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Brief Definitive Report

Serological monitoring of protection of sheep against *Echinococcus granulosus* induced by the EG95 vaccine

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SUMMARY

Although immunity to Echinococcus granulosus *in sheep has been shown to be antibody-mediated and complement-dependent and can be passively transferred in colostrum, in animals vaccinated with EG95, the relationship between protection against an oral challenge infection with* E. granulosus *eggs and anti-EG95 IgG ELISA absorbance values at the time of challenge has not been satisfactorily proven. Using a combination of results from three EG95 vaccination trials, we have found that the IgG ELISA absorbance at the time of challenge infection explains approximately 50% (P ≤ 0·001) of the variability in the percentage protection against an oral challenge with* E. granulosus eggs *(transformed with arcsin).*

Keywords *cystic echinococcosis,* Echinococcus granulosus, *EG95 vaccination, hydatid, serology, sheep*

INTRODUCTION

At least one species of the genus *Echinococcus* occurs on all inhabited continents of the world. Members of the genus are cyclophyllidean cestodes with an indirect, two-host life cycle. Small tapeworms (3–5 mm long) live in the small intestine of carnivores (definitive hosts, usually wild or domestic canids, less commonly felids), and either unilocular or multilocular fluid-filled hydatid cysts (metacestodes) develop in the internal organs (mainly liver and/or lungs) of intermediate hosts (usually herbivorous or omnivorous mammals). Humans are infected directly or indirectly from eggs excreted with dog faeces. *Echinococcus* species are of medical and veterinary importance because infection with metacestodes may cause severe illness and death in the intermediate host. Transmission occurs through predator/prey relationships in the wild or through deliberate feeding of infected livestock or wildlife offal to dogs, or through dogs scavenging carcasses of intermediate hosts.

A vaccine to protect sheep, goats and bovines against hydatid disease caused by the cysts of *Echinococcus granulosus* (1,2) has been prepared as a recombinant fusion protein expressed in *Escherichia coli* (3,4). Solubilized inclusion bodies are injected, together with 1 mg QuilA, subcutaneously on two occasions 1 month or more apart, and induce protection against infection, which lasts for at least 12 months (5). Experimental use of the vaccine in various forms has been described (6,7,8,9,10,).

The correlation between ELISA absorbance against a functional antigen and the potency of the antigen has been validated in some recent publications (11–14). The first validations of the potency of recombinant EG95 protein were carried out by immunizing 8–16-week-old lambs on two occasions 1 month apart with 50 ug of EG95 and challenging the lambs orally with freshly collected *E. granulosus* eggs 2 weeks after the second immunization. Nine months later, the lambs were necropsied and the liver and lungs were finely sliced to determine the degree of protection against challenge infection. Each validation took a minimum of 42 weeks to complete. We report here that serum IgG anti-EG95 antibodies were linearly related to a transformation of the percentage protection against the establishment of a challenge oral infection of *E. granulosus* eggs.

MATERIALS AND METHODS

Trials to provide sera and necropsy data

Trials using EG95 antigen made from inclusion bodies were carried out to determine choice of adjuvants (Trial

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1), dose–response of a chosen formulation (Trial 2) and shelf life of a chosen formulation (Trial 3). These trials provided serology at the time of oral challenge infection with *E. granulosus* eggs and necropsy data 9 months after challenge infection. All trials were approved by the Wallaceville Animal Research Centre Ethics Committee.

Romney lambs, weaned at 3–4 months of age and vaccinated at 5–6 months old, were all raised on the same farm. All immunizations were given on day 1 and day 28, by injecting 1–2 mL of vaccine on each occasion subcutaneously in the neck region. On day 42, a serum sample was obtained from the jugular vein using 8-mL Vacutainer serum separation tubes (SST). On day 42, lambs were also orally challenged with 2000 freshly collected *E. granulosus* eggs (1). The challenge infection was left to develop for 9 months.

Trial 1: trial of adjuvants for EG95

Montanide ISA50 – preblended mineral oil plus emulsifier (water in oil). Seppic, Paris, France. (9·0 mL ISA50 was stirred vigorously on a magnetic stirrer whilst adding dropwise 7·5 mL of antigen. 1·1 mL/dose contained 50 μg EG95.)

