Vaccination against hydatidosis using a defined recombinant antigen

M.W. LIGHTOWLERS1, S.B. LAWRENCE2, C.G. GAUCI1, J. YOUNG1, M.J. RALSTON1, D. MAAS3 & D.D. HEATH1

1Molecular Parasitology Laboratory, The University of Melbourne, Princes Highway, Werribee, Victoria 3030, Australia
2AgResearch, Wallacia Animal Research Centre, PO Box 40063, Upper Hutt, New Zealand

SUMMARY
Echinococcus granulosus is the causative agent of hydatid disease in humans and animals. Natural transmission of the parasite occurs between dogs as definitive hosts and animal intermediate hosts. There is an urgent need for improved methods to control the parasite’s transmission. Here we describe the development of a vaccine based on a cloned recombinant antigen from the parasite egg (oncosphere). Sheep vaccinated with the antigen, designated EG93, are protected (mean 96–98%) against hydatidosis developing from an experimental challenge infection with E. granulosus eggs. The vaccine will provide a valuable new tool to aid in control of transmission of this important human pathogen. It also has the potential to prevent hydatid disease directly through vaccination of humans.

Keywords Echinococcus granulosus, hydatid, echinococcosis, vaccine, recombinant antigen

INTRODUCTION
Hydatid disease is a significant cause of human morbidity and mortality worldwide. The most common form of the disease, cystic hydatidosis, is caused by infection with the larval stage of the dog tapeworm, Echinococcus granulosus. The disease in humans is a zoonosis and herbivorous animals in pastoral countries are potential hosts for transmission of the infection. Despite the availability of methods suitable for control of the parasite, its prevalence appears to be increasing (Matossian, Rickard & Smyth 1977). An effective vaccine against infection with E. granulosus would be a valuable new tool for application in hydatid control campaigns (Gemmell & Soulsby 1968).

The oncosphere is an infective stage in the life cycle of E. granulosus and is contained within the eggs released with the faeces of an infected dog. Oncospheres have been shown to be a potent source of host-protective antigens for both E. granulosus (Osborn & Heath 1982) as well as other taeniid cestode species (Rickard & Williams 1982). In a related parasite, Tænia ovis, a host-protective oncosphere antigen has been characterised (Johnson et al. 1989). The recombinant antigen, designated 45W, can be used as a vaccine to protect sheep against infection with T. ovis eggs. Initial studies with E. granulosus investigated the potential for the T. ovis 45W antigen to be either cross-protective against E. granulosus or for the 45W cDNA to be used to identify an homologous gene in E. granulosus. Vaccination of sheep with T. ovis 45W did not afford any protection against E. granulosus and no homologous gene was apparent in the E. granulosus genome (unpublished observations). Hence, it was necessary to identify the host-protective antigens from E. granulosus oncospheres directly by fractionation studies and vaccine trials in sheep (Heath and Lawrence, submitted for publication).

Here we describe the cloning of antigens from the oncosphere of E. granulosus and the successful vaccination of...
sheep against infection with *E. granulosus* using antigen expressed in *E. coli*.

**MATERIALS AND METHODS**

**Parasite material**

*E. granulosus* of New Zealand sheep origin was maintained at the Wallaceville Animal Research Centre, New Zealand, by passage through sheep and dogs. Eggs and oncospheres were obtained as described previously (Heath & Lawrence 1976).

**Antisera against native host-protective antigens**

Preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was used to fractionate *E. granulosus* oncosphere components and putative host-protective antigens were identified (Heath and Lawrence, submitted for publication). Sheep antisera were prepared against seven antigen fractions based on relative molecular mass of selected antigens. Oncosphere components of 23–25 kDa were identified as candidate host-protective antigens and these were targeted for cloning through recombinant DNA techniques.

**Cloning and immunoassay of recombinant antigens**

Mature *E. granulosus* eggs were activated in vitro (Heath & Smyth 1970) and approximately 4 x 10⁶ oncospheres were solubilized on ice in nuclease-free 6 M guanidine hydrochloride (BRL), 0.1 M sodium acetate, pH 5.2, using a glass/silicon tissue homogenizer and stored at -70°C prior to isolation of RNA. The solubilized oncospheres were thawed on ice and insoluble material was pelleted by centrifugation at 10000 rpm, 30 min at 4°C. The supernatant was layered over a solution containing 4 M caesium chloride (BRL), 10 mM EDTA and RNA was pelleted (Chirgwin *et al.* 1979) by centrifugation at 35 000 rpm, 18 h, 15°C in a SW40 rotor (Beckman). Pelleted RNA was dissolved in 200 µl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE), ethanol precipitated and redissolved in 50 µl TE.

