Comparison of IgG3 responses to carbohydrates following mouse infection or immunization with six species of *Trichinella*

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Abstract

The IgG3 antibody responses to carbohydrate epitopes were compared in BALB/c mice infected or immunized with six species of *Trichinella*: *T. spiralis* (T1), *T. nativa* (T2), *T. britovi* (T3), T6, *T. nelsoni* (T7), and T8. The dynamics of IgG3 responses and antigen recognition following infection or immunization were measured by ELISA and Western blot respectively, using glycosylated and deglycosylated larval crude extracts (LCE) prepared from homologous isolates. A high degree of protein glycosylation was found in all species and with similar profiles. Deglycosylation was completely achieved only in LCE from T1 and T6 isolates. The dynamics of IgG3 responses following infection or immunization significantly differed whereas the antigen recognition profiles appeared similar. Variations in the levels and antigen recognition patterns of IgG3 among the different species were apparent. The highest IgG3 levels were recorded in infections by the T8 isolate and the lowest in infections by the T6 isolate, whereas for immunization the highest IgG3 response was induced by T7 and the lowest by T8. Following antigen deglycosylation, the IgG3 responses were significantly reduced or abrogated and the recognition patterns markedly modified or suppressed in the different species of *Trichinella*.

Introduction

Glycoproteins are abundant components of the *Trichinella* muscle larvae. They are present in surface (Parkhouse *et al*., 1981; Ortega-Pierres *et al*., 1984, 1986; Mclaren *et al*., 1987), excretory–secretory (Silberstein & Despommier, 1984; Gold, 1990) and somatic products (Parkhouse & Ortega-Pierres, 1984). They occur in group II antigens (Denkers *et al*., 1991), composed of six major proteins of molecular weights ranging between 43–68 kDa and 5.0–6.3 isoelectric points, under reducing conditions and are localized in the outer cuticle, granules of the stichocytes, and in the lumen of the alimentary tract (Takahashi *et al*., 1997). These highly glycosylated proteins share a common immunodominant carbohydrate epitope recognized by the monoclonal antibody Tsp 130 and they are therefore included in the TSL-1 group of antigens (Appleton *et al*., 1991; Ortega-Pierrres *et al*., 1996). Chemical characterization of the TSL-1 antigens (Wisnewski *et al*., 1993) revealed the presence of the unusual sugar 3,6-dideoxihexose (tyvelose) and the fucose accounting for 26% and 36% molar of total glycosidic residues, respectively. Monoclonal antibodies have been used to further characterize the *T. spiralis* glycans. Protective antibodies failed to recognize ES antigens after deglycosylation with trifluormethansulphonic acid (TFMS) (Ellis *et al.,* 1994). By contrast, synthetic peptides of the 45 kDa glycoprotein were only recognized by antibodies generated against the

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43 kDa deglycosylated protein (Robinson *et al*., 1995) suggesting that some relevant epitopes could be masked by carbohydrates .

IgG3 subclass isotype responses are related to carbohydrate epitopes (Snapper *et al*., 1992). It has been shown that certain thymus-independent antigens can induce the CD5+ population to undergo a IgG3 class switch (Drabek *et al*., 1997) upon control by the Th1 cytokine γ-IFN (Snapper *et al*., 1992).

Despite the extensive analysis of IgG isotype responses in both experimental and clinical trichinellosis, only a few studies have been concerned with the IgG3 subclass (Almond & Parkhouse, 1986; Ljungstrom *et al*., 1988; Zakroff *et al*., 1989; Robinson *et al*., 1994). Therefore, in the present paper, a comparison of the dynamics of IgG3 responses and antigen recognition patterns on glycosylated and deglycosylated antigens has been made in BALB/c mice following infection and immunization with six species of *Trichinella*.

Materials and methods

Parasites

The following isolates of six *Trichinella* species were used: MFEL/SP/62/GM-1 (*T. spiralis*), MPAN/SU/87/T-2 (*T. nativa*), MCAN/SP76/C-76 (*T. britovi*), MFEL/US/85/T-6-FC , MCRO/KE/75/T-7 (*T. nelsoni*), MPAN/SA/89/T-8. They are reference strains kept in the *Trichinella* Reference Centre (Istituto Superiore di Sanitá, Rome).

Experimental hosts

Female BALB/c mice of 8 weeks of age were used. They were bred in our Animal Breeding Unit under controlled light and temperature conditions. Food and water were supplied *ad libitum*.

