The effect of glycosylation of antigens on the antibody responses against *Echinostoma caproni* (Trematoda: Echinostomatidae)

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SUMMARY

In the present study, we analyse the effect of glycosylation in *Echinostoma caproni* (Trematoda: Echinostomatidae) antigens in antibody responses against the parasite in experimentally infected mice. It has been previously demonstrated that the mouse is a host of high compatibility with *E. caproni* and develops elevated responses of IgG, IgG1, IgG3 and IgM as a consequence of the infection, though the role of glycans in these responses remains unknown. To this purpose, the responses generated in mice against non-treated excretory/secretory antigens of *E. caproni* were compared with those observed after N-deglycosylation, O-deglycosylation and double deglycosylation of the antigens by indirect ELISA and western blot. Our results suggest that *E. caproni*-expressed glycans play a major role in the modulation of the immune responses. The results obtained indicate that IgG subclass responses generated in mice against *E. caproni* are essentially due to glycoproteins and may affect the Th1/Th2 biasing. The reactivity significantly decreased after any of the deglycosylation treatments and the N-glycans appears to be of greater importance than O-glycans. Interestingly, the IgM response increased after N-deglycosylation suggesting that carbohydrates may mask peptide antigens.

Key words: Trematoda, *Echinostoma caproni*, mouse, deglycosylation, glycoprotein, carbohydrates, immunoglobulin, western blot.

INTRODUCTION

Echinostoma caproni (Trematoda: Echinostomatidae) is an intestinal trematode without migration in the definitive host. After infection, metacercariae excyst in the duodenum of the definitive host and the juvenile worms migrate to the posterior third of the small intestine where they attach to the mucosa by the ventral sucker (Fried and Huffman, 1996; Fried *et al*. 2004). *Echinostoma caproni* has a wide range of definitive hosts, and the *E. caproni*-rodent model has been extensively studied to elucidate several aspects of the host-parasite relationships in intestinal infections in the mammalian definitive hosts. In this context, the effect of the host immune response on the course of intestinal helminth infections has been one of the subjects analysed in detail (Toledo and Fried, 2005; Toledo, 2009).

Although several studies on the humoral response generated against *E. caproni* in the definitive host have been performed in the past (Agger *et al*. 1993; Brunet *et al*. 2000; Graczyk, 2000, Sotillo *et al*. 2007), the effect of glycosylation on the antibody response against *E. caproni* has been poorly studied. Simonsen and Andersen (1986) described an intense humoral response in experimentally infected mice against glycosylated antigens, though the extent of the influence in this response of glycans was not determined. Similarly, Sotillo *et al*. (2008) suggested that glycosylated proteins might be mainly responsible for the antibody response against *E. caproni* in mice. In other helminth species, several studies have shown that carbohydrates play an essential role in eliciting the humoral response (Norden and Strand, 1985; Cummings and Nyame, 1996, 1999). It has been observed that the major immune responses to trematode infections are directed to glycan antigens, rather than to peptide epitopes (Eberl *et al*. 2001; Hokke and Deelder, 2001; Nyame *et al*. 2003, 2004), and the response against glycans is biased towards Th2 responses (Faveeuw *et al*. 2003; Tawill *et al*. 2004; Harn *et al*. 2009). Furthermore, the glycosylation of antigens may have important implications in intestinal helminth infections. Several authors have suggested that the parasites could use the carbohydrate epitopes to divert the host immune response away from them (Dell *et al*. 1999). Cummings and Nyame (1999) stated that a better understanding of the carbohydrates present in excretory/secretory

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products (ESP) and their interactions with the host can lead to improved diagnostic procedures and potential glycan-based vaccines.

The aim of the present study is to determine the importance of glycosylation in the humoral systemic response in mice against *E. caproni*. The results obtained may contribute to the understanding of the host-parasite relationship in intestinal trematodiasis and the factors determining long-lasting infections or worm rejection.

MATERIALS AND METHODS

Parasite, host and experimental infection

The strain of *E. caproni* has been previously described by Fujino and Fried (1993). Encysted metacercariae of *E. caproni* were removed from the kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* snails and used to infect ICR male mice. Five male ICR mice were infected by gastric gavage with 75 metacercariae of *E. caproni* each. All animals were necropsied at 12 weeks post infection (wpi) and parasites were employed to make excretory/secretory antigens.

