Parasite Immunology, 1995: **17**: 119–126

Characterization of epitopes on excretory-secretory antigens of *Taenia saginata* metacestodes recognized by monoclonal antibodies with immunodiagnostic potential

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SUMMARY

Four monoclonal antibodies (MoAbs) produced against the excretory-secretory (ES) antigens of Taenia saginata *metacestodes are currently used for the detection of circulating antigen in the sera of cattle with bovine cysticercosis. The epitopes recognised by these MoAb were characterized by periodate oxidation, alkaline borohydride and heat treatment, trichloroacetic acid precipitation, enzymatic proteolysis and deglycosylation of the ES-products. These results together with those of the homologous sandwich, inhibition and competition ELISAs showed that three repetitive epitopes are recognized, which are present on the tegument and on the ES-products of* T. saginata *metacestodes. They are different from phosphorylcholine and seem to be either of carbohydrate or partly carbohydrate/partly protein nature.*

Keywords T. saginata, *metacestode, epitope, excretory-secretory products, monoclonal antibody*

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Received: 11 January 1994
Accepted for publication: 16 November 1994

INTRODUCTION

In a previous report the development of monoclonal antibodies against the excretory-secretory (ES) products of living *Taenia saginata* cysticerci is described (Brandt *et al.* 1992). Four of these monoclonal antibodies (MoAbs) are currently used in a double antibody sandwich ELISA with improved sensitivity for the detection of circulating ES-products in the sera of cattle infected with *T. saginata* cysticerci. This antigen-ELISA is able to distinguish animals harbouring living cysts from those carrying only dead cysticerci. Up to now, however, the nature of the circulating antigens and the specificity of the epitopes recognized by these MoAb are not known. The aim of the present work was to characterize the biochemical and immunological properties of the epitopes on the ES-products recognized by the monoclonal antibodies.

MATERIALS AND METHODS

Excretory-secretory products of *T. saginata* cysticerci

Excretory-secretory (ES) products of 12 week old *T. saginata* metacestodes were obtained and processed as described by Brandt *et al.* (1992). Batches of ES products, collected daily during the first week, were used individually or pooled for various treatments. No significant qualitative differences were observed when the different batches were analysed in SDS-PAGE (silver staining) and in ELISA using hyperimmune sera against ES or somatic antigens of *T. saginata*. The protein and carbohydrate content of the ES-products were determined after dialysis and concentration by the Micro bicinchoninic acid protein assay of Pierce and the method of Monsigny, Petit & Roche (1988) respectively. Unconcentrated ES-products were shown to contain approximately 13–14 μg protein and 2·4 μg carbohydrate per ml.

Heat treatment

ES samples were incubated for 15 min in a water-bath at 100°C, cooled on ice and tested without centrifugation.

Trichloroacetic acid (TCA) treatment

Each ES sample was mixed with an equal volume of 0; 1; 2·5; 5 or 10% TCA and kept on ice for 30 min. The mixture was centrifuged; the supernatant was dialysed against PBS (4°C) and the precipitated material washed in ethanol and resolubilized in the original volume in PBS.

Alkaline borohydride treatment

One volume (100 μl) of ES was incubated with an equal volume of 0·5 M $NaBH_4$ in 0·1 M KOH overnight in the dark at 37°C under nitrogen. Acetic acid 2·0 M (30 μl) was then added till a concentration of 0·25 M to lower the pH. The final concentration of NaBH4 was thus 0·22 M. This mixture was dialysed extensively against PBS at 4°C in the dark. As a control, ES was treated identically with water instead of alkaline borohydride, and also dialysed.

Periodate treatment

ES samples were mixed with identical volumes of 0; 2; 10; 20; 40 or 100 mM H_5IO_6 in 200 mM sodium acetate buffer (pH 4·7) and incubated for one h at room temperature in the dark. Thereafter an equal volume of 150 mM $NaBH_4$ in PBS was added and incubated 30 min at room temperature. The whole was extensively dialysed against PBS at 4°C before evaluation.

