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Glycosylation Pattern of Erythrocyte and Lymphocyte Proteins from Cattle with Tropical Theileriosis

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Abstract: To our knowledge, this is the first study in analysing the glycosylated proteins of erythrocytes and lymphocytes of cattle infected with *Theileria annulata* at high percentage of parasitemia (50-70%). SDS-PAGE separation of extracts of erythrocytes showed that one protein band (28 kDa) was not observed in erythrocytes of bovine theileriosis in comparison to control cells. Interestingly, erythrocytes of bovine theileriosis, analysed by polyacrilamide gels and stained by PAS method, gave much more stronger bands than control erythrocytes although, erythrocytes gave weaker bands on coomassie blue stained polyacrilamide gels in comparison to control erythrocytes. Maackia Amurensis Agglutinin (MAA) lectin staining of semi-dry blotted nitrocelulose membranes after SDS-PAGE analysis of erythrocytes of cattle with theileriosis gave very similar bands with control cells indicating that glycoproteins of erythrocytes of cattle with and without theileriosis possess similar sialic acid structures. Furthermore, stainings of semi-dry blotted nitrocelulose membranes with Dig-glycan detection kit and MAA lectin after SDS-PAGE separation of lymphocytes of cattle with theileriosis gave more prominent glycosylated proteins than control lymphocytes. This result indicates the increased protein glycosylation in lymphocytes of bovine theileriosis when compared with control lymphocytes.

Key words: Cattle, *Theileria annulata*, erythrocytes, lymphocytes, glycosylated proteins, sialic acid

INTRODUCTION

Tropical theileriosis caused by *Theileria annulata* has been reported from the various parts of North Africa, the Mediterranean basin, through the Middle East to the Indian sub-continent and China (Purnell, 1978; Dolan, 1989), as well as in different regions of Turkey (Sayın *et al*., 1994; Eren *et al*., 1995; Sayın *et al*., 2002; Dumanlı *et al*., 2005;). The protozoa *T. annulata* is propagated in cattle with stage-to-stage transmission by *Hyalomma ticks* (Bhattacharyuly *et al*., 1975; Pipano and Shkap, 2000). The main pathological damage as marked hyperplasia and expansion of the infected lymphoblast population in cattle is induced by the schizonte stage (Spooner *et al*., 1989; Pipano and Shkap, 2000). This disease induces important economic losses in the livestock industry and a high mortality in susceptible animals. It threatens exotic *Bos taurus* breeds of European origin particularly and it may cause 40-60% mortality in enzootic areas (Brown, 1990). Therefore, tick-borne infections are difficult to control, where their vectors are readily available.

Glycosylation is an important way for controlling the biological activity and metabolism of numerous proteins: it has been reported that N-glycosylation influences protein folding, stability, antigenicity, intracellular transport and biological activity (Opdenakker *et al*., 1993). Glycoproteins are defined as proteins which contain glycan chains linked to selected amino acid residues. Osides commonly found in the glycans of the glycoproteins including N-acetylneuraminic acid and sialic acid (Hemming, 1991). The sialic acid residues are commonly found at the extremity of the carbohydrate chain covalently linked to galactose, N-acetylgalactosamine or to other sialic acids (Schauer and Kamerling, 1997; Schauer, 2004). Sialic acids and some of the other terminal oses of cellular glycoproteins are often involved in important cell surface communications and infection processes.

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The aim of this study, is to compare the glycosylation pattern of erythrocyte and lymphocyte proteins between cattle with tropical theileriosis and healthy animals for the first time and to use Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Periodic Acid-Schiff (PAS) method, Dig-Glycan detection and differentation (lectin) kits for a better understanding of the pathophysiology of tropical theileriosis.