Excretory/secretory antigens

To obtain the ESP from *E. caproni*, we followed the method described by Toledo *et al*. (2003). Briefly, adult worms were collected from the intestine of the experimentally infected mice at 4 wpi. After collected, the parasites were washed with phosphate buffered saline (PBS, pH 7·4), and maintained at concentrations of 10 worms mL^{-1} for 12 h at 37 °C in PBS containing complete mini EDTA-free protease inhibitor cocktail (Roche). The medium was collected and centrifuged at 15 000 ***g*** for 30 min at 4 °C. After this centrifugation the supernatant was collected and the protein content was measured by the Bio-Rad (Hercules, California) protein assay and adjusted to 1 mg mL^{-1}. The antigens were stored at −20 °C until use.

Serum samples

Blood was collected from each animal by submandibular bleeding biweekly. After clotting of the blood overnight at 4 °C, serum was separated from the clot by centrifugation. The serum samples were stored at −20 °C until use.

Deglycosylation

Deglycosylation was carried out using two different enzymes: N-glycosidase (Roche) and O-glycosidase (Roche). 100 μg of ESP were incubated overnight with 100 mM Na_2HPO_4, 25 mM EDTA, 0·2% SDS, 1% Nonidet P40, 1% β-mercaptoethanol and the adequate amount of enzyme (2U of N-glycosidase and 2,5U of O-glycosidase).

Detection of glycoproteins

In order to assess the correct deglycosylation, the excretory/secretory products and deglycosylated ESP of *E. caproni* were submitted to a 10% SDS-PAGE gel. Glycoproteins were detected using the glycoprotein detection kit (Roche). The kit consisted of a periodate oxidation followed by the Schiff's reagent staining and a reduction by sodium metabisulfite. Glycosylated proteins were stained, whereas non-glycosylated proteins remained unstained. A Coomassie staining of the double deglycosylated antigens was performed in order to determine if the ESP had suffered any degradation after deglycosylation treatment.

Indirect ELISA for antibody detection

In order to detect specific antibodies against *E. caproni* ESP, an indirect ELISA was carried out as described by Sotillo *et al*. (2007). This method was designed to detect IgM, IgG, IgG1 and IgG3 in serum samples from mice. Briefly, polystyrene microtitre plates (Nalgene, Naperville, IL) were coated overnight at 4 °C with 100 μL $well^{-1}$ of a 30 μg mL^{-1} solution of glycosylated (ESP) and N-deglycosylated (NG), O-deglycosylated (OG) and double-deglycosylated (DG) antigen of *E. caproni*. After thorough washings plates were blocked with a 4% non-fat dry milk-PBS solution. Then, plates were incubated with serum at adequate dilutions for 2 h at RT. In the next step, plates were incubated with goat anti-mouse IgG (Bio-Rad), goat anti-mouse IgG1 (Nordic), goat anti-mouse IgG3 (Nordic) and goat anti-mouse IgM (OEM concepts, Toms River, NJ) coupled to horseradish peroxidase at adequate conditions for 2 h at RT. Finally, 100 μL of the substrate solution was added to the plates and these were read at 492 nm in a Bio-Rad iMark Microplate Reader.

1D SDS-PAGE and western-blot analysis

For the analysis of the glycosylation pattern recognition, a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was performed using the method of Sambrook *et al*. (1989). Equal amounts of glycosylated and non-glycosylated proteins were loaded into each lane of a 10% resolving gel and 4% stacking gel, using a vertical slab gel system (Bio-Rad). Electrotransfer of polypeptide from gel to nitrocellulose membrane (0·45 mm) was performed as described by Hillyer and Soler de Galanes (1988). After blocking with 5% skimmed milk at RT for 2 h, a 1/200 diluted serum was added to the nitrocellulose and incubated for 2 h at RT. For better results, only the sera of 0, 2 and 8 wpi were analysed. The nitrocellulose was then washed and probed with goat anti-mouse IgG (Bio-Rad), goat anti-mouse IgG1