Montanide ISA70 – preblended mineral oil plus emulsifier (water in oil). Seppic, Paris, France. (16·0 mL ISA70 was stirred vigorously on a magnetic stirrer whilst adding dropwise 4·0 mL of antigen. 2·0 mL/dose contained 50 μg EG95.)

Tasgel – preformed aluminium hydroxide gel, used in most veterinary clostridial vaccines. Product Batch PM-NZ, Upper Hutt. #1891. (7·5 mL of antigen was added to 2·50 mL Tasgel and stirred gently for 30 min. 2 mL/dose contained 50 μg EG95).

Saponin – HP2 was obtained from Proteus International plc (Cheshire, UK). Batch P2000 was stored at room temperature as a dry powder. (A stock solution of 10 mg/mL was made by dissolving in saline and filtering through a 0·2-μm filter. A 1-mL dose contained 10 mg saponin and 50 μg EG95).

QuilA – obtained as a dry powder from Superfos Biosector (Frederikssund, Denmark). Batch L77–153. (10 mg of QuilA were dissolved in 10 mL of antigen solution and passed through a 0·2-μm filter. A 1-mL dose contained 1 mg QuilA and 50 μg EG95).

Drakeol 6VR/Montanide 888 – Pitman Moore footrot vaccine adjuvant. (9·0 mL mineral oil Drakeol 6VR was blended with 1 mL Montanide 888 on a magnetic stirrer. During vigorous mixing, 10 mL of antigen was slowly added and mixing continued for 10 min. A 2-mL dose contained 50 ug EG95).

STM – Span/Tween/Marcol – (Boukhout,1981) Span 85 [54 parts + Tween 85 (46 parts)]. (The emulsion is made by mixing 8 mL aqueous antigen with 9 mL Marcol 52 and 1 mL of the Span/Tween mixture. Ultraturrax homogenizer for 30 s. 2 mL of emulsion/dose contained 50 μg EG95).

DEAE Dextran – high molecular weight polysaccharide anion exchanger – Pharmacia, Lot 12 114. Average *MW* = 500 000 [10 g DEAE Dextran (DDX) was dissolved in 50 mL 0·25 M Tris/HCl pH8·2 and autoclaved at 115°C for 25 min. After autoclaving, pH was 7·2. 7·5 mL of DDX was mixed with 7·5 mL antigen and held at 4°C. A 2 mL dose contained 50 μg EG95 and 10% DDX].

Trial 2: a trial of shelf life after 12 months

A liquid vaccine (batch 005) was formulated under aseptic conditions and was end-filtered through a 0·2-um membrane into 20 mL freeze-drying vials and capped. Each vial contained 100 doses in 10 mL (5 mg of EG95 and 100 mg of QuilA). Some vials were freeze-dried in a sterile environment before capping. Vials containing liquid vaccine were stored for 12 months at ambient temperature (5–25°C), at 4°C or at −18°C, and vials containing freeze-dried vaccine were stored at 4°C. After 12 months, vaccines were diluted aseptically to 1 mL per dose with 0·85% NaCl and stored at 4°C for injections given subcutaneously at day 0 and at day 28.

Trial 3: dose–response of a vaccine

. The same antigen batch as for trial 2 was used. Formulations were made to contain 10, 15, 20 or 50 μg of EG95, but each formulation contained 1 mg of QuilA per 1 mL dose.

Necropsy

Nine months after challenge infection, the sheep were killed by stunning with a captive bolt and then exsanguination. The liver and lungs of each animal were finely sliced (3 mm for liver and 5 mm for lungs), and each slice was inspected and palpated to find the cysts (2–4 mm diameter in the liver and 3–6 mm in the lung). All cysts were sliced through the middle with a sharp scalpel to confirm that they were *E. granulosus* with a fluid-filled central cavity.

ELISA procedures

The EG95 antigen for ELISA was prepared by expressing EG95-6HIS in *E. coli* and purifying the construct on Ni-NTA resin (Qiagen Pty Ltd., Melbourne, Australia).

All recombinant DNA techniques and media were as described (15,16).

A PCR product was amplified from the EG95 pGEX-3X construct using the Invitrogen primers (Invitrogen New Zealand Ltd., Auckland, New Zealand).

