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RESEARCH NOTE

Toxocara canis glycans influence antigen recognition by mouse IgG1 and IgM antibodies

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Abstract

The impact of sugar moieties of *Toxocara canis* glycoprotein antigens on their recognition by infected mouse antibodies was investigated in this study. Native TES and recombinant *Toxocara* mucins generated in *Pichia pastoris* yeast as well as their deglycosylated forms were used in ELISA. TES and recombinant mucins were equally recognized by *T. canis* infected mouse IgG1 antibodies. IgM immunoglobulins predominantly recognized TES antigens. Among mucins recognition of *Tc*-MUC-4 was the most significant. Deglycosylation of antigens resulted in significant loss of IgM and IgG1 reactivity to TES, mucins, *Tc*-MUC-3 and *Tc*-MUC-4. The presence of sugar moieties had no influence on IgE binding to native or recombinant *T. canis* antigens. Our results suggest that glycans are involved in epitope formation what should be taken into consideration in production of recombinant helminth antigens for diagnostic purposes.

Keywords

Toxocara canis, glycoproteins, recombinant antigens, antibodies

Toxocara canis is one of the most prevalent zoonotic parasites. Human toxocariasis is present worldwide. As reviewed by Maizels (2013), the seropositivity in Europe is noted in 3 to 7% of adults and 7 to 23% of children (Gillespie *et al*. 1993). In Poland prevalence of 14.5% was noted in teenagers (Jarosz *et al*. 2010), but the number of reported clinical cases of *Toxocara* infection in children has increased recently (Gawor *et al*. 2015). In USA almost 14% of examined children were positive for anti *Toxocara* antibodies (Won *et al*. 2008). The seroprevalence is much higher in tropical regions (Hayashi *et al*. 2005).

To date most diagnostic tests are based on *Toxocara* excretory – secretory (TES) antigens. These tests have some limitations, such as crossreactivity with antibodies from common human helminth infections (Smith *et al*. 2009). The sensitivity and specificity of diagnostic tests may be increased with use of recombinant antigens. So called low molecular weight antigens produced in *Escherichia coli* were tested with success (Yamasaki *et al*. 2000; Coelho *et al.* 2005; Mohamad *et al*. 2009). These antigens however lack their native glycoprotein structure. TES products also contain higher molecular mass heavily glycosylated proteins which have been identified as mucins (Gems and Maizels 1996, Loukas *et al*. 2000, Doedens *et al*. 2001). One of these was produced in yeast as a recombinant glycoprotein and it was proved that it can potentially be used as a diagnostic antigen (Fong and Lau 2004). Other mucins (*Tc*-MUC-2, -3, -4, -5) were not yet investigated in the context of antibody reactivity.

It has been shown that sugar moieties constitute 40% of total TES weight (Meghji and Maizels 1986). Synthetic analogues of these sugars, as shown in other studies, are recognized by antibodies from *T. canis* infected humans and mice (Schabussova *et al*. 2007, Koizumi *et al*. 2012). The aim of this study was therefore to evaluate the antigenicity of native TES and four recombinant *Toxocara* mucins produced in yeast versus their deglycosylated forms.

TES antigens were collected from an *in vitro* L3 larvae culture. Larvae were hatched from fully embryonated eggs by the method described by Oaks and Kayes (1979) and maintained in Minimal Essential Medium (Sigma Aldrich) supplemented

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with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) at 37°C, 5% CO_2. Medium containing TES antigens was collected, concentrated and dialysed into sterile phosphate buffered saline (PBS). Antigen concentration was determined using BCA Protein Assay (Thermo Scientific).

Recombinant mucins were produced in *Pichia pastoris* expression system (Life Technologies) as described elsewhere (Długosz *et al*. 2015). Briefly, cDNA fragments encoding mucins (*Tc*-muc-2, -3, -4 and -5) were cloned into pPICZα vectors. *P. pastoris* X33 strain were then transformed with recombinant vectors and protein expression was carried out in Buffered Minimal Methanol Medium at 28°C for 96 h. Proteins were purified from culture media using affinity chromatography with HIS Select HF Nickel Affinity Gel (Sigma Aldrich).