Pronase digestion

One volume of ES was mixed with one volume of pronase solution (Boehringer Mannheim, Brussels; 8 u/ml) in PBS, pH 7·4 and the whole was incubated at 37°C for 0, 10 or 30 min and for 1, 2, 4 or 16 h. Enzyme activity was stopped by boiling the samples for 5 min.

Enzymatic deglycosylation

The removal of N-linked carbohydrate chains was done mainly according to the procedures advised by Boehringer Mannheim. SDS was added to ES samples till a final concentration of 1% and boiled for two min. Then 9 volumes (sodium phosphate buffer, 20 mM, pH 7·2, sodium azide, 10 mM, EDTA, 50 mM, n-Octyl-glucoside 0·5% w/v) were added and boiled again for 2 min. After cooling, 2 units N-Glycosidase F were added and the whole was incubated for 18 h at 37°C. As a control ES was treated similarly, but without enzyme. After extensive dialysis against distilled water at 4°C, the diluted ES sample was reconcentrated to the original volume and its reactivity to the monoclonal antibodies was tested in DOT-ELISA.

The simultaneous removal of N- and O-linked carbohydrate chains was done in a similar way as for the removal of the N-linked carbohydrates, except that after the initial denaturation 5 mU O-Glycosidase and 10 mU Neuraminidase were added in addition to 2 U of N-Glycosidase F.

Monoclonal antibodies

MoAbs were produced against the ES-products of *T. saginata* metacestodes as described by Brandt *et al.* (1992). Four MoAbs were used in the present experiment: 2H8, 12G5, 153G7 and 158C11. They all belong to the IgM isotype, except 158C11, which is an IgG_1.

Indirect and sandwich ELISA

Microtitre plates (Nunc® Maxisorp, Intermed, Denmark) were coated overnight at 4°C with 200 μl MoAb or ES antigen diluted in 0·5 M carbonate buffer, pH 9·6, respectively for the sandwich-ELISA or the indirect ELISA. Blocking was done with 350 μl PBS containing 0·05% casein (w/v) for 1 h at room temperature.

In the indirect ELISA 200 μl MoAb diluted in PBS, containing 0·1% casein, was added after washing with PBST (PBS + 0·05% Tween 20, v/v) and incubated for 1 h at room temperature. The plates were washed with PBST afterwards.

In the sandwich-ELISA 200 μl ES antigen was incubated in the MoAb (20 μg/ml) coated wells for 1 h at room temperature. After washing 200 μl biotinylated MoAb, diluted at 10 μg/ml in PBS + 0·1% casein, was added for 1 h at room temperature. Again the plates were washed with PBST, followed by the incubation with a streptavidin-biotin-alkaline phosphatase complex (Dakopatts K391) diluted 1/50 in PBS, for 1 h at room temperature. The plates were washed first with PBST and then with substrate buffer (10 mM ethylaminoethanol; 0·5 mM $MgCl_2$; pH 9·8). Finally 200 μl of the substrate solution (1 mg 4-Nitrophenyl phosphate (PNPP)/ml substrate buffer) was added. The plates were read using an automated spectrophotometer (Titertek Multiskan

EIA Reader) at 405 nm after incubation in the dark at room temperature.

In the indirect ELISA, bound MoAbs were detected with different conjugates according to their isotypes. IgG monoclonal antibodies were detected with an alkaline phosphatase (AP) conjugated anti-mouse IgG (Sigma A-1902) diluted 1/1000 in PBS. IgM monoclonal antibodies were detected by a double incubation with rabbit-anti-mouse IgM (Dakopatts Z457) diluted 1/1000 in PBS + 0·1% casein and with an AP conjugated anti-rabbit IgG (Sigma A-8025), diluted 1/3000 in PBS. The reagent volumes, washings, substrate incubations and absorbance readings were as described for the sandwich-ELISA.