EG95-X1

3′ – CGGGATCCATATGGCTTCTCAGTTATGTCT-CATTTTGTTTGC – 5′

EG95-X2

3′ – CTTGCGGCCGCAGTAAGGACAACCACTATGC – 5′

The PCR product was digested with the New England Biolabs restriction enzymes NdeI and NotI and cloned into NdeI-NotI digested AY2·4 (17). The construct produced (EG95-ETag-6xHis) was then modified so that the recombinant protein produced is EG95-6xHis minus the E-Tag epitope.

Briefly, the transformed *E. coli* were grown in LB-Amp and induced with 0·2% arabinose. Inclusion bodies were purified from lysed cells and solubilized in 8 M urea.

Urea was diluted to 2 M and reacted with Ni-NTA resin. EG95-6HIS was eluted with imidazole containing 0·1% sodium dodecyl sulphate (SDS).

The antigen was titrated with chequer-board dilutions of positive and negative sera and laid down at −80°C at a concentration where it could be diluted 1 : 1000 with coating buffer for coating Nunc Immunosorb ELISA plates with 1 μg per well. The antigen was thawed and diluted with carbonate/bicarbonate coating buffer pH9·0 (Sigma-Aldrich Pty Ltd., Sydney, Australia). Wells were sensitized with 50 μL of the antigen at room temperature (RT) overnight. All tests were carried out in duplicate on two separate plates. The following day, the coating buffer was poured off and plates were washed three times for 3 min each time with washing buffer [0·15 M phosphate-buffered saline containing 0·3% Tween 20 (Sigma)]. Plates were then blocked with 350 μL/well of 5% skim milk powder (Blotto) in washing buffer for 1 h at RT or 37°C if the ambient temperature was very cold. The blocking buffer was poured off and plates washed three times. Then 100 μL of the test sera, diluted 1 : 400 in Blotto (1), was added to each well, and plates were incubated at RT for 2 h. Plates were then washed three times, and 100 μL of donkey anti-sheep IgG Horse Radish Peroxidase (HRP) conjugate (Sigma) 1 : 4000 in Blotto was added, and plates were left for 1 h at RT. Plates were washed three times, and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB), 0·1 mg/mL in 100 mM citric acid/sodium acetate, pH 5·2 with 12 μL of 30% hydrogen peroxide per 10 mL of substrate added at the last moment, was added to each well. Plates were incubated in the dark for 30 min. The reaction was stopped by the addition of 50 μL of 1 M sulphuric acid to each well. Plates were then read at 450 nm using an automated ELISA plate reader. The reading was the mean of the two results. Where duplicate results differed by >0·3 absorbance units, the sample was retested in duplicate.

Statistical analysis

The relationship between ELISA IgG absorbance of serum at the time of challenge infection and the degree (expressed as a percentage) of protection against challenge infection as revealed at necropsy of nonvaccinated and of vaccinated lambs was modelled linearly in a regression-type analysis, after transforming the percentage protection by the arcsin function. The effect of trial and adjuvant within trial group was also accounted for and estimated in a mixed-effects model, which was fitted in GenStat v13 (18).

A total of 97 vaccinated lambs and 24 controls were examined. The controls enabled the calculation of percentage protection for each trial, but were not included in the statistical analysis. The average cyst count for the control group from each trial was used to calculate the percentage protection achieved for each treated animal.

RESULTS

Trial 1: adjuvant trial

Results are shown in Table S1. Best results were obtained with Saponin, QuilA and ISA70, although only QuilA maintained solid protective immunity for 7·5 months after challenge as measured by oncosphere-killing test (1) (data not shown).

ISA70 resulted in unacceptable vaccination lesions in some animals.

The saponin used in this trial was selected because of the variability between batches. Some batches induced unacceptable pyrexia and lassitude, and others were not good adjuvants. Each batch from each supplier required quality testing.

Trial 2: dose–response trial

Results are shown in Table S2. The dilution of vaccine to well-below optimum resulted in suboptimal results.

Trial 3: 12-month shelf life

Results are shown in Table S3. Best results were achieved when the vaccine was freeze-dried and stored at 4°C or when the liquid vaccine was stored frozen at −18°C.

IgG and necropsy results

These are shown in Tables S1–S3. Only viable cysts were counted – those in which a patent central cavity could be observed.