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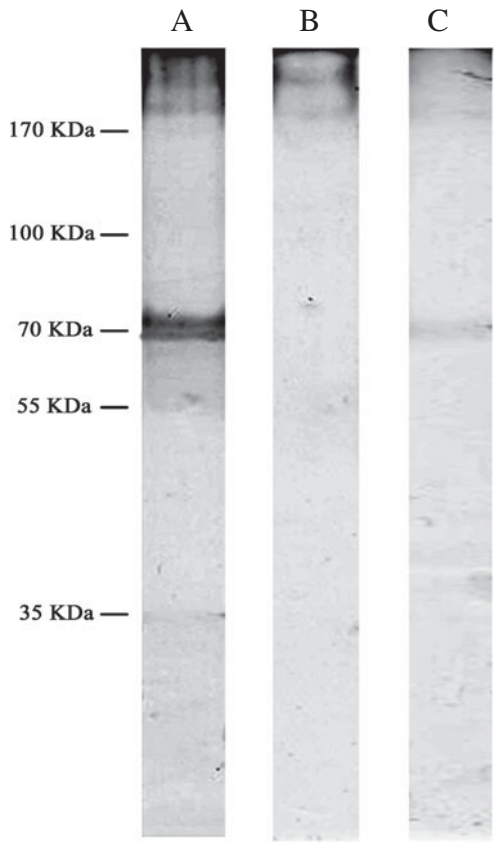

Fig. 1. Detection of glycoproteins in ESP (A), N-deglycosylated (B) and O-deglycosylated (C) *Echinostoma caproni* antigens using the Roche glycoprotein detection kit.

(Nordic), goat anti-mouse IgG3 (Nordic) and goat anti-mouse IgM (OEM concepts, Toms River, NJ) coupled to horseradish peroxidase at adequate conditions for 2 h at RT.

Statistical analysis

Each ELISA assay was performed in triplicate and the absorbance readings from wells with the same sample were expressed as the mean ± S.D. The difference between the optical density (OD) values obtained for each treatment in serum specimens on each point in time was calculated and tested by the use of Student's *t*-test. $P < 0{\cdot}05$ was considered as significant.

RESULTS

Detection of glycoproteins in E. caproni *ESP*

The pattern of protein glycosylation in the ESP of *E. caproni* is shown in Fig. 1. Most of the glycosylated proteins were detected at a molecular weight (MW) higher than 170 kDa. Moreover, a prominent band was observed at 70 kDa and additional fainter bands at low MW (Fig. 1A). Deglycosylation was achieved almost completely using N-glycosidase and only some bands remained at high MW after the treatment (Fig. 1B). After O-deglycosylation some bands at high MW and also the 70 kDa band remained glycosylated (Fig. 1C). The Coomassie staining of the double deglycosylated ESP shows most of the proteins in the range of 12–48 kDa, although some bands could be seen between 50–199 kDa, confirming the lack of degradation after double-deglycosylation of the antigens (Supplementary Fig. 1).

Kinetics of IgG, IgG1, IgG3 and IgM to glycosylated and non-glycosylated E. caproni *ESP*

As mentioned above, the *E. caproni* infection in mice is characterized by significant responses of IgG, IgG1, IgG3 and IgM. Herein, we analyse the effect of glycosylation in these responses.

IgG. The IgG responses against deglycosylated and non-deglycosylated antigens are shown in Fig. 2A. The antibody levels against non-deglycosylated ESP increased over the course of the infection to reach a peak at 10 wpi (1·094 ± 0·223). The responses against NG and OG were similar until 6 wpi, when the values decreased abruptly. In the case of the OG, the values were significantly lower than those in ESP from that week until the end of the experiment, whereas in the case of NG the IgG level was recovered at 12 wpi. The antibody levels against DG were significantly lower than those observed against ESP for each week from the 3 wpi until the end of the experiment (Fig. 2A). The maximum value using NG antigens was found at 12 wpi (1·033 ± 0·08). Using OG and DG antigens, the maximum values were found at 12 wpi (0·62 ± 0·21) and at 6 wpi (0·795 ± 0·137), respectively.

IgG1. The response mediated by IgG1 was strongly determined by the glycosylation of antigens. The responses against glycosylated antigens were elevated reaching a maximum at 8 wpi (1·025 ± 0·355). In contrast, the values against deglycosylated antigens were significantly lower from 4 wpi until the end of the experiment ($P < 0{\cdot}05$) for all the treatments (Fig. 2B). The maximum OD values when using NG, OG and DG antigens were found at 12 wpi (0·59 ± 0·2), 8 wpi (0·47 ± 0·28) and 8 wpi (0·504 ± 0·41), respectively.

