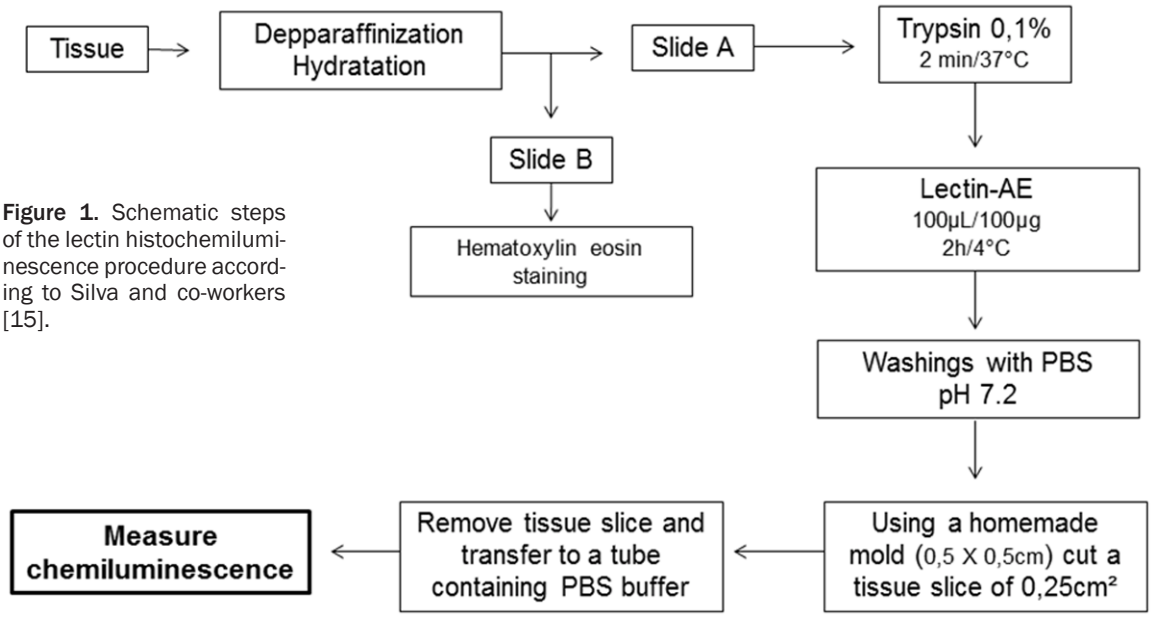

Figure 1. Schematic steps of the lectin histochemiluminescence procedure according to Silva and co-workers [15].

by a dip in molten paraffin at 60°C. This Lectin histochemiluminescence procedure was in accordance to Silva [15]. Two paraffin sections of samples were sequentially cut sizing 8 µm deep each, transferred to glass slides, deparaffinized in xylene (once 5 min, and three-fold 1 min) and rehydrated in graded alcohols (three-fold 100% and once 70%-1 min each). The slides were differently processed: Slide B was traditionally stained with hematoxylin-eosin; whereas Slide A was treated with 0.1% (w/v) trypsin at 37°C for 2 min. The Slide A was further processed as follows: washed (twice, 5 min each time) with PBS and was incubated with lectins-AE (100 µL containing 100 µg of protein) for 2 h at 4°C, followed by washing (three-fold 5 min) with 15 mL of PBS. The area corresponding to tissue section (square-shaped; 0.25 cm^2) containing the hepatic granuloma identified in the hematoxylin-eosin stained (Slide B) was cut using a homemade mold. Then this 0.25 cm^2 tissue piece was removed with a bistoury from the glass slide and transferred to a polypropylene test tub containing 50 µL of PBS. Finally, chemiluminescence from tissue slices was assayed as described above. Triplicate measurements were carried out throughout in this study. Lectin binding inhibition assays were accomplished by incubating each lectin solution with 300 mM methyl-α-D-mannoside (Con A) and N-acetyl-D-glucosamine (WGA) for 45 min at 25°C prior to their incubation with tissues. The following steps were as described above.

