Protection induced by vaccinating sheep with a thiol-binding extract of *Haemonchus contortus* membranes is associated with its protease components

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**SUMMARY**

Previous work has shown that a protein extract enriched for cysteine protease activity (TSBP) prepared from adult *Haemonchus contortus* using thiol sepharose affinity chromatography confers substantial protection against a single challenge infection. TSBP comprised proteases and other proteins. Here, TSBP were further fractionated using anion-exchange chromatography and fractions pooled on the basis of containing (1) protease activity, (2) a prominent non-protease peptide and (3) material which did not bind to the column. A protection trial showed that only the protease-enriched material conferred protective immunity and this was comparable to that observed in a TSBP-immunized positive control group. Immunization stimulated a marked IgG response with the IgG2 isotype predominating.

**Keywords** *Haemonchus contortus*, proteases, protection, vaccination

**INTRODUCTION**

A cysteine protease enriched fraction [thiol sepharose binding proteins (TSBP)], prepared from membrane extracts of adult *Haemonchus contortus*, conferred substantial protection against a single challenge infection with *H. contortus* in vaccine trials (1). Mean reductions in daily faecal egg outputs of 77% and final worm burdens of 47% were observed over three trials. TSBP comprised a mixture of a small number of proteins which were clearly localized to the microvillar surface of the intestinal cells as judged by immunohistochemistry (1). The microvillar surface of worms surviving in vaccinated lambs was coated with sheep immunoglobulin, suggesting that antibody may be the effector of protection (2).

TSBP comprise a prominent 60 kDa protein and several minor bands between 35 and 45 kDa and 97–120 kDa. Protease activity is mostly attributable to cysteine proteases, although serine/metallo proteases have also been identified (1). Expression library immunoscreening revealed that the prominent 60 kDa component of TSBP was a glutamate dehydrogenase (GDH) homologue (3) and that the cysteine protease activity could be attributed to the protein products of three distinct cathepsin B-like genes (4).

The expression of the GDH and cysteine protease encoding genes coincides with the onset of blood feeding, and immunolocalization studies showed that the former was expressed in the cytoplasm of the intestinal cells (3) while the latter were expressed on the microvillar surface (4). Here, TSBP were further fractionated using anion exchange chromatography, and the protective efficacy of fractions containing the bulk of the cysteine protease activity was compared with fractions containing the GDH and with material which did not bind to the column.
The table shows the individual faecal egg outputs averaged over the period of the experiment, individual worm burdens recovered at necropsy, the group means and the percent reductions compared to the controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>Eggs (individual values)</th>
<th>Mean (SE)</th>
<th>% Protection</th>
<th>Worms (individual values)</th>
<th>Mean (SE)</th>
<th>% male (SE)</th>
<th>% protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. TSBP</td>
<td>2897, 67, 1652, 354, 2990, 728, 648</td>
<td>1334 (455)</td>
<td>54</td>
<td>1326, 177, 2994, 338, 3220, 341, 1698</td>
<td>1442 (455)</td>
<td>64·5 (3·9)</td>
<td>43</td>
</tr>
<tr>
<td>2. Unbounda</td>
<td>2125, 2847, 3090, 3785, 2859, 2803</td>
<td>2918 (535)</td>
<td>−0·96</td>
<td>3238, 3452, 3114, 2922, 2695, 3480</td>
<td>3152 (116)</td>
<td>51·5 (1·0)</td>
<td>−24</td>
</tr>
<tr>
<td>3. Protease-rich</td>
<td>1854, 952, 2372, 383, 1446, 557, 1361</td>
<td>1275 (267)</td>
<td>56</td>
<td>2195, 950, 2820, 523, 1631, 977, 2828</td>
<td>1703 (353)</td>
<td>65·3 (4·8)</td>
<td>33</td>
</tr>
<tr>
<td>4. 60 kDa-rich</td>
<td>2532, 3676, 2790, 2478, 3475, 4111, 1058</td>
<td>2874 (382)</td>
<td>0</td>
<td>1301, 2375, 2490, 3696, 3183, 2390, 547</td>
<td>2283 (403)</td>
<td>55·4 (5·8)</td>
<td>10</td>
</tr>
<tr>
<td>5. Control</td>
<td>3807, 2190, 2084, 2057, 3207, 3097, 3791</td>
<td>2890 (294)</td>
<td>0</td>
<td>3253, 1056, 2229, 2866, 2820, 2867, 2618</td>
<td>2530 (272)</td>
<td>48·1 (1·9)</td>
<td>0</td>
</tr>
</tbody>
</table>

The table shows the individual faecal egg outputs averaged over the period of the experiment, individual worm burdens recovered at necropsy, the group means and the percent reductions compared to the controls.