IgG3. The levels of IgG3 against ESP in serum of mice rapidly increased reaching a maximum OD value at 8 wpi (0·682 ± 0·187) and decreased thereafter. Using NG and DG antigen, IgG3 responses were significantly lower than those obtained against ESP for each week from 2 to 10 wpi. Using OG antigen the values were significantly lower than ESP only at 8 and 10 wpi (Fig. 2C). The maximum OD values using NG, OG and DG antigens were

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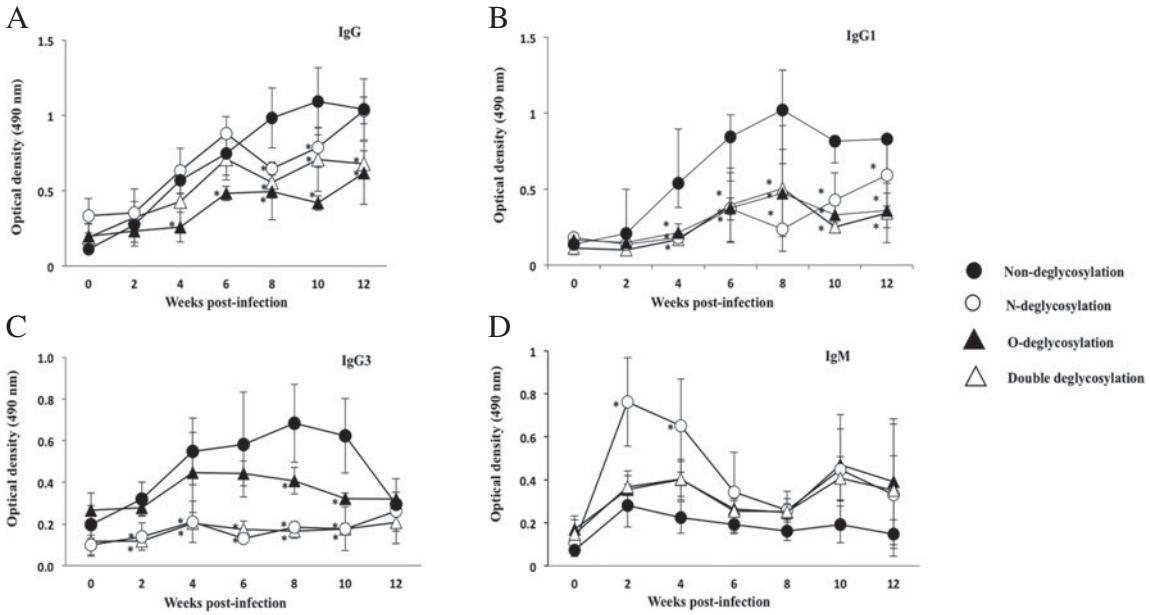

Fig. 2. Detection of serum IgG (A), IgG1 (B), IgG3 (C) and IgM (D) against *Echinostoma caproni* excretory/secretory antigens by ELISA in experimentally infected mice over the course of the experiment. Vertical bars represent the S.D. Asterisks represent significant differences between each treated antigen and non-treated *Echinostoma caproni* excretory/secretory antigens ($P < 0{\cdot}05$).

observed at 12 wpi (0·26 ± 0·15), 4 wpi (0·44 ± 0·19) and 12 wpi (0·207 ± 0·041), respectively.

IgM. Contrary to that observed in the other immunoglobulins, the OD values increased using deglycosylated antigens. Interestingly, the IgM values against ESP were higher using NG than in non-treated ESP or in the other treatments (Fig. 2D). Using NG, the values rapidly increased to reach a maximum at 2 wpi (0·761 ± 0·204) and thereafter decreased. The values were significantly higher than those observed using non-treated ESP at 2 and 4 wpi. In contrast no significant IgM responses were detected against non-treated ESP, OG and DG.

Immunoglobulin recognition patterns before and after deglycosylation

The results obtained for IgG, IgG1, IgG3 and IgM recognition by western blot after deglycosylation of the ESP are shown in Fig. 3.

IgG recognition pattern. The strongest reaction was detected when using ESP antigens, with several bands between 25–105 kDa and a denser area of bands at 85–90 kDa at 2 and 8 wpi. Almost all the bands recognized by sera when ESP antigen was used disappeared after deglycosylation and only fewer and weaker bands were observed. A single band at 45–47 kDa was also recognized after deglycosylation at 2 and 8 wpi except in DG (only at 8 wpi). Another single band at 70–80 kDa was recognized when N-deglycosylating, but it was not recognized when O-deglycosylating or double-deglycosylating.