MATERIALS AND METHODS

Animal and protocol design: A total of 16 one year old Holstein heifers from the Ankara region (Turkey) were included in this study: 8 cows were naturally infected with *Theileria annulata* and the others were clinically healthy. All the diseased animals exhibited clinical signs compatible with tropical theileriosis, i.e., fever, enlargement of lymph nodes, haemolytic anaemia, inappetence, drooling from mouth, serous nasal discharge and swelling of the eyelids. After puncture of the jugular vein, blood samples were collected into EDTA containing vacutainer tubes and blood smears were prepared and stained with Giemsa method (methanol-fixed smears were stained with 5% of Giemsa solution (Merck) for 30 min) and examined under microscope at 100×magnification for the presence of Theileria piroplasms. Totally, 8 diseased animals with high percentage of parasitemia (50-70%) were selected for analysing glycosylated proteins from erythrocytes and lymphocytes.

Biochemical analysis of the glycosylated proteins: Separation of erythrocytes and lymphocytes from enfected and nonenfected blood samples were performed using ficoll paque (Pharmacia) as described in detail by Brown (1987). Equal amounts of cellular (erythrocytes and lymphocytes) extracts from enfected and nonenfected blood samples were mixed 1-1 in volumes with sample application buffer (0.0625 M Tris-HCl, pH 6.8, 2% (w v^{-1}) SDS, 5% (v v^{-1}) 2-mercaptoethanol, 10% (v v^{-1}) glycerol, 0.02% (w v^{-1}) bromophenol blue) and boiled in water bath at 100°C for 5 min before their application to the electrophoresis.

Cellular preparations were submitted to Sodium Dodecyl Sulphate Polyacrylamide-gel Electrophoresis (SDS-PAGE) under reducing conditions using 10% polyacrylamide gels with a Mighty Small II SE250 Vertical Slab Elecrophoresis unit (Hoefer Scientific Instruments, USA) according to the procedure described by Laemmli (1970). Electrophoresis with rutin running (tris-glycine) buffer system was performed at a constant current of 40 mA (20 mA gel^{-1}) at room temperature with cooling system for 3-4 h until the dye-front had reached the bottom of the gel. After electrophoresis, one gel was stained with Coomassie G-250 by the method of Candiano *et al.* (2004) to see the general protein profile and the other gel was Western blotted onto the nitrocellulose membrane for glycoprotein analysis. Separated proteins on gels by SDS-PAGE were transferred (blotted) onto nitrocellulose membranes as previously described (Burnettte, 1981). General detection of glycoproteins on gels was performed using Periodic Acid/Schiff (PAS) staining (Zacharius *et al.*, 1969; Kapitany and Zebrowski, 1973). Total glycoproteins on membrane blots were analysed using the DIG Glycan Detection Kit and the carbohydrate moieties of glycoproteins using the DIG Glycan Differentation Kit (Roche Molecular Biochemicals, Germany) after the separation of proteins by SDS-PAGE and transfer to nitrocellulose membranes, according to the manufacturer's instructions. Membranes were incubated with Digoxigenin-labeled lectins (DIG glycan differentiation kit, Roche Diagnostics Germany). DIG labelled lectins MAA (Maackia amurensis agglutinin; specific for sialic acid α-2-3-linked to galactose) and PNA (Peanut agglutinin; specific for the disaccharide galactose β (1-3) N acetylgalactosamine) were used from the kit. The bound lectins were immunologically detected using an anti-digoxigenin Fab-fragment conjugated to alkaline phosphatase using the protocol supplied with the kit.

RESULTS AND DISCUSSION

Erythrocytes from bovine theileriosis infected animals analysed by polyacrylamide gels and stained with PAS gave much more intensely stained bands than control cells although, they gave weaker bands on Coomassie stained polyacrylamide gels in comparison to control erythrocytes (Fig. 1). Specific binding of lectins to carbohydrate moieties was used to identify these structures in glycoproteins. The Maackia Amurensis Agglutinin (MAA) lectin staining of semi-dry blotted nitrocellulose membranes after SDS-PAGE analysis of erythrocytes from cattle with theileriosis gave very similar bands with control cells (Fig. 2a). Nevertheless, the PNA lectin probe failed to label glycoproteins from erythrocytes of diseased heifers whereas galactose β (1-3) N-acetylgalactosamine in O-glycosylated linked carbohydrate chains was detected in proteins from healthy cows (Fig. 2b). Lymphocyte proteins of bovines with and without infection were analysed by SDS-PAGE (Fig. 3).