Preparation of larval crude extracts (LCE)

First stage (L1) muscle larvae were obtained after artificial digestion using the method of Wakelin & Lloyd (1976). After selection of live larvae by the Baermann method, they were washed ten times by sedimentation in PBS pH 7.2–7.4. After washing, the settled larvae were sonicated in a Virsonic 5 sonicator for several 10 s pulses at 70% power. The crude extract was allowed to extract overnight at 4°C and then centrifuged at 40.000 *g* for 1 h. The supernatant was collected and the protein content estimated by the Bradford (1976) method. Once aliquoted the samples were stored at –80°C.

Infection

Mice were divided into six groups of six mice each. Each group was infected with 600 larvae of each of the *Trichinella* species. Larvae were administered orally using an insulin syringe coupled to a blunted needle.

Immunization

Groups of six mice were prepared and each group was immunized intramuscularly with LCE from one of the *Trichinella* species. The antigen was emulsified in an equal volume of Freund complete adjuvant, to provide a dose of 500 μg per mouse.

Serum samples

Infected and immunized mice were bled every two weeks for 18–21 weeks, from the retro-orbital venous sinus under ether anaesthesia. Blood samples were allowed to clot and then centrifuged at 900 *g* for 15 min. Sera samples from each mouse group were pooled, aliquoted and stored at –30°C until use.

Detection of glycoproteins in LCE

Larval crude extracts from *Trichinella* strains were submitted to SDS-PAGE in 5–20% gradient gels (Laemmli *et al*., 1970) and blotted onto nitrocellulose paper. Glycoproteins were detected using a commercial kit (BioRad). The process consists of a periodate oxidation followed by biotinylation with hydrazide solution in sodium acetate/EDTA. The reaction was developed by streptavidin-alkaline phosphatase with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate.

Enzymatic deglycosylation of LCE

Deglycosylation was carried out using a commercial kit (BioRad) following the instructions from the manufacturer. Briefly, the LCE samples from each strain were freeze-dried to adjust to an initial concentration of 10 mg ml^{-1} and then treated by NaNase II and O-glycosidase for 1 h at 37°C. Following enzymatic treatment by PNGase F for 3 h at 47°C, samples were boiled in 2% sodium dodecyl-sulphate (SDS) + 1 M β-mercaptoethanol. Deglycosylation was assessed using the glycoprotein detection kit from BioRad as previously described.

ELISA for measurement of specific antibodies

Each of the 96 well microtitre plates (Nunc-Immunoplate Maxi Sorp ™) was coated with 100 μl of glycosylated or deglycosylated LCE from each isolate at a concentration of 2 μg ml^{-1} diluted in 0.1 M carbonate buffer at 9.6 pH and maintained overnight at 4°C. Several wells were kept uncoated as controls for non-specific reactions. After washing for three times with 0.05% PBS-Tween 20, the post-coating was carried out by adding 250 μl per well of 0.1% BSA in PBS-Tween and then incubated for 1 h at 37°C. After washing, 100 μl of the 1/100 PBS-Tween 0.1% BSA diluted sera samples were added in quadruplicate and the plates were incubated at 37°C for 2 h. Sera samples taken on the day of infection or immunization were used as negative controls. Once the plates were washed, 100 μl per well of goat affinity isolated, horseradish peroxidase conjugated antibody specific to mouse IgG3 (Caltag Laboratories, San Francisco, California), at the appropriate dilution in PBS-Tween, 0.1% BSA, were added and incubated for 1 h at 37°C. After washing, 100 μl per well of substrate (o-phenylenediamine, Sigma) was added at 0.04% in a phosphate citrate buffer (pH 5.0) with 0.04% hydrogen peroxide. The reaction was stopped with 50 μl 3 N sulphuric acid and the plates were read at 492 nm. Results were expressed as optical densities of sera

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test-optical densities of control sera values once the values corresponding to non-specific wells were subtracted.