In order to remove glycan moieties TES and recombinant mucins were treated with sodium metaperiodate as described by Tawill *et al*. (2004). Samples were then dialyzed into PBS and the protein concentration was determined with BCA Protein Assay (Thermo Scientific). Investigated antigens before and after metaperiodate treatment were separated by SDS-PAGE in 12.5% polyacrylamide gels and stained with Coomassie Blue and Glycoprotein Staining Kit (Pierce). As shown in Fig. 1 and Fig. 2 sugar moieties were removed from TES and recombinant mucins.

8-week-old male BALB/c mice (n = 6) were experimentally infected with 500 *T. canis* infective eggs. Non infected mice (n = 6) were used as controls. Three weeks after infection animals were euthanized and sera were collected. The experiment was approved and conducted according to the guidelines of the Third Local Ethical Committee at Warsaw University of Life Sciences – SGGW.

Antibody reactivity to *Toxocara* antigens was measured with ELISA. Plates were coated with TES antigens (5 µg/ml), deglycosylated TES antigens (5 µg/ml), a mixture of recombinant mucins (5 µg/ml) (*Tc*-MUC-2, -3, -4 and -5 – 1.25 µg/ml each) and deglycosylated recombinant mucins (5 µg/ml) (*Tc*-MUC-2, -3, -4 and -5 – 1.25 µg/ml each) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) for 12 h at 4°C. Each mucin was also analyzed separately (1.25 µg/ml). The plates were then washed 3 times with PBS/0.05% Tween (PBS-T) and blocked with 5% skimmed milk in PBS-T (1 h, RT). After washing, the plates were incubated with diluted mouse sera (1:1000) for 1 h, washed and incubated with HRP conjugated goat anti mouse IgE, IgM, IgG1 and IgG2a (AbD Serotec) (1:4000) (1h, RT). Plates were developed with TMB substrate (Sigma Aldrich) and OD was read at 450 nm. Cut off values for each antigen or antigen mixture were calculated as an arithmetic mean of the optical densities of non infected serum samples + 3 ´ SD and were subtracted from each OD result from the infected group. Statistical analysis was performed using non-parametric Mann-Whitney test. A value of P<0.05 was considered to be significant.

T. canis antigens were recognized by mouse IgG1, IgM and IgE antibodies (Fig. 3). IgG2a reactivity to parasite antigens was very low (data not shown). IgM antibodies showed the highest reactivity to TES antigens. Deglycosylated TES proteins, mucins and deglycosylated mucins recognition by IgM antibodies was significantly lower. The presence of glycans on recombinant mucins also affected their antigenicity, as their glycoprotein forms showed higher reactivity comparing to deglycosylated forms. Among mucins *Tc*-MUC-4 was predominantly recognized by IgM and deglycosylation of this antigen led to significant loss of IgM reactivity.

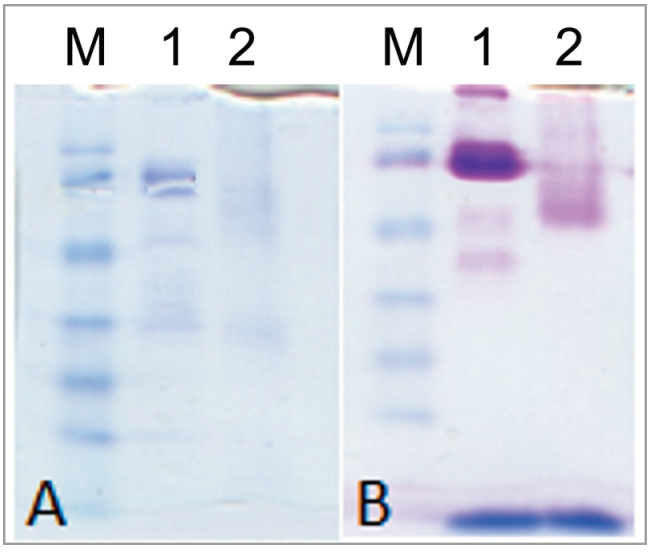


Fig. 1. SDS-PAGE analysis of TES and deglycosylated TES antigens. TES (lane 1) and deglycosylated TES (lane 2) were separated in 12.5% polyacrylamide gels and stained with Coomassie Blue (A) and with Glycoprotein Staining Kit (B), lane M – molecular weight marker