Poly(A)+ mRNA was isolated on oligot(dT) cellulose Type 7 (Pharmacia) according to the manufacturer’s instructions, essentially as described by Aviv & Ledder (1972). Eluted poly A+ mRNA was ethanol precipitated and redissolved in 50 µl TE. Quality of the mRNA was assessed by *in vitro* translation of a 5 µl aliquot using rabbit reticulocyte lysate (Amersham) in a total reaction volume of 25 µl according to the manufacturer’s instructions. The products were radiolabelled using 37.5 µCi ³²S-methionine (Amersham) and shown by SDS PAGE/fluorography to include abundant labelled proteins including products of greater than 100 kDa (data not shown).

Thirty seven µl of the mRNA solution in TE was reverse transcribed in the presence of α-³²P-dATP (Amersham) using Molaroney-Murine Leukemia Virus Reverse Transcriptase (ZAP-cDNA Synthesis Kit, Stratagene). cDNA was cloned into the bacteriophage vector Uni-ZAP XR (ZAP) according to the Uni-ZAP XR Cloning protocols (Stratagene). The entire cDNA sample was ligated to 1 µg vector DNA in a total reaction volume of 5 µl and the reactants packaged into infective phage using three Gigapack II Gold packaging reactions (Stratagene). The resultant library of cDNA clones in ZAP was titred on *E. coli* PLK-F' (Stratagene) and assessed to comprise approximately 100 000 primary clones. The entire cDNA library was amplified on *E. coli* PLK-F' cells at a density of 5000 plaque forming units (pfu) per 150 mm diameter plate. Amplified phage were harvested overnight into 10 ml 100 mM NaCl, 8 mM MgSO₄, 50 mM Tris pH 7.5, 0.01% w/v gelatin per plate. The pooled, amplified library was titred on *E. coli* XL1-Blue cells (Stratagene) in the presence of isopropyl β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside and assessed to comprise 1.7 x 10¹¹ pfu with >99% recombinants.

The amplified library was screened in plaque immunoassay (Sambrook, Fritsch & Maniatis 1989) with antibodies from sheep hyper-immunized with whole *E. granulosus* oncospheres and which had been demonstrated to be immune to challenge infection with *E. granulosus*. Serum samples were depleted of 'background' reactivity to *E. coli* and/or β-galactosidase by affinity depletion as follows. Non-recombinant ZAP were plated at near-confluence on *E. coli* XL1-Blue (Stratagene). Nitrocellulose filters (Schleicher & Schuell) impregnated with 10 mM IPTG were overlayed over plaques after growth at 42°C for four hours and plates incubated subsequently at 37°C overnight. The filters were removed, rinsed in TNT (150 mM NaCl, 0.05% Tween 20, 10 mM Tris, pH 8.0) and incubated in 5% skim milk powder/TNT for 1 h. Affinity depletion and other immunoassay incubations were performed at room temperature on a rocking platform. Serum samples were diluted in TNT plus 20% foetal calf serum and depleted by incubation for two h with nitrocellulose filters prepared as above, two filters per 20 ml diluted serum.

The cDNA library was screened for clones expressing oncosphere antigens by plaque immunoassay (Sambrook *et al.* 1989). Library phage were plated at 5000 pfu per 150 mm petri dish on *E. coli* XL1-Blue. Clones binding
antibody in the test sheep sera (1:100 dilution) were detected using rabbit anti-sheep IgG (heavy and light chain) antibody conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories) in TNT containing 5% skim milk powder and bromochloroindolyl phosphate/nitroblue tetrazolium substrate. Clones selected on primary screening were replated and selected by immunosassay a further two times at low plaque density. Confirmed antigen positive clones were further screened in plaque immunosassay with affinity purified anti-23/25kDa antibodies (Heath and Lawrence, submitted for publication).

**DNA hybridization**

Sibling analysis was performed on antigen-expressing clones (Sambrook et al. 1989) by hybridization of digoxigenin-labelled (Boehringer Mannheim) insert cDNA. Plaque arrays were overlayed with nylon membranes (Boehringer Mannheim) and hybridization of labelled cDNA detected using the DIG System (Boehringer Mannheim) chemiluminescent substrate.

**Expression of recombinant antigens**

Selected clones were subcloned into a pGEX vector (Smith & Johnson 1988) for expression and purification of recombinant antigens. Recombinant pBluescript plasmids were generated from λZAP clones by phagemid rescue according to Stratagene protocols and the EcoRI–XhoI inserts subcloned into pGEX-3 (Pharmacia) modified to accept directional EcoRI–XhoI insert sequences (pGEX–EX). E. coli B(+) (Stratagene), transformed with EG95 pGEX 3-EX, were grown overnight and induced with IPTG for two h before purification of the GST fusion protein according to Smith & Johnson (1988).