Immunoblotting

Following separation of glycosylated and deglycosylated LCE from each species by SDS-PAGE in 5–20% gradient (Laemmli *et al*., 1970) the protein bands were transferred to nitrocellulose paper (0.2 μm size pore) in a semi-dry system. The paper was blocked overnight at 4°C with 5% skimmed milk in PBS pH 7.2, and cut into appropriate strips. These were then incubated with the corresponding sera samples taken from infected or immunized mice on weeks 0, 8 and 15 post-infection or immunization, at 1/100 dilution in 1% skimmed milk, 1% Triton-X-100 in PBS-Tween 20 and incubated for 3 h at 37°C. After washing for three times in PBS-Tween 20, the strips were incubated with goat affinity isolated, horseradish peroxidase conjugated antibody specific to mouse IgG3 (Caltag Laboratories, San Francisco, California), at the appropriate dilution in 1% skimmed milk, 1% Triton-X-100 in PBS-Tween 20 for 3 h at room temperature. After washing, the bands were developed by adding the substrate (6 μl H_2O_2 in 10 ml PBS plus 0.03 g 4-chloronaphthol (Sigma) in 10 ml methanol, in darkness at room temperature.

Results

Detection of glycoproteins in LCE of Trichinella *species*

A high degree of protein glycosylation is present in LCE from all species with prominent bands at 45 kDa (fig. 1). Profiles were similar amongst species with only minor differences at low molecular weight (A, lanes a–f). Deglycosylation was achieved almost completely in *T. spiralis* and T6 (B, lanes a, d), whereas for the other species a prominent band of 58 kDa and fainter bands of lower MW remained in some preparations (B, lanes b, c, e, f).

IgG3 responses following infection with species of Trichinella *using ELISA with homologous glycosylated and deglycosylated LCE*

The IgG3 responses in the sera from animals infected with different species, as measured by the ELISA method with LCE prior and after deglycosylation are shown in fig. 2A–F. Significant differences were seen amongst the species in the dynamics of IgG3. The highest IgG3 response was recorded in infections by the T8 isolate with values near 0.9 OD as early as week 5 post-infection (p.i.) then slightly increasing up to 1.3 OD on week 19 p.i. IgG3 responses following *T. nelsoni* infection were also shown from week 5 p.i. although at lower levels (OD values about 0.3) with a maximum being achieved at week 9 p.i. The patterns observed following infections by *T. spiralis* and *T. nativa* were similar with the highest values (OD about 0.6 and 0.9) achieved on weeks 9 and 7, respectively. The lowest IgG3 responses were recorded in *T. britovi* and T6 infections with OD values below 0.3 and 0.4 respectively throughout the observation period (week 19 p.i.). The response was particularly delayed in infections by T6 isolate with positive values above 0.2 OD achieved on week 15 p.i.

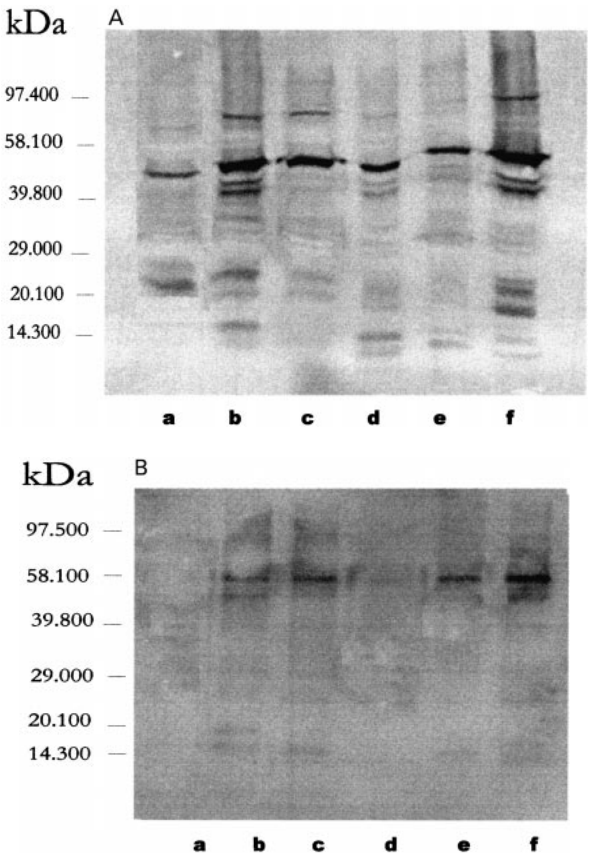

Fig. 1. Detection of glycoproteins in the LCE of *Trichinella* species before (A) and after (B) deglycosylation by the BioRad deglycosylating kit. a, *T. spiralis*; b, *T. nativa*; c, *T. britovi*; d, T6; e, *T. nelsoni*; f, T8.