Competition ELISA

Non-biotinylated homologous and heterologous MoAbs were diluted in a previously determined sub-saturating-level dilution of biotinylated MoAb, to analyse the binding-inhibition of the marked antibody in a homologous sandwich-ELISA (Harlow & Lane 1988). Briefly, after coating of the microtitre plates with a MoAb, blocking and adding the ES antigen (see supra) a mixture of the same biotinylated MoAb and increasing concentrations of homologous or heterologous MoAb were added. Streptavidin-biotin-alkaline phosphatase complex was used as conjugate and PNPP as substrate, as described earlier. Decreasing optical densities when using increasing concentrations of a competing MoAb indicate a competition for the same or a closely related epitope.

DOT-ELISA

Droplets of 1 μl of ES antigen were deposited on a nitrocellulose membrane strip with pore size 0·22 μm (Schleicher & Schuell, BA 83) and dried on the air. The membrane was blocked with 5% BLOTTO in Tris buffered saline (TBS, 20 mM Tris-HCl, 500 mM NaCl, pH 7·5) for 1 h at room temperature. Individual strips were incubated with 1 ml of MoAb at a final concentration of 10 μg/ml for one h. After four washings with TBS, a second antibody and conjugate were used as for the plate ELISA. Each incubation step was followed by four washings with TBS, with an additional wash with AP-buffer (100 mM NaCl, 100 mM Tris-HCl, 5 mM $MgCl_2$, pH 9·5) before the incubation in substrate solution (0·03% NBT + 0·015% BCIP in 10 ml AP-buffer) for 30 min. The reaction was stopped by rinsing the strips with water.

Inhibition ELISA

The reactivity of the individual MoAbs against ES antigen was investigated by incubating them in a range of increasing concentrations (10^{-10} M to 10^{-1} M) of free phosphorylcholine (Sigma P-0378) and choline (Sigma C-1879), both in indirect and in sandwich-ELISA.

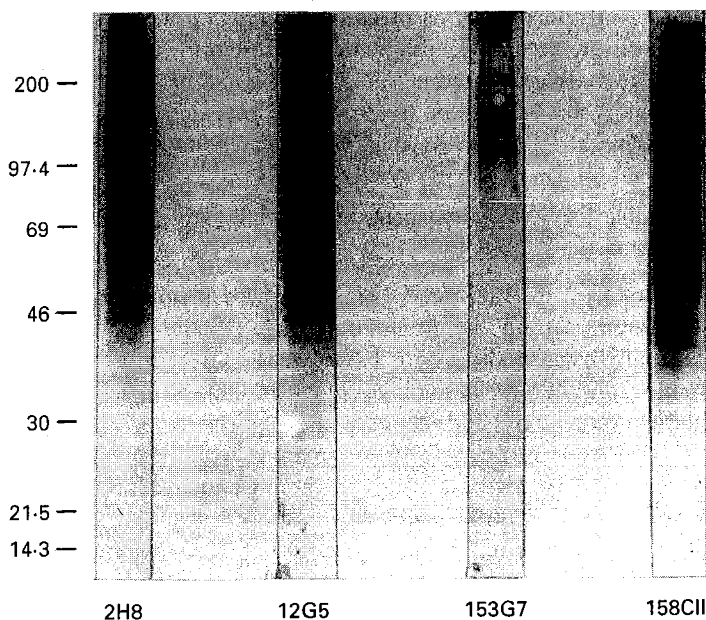


Figure 1 Immunoblottings of ES antigen of *T. saginata* metacestodes reacting with different monoclonal antibodies. The positions of the molecular weight standards (×1000) are indicated.