IgG1 recognition pattern. The pattern observed with IgG1 was similar to that obtained with IgG. The recognition in ESP was focused in an area between 100–130 and 30–130 kDa at 2 and 8 wpi respectively. A strong band also was observed at 100 kDa using ESP antigen, and this band remained using NG or OG antigens though it was not observed in DG antigen. Apart from a band at 150 kDa (which strikingly was not observed with ESP, NG and OG) some other bands were observed under 37 kDa in DG. Using NG antigen, only three bands were observed, whereas more than five bands were seen when using OG antigen.

IgG3 recognition pattern. The IgG3 recognition was strong beginning at 2 wpi. Bands were almost at each MW (30–120 and 20–154 kDa at 2 and 8 wpi, respectively) using ESP antigen. The number of recognized bands diminished using NG antigen. Only two bands were seen at 2 wpi at 70 and 60 kDa. Additional bands were detected at 8 wpi at a MW range of 20–150 kDa. No bands were seen when using OG or DG antigen.

IgM recognition pattern. In contrast to that observed by ELISA, the recognition of bands was higher in ESP than in the deglycosylated antigens. Lesser number of bands, though more intense, were recognized using NG antigen, which was in concordance with the results obtained with ELISA. A single band at 70 kDa was observed at 2 and 8 wpi

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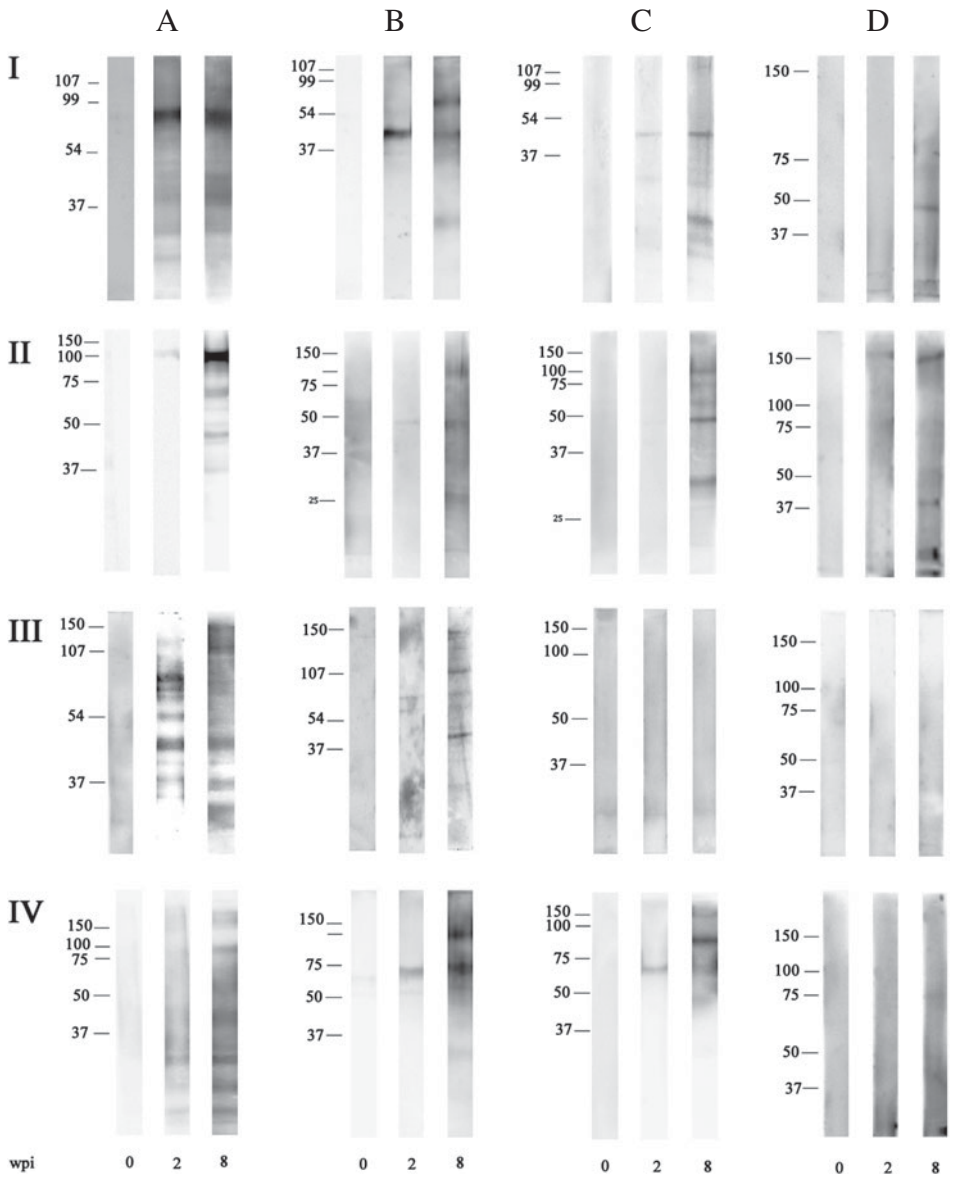

