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The majority of sialylated glycoproteins in adult *Ixodes ricinus* ticks originate in the host, not the tick

Jan Sterba [a,b,*], Marie Vancova [a,b], Jarmila Sterbova [a,b], Lesley Bell-Sakyi [c], Libor Grubhoffer [a,b]

[a] Institute of Parasitology, Biology Centre of the ASCR, CZ-37005 České Budějovice, Czech Republic
[b] Faculty of Science, University of South Bohemia, CZ-37005 České Budějovice, Czech Republic
[c] The Pirbright Institute, Pirbright, Surrey GU24 0NF, United Kingdom

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ABSTRACT

The presence of sialylated structures in tick organs was observed previously using lectin staining. Recently, we demonstrated the presence of sialylated *N*-glycans using mass spectrometry in tick salivary glands and the gut. However, we proposed a host (blood) origin for these glycans and mapped the transport of sialylated molecules from the gut to the salivary glands. In this report, we performed quantitation of whole sialic acid and of metabolically incorporated sialic acid (*N*-azido neuraminic acid) in *Ixodes ricinus* tick samples. We show that the majority of sialylated molecules in the adult tick originate in the host (blood) and are not synthesized by the tick. Similar results were observed for tick cell cultures. The almost complete absence of tick sialylated molecules and the specific transport and localization of host structures into the tick salivary glands and the saliva raises many questions on the role of these molecules in the physiology and, specifically, the blood-feeding of ticks.

1. Introduction

Sialic acids (Sia) are a group of saccharides providing many functions to glycoproteins in a wide range of organisms, mainly higher Eukaryotes.[1,2] Moreover, they can be found in some pathogens and parasites where they serve as mimics protecting the microorganisms from the immune system of the host organisms.[3] Sialylated structures in the mammalian host cells often serve, on the other hand, as receptors for the pathogens.[2] While sialylated glycans are widely present in eukaryotes, arthropod glycans are not usually sialylated.[4,5]

Sia was detected previously in several arthropods including blood-feeding parasites. The best-described species in this regard is the *Drosophila* fly, in which sialylated glycans were described in detail using mass spectrometry.[6] Here, sialic acid was shown to be present in the embryo, where it occurs in the development of the neuronal system.[6,7]

The responsible sialyltransferase, a single vertebrate-type sialyltransferase, was identified in *Drosophila melanogaster*.[8] Putative sialyltransferases were next identified in other *Drosophila* species, and also in the mosquitoes *Aedes aegypti* and *Anopheles gambiae*, and in other arthropods (*Acyrthosiphon pisum*, *Strongylocentrotus purpuratus*, *Tribolium castaneum* and others).[9,10]

The α2,6-sialyltransferase of *Drosophila* was further studied in respect to its specificity and it has been shown to be specific for glycoproteins and oligosaccharides, while no activity has been shown for glycolipids.[8] Both sialic acid synthesis and sialyltransferase activity are required for the development and function of the nervous system.[11,12]

Sia-specific lectins (MAA, SNA) have been used for confirmation of the presence of Sia in blood-feeding parasites such as ticks,[13] and oxidation using sodium metaperiodate suggested the presence of Sia in the gut of mosquitoes.[14] Direct confirmation of Sia in blood-feeding parasites using mass spectrometry was missing until recently, when sialylated *N*-glycans were described in the ticks *I. ricinus* and *D. marginatus*.[15] However, the analysis of the *N*-glycans cleaved off the glycoproteins did not prove their tick origin. This is particularly the case for obligatory blood-feeding parasites, in which the ingestion of huge amounts of mammalian blood takes place. The presence of intact host proteins from the tick gut in the haemolymph and other organs including salivary glands was shown previously.[16–19] Thus it is possible that sialylated host proteins could also be present in the tick haemocoel and internal organs. Accordingly, we proposed a host origin for the observed sialylated glycans and showed the probable route of transport of

* Corresponding author. Tel.: +420 38 7775465; fax: +420 38 5310388.
E-mail addresses: sterbj02@prf.jcu.cz (J. Sterba), vancova@paru.cas.cz (M. Vancova), dupejj00@prf.jcu.cz (J. Sterbova), lesley.sakyi@pirbright.ac.uk (L. Bell-Sakyi), liborex@prf.jcu.cz (L. Grubhoffer).

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sialylated molecules from the gut through the tick haemocoel into the salivary glands, from where these molecules could be released back into the host.[15]

In the present study we performed the quantification of sialylated molecules in tick organs, whole ticks and tick cell cultures and compared the overall amount of Sia to that of bioorthogonally labelled sialylated molecules. Several approaches were used for the quantitation of sialylated molecules, while metabolically labelled Sia (using *N*-azido-mannosamine, ManNAz, which is metabolized to *N*-azido-neuraminic acid, NeuNAz[10]) was reacted with Alexa Fluor® 488-alkyne through the Click reaction[20] and was subsequently labelled and quantified using anti- Alexa Fluor® 488 antibodies.

Our results show that the majority of sialylated molecules in the adult tick originate in the host (blood) and are not synthesized by the tick. In cultures of a continuous embryo-derived tick cell line, bioorthogonally labelled structures were not observed at all. Fluorescence microscopy (FM) of bioorthogonally labelled tick organs and cultured cells supported these results.