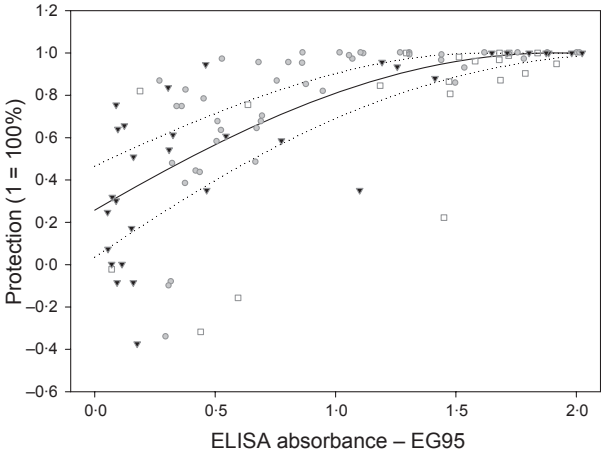

Figure 1 Back-transformed data from the three trials. Trial 1: adjuvant trial (Circles); Trial 2: dose–response trial (Triangles); Trial 3: 12-month shelf life (Squares).

Statistical analysis

The relationship between ELISA IgG absorbance of serum at the time of challenge infection (X) and the degree of protection (expressed as a proportion and transformed using the arcsin function) against challenge infection as revealed at necropsy of nonvaccinated and of vaccinated lambs (Y) may be described by the equation:

Y (in radians) = 0·261 + 0·684X. The standard error of the intercept is 0·1110 and of the slope is 0·0673.

Approximately 50% of the variation is explained by the model. A graph of the data is shown in Figure 1, together with fitted mean of Y and 95% confidence intervals for that mean, over the observed range of X. The mean and confidence intervals have been back-transformed to proportions, resulting in the curvature of the fitted lines and decreasing width of the confidence intervals as 100% protection is approached.

DISCUSSION

Because the oncosphere is known to be associated with the protective immune response to *E. granulosus* infection of intermediate hosts, understanding the mechanisms whereby protective antibodies act against the oncosphere is of fundamental importance in developing highly effective vaccine against *E. granulosus* (19–21).

The EG95 vaccine against hydatid disease cystic echinococcosis (CE) caused by the larval stage of *E. granulosus* has proven to be highly effective (22). The predominant role of antibody in protection of sheep against infection with the eggs of *E. granulosus* has been established for sheep (1,2,5–10) and confirmed for rodents as well (20). Further work has attempted to define the structure of the epitope recognized by protective antibody (23,24). In one study (23), all EG95-vaccinated sheep were highly protected against challenge infection with *E. granulosus*, and this did not allow correlation of antibody titre with the degree of protection against infection. Protective antibody is defined as antibody that binds to the tegument of the oncosphere, and in the presence of complement, is able to create damage to the tegument resulting in lysis of the oncosphere. In the absence of complement, it precipitates on the microvilli of the developing metacestode and is sloughed when the first layer of the laminated membrane develops (25). Protective antibody of the IgG1 class against EG95 is transferred in colostrum of sheep and passively protects lambs against infection for 1–3 months (5). However, we have also shown oncosphere killing in the IgG2 class (26), and therefore decided to monitor total IgG rather than the subclasses.

Although many trials have shown that immunity to hydatid infection stimulated by vaccination with EG95 is antibody-derived and complement-dependent, there is also the possibility that some immunity is T-cell mediated. For instance, in the adjuvant trial, the best adjuvants were those known to stimulate high antibody and also to stimulate a strong cell-mediated immunity. Every newly immunized animal is slightly different genetically, and some animals may direct a lot of their protective immunity into the cell-mediated arm of the immune response. We have not analysed cell-mediated immunity to *E. granulosus* oncospheres *in vitro*, so cannot give a specific example.

Although there is variability in the results within groups in each trial, this variability could also be due to different numbers of eggs hatching and oncospheres establishing, brought about by variations in intestinal physiology or the innate immune status of each individual animal, especially regarding complement levels. Overall, Figure 1 indicates the relationship between percentage protection of lambs against *E. granulosus* (transformed with inverse sin) and anti-EG95 IgG ELISA absorbance.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article:

Table S1. Comparison of various adjuvants to enhance the effect of EG95 vaccine.

Table S2. Testing of Dose-Response to various amounts of EG95 vaccine.

Table S3. Comparison of the effect of various storage conditions on shelf-life.

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