IgG3 responses fell to basal values in ELISA with deglycosylated antigen when tested against sera from mice infected with *T. spiralis*, *T. nativa*, *T. britovi* and T6. For *T. nelsoni* and T8 infections, the IgG3 responses decreased with respect to those before deglycosylation although they remained above 0.1 to 0.5 OD.

IgG3 responses following immunization with LCE from species of Trichinella *using ELISA with homologous glycosylated and deglycosylated LCE*

The dynamics of IgG3 responses to immunization with LCE as measured in ELISA before and after deglycosylation is shown in fig. 3A–F. The highest IgG3 response was recorded following immunization with *T. nelsoni* with OD values above 1.0 from week 5 post immunization (p. im.) reaching the maximum (near 2.0 OD) on week 9 p.im. which was maintained until week 21. A similar pattern was observed for *T. britovi* although the OD values were slightly lowered (maximum near 1.8). Immunization by *T. spiralis* and *T. nativa* LCE induced similar IgG3 responses with similar OD values from week 7 p.im. remaining up to the end of the experiment although the response was earlier following immunization by *T. nativa*, with significantly higher values in week 3 and 5 p.im. The lowest

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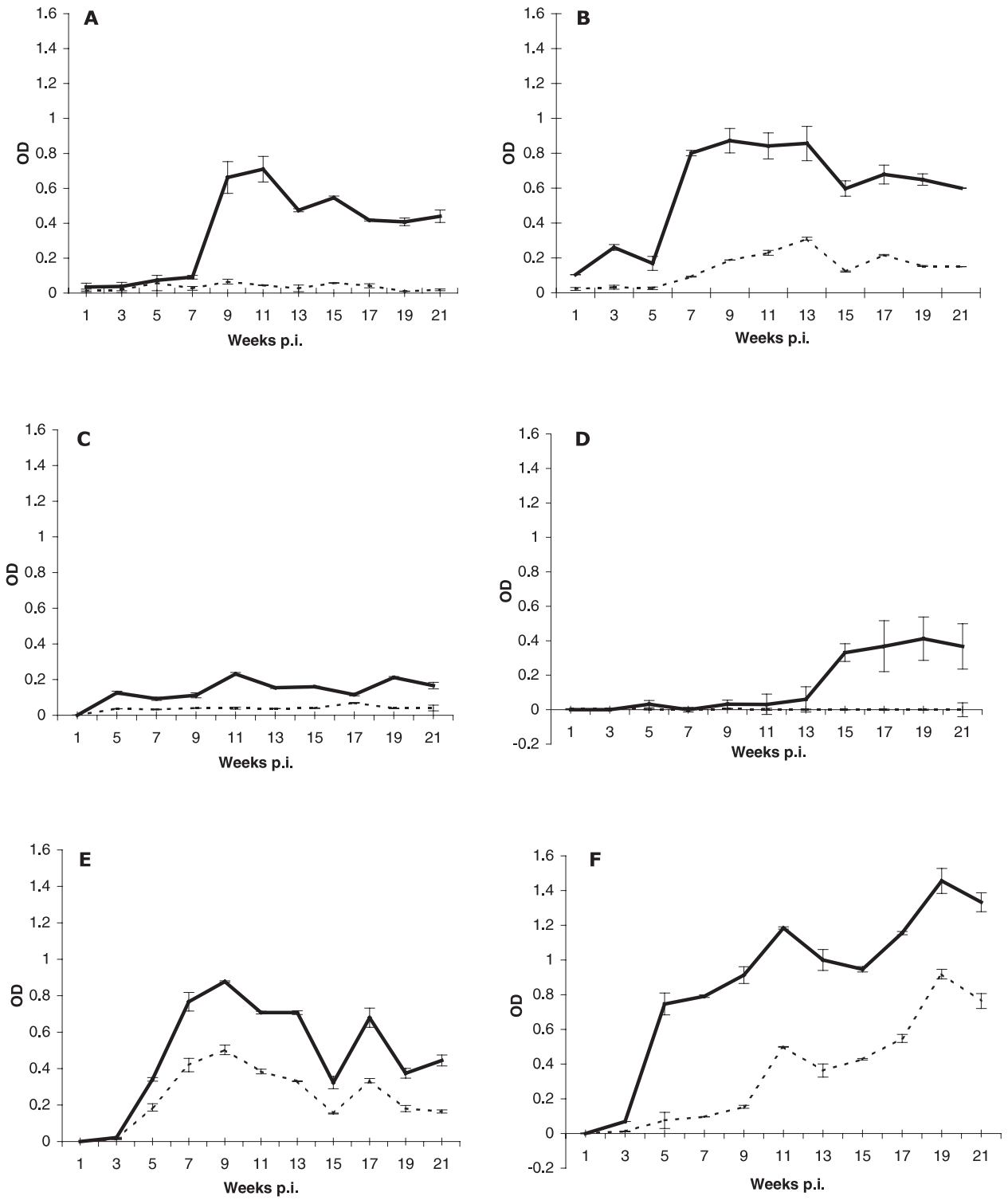