Statistical analysis

Software Origin Pro8 (Origin Lab Corporation, One Roundhouse Plaza, Northampton, MA 010-60 USA) was used for the statistical analysis and data were expressed as mean ± standard deviation (s.d.). Obtained data were confronted using ANOVA test followed by post-hoc Tukey test for comparison between infected and control tissues to Con A-AE, WGA-AE and SNA-AE tests ($P < 0.05$).

Results

The steps of the lectin histochemiluminescence procedure as described by Silva [15] are schematically displayed in **Figure 1**.

Figure 2 displays the chemiluminescence obtained from the hepatic granuloma tissues from infected mice (0.25 cm^2) treated with the lectin-AE compared with the control tissues. Firstly, the Con A-AE treated tissues showed about three-fold higher values (1,501,182 ± 163,450 RLU) compared to the control tissues (575,280 ± 97,216 RLU), namely, hepatic tissues from non-infected mice. This approximated three-fold superior value was statistically significant. Regarding the WGA-AE values again were higher (189,654 ± 20,686 RLU) than those found for the control tissue samples (82,878 ± 24,411), about twice higher and statistically significant. Finally, concerning the results for the SNA-AE no statistical difference

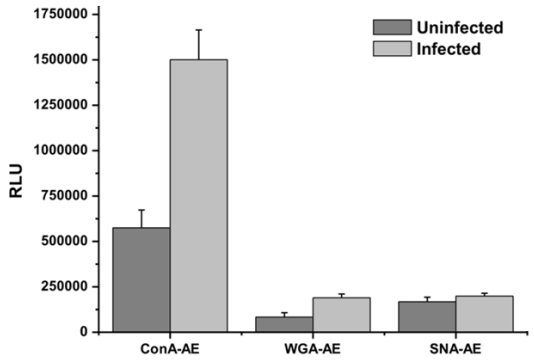

Figure 2. Comparison between chemiluminescence of hepatic tissue (n=10) and tissue infected by *S. mansoni* (n=10) for three lectin conjugates, respectively, Con A-Acridiunium Ester (AE), WGA-AE and SNA-AE.

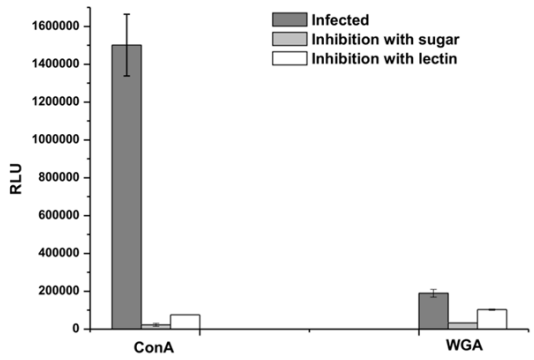

Figure 3. Inhibition assay. Chemiluminescence of infected tissues incubated with labeled lectins (Con A-AE and WGA-AE) and previously treated with specific carbohydrates, methyl-α-D-mannoside (Con A) and N-acetyl-D-glucosamine (WGA), and unlabeled lectins.

was found between the granulomatous tissues (198,990 ± 15,131) and the control ones (167,290 ± 25,194).

Figure 3 presents the specificity evaluation of the carbohydrate/glycoconjugates and the respective lectin-AE complex formation by using two inhibition procedures: previously incubating the tissues with either the specific carbohydrate (300 mM) for the lectin-AE or the non-labeled acridinium ester lectin. Then the tissues chemiluminescences were measured. The inhibition assays with the specific carbohydrates of Con A-AE and WGA-AE decreased the RLU values in 98, 47%, and 82, 94% respectively. Meanwhile the decreases using the non-labeled acridinium ester lectin were 94.97% (Con A-AE) and 45.48% (WGA-AE). No inhibition evaluation assays were carried out for the SNA-AE because this lectin did not show difference between the granulomatous and control tissues.

Discussion

Previous studies using Con A and WGA conjugated to peroxidase to evaluate the glycode of the egg-granuloma [8, 9] did not show expressive difference compared to the non-infected hepatic tissues, as observed in the present work. Histological reactivity was absent and weak stained for WGA and Con A in egg-granuloma and hepatic tissue (control), respectively. It is important to register that these studies used an attempt procedure to quantify the findings.