Fig. 3. Immunoblots against mouse anti-IgG (I), anti-IgG1 (II), anti-IgG3 (III) and anti-IgM (IV) of *Echinostoma caproni* antigens: (A) non-treated; (B) N-deglycosylated; (C) O-deglycosylated; and (D) double-deglycosylated antigens. Only blots at 0, 2 and 8 weeks post-infection (wpi) for each isotype and treatment are shown.

as well as some other bands at 100, 60 and 25 kDa at 8 wpi. The intensity of bands was correlated with the data obtained by ELISA. Using OG antigen the intensity of bands markedly diminished, though similar bands to NG were observed. Very few bands were seen when using DG antigen.

DISCUSSION

Although it is well known that *E. caproni* induces strong antibody responses in mice, little is known about the factors determining the induction and the modulation of these responses. In the present study, we analysed the effect of the glycosylation of the antigens of *E. caproni* in the regulation of the antibody responses.

Glycoconjugates play a crucial role in several aspects of the host-parasite relationships in helminth infections, including the generation and modulation of the immune responses (Cummings and Nyame, 1996, 1999; Eberl *et al*. 2001; Hokke and Deelder, 2001; Nyame *et al*. 2003, 2004; Van Diepen *et al*. 2012). Herein, we have shown that infection of mice with the intestinal helminth *E. caproni* generates an important antibody response that is markedly modulated by the presence of carbohydrate residues. Our results in relation to the antibody response against *E. caproni* in mice are consistent with previous studies (Sotillo *et al*. 2007). Serum responses in mice are characterized by elevated levels of IgG, IgG1 and IgG3, together with an earlier and transient IgM response. Moreover, we have demonstrated that these responses can be largely attributed

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to the presence of carbohydrate structures. In general, the treatment of ESP with either O-, N-or both glycosidases resulted in a significant reduction or even the abrogation of the reactivity. Our results suggest that *E. caproni* glycosylation profile is similar to other parasitic helminths such as *Schistosoma* spp. (Hokke *et al*. 2007), *Taenia solium* (Haslam *et al*. 2003) or *Opisthorchis viverrini* (Talabnin *et al*. 2013) in which N-glycosylation is dominant.

The total IgG response was markedly reduced after double deglycosylation as demonstrated by ELISA. Furthermore, almost all the bands recognized by total IgG in the non-treated ESP disappeared after double deglycosylation. In the treated ESP only a discrete band at 45–47 kDa was recognized, indicating that this is a non-glycosylated antigen. Interestingly, the MW of this band coincides with non-glycosylated forms of enolase, which is known to be one of the most immunogenic proteins in the *E. caproni* infections in mice (Sotillo *et al*. 2008). Sotillo *et al*. (2008) showed that a glycosylated enolase was only recognized by IgM whereas the deglycosylated antigen was recognized by IgG, suggesting the role of glycosylation in the different recognition pattern. It is remarkable that the antigenicity of the ESP was significantly more reduced after O-deglycosylation than after N-deglycosylation, confirming the importance of the O-glycosylation in the IgG response against *E. caproni*. A discrete band at 70 kDa was exclusively recognized by serum in the N-deglycosylated ESP suggesting that this is an O-glycosylated antigen. Some other bands also were detected at low MW in O-deglycosylated antigens, probably as a consequence of the degradation of glycosylated antigens of higher MW.