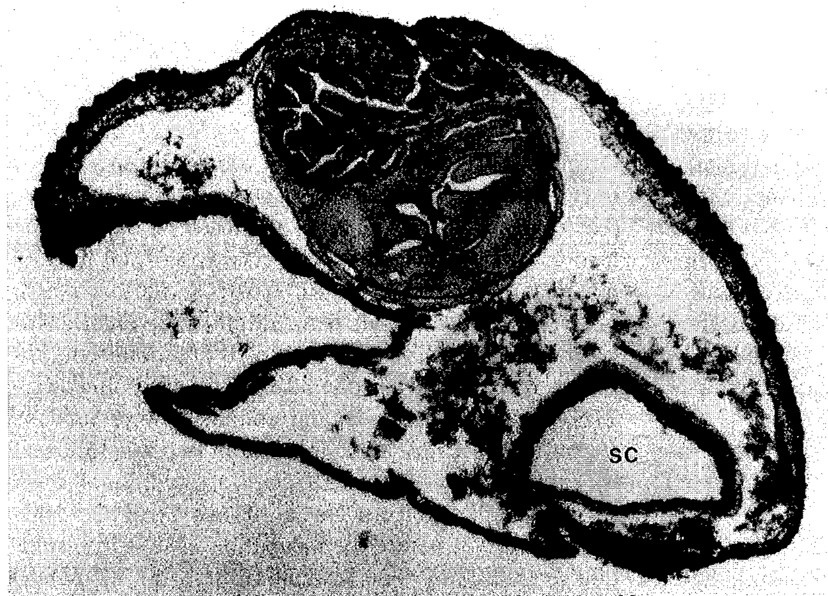


Figure 2 Immunoperoxidase staining of a histological section of a *T. saginata* metacestode showing the reaction of the MoAb 2H8 with the outer tegument and the tegument of the spinal canal (sc). Magnification ×56 *ca.*

Immunoblotting

Immunoblotting was carried out as described by Brandt *et al.* (1992). Briefly 10 μg of ES antigen was loaded per well and run on 10% SDS-PAGE gels. After transfer to a nitrocellulose membrane with 0·22 μm pore size, immunodetection was performed with the 4 MoAbs diluted to 10 μg/ml. The same conjugates were used as in the ELISA.

Immunoperoxidase staining of histological sections

Paraffin-embedded sections of living *T. saginata* metacestodes were prepared and treated with biotinylated MoAb as described by Brandt *et al.* (1992).

RESULTS

Immunoblotting and immunoperoxidase staining of metacestodes

The MoAbs 2H8, 12G5 and 158C11 showed a similar recognition pattern in immunoblotting (Figure 1). Essentially each of these MoAbs showed a smear in a broad molecular weight range of <200 000–40 000, although individual bands could be distinguished.

The MoAb 153G7 clearly was less reactive with the ES antigens. It showed some bands in the high molecular weight range of <200 000–60 000.

Immunoperoxidase staining of sections of *T. saginata* metacestodes showed that all four MoAb only reacted with the tegument of the parasite. Figure 2 illustrates that the reaction of MoAb 2H8 is limited to the outer tegument and the tegument of the spinal canal of the metacestode.

Sandwich, competition and inhibition ELISAs

Sandwich ELISAs were used to demonstrate the presence of repetitive epitopes. For the four MoAbs tested it was possible to use the same MoAb for coating of the microplate wells as for biotin-labelled indicator, which means that the recognized antigens had more than one binding site for each of the tested MoAb.

The results of the competition ELISAs are summarized in Table 1. Three out of four investigated MoAbs showed a mutual competition to bind the epitopes on ES antigen. However, the MoAb 153G7, with IgM isotype, was clearly distinct from the other three

Table 1 Screening of MoAbs for competition in homologous sandwich ELISAs

	Competing antibody			
Homologous ELISA	2H8	12G5	153G7	158C11
2H8/2H8-Biotin	+	+	−	+
12G5/12G5-Biotin	+	+	−	+
153G7/153G7-Biotin	−	−	+	−
158C11/158C11-Biotin	+	+	−	+

+ = competition; − = no competition.