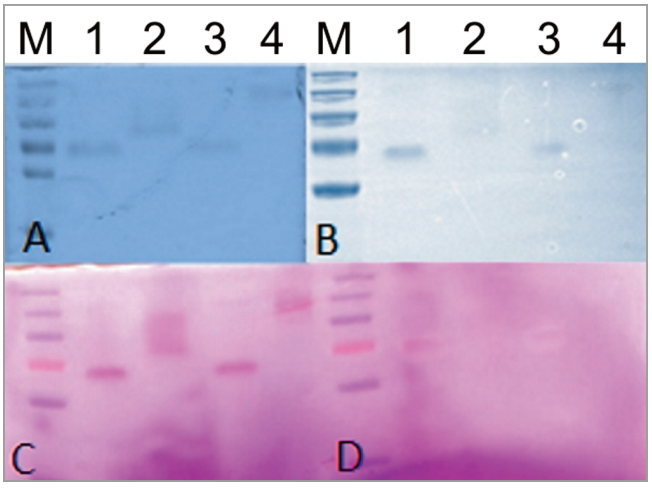


Fig. 2. SDS-PAGE analysis of recombinant and deglycosylated mucins. Recombinant mucins (A): *Tc*-MUC-2 (lane 1), *Tc*-MUC-3 (lane 2), *Tc*-MUC-4 (lane 3) and *Tc*-MUC-5 (lane 4) and their deglycosylated forms (B, lanes 1-4) were separated in 12.5% polyacrylamide gels and stained with Coomassie Blue (A, B) and Glycoprotein Staining Kit (C, D), lane M – molecular weight marker

It has been reported previously that murine IgM antibodies generated to *T. canis* infection recognize synthetic *Toxocara* trisaccharides (Schabussova *et al.* 2007). Authors found this polyclonal IgM response to be highly sensitive to the distribution and linkage of methylated modifications on parasite carbohydrates. This extreme sensitivity may explain why mouse IgM immunoglobulins recognized recombinant mucins much weaker than TES antigens. *Pichia* yeast carry out both *O*- and *N*- glycosylation (Ahmad *et al.* 2014), however it is possible that glycoproteins produced in yeast differ from native helminth glycoproteins in sugar structures. This expression system has been successfully used to generate parasitic antigens (Fong and Lau 2004, Bąska *et al*. 2013a, Rogé *et al*. 2013, Zawistowska-Deniziak *et al*. 2013), although in some cases it is not effective (Bąska *et al*. 2013b).

Sugar moieties are also involved in formation of epitopes recognized by IgG immunoglobulins. Synthetic *Toxocara* glycans are recognized by infected human serum IgG antibodies (Koizumi *et al*. 2012). Our results show that murine IgG1 antibodies react equally with TES and recombinant mucins and that glycosylation of these antigens affects the binding of immunoglobulins. Analysis of IgG1 reactivity to particular mucins showed no significant differences. Deglycosylation of *Tc*-MUC-3 significantly decreased its recognition by IgG1 immunoglobulins.

IgE production is a hallmark feature of the immune response against helminth parasites. In case of *Toxocara* infections detection of specific IgE has been considered as a diagnostic method in ocular toxocariasis (Fillaux and Magnaval 2013). However not all patients develop *Toxocara* specific IgE (Mohamad *et al*. 2009), they are absent in 20% of infected individuals (Fillaux and Magnaval 2013). In our experiment we have also noted high differences in reactivity of IgE originating from individual mice. It seems that IgE production toward *Toxocara* molecules is not affected by the form of the antigen and that TES and recombinant mucins do not differ in their antigenicity to IgE antibodies.