As far as lymphocyte glycoproteins were concerned. Further more, staining of semi-dry blotted nitrocellulose membranes with Dig-glycan detection kit (Fig. 4) after

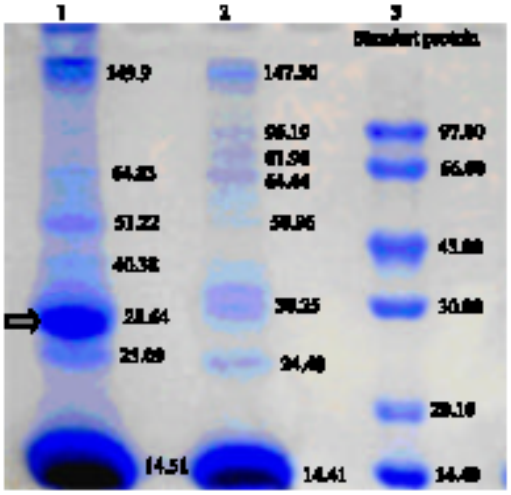

Fig. 1: Separation of erythrocyte proteins by SDS-PAGE. Proteins of erythrocytes were separated on 10% polyacrilamide gel and stained with Coomassie G-250 as indicated in Materials and methods. Lane 1 on the left; control erythrocytes of cattle (theileria negative). Lane 2 in the middle; *T. Annulata* infected erythrocytes cattle with high percentage of parasitemia (50-70%). Lane 3 on the right; Mixture of standard protein markers with actual molecular weights indicated to the right

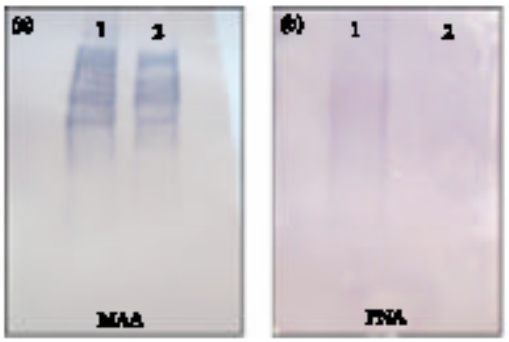

Fig. 2: Identification of terminal sugars of erythrocyte glycoproteins by SDS-PAGE. Proteins of erythrocytes were separated on 10% polyacrilamide gel and transferred onto nitrocellulose membranes by semi-dry blotting technique. Terminal carbohydrate moieties of erythrocyte glycoproteins on blotted membranes were analysed by use of digoxigenin-labelled lectins (MAA and PNA) assay. Lane 1; control erythrocytes of cattle (theileria negative). Lane 2; *T. annulata* infected erythrocytes cattle with high percentage of parasitemia (50-70%)

SDS-PAGE analysis of lymphocyte extracts from cattle infected with *T. annulata* gave more prominent

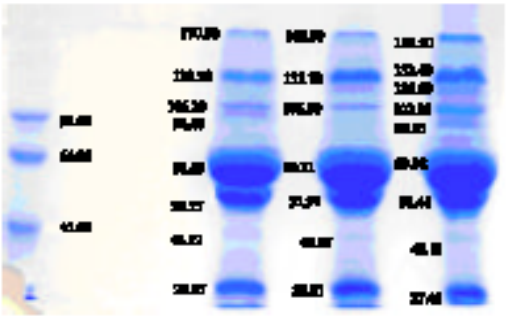

Fig. 3: Separation of lymphocytes proteins by SDS-PAGE. Proteins of lymphocytes were separated on 10% polyacrilamide gel and stained with Coomassie G-250 as indicated in Materials and methods. Lane 1 on the left; Mixture of standard protein markers with actual molecular weights. Lane 2 and 4; *T. annulata* infected lymphocytes of cattle with high percentage of parasitemia (50-70%). Lane 3; control lymphocytes of cattle (theileria negative)

