Parasite Immunology, 1996: **18:** 347–357

Antigenic polypeptides of *Echinococcus granulosus* oncospheres and definition of protective molecules

DAVID D.HEATH & STEPHEN B.LAWRENCE

AgResearch, Wallaceville Animal Research Centre, PO Box 40063, Upper Hutt, 6007, New Zealand

SUMMARY

Immunoblotting and in vitro *oncosphere-killing were used to identify a putative protective molecule in* Echinococcus granulosus *mature oncospheres. A range of sera from sheep that had been shown to be protected against* E. granulosus, *and from those that were not, were tested. The sera used were obtained from sheep hyperimmunized with* E. granulosus *oncospheres, or immunized with oncosphere non-denatured extract, with immature oncosphere extract or with denatured extracts of oncospheres. Results indicated the involvement of native antigens of 23, 25, 30, 34 and 40 kDa in the protective response to* E. granulosus *infection. The rapid appearance of antibodies to the 23, 25 kDa antigens, their association with early onset of protection and* in vitro *oncosphere lysis by affinity-purified antibodies obtained from these fractions, indicated that these antigens contained protective epitopes. Final confirmation was provided by immunization of sheep with fractions prepared by preparative SDS/PAGE, and challenge infection. Only the fraction containing the 23 and 25 kDa molecules was able to stimulate protection. Antisera against this pair of molecules should provide a useful probe for screening an* E. granulosus *oncosphere cDNA library to identify clones expressing protective molecules.*

Keywords Echinococcus granulosus, *protective molecules, protection, immunity, vaccination, mature oncosphere*

Correspondence: David D.Heath
Received: 11 December 1995
Accepted for publication: 6 March 1996

INTRODUCTION

Hydatid disease is caused by infection with the larval (metacestode stage) of *Taeniid* tapeworms belonging to the genus *Echinococcus.* Transmission occurs in predator-prey mammalian cycles between carnivorous definitive hosts and herbivorous intermediate hosts.

Of *Echinococcus* tapeworms, *Echinococcus granulosus* is the most significant. *E. granulosus* is the cause of cystic hydatid disease (CHD) and is most often typified by transmission between the domestic dog and sheep. As a consequence, *E. granulosus* is geographically widespread and its distribution closely parallels the areas of the world where grazing of animals is the main occupation. CHD is an important zoonotic disease over large parts of South America, East Africa, Australasia, Central Europe, Central Asia and the Mediterranean littoral including North Africa.

Most countries are now attempting control of CHD through education of the dog owner and regular anthelmintic treatment of the dog as definitive host. However, while some programmes have been partially successful, eradication has proved more difficult to achieve. In other cases, the enthusiasm and motivation for regular treatment of dogs has waned after the first year, and no consistent change in endemicity of the parasite in intermediate hosts has been observed.

An alternative and desirable approach to control CHD involves the use of a vaccine against acquisition of the cystic stage of the life cycle of *E. granulosus* by the intermediate host. This approach has the considerable advantage that transmission of the infection to dogs through consumption of cyst-containing offal from the intermediate host is prevented. As a consequence, a high degree of control of the disease becomes a realistic possibility.

For a recombinant vaccine against CHD to be developed, it is essential that specific protective antigens against infection with *E. granulosus* be identified. While investigations to date (Gemmell 1966, Heath *et al*. 1981, Osborn & Heath 1982) have clearly shown that the oncosphere of *E. granulosus* is a potent source of protective antigens, no such specific

E. granulosus antigens have been identified as conferring protection.

MATERIALS AND METHODS

Echinococcus eggs

Eggs were obtained from experimentally-infected dogs, as previously described (Heath & Lawrence 1976, 1991).

Activation of oncospheres

E. granulosus eggs with fully-developed embryophores were counted and the required number of eggs (assuming a 20% yield of activated oncospheres) were placed in a 15 ml disposable plastic centrifuge tube.

No more than 500 000 were placed in each tube. Eggs were centrifuged at 1000 ***g*** for 2 min, the supernatant was discarded, and 10 ml of artificial gastric fluid (AGF) at 37°C (Heath & Smyth 1970) which had been passed through a 0·2 μm membrane, was added. Tubes were mixed on a rotator at 37°C for 1 h.