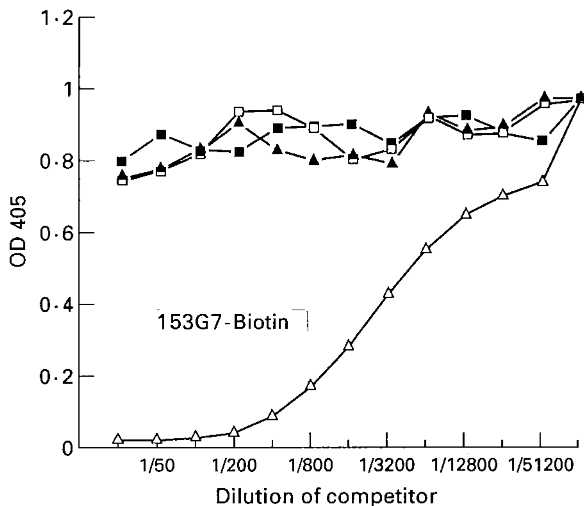


Figure 3 Competitive sandwich ELISA using 153G7 as capturing and as biotinylated detector antibody. Increasing concentrations of non biotinylated competing MoAb are added. ■ IgM 2H8; □ IgG 158Cll; △ IgM 153G7; ▲ IgM 12G5. Competitor MoAb: starting conc. 1 mg/ml; 153G7-Biotin 10 μg/ml.

MoAbs, independently from whatever biotin-labelled MoAb was chosen for the homologous sandwich-ELISA. Figure 3 clearly illustrates that MoAb 153G7 is reacting with a different epitope than 12G5, 2H8 and 158C11, since adding increasing concentrations of each of these latter MoAbs did not result in significantly decreasing optical densities.

Neither phosphorylcholine nor choline could inhibit the reactivity of the four MoAb in ELISA, indicating that the specific epitopes did not contain phosphorylcholine.

Enzymatic treatments of ES antigen

Enzymatic proteolysis of ES antigen by pronase digestion did not reduce the reactivity of the MoAbs 2H8 and 12G5 in a conclusive way as shown in Figure 4. The reactivity of the MoAbs 153G7 and 158C11, however, dropped almost to zero, even after short incubation times with pronase. These findings strongly suggest that 2H8 and 12G5 are not reactive with peptidic epitopes.

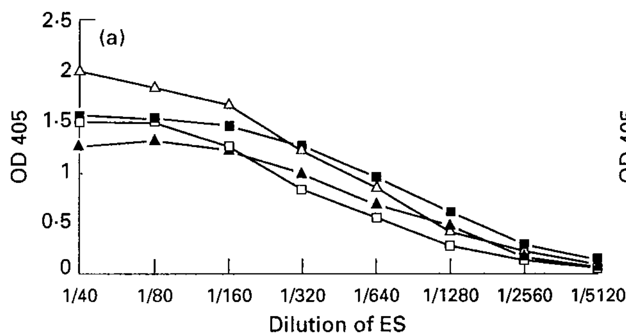
Enzymatic deglycosylation was analysed by DOT-ELISA. Twofold dilutions of digested and undigested ES antigen were blotted on nitrocellulose strips and the reactivities of the individual MoAbs were compared. It was assumed that the cleaved carbohydrates moieties would not or poorly bind to nitrocellulose and as such would not contribute to the reactivities of the MoAb. Cleavage of N-linked carbohydrates alone or the N- and O-linked carbohydrates together reduced the reactivity of the four tested MoAbs.

Oxidation of carbohydrates

Three out of the four MoAbs maintained their reactivity towards ES antigen treated with six different concentrations of periodate. MoAb 2H8 and 12G5 showed no reduction of their reactivity. 158C11 had an increased reactivity to ES antigen treated with a high concentration of periodate. Possibly the epitopes became unshielded after the treatment as was already suggested by Bara *et al.* (1992). The reactivity of MoAb 153G7 to ES antigen treated with a final concentration of 100 mM periodate was completely obliterated (Figure 5). If dealing with different carbohydrate epitopes, the epitope recognized by 153G7 must be the only one sensitive to periodate.

Heat treatment

Heat treatment of ES antigen did not alter its recognition by the MoAbs. This heat resistance indicates that the recognized epitopes are not conformational polypeptide epitopes which would be denatured after boiling.