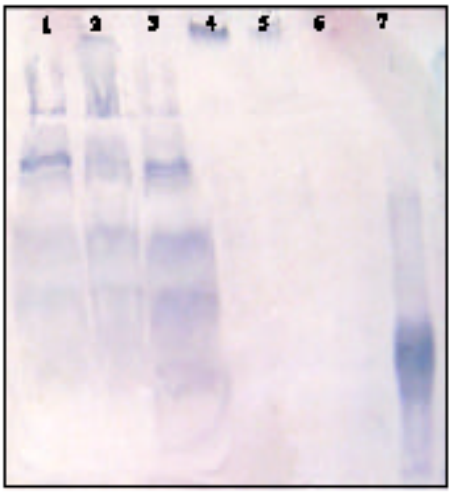

Fig. 4: Glycosylated proteins of lymphocytes are shown by staining with Glycan detection kit on blotted membranes following SDS-PAGE analysis. Lanes 4-6; control lymphocytes (no infection with *T. annulata*). Lanes 1-3; *T. annulata* infected lymphocytes cattle with high percentage of parasitemia (50-70%). Lane 7; transferrin as positive control for glycoprotein detection

glycosylated proteins than with control lymphocytes. The staining of blotted nitrocellulose membranes with the MAA probe revealed that lymphocyte glycoproteins from heifers with theileriosis were especially enriched in sialic acid residues (α-2-3-linked to galactose) contrary to those from controls (Fig. 5).

Fig. 5: Identification of terminal sialic acid in lymphocyte glycoproteins. Terminal sialic acid residues of erythrocyte glycoproteins were analysed by use of digoxigenin-labelled lectin (Maackia Amurensis Agglutinin MAA) assay. MAA is specific for sialic acid (α-2-3-linked to galactose) in complex sialylated O-glycan chains. Lane 1; control lymphocytes of cattle (no infection with *T. annulata*). Lane 2; *T. annulata* infected lymphocytes of cattle with high percentage of parasitemia (50-70%)

In the present study, the electrophoresis analysis of extracts of erythrocytes and lymphocytes from heifers infected by *T. annulata* showed that glycoproteins were globally more abundant in diseased animals than in healthy cows. Besides, the sugar composition of the carbohydrate chains was markedly modified and these changes were not always identical in erythrocytes and in lymphocytes. Interestingly, a specific protein band (around 28 kDa) seen in control erythrocytes was not evidenced in cell extracts from bovine theileriosis infected cows (Fig. 1).

The galactose β (1-3) N-acetylgalactosamine motif was surprinsingly absent in the glycoproteins from red blood cells of heifers with theileriosis. Moreover, whereas the occurrence of sialic acid (α 2-3 linked to galactose) structures was not affected in erythrocyte glycoproteins, lymphocytes from infected cattle exhibited numerous glycoproteins with this motif compared to control cells as evidenced by the use of the MAA probe.

The protozoa *T. annulata* is very pathogenic and the pathogenicity of a *Theileria* sp. is related to the density of schizontes in lymphocytes and piroplasms in erythrocytes (Soulsby, 1984; Kettle, 1995). Similarly, severely diseased animals selected in the present study exhibited a high density of schizontes in lymphocytes and of piroplasms in the erythrocytes. Glycosylation is the most common posttranslational modification of protozoa proteins and contributes to protein antigenic proteins (Lisowska, 2002). The diversity of protein glycosylation plays an important role in the biosynthesis and biological activity of the glycoproteins involved in antigen recognition (Rudd *et al*., 2001).

The results obtained in this study, suggest that increased protein glycosylation, especially with sialic acid, may have a significant role in the development of schizontes in lymphocytes during infection process of cattle with *T. annulata*. The identification and characterisation of these specific erythrocyte and lymphocyte glycoproteins from cattle with tropical theileriosis could be useful to design new vaccines for bovine theileriosis.

CONCLUSION

The glycosylation pattern of proteins from *T. annulata* infected erythrocytes and lymphocytes revealed by sugar specific staining polyacrylamide gels and the use of DIG specific lectins was markedly modified compared to that obtained from not infected cells. Glycoproteins were globally more numerous in these 2 cell types and also the sugar composition (for example, the frequency of sialic acid residues) in carbohydrate chains was notably changed according to the cellular parasite stage. This altered protein glycosylation could be particularly important for the development of the protozoa. Future studies should focus on the consequences of the altered protein glycosylation in the infected cells for the parasite and for the recipient animal.

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