*One individual died before the end of the experiment through causes unrelated to the experimental procedures.

**MATERIALS AND METHODS**

**Antigen preparation**

TSBP were prepared by a combination of peanut lectin and Thiol Sepharose (Pharmacia) affinity chromatography as described previously (1) and then fractionated further by chromatography on Mono Q Sepharose. The column was equilibrated in 10 mM Tris-HCl, pH 7·4 containing 0·1% Triton X-100. TSBP, diluted in the same buffer, were applied to the column and, after the unbound fraction was collected, proteins bound to the column were eluted sequentially in a stepwise manner with 100, 200, 300, 500 mM and 1 M NaCl in the same buffer. The proteins eluted in each step of the gradient were analysed using 10% SDS-PAGE and protease activity monitored using gelatin-substrate gels, these being incubated overnight at 37°C in 0·1 M acetate buffer, pH 5·0, containing 5 mM DTT prior to washing and staining (1).

**Protection trial**

Thirty-five 3–4 month old Greyface × Suffolk worm-free lambs were allocated to five equal groups balanced for sex and weight and the treatment administered to each group is summarized in Table 1. Groups 2, 3 and 4 each received an amount of antigen equivalent to 200 µg TSBP separated into the respective fractions. Antigens were administered to the lambs as three injections at 3-weekly intervals, being emulsified in an equal volume of Freund’s complete adjuvant for the first injection and in incomplete Freund’s for the two booster doses. The first vaccine dose was administered as four subcutaneous injections, each of 0·5 mL, two on each flank, and the boosters given intramuscularly as two 2 mL injections, one into each hind leg. Antibody responses to vaccination were assessed, by immunoblotting and ELISA, in blood samples taken immediately prior to challenge by jugular venepuncture. Two weeks after the final immunization, lambs were challenged with 5000 infective *H. contortus* larvae. Faecal egg counts were determined three times a week from 14 days after challenge using a modified McMaster technique and expressed as eggs per gram fresh faeces. Worm counts were performed on aliquots of gastric washings and mucosal digestes.

**SDS-PAGE and Western blotting**

Antigens (5 µg) were fractionated by 10% SDS-PAGE under reducing conditions and visualized by Coomassie blue staining or electrophoretically transferred onto Immobilon P membrane (Millipore). After transfer, the membrane was cut into strips prior to blocking overnight at +4°C in 0·1% Tris, 137 mM NaCl, 2·7 mM KCl, pH 7·4, 0·1% (v/v) Tween 20 (TBST) containing 10% (w/v) dried milk powder (Marvel). Blot strips were treated with sodium periodate to oxidize carbohydrate epitopes before blocking (5). They were then incubated with pre-immune or immune sera from each immunized animal diluted 1/500 in TBST +10% (w/v) milk powder for 2 h at room temperature. Following extensive washing in TBST, blot strips were incubated in peroxidase-conjugated donkey anti-sheep IgG (Sigma) at a 1/1000 dilution in TBST +10% (w/v) milk powder for 1 h, re-washed and recognized antigens visualized using Fast™ 3,3′-diaminobenzidine tablet sets (Sigma).

**Enzyme-linked immunosorbant assay (ELISA)**

The systemic IgG (total IgG, IgG1 and IgG2) response to vaccination was evaluated for individual animals using the ELISA method described previously (6), with each microtitre
plate well being coated with 0.1 µg TSBP. Mouse anti-bovine immunoglobulin (Ig) G1 was kindly supplied by Peter Geldhof (University of Ghent) whilst mouse anti-sheep IgG2 was prepared in-house at Moredun, the latter showing approximately double the affinity for the purified target isotype compared to the anti-IgG1 (G. Newlands, personal communication). Plates were read at 490 nm and values expressed as the dilution factor required to obtain the mean of a 1/20 dilution of the group pre-immune serum values. The ratios of IgG1:IgG2 titres were calculated and expressed as percentages.