**Vaccination trials and assessment of the level of protection**

Sheep were Romney and Dorset breeds and approximately eight months old at the time of initial vaccination. Each received 50 μg of GST–fusion protein, attached to glutathione agarose, in adjuvant injected half intramuscularly and half subcutaneously, repeated after one month.

**Trial A**: Antigen was formulated in oil adjuvant (Bokhour, Van Gaalen, & Van der Heijden 1987) 1:1 to a total volume of 2 ml.

**Trial B**: Antigen was made up to a final volume of 2 ml containing either 9 mg Saponin (Saponine PDR 5012, Quest International), 1 mg Quil A (Superfos Biosector a/s) or 70 parts Montanide ISA7030 parts aqueuous antigen. Controls were vaccinated with GST expressed by non-recombinant pGEX-3 with saponin adjuvant. Two weeks after the second injection each sheep was challenged per os with 1000 E. granulosus eggs. Six months later the animals were autopsied, the liver and lungs sliced at 2–4 mm intervals and inspected for hydatid cysts.

**DNA sequencing**

The EG95 cDNA in pBluescript was sequenced on both strands by the di-deoxy chain termination method (Sanger, Nicklen & Coulson 1977). Initially T3 and T7 vector primers were used and subsequently specific internal primers as necessary.

**SDS PAGE and immunoblotting**

E. granulosus oncosphere antigens and EG95 fusion protein were resolved in SDS PAGE (13% acrylamide) under reducing conditions and transferred to nitrocellulose membranes (Schleicher & Schuell) using Transblot electrophoresis (BioRad). Reactivity of sheep sera (1:500 dilution) on blots was revealed using rabbit anti-sheep IgG antibody conjugated to horseradish peroxidase (Silenus) and ECL substrate (Amersham).

**RESULTS AND DISCUSSION**

Eighty clones were selected on primary screening of the cDNA library using hyperimmune sheep anti-E. granulosus oncosphere serum and confirmed as expressing antigens following secondary and tertiary immunosassay. Immunoblotting of plaque arrays with affinity purified antibodies to 23/25kDa native oncosphere antigens identified ten clones showing unequivocally positive reactivity of which seven were shown to be members of the same sibling family by cross hybridization of cDNAs. Three of these clones (EG48, EG95, EG119) and three clones representing other oncosphere antigens (EG3, EG101, EG123) were selected for sub-cloning into pGEX and expression of antigen for vaccine trials.

Antigens expressed in pGEX by clones designated EG53, EG48, EG95, EG101, and EG119 induced significant protection against challenge infection with E. granulosus eggs (P<0.01, except EG101, P<0.05; Mann-Whitney U-test). Clone EG95 induced the highest level of protection and was selected for testing in a second vaccine trial. Protection with antigen expressed by EG95 was confirmed with 96–98% protection achieved using three different adjuvant formulations (Table 1). Seven of the 15 animals vaccinated with
Table 1. Vaccination of sheep against *E. granulosus* with recombinant oncosphere antigens

<table>
<thead>
<tr>
<th>Antigen group</th>
<th>Number of cysts in individual sheep</th>
<th>Mean</th>
<th>Protection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>70, 105, 108, 122, 171, 180, 227, 270</td>
<td>156.6</td>
<td></td>
</tr>
<tr>
<td>EGS3</td>
<td>0, 53, 66, 72, 99</td>
<td>58.4</td>
<td>63</td>
</tr>
<tr>
<td>EG48</td>
<td>0, 12, 17, 46, 58</td>
<td>26.6</td>
<td>68</td>
</tr>
<tr>
<td>EG95</td>
<td>0, 0, 2, 2, 3, 3, 5, 13, 14, 16</td>
<td>5.8</td>
<td>96</td>
</tr>
<tr>
<td>EG101</td>
<td>30, 64, 79, 100, 108</td>
<td>76.2</td>
<td>51</td>
</tr>
<tr>
<td>EGI19</td>
<td>0, 13, 29, 40, 53</td>
<td>27.0</td>
<td>83</td>
</tr>
<tr>
<td>EGI23</td>
<td>8, 20, 40, 100, 305</td>
<td>94.6</td>
<td>40</td>
</tr>
<tr>
<td>Mix of 6 clones</td>
<td>0, 8, 27, 74, 105</td>
<td>42.8</td>
<td>73</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GST</td>
<td>33, 196, 221, 386, 445</td>
<td>256.0</td>
<td></td>
</tr>
<tr>
<td>EG95 - saporin</td>
<td>0, 0, 9, 11, 22</td>
<td>8.4</td>
<td>97</td>
</tr>
<tr>
<td>EG95 - Quil A</td>
<td>0, 0, 1, 3, 44</td>
<td>9.6</td>
<td>98</td>
</tr>
<tr>
<td>EG95 - ISA 70</td>
<td>0, 0, 6, 9, 11</td>
<td>4.6</td>
<td>98</td>
</tr>
<tr>
<td>EG95 - no adjuvant</td>
<td>41, 100, 161, 333, 413</td>
<td>209.6</td>
<td>18</td>
</tr>
</tbody>
</table>