Fig. 2. Dynamics of IgG3 responses following infection by *Trichinella* species as measured in ELISA with the homologous LCE before and after deglycosylation. Sera samples were taken on weeks 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 post-infection (p.i.). Results are expressed as mean optical densities (OD) from four values. A, *T. spiralis*; B, *T. nativa*; C, *T. britovi*; D, *T6*; E, *T. nelsoni*; F, T8. ——, control; · · · · · ·, deglycosylated.

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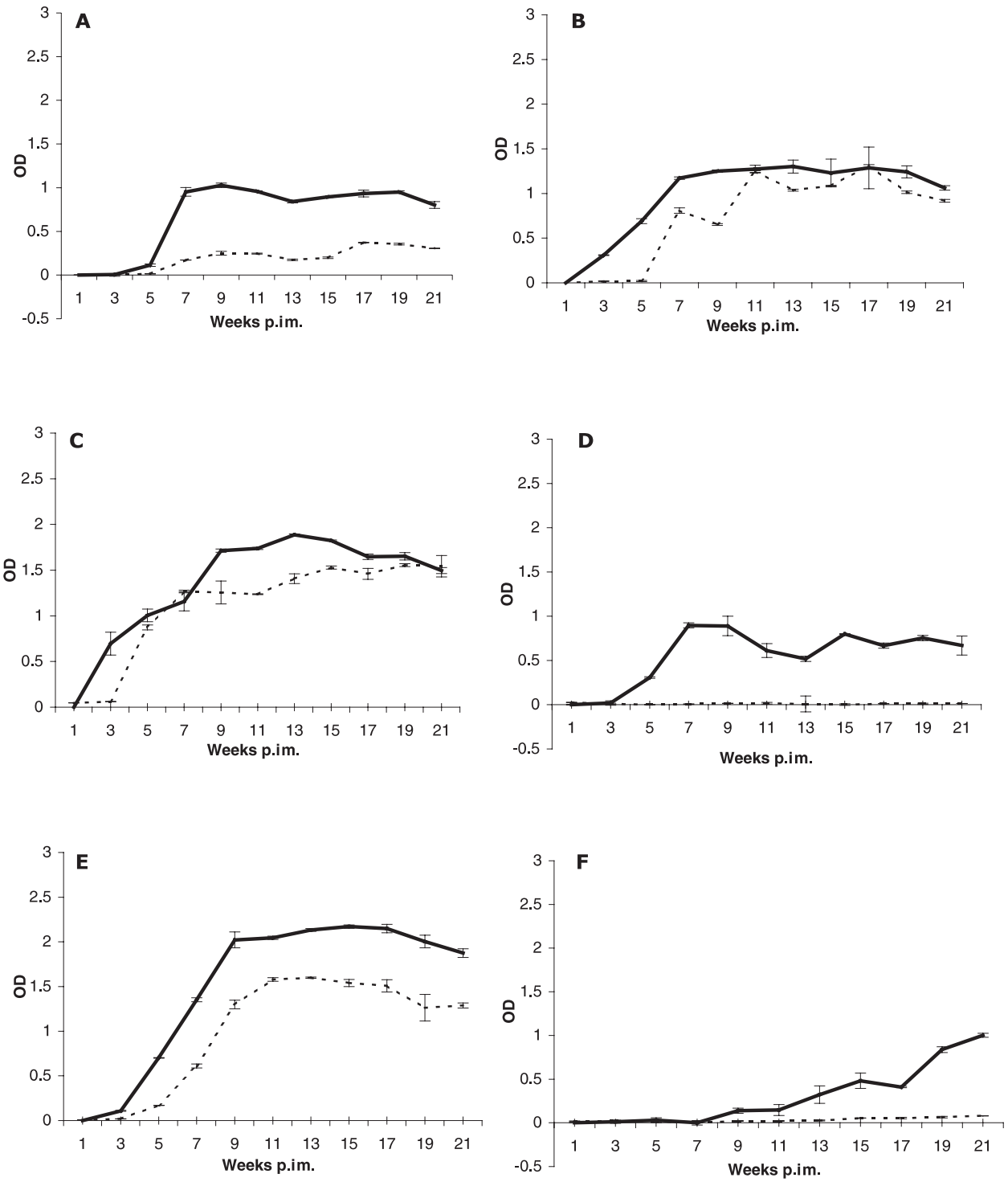