For total IgG analysis, 50 µL of peroxidase-conjugated anti-sheep IgG (Sigma), was added directly to bound sheep Ig, and developed as above.

Statistical analyses

All parasitological parameters were log-transformed and analysed using a two-sample t-test assuming unequal variance.

RESULTS

Protein and protease profiles of TSBP fractions eluted from Mono Q

The protein and protease profiles of TSBP fractions eluted from the Mono Q column with increasing salt concentration are shown in Figure 1a and 1b respectively. The prominent 60 kDa component of TSBP (Figure 1a, lane 1) was mostly eluted by 200 mM NaCl (Figure 1a, lane 4) and did not contain any protease activity. The 60 kDa protein was not evident in the 500 mM and 1.0 M eluates (Figure 1a, lanes 6 and 7) which contained the bulk of the protease activity (Figure 1b, lanes 6 and 7). The unbound fraction (Figure 1a,b, lane 2) contained relatively few peptides and some protease activity. Serine/metallo protease activity at alkaline pH, shown previously (1) to be present in TSBP, partitioned equally across all the fractions (not shown). Pooled fractions 6 and 7 (protease-rich) and fraction 4 (GDH-rich) as well as the unbound material were used as immunogens in the protection trial.

Outcome of protection trial

Only lambs immunized with TSBP or the protease-rich sub-fraction showed significant reductions in faecal egg output (54 and 56% respectively) and final worm burdens (43 and 33% respectively), with a higher proportion of males compared to control animals following the single challenge infection (Table 1). Of note, the reductions in these parameters in the protease-enriched group were almost identical to those in the TSBP vaccinates. Moreover, animals vaccinated with the unbound fraction actually showed higher faecal egg output and worm numbers than the controls, the latter being statistically significant. Vaccination with the 60 kDa protein-rich fraction had no effect on infection outcome.

Immunological responses to vaccination

Immunoblot analyses (Figure 2a) showed that lambs vaccinated with TSBP had a fairly uniform antigen recognition profile, with proteins at ~60 kDa being particularly prominent. The recognition profile of the 60 kDa vaccinates and the unbound were surprisingly similar to whole TSBP, while a limited subset of TSBP was only weakly recognized by sera from animals immunized with the protease-enriched fraction. Here, some variation was evident in the antigen profile recognized by individual sera. Sera from the control group did not recognize any antigens at the dilution tested, in agreement with the ELISA data (Figure 2b). Pre-immune sera did not recognize any antigens in the various immunogens (not shown).
With the exception of the protease-enriched fraction, vaccination induced significantly elevated total IgG responses, with a clear bias towards the IgG2 isotype in all vaccinate groups compared to the adjuvant-only controls (Figure 2b).

**DISCUSSION**

The outcome of the protection trial described here clearly suggests that protection conferred against *H. contortus* infection in lambs by immunization with TSBP is likely to be attributed to the protease components of this antigen extract and not to the other major peptide components. A very limited subset of the total TSBP peptides partitioned in this fraction compared to the unbound and 60 kDa-enriched fractions (Figure 1a). The level of protection observed in lambs immunized with the protease-rich material (fractions 6 and 7, Figure 1a,b) was not statistically different from that observed when whole TSBP was used as immunogen. In particular, the trial showed that the prominent 60 kDa protein component of TSBP (Figure 1a, fraction 4), known to be a glutamate dehydrogenase (3), was not a protective antigen and no protective antigens partitioned in the material which did not bind to Mono Q (Figure 1a, lane 2). Intriguingly, this material did contain some protease activity (Figure 1b, lane 2) and this result may indicate that not all the proteases in TSBP are protective, possibly being products of distinct genes or products of the same gene but carrying distinct post-translational modifications which affect immunogenicity and binding to Mono Q.