2. Materials and methods

2.1. Ticks and in vitro feeding

I. ricinus females were obtained from the tick rearing facility of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic in České Budějovice. The ticks were allowed to feed using artificial in vitro feeding on ovine blood.[21] In brief, 3.1 mL aliquots of ovine blood were supplemented with ATP (1 mM), gentamicin (5 μg/mL), Nystatin (100 i.u./mL, all from Sigma–Aldrich, Saint Louis, MO, USA) and 3.1 μL of 25 mM *N*-azido-mannosamine (Life Technologies, Carlsbad, CA, USA) in DMSO; this mixture was placed in wells of a 6-well cell culture microplate (TPP, Trasadingen, Switzerland). In the control experiments, 3.1 μL of DMSO without ManNAz was used. The plate was placed into a water bath at a temperature of 37 °C. Unfed *I. ricinus* females were put into feeding units[21] (ten ticks in each unit) and the units were placed in the wells of the plate. The blood was replaced with fresh supplemented blood every 12 h during the feeding. To avoid bacterial or fungal contamination, feeding units were washed with sterile phosphate buffered saline (PBS) before each blood change and with Nystatin solution (100 i.u./mL) every two days. After five days of feeding, ticks were collected and dissected if required. Tick organs (salivary glands, gut, Malpighian tubules and ovaries) or whole ticks were homogenized or processed for fluorescence microscopy.

2.2. Tick cell line

The *I. ricinus* embryo-derived cell line IRE/CTVM19[22] was grown in L-15 medium supplemented with 20% foetal calf serum, 10% tryptose phosphate broth, 2 mM glutamine and 1% of Antibiotic–Antimycotic Solution (all PAA Laboratories, Pasching, Austria) at 28 °C in flat-sided cell culture tubes (Nunc, Thermo Scientific, Waltham, MA, USA). For metabolic labelling of Sia, the medium was supplemented with 25 μM ManNAz and the cells were grown for at least 4 days. Thereafter, cells were detached by pipetting and the cell suspension was centrifuged at 400*g* for 10 min at 4 °C to pellet the cells. The pelleted cells were washed 3× with PBS to remove the remaining medium, sonicated and centrifuged at 14,000*g* for 10 min at 4 °C to clear the lysates. Lysates of cells from three different culture tubes were analysed by each of the quantitation methods (2.4–2.7); three replicate samples were prepared from each lysate for each method.

2.3. Preparation of homogenates from tick tissues or whole ticks

Tick tissues were dissected from in vitro partially fed females in ice cold distilled water containing 10 μL/mL Halt™ Protease Inhibitor Single-Use Cocktail (Thermo Scientific). The tissues were carefully removed to prevent contamination from the contents of the gut and separately rinsed thoroughly in water. The gut tissues were rinsed to remove most of the lumen contents. Tissues of three to five ticks were pooled, sonicated and centrifuged at 14,000*g* for 10 min at 4 °C to clear the lysates. The protein concentrations in the supernatants were measured using a Qubit® fluorometer (Life Technologies). The lysates were stored at −20 °C. Three different lysates were analysed by each of the quantitation methods (2.4–2.7); three replicate samples were prepared from each lysate for each method.

2.4. Quantitation of Sia using aminooxy-biotin

In this approach we used the combination of periodate oxidation of Sia with aniline-catalysed oxime ligation (PAL).[24,25] Quantitation was performed in white 96-well ELISA microplates (Greiner Bio-one, Frickenhausen, Germany). The wells were coated with tick cell lysates, tick organ lysates or control proteins (bovine serum albumin BSA, fetuin) using approximately 20 μg of proteins/well in a coating buffer (0.1 M sodium carbonate buffer, pH 9.6) for 2 h at ambient temperature. After coating, the wells were washed 3× with Wash buffer (0.05% Tween 20 in phosphate buffered saline PBS, pH 7.4), with the last wash performed on ice. Sia was oxidized with 100 μL of 1 mM sodium periodate/well for 30 min on ice[23] and the reaction was stopped by the addition of 50 μL of 50 mM sodium thiosulphate/well. The wells were washed and subsequently blocked with 3% BSA/PBS overnight at 4 °C. Next, the wells were washed and the aldehyde groups of the Sia were reacted with aminooxy-biotin (100 μL of 100 mM aminooxy-biotin, 10 mM aniline in PBS/well) for 90 min on ice. Each well was then washed with 1% BSA and the biotin molecules were labelled with STREP-HRP (Vector Laboratories, Burlingame, CA, USA, 33.3 pmol conjugate in 1% BSA/well) for 1 h at ambient temperature. After three washing steps, luminescence was measured using the DuoLux substrate (Vector Laboratories). The standard curve was constructed using the signal from wells with serial dilutions of STREP-HRP. Chemiluminiscence measurements were performed on an Infinite M200 Pro microplate reader. For each tick sample, the content of Sia was expressed as the mean amount of Sia in μmoles per g of proteins. Measurement of each replicate was performed three times.

2.5. Quantitation of Sia using thiobarbituric acid assay

This method for Sia quantitation uses the coupling reaction of thiobarbituric acid (TBA) with the aldehyde formed during Sia oxidation.[26] Sia was released from the control proteins and tick samples (approx. 20–100 μg of total proteins) using 25 U α2-3,6,8-neuraminidase from *Clostridium perfringens* (New England Biolabs, Ipswich, MA, USA) or 0.05 M H_2SO_4.[27] A 40 μL aliquot of the neuraminidase-treated sample was oxidized by the addition of 20 μL of 0.2 M sodium metaperiodate in 9 M phosphoric acid for 20 min at ambient temperature. The unreacted metaperiodate was reduced using 200 μL of 10% sodium arsenite in 0.5 M sodium sulfate and 0.1 N sulfuric acid. The aldehyde formed during the oxidation step was coupled to TBA by addition of 600 μL of 0.6% TBA in 0.5 M sodium sulfate and boiling the reaction mixture for 15 min. The mixture was then cooled for 5 min on ice and the coloured product was extracted into 860 μL acidic butanol, the upper organic phase was removed and the absorbance of the product was