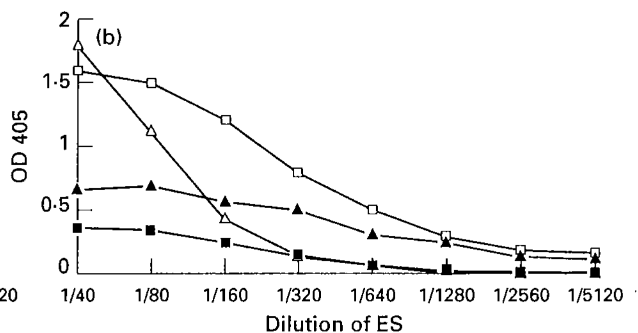


Figure 4 Reactivity of different MoAbs in ELISA after pronase treatment of ES antigen of *T. saginata* metacestodes. (a) □ 2H8 untreated; △ 12G5 untreated; ▲ 2H8 (10 min treatment); ■ 12G5 (10 min treatment). (b) □ 153G7 untreated; △ 158Cll untreated; ▲ 153G7 (10 min treatment), ■ 158C11 (10 min treatment).

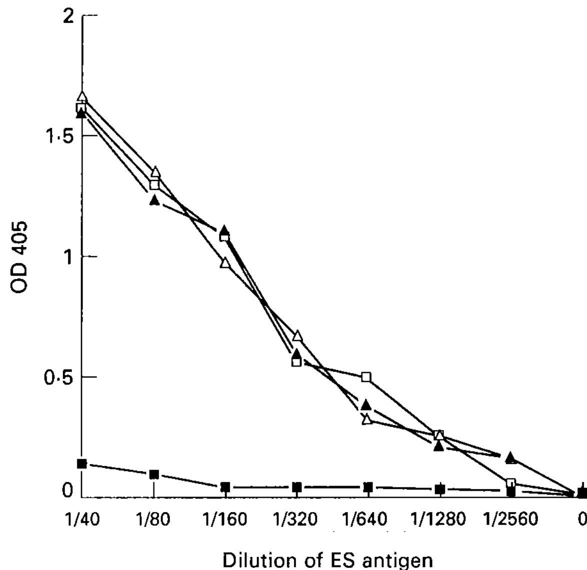


Figure 5 Reactivity of MoAb 153G7 in ELISA after periodate treatment of ES antigen of *T. saginata* metacestodes. Conc. of periodate: □ 0 mM; △ 5 mM; ▲ 20 mM; ■ 100 mM.

Trans-elimination by alkaline borohydride treatment

This treatment of ES antigen resulted in a partial (MoAbs 2H8 and 12G5) or strong (MoAbs 153G7 and 158C11) reduction of the reactivity of the MoAbs, suggesting that the specific epitopes are present on O-linked carbohydrates which are partially or completely removed by the above treatment.

TCA precipitation

This treatment preferentially precipitates proteins in a concentration-dependant manner. Both TCA soluble fractions and the precipitated material were tested. All results were conclusive and indicate the TCA solubility of the MoAb-specific epitopes, which suggests the non-protein nature of the epitopes.

The results are summarized in Table 2.

DISCUSSION

All four MoAbs, prepared against the ES products of *T. saginata* metacestodes, reacted more or less strongly with the larval tegument. This was to be expected since the ES products are produced by the tegumental surface of the parasite.

Due to the glycoprotein nature of the ES products (Joshua, Harrison & Sewell 1989) it was difficult to obtain clearcut bands in immunoblotting. All four MoAbs reacted, however, with antigenic components in a broad range of molecular weights between 40 000–60 000 and 200 000. Due to a better resolution it was possible to detect several faint individual bands in the smears on the nitrocellulose paper, which had not been possible previously (Brandt *et al.* 1992). This feature together with the results of the homologous sandwich ELISAs indicate the repetitive nature of the recognized epitopes.

From Tables 1 & 2 it is clear that the MoAbs 12G5 and 2H8 react with epitopes showing similar biochemical and immunological properties. Furthermore, they are

Table 2 Effect of various treatments on the epitopes of ES-products of *T. saginata* metacestodes recognized by monoclonal antibodies