Fig. 3. Dynamics of IgG3 responses following immunization with LCE from the *Trichinella* species as measured in ELISA with the homologous LCE before and after delgycosylation. Sera samples were taken on weeks 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 post-immunization (p.im.). Results are expressed as mean optical densities (OD) from four values. A, *T. spiralis*; B, *T. nativa*; C, *T. britovi*; D, *T6*; E, *T. nelsoni*; F, T8. ——, control; · · · · · ·, deglycosylated.

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IgG3 response was recorded after immunization by T6 and by T8 although the patterns were clearly different. For T6, values reached a maximum by week 7 (above 0.8 OD) and then remained up to the end of the observation period without much variation whereas for T8, a progressive increase was observed from week 9 when responses were positive up to week 21 when the maximum was reached (near 0.9 OD).

Following deglycosylation, the IgG3 response was practically abrogated in mice immunized by T6 and T8 and markedly reduced in those immunized by *T. spiralis* with maximum values below 0.4 OD. A reduction was also observed in mice immunized with *T. nelsoni* although to a lesser extent than in those immunized by *T. spiralis*. The effect of deglycosylation on IgG3 recognition was minimal in mice immunized with *T. nativa* and with *T. britovi*.

IgG3 recognition patterns following infection or immunization by species of Trichinella *in Western blot with homologous glycosylated and deglycosylated LCE*

Results are summarized in fig. 4I for infections and 4II for immunizations. Overall, the IgG3 recognition profiles as measured in Western blot on days 0, 8 and 15 p.i or p.im. were similar for each species in both infection and immunization experiments. The strongest reaction was observed for IgG3 against immunization with *T. britovi* followed by *T. nelsoni* when tested with homologous LCE before deglycosylation, with a dense area of bands between 46 and 64 kDa (fig. 4IIC and 4IIE). For the remaining species, fewer and fainter bands were observed. Marked differences appeared among species with profiles appearing almost unique for each one tested. For *T. spiralis*, the IgG3 recognition resulted in faint bands of between 40 and 60 kDa which were completely abrogated after deglycosylation in infected animals (fig. 4IA) whereas a faint band of about 45 kDa remained for immunized mice (fig. 4IIA). For *T. nativa*, few but clear bands appeared between 50 and 60 kDa before deglycosylation which changed in molecular weight (mw) after deglycosylation giving rise to new bands of lower mw (fig. 4IB and 4IIB). In *T. britovi*, the reaction was strong following immunization and light following infection (fig. 4IIC and 4IC). Deglycosylation did not abrogate IgG3 epitopes but the recognition patterns clearly changed after infection with only three faint bands around 50 kDa (fig. 4IC). A similar situation to that described for *T. britovi* was observed for *T. nelsoni* although the recognition profiles were somewhat different (fig. 4IE and 4IIE). The IgG3 epitopes in mice infected or immunized by T6 were identical with three main clear bands of about 36, 45 and 52 kDa before deglycosylation which completely disappeared afterwards (fig. 4ID and