The IgG1 response was the most affected by the glycosylation. This response was reduced after any of the deglycosylation treatments suggesting that the response to *E. caproni* mediated by the IgG1 subclass is almost completely due to glycan antigens. Moreover, the analysis by western blot showed that the 100 kDa band was recognized on non-treated ESP and remained after O- and N-deglycosylation, though the band disappeared after double-deglycosylation. This fact also occurs with other bands between 40–80 kDa. This suggests glycans in *E. caproni* ESP are mainly responsible for the immune response biased toward a Th2 phenotype, which is the systemic response that characterizes the infection in mice (Sotillo *et al*. 2007; Trelis *et al*. 2011). The IgG1 response has been commonly correlated to glycans. It has been shown that the immune responses biased towards a Th2 response are largely due to helminth expressed glycans (Faveeuw *et al*. 2003; Tawill *et al*. 2004; Thomas and Harn, 2004; Perrigoue *et al*. 2008; Harn *et al*. 2009; Tundup *et al*. 2012). Tawill *et al*. (2004) proposed that glycans of helminths might potentiate the Th2 arm by binding to receptors in IL-4 producing cells or by the recruitment of cells that produce suppressive cytokines such as IL-10 or TGF-β. Our results in previous studies do not support a role for IL-10 or TGF-β since the levels of these cytokines are unaltered in the spleen of mice infected with *E. caproni* (Sotillo *et al*. 2011; Trelis *et al*. 2011). Probably, the production of IL-4 and/or IL-13 in relation to the carbohydrates in the ESP of *E. caproni* are of more importance (Trelis *et al*. 2011).

Sotillo *et al*. (2007) showed that *E. caproni* infection in mice is characterized by strong and maintained IgG3 responses. Our results support the notion that the IgG3 response in mice is related to carbohydrate epitopes and mainly to N-linked epitopes. After N- and double-deglycosylation, the IgG3 reactivity was markedly reduced and most of the bands in the immunoblottings disappeared. In contrast, we only observed a slight decrease in the response against IgG3 after O-deglycosylation whereas no bands remained in the western blots. The fact that the IgG3 subclass may recognize glycan epitopes and carbohydrates is well documented (Snapper *et al*. 1992; Dea-Ayuela *et al*. 2000). It has been shown that certain thymus-independent antigens induce the CD5+ populations to undergo an IgG3 class switch (Drabek *et al*. 1997) upon control of Th1 cytokine IFN-γ (Snapper *et al*. 1992). The intense IgG3 response observed in mice may be related to the N-glycosylation of the antigens in ESP of *E. caproni*. This is also supported by the fact that *E. caproni* infection generates significant increases of IFN-γ in the spleen of infected mice (Trelis *et al*. 2011).

One of the most striking features observed in our study is the IgM reactivity against deglycosylated ESP of *E. caproni*. The reactivity of IgM against N-deglycosylated antigens was significantly higher than against non-treated ESP or the remainder of the deglycosylation treatments. This suggests that carbohydrates mask some peptide epitopes, which may be a mechanism to evade the antibody response. It has been proposed that the carbohydrates present on the surface of helminths could act as a smoke screen to divert the immune attention to these molecules and away from the parasite (Maizels *et al*. 1993). These authors suggested that the anti-glycan responses would mask vulnerable peptide epitopes or larva-specific glycan epitopes that could mediate a protective response and, thus, the immune response would be biased towards the carbohydrates excreted rather than towards the tegument and the parasite (Maizels *et al*. 1993; Eberl *et al*. 2001). Although we can only speculate that carbohydrates bias the immune response in *E. caproni* infections, the fact that different antibody responses are driven by the ESP glycans together with the rapid tegumental turnover that occurs in *E. caproni* (Andresen *et al*. 1989) may explain, at least in part, the resistance of the adult worms to the elevated antibody responses generated

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by this parasite in mice, even at the local level (Sotillo *et al*. 2007).

In summary, we have shown that antibody response against *E. caproni* in mice is essentially generated by glycoconjugates. Furthermore, *E. caproni*-expressed glycans appear to play a major role in the modulation of the host immune response by the biasing of the response toward a systemic Th2 phenotype, and the expression of glycoconjugates by the parasite may serve to evade the antibody response by masking peptide epitopes.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182014000596.

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