	MoAb (isotype)			
	2H8 (IgM)	12G5 (IgM)	153G7 (IgM)	158C11 (IgG_1)
Heat treatment	RES	RES	RES	RES
TCA precipitation	SOL	SOL	SOL	SOL
$NaBH_4$ treatment	PRed	PRed	SRed	SRed
Periodate treatment (<100 mM)	RES	RES	RES	RES
Periodate treatment (100 mM)	RES	RES	SRed	RES
Proteolysis (pronase)	RES	RES	SRed	SRed
Deglycosylation N-Glyc.F	PRed	PRed	PRed	PRed
Deglycosylation N-Glyc.F O-Glyc. Neuraminidase	PRed	PRed	PRed	PRed
Choline inhibition	RES	RES	RES	RES
Phosphorylcholine inhibition	RES	RES	RES	RES

SOL: soluble; RES: resistant; PRed: partial reduction; SRed: strong reduction.

completely inhibiting each other in a competition ELISA, which is a strong indication that they are specific for the same epitope. This epitope seems to have a carbohydrate nature as is suggested by its TCA-solubility, its pronase resistance and susceptibility to alkaline borohydride treatment and enzymatic deglycosylation of the targeted antigens.

Equally the epitopes recognized by 153G7 and 158C11 share some similar features. They differ from those recognized by 2H8 and 12G5 by the fact that they are not resistant to pronase treatment and show a strong reduction after NaBH4 treatment. This indicates that both 153G7 and 158C11 react with epitopes, which might be partly protein and partly carbohydrate. The epitope recognized by 153G7 is periodate sensitive (100 mM treatment), which is not the case for the epitope recognized by 158C11. Furthermore there is no inhibition in a competition ELISA between MoAbs 153G7 and 158C11. Consequently the epitopes recognized by these two MoAbs cannot be considered as identical. The epitope recognized by 158C11 is probably situated very closely to the one, recognized by 12G5 and 2H8, because the latter MoAbs are able to inhibit 158C11 in a competitive ELISA (Table 1).

Inhibition-ELISAs using phosphorylcholine or choline on the other hand prove that the recognized epitopes are not the phosphorylcholine hapten, a widely distributed cross-reactive determinant in nature, equally reported in cestodes (Maizels *et al.* 1987). Neither are these epitopes stage-specific since the MoAbs also are strongly reactive with somatic extracts of adult *T. saginata* in ELISA and Western blot (data not shown).

Comparison of these results with those obtained by Harrison *et al.* (1989), who examined the epitopes recognized by 2 IgM-MoAbs (HP8 and HP10) produced after immunization of mice with living cysticerci, show a striking similarity. The MoAbs HP8 and HP10 recognize respectively a partly protein/partly carbohydrate determinant and a carbohydrate determinant present on surface enriched detergent extracts of *T. saginata* cysticerci. Both MoAbs were also able to detect circulating surface/secreted antigens of viable cysticerci in the sera of infected cattle. It would be worthwhile to investigate whether the MoAbs described by Harrison *et al.* (1989) react with identical epitopes as the MoAbs examined in this study.

It can be concluded that the four MoAbs—2H8, 12G5, 153G7 and 158C11—react with three different repetitive epitopes with a carbohydrate or partly carbohydrate/partly protein nature, which are present on the metacestode tegument and also on the ES products of *T. saginata* metacestodes. The combined use of these four MoAb allowed to improve the sensitivity of the ELISA for the detection of circulating ES-antigens in the sera of cattle infected with *T. saginata* metacestodes (Brandt *et al.* 1992). Indeed, the minimum detection rate as expressed by living cysticerci per animal rose from 88 (Brandt *et al.* 1992) to 22 (unpublished results).

The immunobiochemical properties of the circulating antigens detected with the antigen-ELISA in infected calf sera have so far not been systematically investigated. Based on the above mentioned results, however, calf sera were extracted with 10% TCA. Similar results were obtained when these TCA treated sera were compared with untreated sera in an antigen detection ELISA. TCA pretreatment of calf sera before coating on nitrocellulose, however, significantly improved the results in a DOT-ELISA (Draelants *et al.* 1995).

ACKNOWLEDGEMENTS

The authors wish to thank R. Beudeker, W. Vansteenkiste and F. Ceulemans for their valuable technical assistance. This work was supported by a financial grant from the Institute for Scientific Research in Industries and Agriculture (IWONL), Brussels.

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