The level of protection reported here following vaccination with TSBP is somewhat lower than that reported previously (1). This may reflect batch differences in the amount of actual protease protein in TSBP, the thiol-affinity purification yield being dependent on free –SH groups on the target protein. Our recent data indicate that the cysteine protease components constitute less than 2% of total TSBP protein. We have sought evidence that antibody harvested from sera of vaccinated lambs inhibits TSBP protease activity, with highly equivocal results. Recent experiments showed that vaccination of lambs with bacterially expressed recombinant TSBP cysteine proteases which were enzymically inactive stimulated significant protective immunity (30–40% reductions in worms and egg output; Redmond and Knox, unpublished). A follow-up trial, in which we tested the protective efficacy of the GDH component almost to homogeneity, confirmed that this component was not protective (mean final worm burdens and faecal egg counts of 4643, 3783 [worms] and 16 149, 16 367 [cumulative egg output], respectively, in GDH vaccinates compared to controls).

Proteases are released during *in vitro* culture of many parasitic helminths and have long been proposed as vaccine candidates because of suggested roles in the penetration of host tissues barriers, immunoevasion (e.g. (7)) and, in the case of blood-feeding parasites, blood meal digestion (e.g. (8)). Several studies in a variety of helminth species have provided evidence to support this proposal in recent years (e.g. (1,9–14)) although levels of protection observed have been variable depending on target helminth species and the protease selected for vaccination.

A 35 kDa cysteine protease has been implicated in protection conferred to lambs following immunization with a glycerol extract from adult *H. contortus* (9). The protease had anti-coagulant properties and was capable of degrading fibrinogen (15), but it was not clearly established if the protease was an excretory/secretory (ES) component or a gut-associated enzyme. As noted above, Knox *et al*. (1) reported several trials where the protective efficacy of cysteine protease-enriched extracts from the adult parasite was analysed, enrichment being achieved by Thiol-Sepharose affinity chromatography. The membrane-bound TSBP were effective immunogens and clearly localized to the luminal surface of the intestinal cells in adult worms (1). The work here provides strong evidence that protection could be attributed to the cysteine protease components of TSBP. By contrast, water-soluble and membrane-associated cysteine proteases were without effect as immunogens (1), and these...
extracts may have contained cysteine proteases known to be present in adult parasite in vitro ES (16). This implies that ES cysteine proteases may not be useful immunogens against Haemonchus, but this would require confirmation by conducting vaccine trials with proteases purified from ES alone. This was attempted recently (14) when adult ES material was fractionated using Thiol-Sepharose. A cysteine-eluted fraction containing the bulk of the protease activity did confer some protection to vaccinated lambs but proteomic analysis of this material did not identify any cysteine proteases. So the situation regarding the protective efficacy of H. contortus ES cysteine proteases remains unclear.

In the present trial, immunization stimulated a predominant IgG response in all groups, although this was surprisingly weak in the case of lambs immunized with the protease-enriched fraction (Figure 2b). The total IgG titres in the unbound, 60 kDa-enriched and TSBP groups were comparable and the same was true when the antigens recognized were compared by immunoblot (Figure 2a). However, it was clear that a very limited subset of proteins was recognized by lambs receiving the protease-enriched fraction and there was some evidence for heterogeneous recognition between individual lambs.

The IgG2 response predominated in all groups with the exception of the protease-enriched group where IgG1 was the only isotype detected, albeit at low levels (Figure 2b). The IgG1: IgG2 ratio did not show any correlation with protection. In addition, IgG purified from these sera did not inhibit TSBP protease activity in a manner which correlated with protection as judged using an azocasein-based assay (D. Knox, unpublished; (17)). These data contrast to the situation when lambs are vaccinated with the two lead vaccine candidates for Haemonchus, namely H11, an aminopeptidase (18) and H-galGP, a complex of a number of proteins including proteases (6), where IgG titres stimulated by vaccination closely correlated with protection (6,18). One possibility is that protection correlates to the actual avidity of the antibody for the target antigens. When analysing the antibody responses of cattle immunized against Fasciola hepatica infection using a combination of the fluke-derived antigens cathepsin L2 and haemoglobin, the authors (19) noted that there was no relationship between fluke-specific antibody in immunized animals and antibody titre. However, when the response was analysed in more detail, a relationship between the amount and avidity of IgG2 and fluke burdens became apparent.

In summary, the present study provides compelling evidence that the protection conferred against Haemonchosis in lambs following vaccination with TSBP is attributable to the protease components of the vaccine. It also shows that the bulk of the peptide components of TSBP, in particular the predominant 60 kDa glutamate dehydrogenase, do not confer significant protective immunity.

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REFERENCES

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