The use of WGA-AE, Con A-AE and SNA-AE allowing the chemiluminescence approach combines the specificity of the lectins with the sensitivity of this method. Exposure of lectin-AE to an alkaline hydrogen peroxide solution triggers a flash of light. AE forms an unstable dioxetane yielding N-methylacridone and produces light at a wavelength of 470 nm [16]. Inhibition assays carried out in this contribution demonstrated that this procedure is specific to the carbohydrate-lectin complex detection. Therefore, one can assume that WGA-AE and Con A-AE probes demonstrated that N-acetyl-D-glucosamine and glucose/mannose residues, respectively, are higher in the schistosome egg-granuloma complex compared to the control tissues. On the other hand, N-acetylneuraminic acid remained virtually unchanged provided that there was no change in the SNA-AE between egg-granuloma and hepatic tissue (control) analysis.

Araújo and co-workers [17] treated *S. mansoni* infected mice with an encapsulated sulfated polysaccharide α-D-glycan and employed WGA-peroxidase and Con A-peroxidase for assessing the profile of carbohydrates in animals treated and untreated. The analysis of the tissues allowed realizing that both animals under the treatment and control were well stained with the WGA lectin. On the other hand, Con A lectin only labeling and staining the tissues of untreated animals. Rêgo and co-workers [18] made a brief study on lectin histochemistry of human colon lesions induced by schistosomia-

sis. Although the expression of N-acetyl-D-glucosamine residues via WGA recognition was effective and had intense staining, Con A had failed to recognize mannose and glucose residues. The authors did not report an absence of these carbohydrate residues, but that if they were present, the Con A was unable to access them, even after treatment with trypsin.

Studies about a comparative analysis of glycans secreted by eggs and cercariae reported that both expressed a sweeping complex of glycans. N-glycans from proteins secreted by the eggs are rich in mannose structures mainly, and N-acetyl-D-glucosamine residues. These both glycans have Lewis^x antigens as common component, which has been shown to induce the production of immune mediators such as Interleukin 10 and Prostaglandin E2, which promote Th2 response [3].

N-acetylneuraminic acid (α-NeuNAc-[2→6] Gal/GalNAc), generically termed sialic acid, is a group of carbohydrates with nine carbons each widely distributed among the living beings in the form of oligosaccharides. It can bind to proteins and lipids of the plasmatic membrane and participate in biological processes. It has several functions as cell renewal, receptors for bacteria and viruses and cell-cell recognition/adhesion [19]. Despite being the most common terminal monosaccharide of human glycans, every *S. mansoni* stages do not produce sialic acid [7, 20], but they incorporate from the host which is believed to be one of escape mechanism from the host immune system [21]. Therefore, for not producing sialic acid the SNA-AE results in infected liver tissues were too close to the not-infected ones.

It is important to point out that WGA is also able to recognize N-acetylneuraminic acid [22]. However, it was not noticed the presence of sialic acid through specific use of SNA, therefore it could be concluded that WGA recognized only N-acetyl-D-glucosamine.

Besides the glycophenotypic point of view, from the morphogenetic aspect the hepatic granuloma is subdivided in two pre-granulomatous phases (weakly and/or initial reactive and exudative) and three granulomatous phases (exudative-productive, productive and involutional) [23]. It should be noted that depending on which stage the granuloma is found the carbohydrates pattern on egg-granuloma system may modify. In our study mice were euthanized after 60 days of infection, stage when the granulomas are in the productive phase [23].

The evaluation of glycoconjugates has proven important to contribute to the study of the immunopathology of schistosomiasis and immunomodulatory processes involving this disease. Identification of many of these antigenic glycoconjugates by histochemical techniques helps in better understanding the biology of the parasite and the disease itself. The understanding of schistosome glycophenotype is relevant for the development of new diagnostic methods for schistosomiasis, design of new drug targets and preparation of glycan-based vaccines.

This study allowed to attend that Con A, WGA and SNA conjugated to acridinium ester demonstrated that there is a significant increase in glucose/mannose residues and N-acetyl-D-glucosamine in hepatic granuloma of *S. mansoni*, while the N-acetylneuraminic acid remains virtually unchanged.

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Disclosure of conflict of interest

None.

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