Sheep received two immunizations with recombinant antigens designated EGS3, EG48, EG95, EG101, EGI19, EGI23 or with glutathione S-transferase (GST) as a negative control. After the second immunization each animal was infected with *E. granulosus* eggs and the number of hydatid cysts developing from the infection was assessed six months later. The level of protection was calculated as a percentage of the mean number of cysts in GST controls.

**Figure 1** Immunoblot analysis of *E. granulosus* oncosphere antigen (Panel a) and EG95 fusion protein (Panel b). Lane 1, probed with hyper-immune sheep anti-*E. granulosus* oncospheres; Lanes 2-3, probed with sera from two individual sheep vaccinated with EG95; Lane 4, Coomassie stained, Trion X-100/lysozyme soluble proteins of EG95-pGEX transformed *E. coli* and, Lane 5, glutathione agarose affinity purified EG95 fusion protein. Lanes 6-11, Western blots of EG95 fusion protein probed with: Lane 6, anti-glutathione S-transferase; Lane 7, sheep serum taken prior to vaccination with EG95; Lanes 8-11, serum from individual sheep vaccinated with EG95 fusion protein and subsequently shown to be immune to *E. granulosus* infection.

EG95 plus adjuvant were completely protected against the challenge infection. The sheep were not maintained for a sufficient length of time after the challenge infection to determine any effect that EG95 vaccination may have on the fertility of the small number of cysts found in some vaccinated animals. Immunization with EG95 without the addition of any adjuvant did not induce protection against hydatid infection. Sheep immunized with EG95 raised specific antibodies to the immunizing antigen and to native oncosphere antigen(s) of approximately 23kDa (Figure 1).

DNA sequencing of the EG95 cDNA revealed a 715 bp insert (Figure 2) encoding a predicted protein of 16592 Da. This is smaller than the size of the associated native antigen seen in SDS PAGE immunoblots. However the EG95 cDNA contains no 5' untranslated sequence nor an initiation methionine, suggesting that it is an incomplete copy of the associated mRNA. Post-translational modifications of the native protein may also contribute to the size of the antigen. Homology searches of DNA and protein databases revealed no significant homology with previously described sequences including previously sequenced oncosphere antigens from other taeniid cestodes (Johnson et al. 1989, Cougill et al. 1991, Waterkeyn et al. 1995). Southern blots suggest that EG95 is a member of a family of genes in *E. granulosus* and that a close homologue exists in *E. multilocularis* (unpublished observations).
Clones EG48 and EG119 were found to contain cDNA inserts of identical sequence to EG95 but shorter at their 5' ends by 9 nucleotides. The superior level of protection afforded by EG95 may indicate that an important host-protective epitope occurs at the amino terminus of the parasite encoded sequence in EG95. Significant levels of protection were induced by antigens expressed by clones representing other, uncharacterized antigens (EGS3, EG101).

The EG95 vaccine against *E. granulosus* and the 45 W vaccine against another taeniid cestode, *T. ovis* (Johnson et al., 1989) induce the highest level of immunity yet described for recombinant antigen vaccines against parasitic infections. Some of the characteristics of the host-parasite relationship in the taeniid cestodes, particularly the role of immunity in the natural regulation of transmission of these parasites and the importance of host protective antibody, facilitate the development of recombinant vaccines. Nevertheless, these vaccines against *E. granulosus* and *T. ovis* indicate that it is possible to induce very high levels of protection against complex metazoan parasites using recombinant antigens.

The EG95 vaccine may assist in the control of hydatid disease transmission through domestic livestock. Before the vaccine can be applied in control campaigns, several operational characteristics will need to be defined including the duration of immunity, effectiveness against different *E. granulosus* strains and in different host species, protection of young animals and effectiveness against naturally acquired infection.

The EG95 vaccine also has the potential to be used directly in humans. Vaccination of the human population may have a place in areas of high endemicity for human *E. granulosus* infection, such as parts of Africa (Nelson 1986) or China (Craig, Deshan & Zhaoxun 1991) or where transmission through sylvatic life cycles complicates the control of hydatidosis by more conventional methods.

ACKNOWLEDGEMENTS

Funded by research grants from the National Health and Medical Research Council of Australia and MAFTechology, New Zealand.

REFERENCES