The diagnostic potential of *T. canis* recombinant antigens has already been shown (Yamasaki *et al*. 2000; Coelho *et al*. 2005; Mohamad *et al*. 2009). Most studies however concerned antigens produced in bacterial expression systems therefore lacking their glycan moieties. Fong and Lau (2004) generated recombinant TES-120 antigen in *P. pastoris* and proved its antigenicity to human antibodies. Our results are in line with their result. Moreover we show that the presence of glycans increases the reactivity of recombinant antigens with IgG1 and IgM antibodies. Recombinant yeast glycoproteins show therefore a great potential in diagnosis of toxocariasis.

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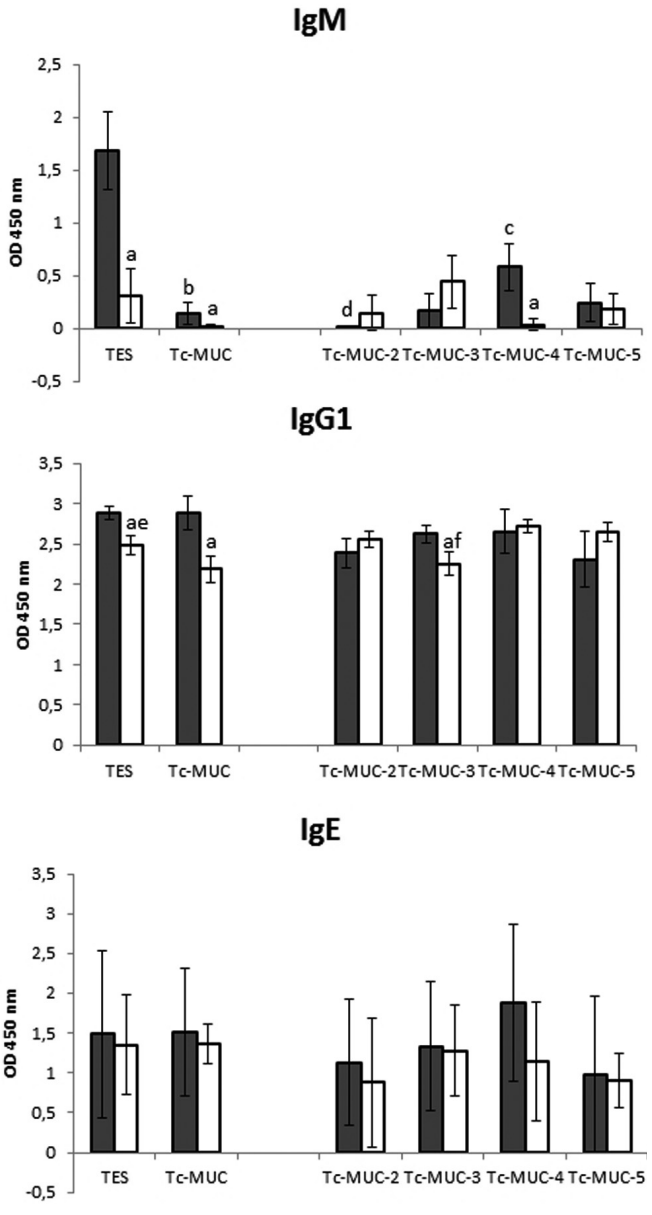


Fig. 3. Reactivity of *T. canis* infected mouse antibodies with *Toxocara* excretory-secretory antigens (TES), mixture recombinant mucins (*Tc*-MUC) and particular mucins (*Tc*-MUC-2, -3, -4 and -5) (black bars) and the corresponding deglycosylated antigens (white bars) determined by ELISA. Data is presented as mean ± SD from six animals. Cutoff OD values for each antigen were calculated as the arithmetic mean of the optical densities from corresponding non infected group + 3SD and were subtracted from each OD result from infected group. Statistical analysis was performed using non-parametric Mann-Whitney test. A value of $P<0.05$ was considered to be significant. Differences were marked with: a - different form the corresponding deglycosylated antigen; b – different from TES; c – different from *Tc*-MUC-2, -3 and -5; d – different from *Tc*-MUC-4 and -5; e – different from deglycosylated *Tc*-MUC; f – different from deglycosylated *Tc*-MUC-2, -4 and -5

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