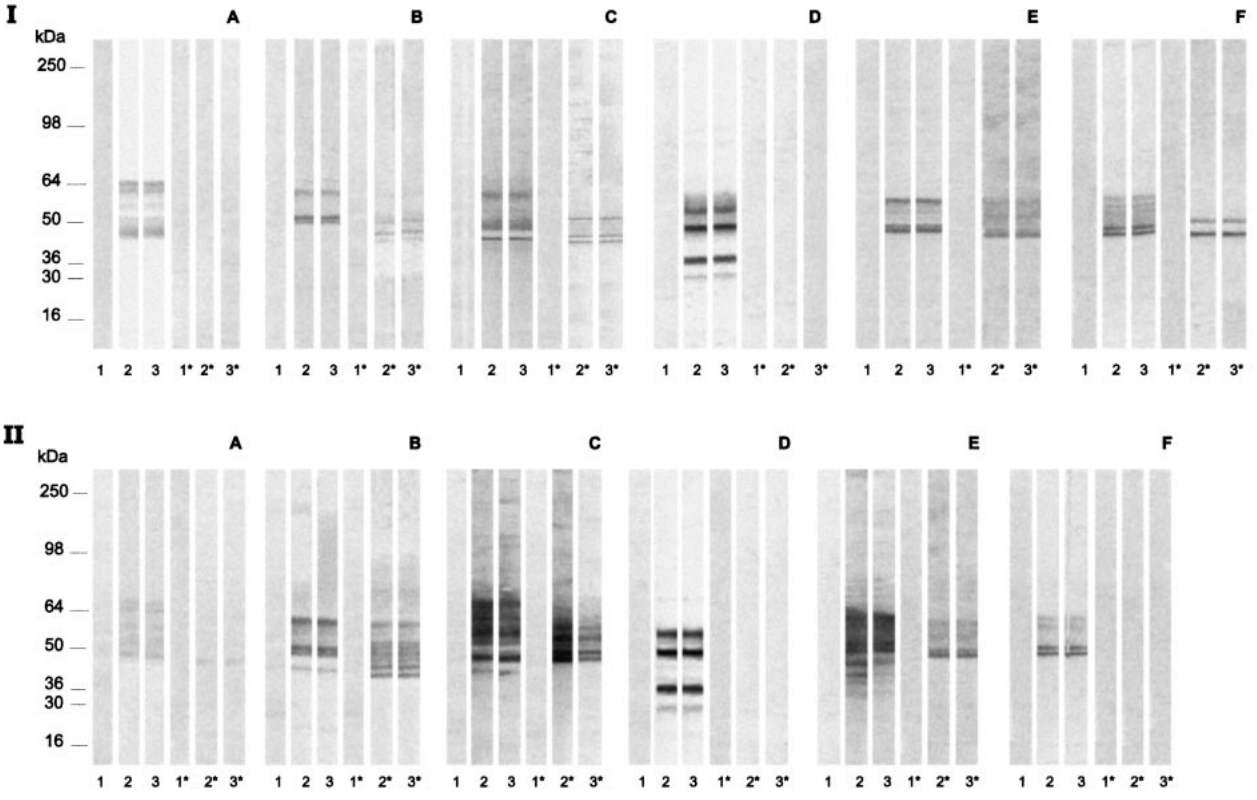

Fig. 4. Antigen recognition profiles of IgG3 anti-infection (I) or anti-immunization (II) by various *Trichinella* species as measured in Western blot with homologous LCE before and after (*) deglycosylation. Sera samples were analysed on weeks 0, 8 and 15 post-immunization. A, *T. spiralis*; B, *T. nativa*; C, *T. britovi*; D, *T6*; E, *T. nelsoni*; F, T8.

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4IID). The IgG3 epitopes following infection or immunization by T8 were also similar before deglycosylation with a prominent doublet at 52 kDa which later disappeared in immunized mice (fig. 4IIF) whereas after infection, two clear bands of 52 and 55 kDa respectively, remained (fig. 4IF).

Discussion

Carbohydrates play important roles in the survival strategy of parasites such as *Schistosoma* spp. (Dunne *et al*., 1990) and *Trypanosoma brucei* (Ferguson *et al*., 1988). In *Trichinella* species, relevant glycans bound to proteins of TSL-1 antigens have been identified (Denkers *et al*., 1991; Ellis *et al*., 1994; Romaris, unpublished results) that may be involved in muscle invasion and nurse cell development. Peptide epitopes alone can generate protective immune responses as shown by inhibition in recognition of 43–55 kDa TSL-1 antigens by sera raised against re-infection, following antigen oxidation by sodium periodate (Jarvis & Pritchard, 1992). Fine structural analysis of carbohydrate moieties (Wisnewski *et al*., 1993) combined with recognition assays by monoclonal antibodies before and after non-selective (TFMS) or selective (PNGase or O-glycosidases) antigen deglycosylations (Ellis *et al*., 1994, 1997) have led to the identification of a novel glycan (tyvelose) that mediates antibody protection against *Trichinella spiralis* intestinal infections (McVay *et al*., 1998).

In the present work, glycoproteins are represented by a major band at 45 kDa present in the LCE from all species as well as by less relevant bands both above and below 45 kDa down to a very low mw (below 14.3 kDa) in some species. Following deglycosylation, carbohydrates are completely removed from glycoproteins of LCE from *T. spiralis* and T6 whereas for the remainder (*T. nativa*. *T. nelsoni*, *T. britovi* and T8) a prominent band of about 58 kDa was present. This prominent band, absent in the glycosylated extracts, may result from higher molecular weight glycosylated proteins losing some bound sugars as a consequence of the deglycosylation process. Although more controlled assays are required, the variation in deglycosylation effectiveness in the LCE from the various species of *Trichinella* may reflect relevant changes in protein N- or O-glycosylation rates. Similar profiles were obtained before and after antigen deglycosylation in Western blot using a monoclonal antibody that recognizes a peptide on the 53 kDa TSL-1 antigen (Romaris *et al*., unpublished results) thus indicating that this procedure should not significantly affect peptide epitopes.

In mice, IgG3 subclass antibodies predominate in humoral responses to bacterial polysaccharide antigens with enhanced binding capacity to N-acetylglucosamine (GlcNAc) residues (Greenspan & Cooper, 1992). In *T. spiralis*, a high proportion of GlcNAc and GalNAc was also found in the TSL-1 antigens (Wisnewski *et al*., 1993). In the present study, when the glycosylated and deglycosylated antigens were assayed in ELISA for an IgG3 isotype, a clear correlation was observed between IgG3 responses and the presence of carbohydrates in LCE used in the assay as the strong response in ELISA against crude antigens was markedly reduced or completely abrogated in *T. spiralis* and T6 LCE, where deglycosylation was apparently complete. For the remainder, *T. nativa, T. britovi* and *T. nelsoni*, the IgG3 responses were similar with much reduction following deglycosylation in both infected and immunized mice when tested against deglycosylated LCE, although to a lesser extent in the immunized group.

The differences observed in the dynamics of IgG3 responses between the infection and immunization processes, where the T8 isolates induced the highest response following infection while the lowest in immunization, could be due to the presence of different IgG3 inducing epitopes in infection and immunization as live larvae can expose particular surface components while hiding some somatic ones (Ortega-Pierres *et al*., 1984) which are available in the LCE. However, the similarity in the IgG3 recognition profiles observed for most species (*T. spiralis*, *T. nativa*, T6 and T8) before deglycosylation does not support this hypothesis and probably these differences are due to the way in which epitopes are delivered in both procedures. Furthermore, IgG3 responses in mice immunized with the T8 isolate were nearly abrogated following deglycosylation while markedly reduced in infected mice. This was consistent with the presence of two bands only recognized by the sera from immunized animals. This pattern was also shown by the *T. spiralis* isolate but in the reverse mode, where a faint band of about 45 kDa was present only following infection. This is difficult to explain as no particular glycoprotein bands which could be recognized by IgG3 are present in the LCE from these species, either before or after deglycosylation. The presence of IgG3 non-carbohydrate epitopes in the *Trichinella* larval antigens should also be considered.

The infectivity and the dynamics of the infection by different species can affect both the amount of antigen released and the production of antibodies. Worm burdens in the intestine on day 5 and in the muscles by week 18–21 post-infection or -immunization (data not shown) varied among species. The highest burdens in the intestine and muscles were recorded for *T. spiralis* and *T. nativa* whereas for the remaining four species the infectivity was markedly lower and varied to some extent. Therefore, no correlation was observed between infectivity and quantitative IgG3 responses as shown by its dynamics in infections by *T. nelsoni* and T8 compared to those by *T. spiralis* and *T. nativa* and by *T. britovi* and T6. This suggests that other factors such as the duration of the intestinal infection, female fecundity and rhythm of larviposition as well as the duration of muscle encapsulation (Pozio *et al*., 1992) may affect antigen release and lymphocyte triggering.

The variation which exist between the species in the dynamics of IgG3 responses is also in accordance with the IgG3 recognition patterns shown in Western blots of sera from infected or immunized mice with LCE from the homologous species, thus supporting the fact that IgG3 epitopes in *Trichinella* species whether glycosylated or not are present in proteins of different molecular weights.

Nevertheless, the predominance of IgG3 carbohydrate epitopes in the LCE of *Trichinella* species is also confirmed by the fact that following deglycosylation IgG3 recognition

profiles were dramatically modified (*T. nativa*, *T. britovi* and *T. nelsoni*) or partially (*T. spiralis* and T8) and completely abrogated (T6).

In summary, our results suggest that, as in other parasites, carbohydrates bound to proteins capable of eliciting high IgG3 subclass responses are abundant in *Trichinella* crude larval antigens with significant variation amongst the species.

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