Eggs were centrifuged at 1000 ***g*** for 2 min, the supernatant withdrawn, and replaced by 10 ml of artificial intestinal fluid (AIF) at 37°C (Heath & Smyth 1970) which had been passed through a 0·2 μm membrane. Eggs were mixed on a rotator at 37°C for 30 min, followed by centrifugation at 1000 ***g*** for 2 min. The supernatant was discarded, and replaced by 15 ml of Percoll (Pharmacia, Sweden) diluted aseptically 9 : 1 (v/v) with 10× concentrated NCTC135 (Gibco, BRL Life Technologies Inc., Gaithersberg, Maryland, USA). The embryophoric blocks and activated and unactivated oncospheres, which all constitute the pellet, were mixed with the Percoll by inversion. The tube was then centrifuged at 1000 ***g*** for 10 min, after which the supernatant, containing the oncospheres, was tipped into a sterile 15 ml centrifuge tube. Half of the supernatant (7·5 mls) was then tipped into another sterile centrifuge tube, and to each of the two tubes was added 7·5 ml of NCTC135. The tubes were mixed by inversion, and centrifuged at 1000 ***g*** for 5 min. The supernatants were removed, and the pellet containing oncospheres was washed twice in NCTC135, centrifuging for 2 min at 1000 ***g*** each time. The pellet was finally suspended in an appropriate volume of NCTC135 that would give 10 μl samples containing between 40 and 200 oncospheres. The number of activated and unactivated oncospheres was then estimated by counting the entire volume of 4 × 10 μl samples dispensed onto both sides of two haemocytometer slides (improved Neubauer), using 200× magnification to distinguish unactivated oncospheres from those free of their oncospheral membrane (activated oncospheres).

In vitro culture of oncospheres

Cultures were established in 96-well flat-bottomed culture plates (Falcon Microtest III, Falcon Plastics, Onnard, CA, USA). To each well was added 150 μl of test serum (inactivated at 56°C for 30 min), or 150 μl of affinity-purified antibody, plus 150 μl of NCTC135 containing 50 activated oncospheres, plus 50 μl of foetal lamb serum complement (FLSC). All cultures were established in duplicate. Plates were kept in a humidified CO_2 incubator at 37°C, and results were assessed at 24 h and at nine days, using an inverted microscope. Wherever NCTC135 is mentioned, it was supplemented with 300 mg/l of cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) and 50 μg/ml of gentamicin sulphate (Serva, Heidelberg, Germany).

Antigen

The basic freeze-thaw-sonicate (Oncosphere FTS) was prepared by adding enzyme-inhibitors to the sum of activated and un-activated oncospheres, at 1 million/ml, and freezing at −20°C. The enzyme-inhibitors (all from Sigma), were made up in 20 mM Tris/HCl, pH 8·0, so that when diluted v/v with oncospheres, the final concentrations were as follows: iodoacetamide, 20 mM; aprotinin, 10 μl/ml; pepstatin, 2 μg/ml; N-tosyl-1-phenyl-alanine chloro methyl ketone (TCLK), 50 μg/ml; Na-p-tosyl-1-lysine chloro methyl ketone (TLCK) 50 μg/ml; ethylene diamine tetra-acetic acid (EDTA), 2 mM; phenyl methyl sulphonyl fluoride (PMSF), 1 mM. After thawing, oncospheres were sonicated on ice in 20 s bursts until microscopic examination showed that all had ruptured.

For FTS antigen, 0·15 M NaCl was added after sonication to a final concentration of oncospheres of 0·5 million/ml.

For urea-solubilized antigen, (Oncosphere FTS/urea), urea was added before sonication so that the final concentration was 0·5 million/ml in 3 M urea.

For SDS-solubilized antigen, (Oncosphere FTS/SDS), after sonication the oncosphere homogenate was boiled for 4 min in SDS sample-buffer so that the final concentration was 0·5 million/ml. The sample-buffer was that described for SDS/polyacrylamide gels (Laemmli 1970) except that the mercaptoethanol was replaced by 10 mg/ml of dithiothreitol (DTT) (Sigma).

All antigen preparations were centrifuged at 2000 ***g*** to remove unsolubilized material, and were then stored at −20°C in 1·5 ml eppendorf tubes.

For somatic antigens of immature worms, (Immature worm ext) these were harvested from a 34 day infection. All worms had the fourth and fifth segment full of eggs but without fully-developed embryophores. Worms were

homogenized with enzyme inhibitors, and the homogenate spun at 100 000 ***g***. The final concentration was equivalent to 0·3 ml of packed worms/ml. The Oncosphere FTS was centrifuged at 100 000 ***g*** to match the treatment given to Immature worm ext.

Polyacrylamide gel electrophoresis

Parasite antigens were separated by SDS PAGE according to published methods (Laemmli 1970, Hames & Rickwood 1981). Samples were solubilized by boiling for three min in sample buffer as described above for Oncosphere FTS/SDS. Proteins were routinely separated on gradient gels (T = 5−25%) using a vertical electrophoresis system (Bio-Rad Protean II, Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions.

Preparative polyacrylamide gel electrophoresis

Seven million hatched oncospheres were boiled for 4 min in 1 ml of SDS Sample Buffer, followed by centrifugation at 100 000 ***g*** for 15 min. A Bio-Rad Model 491 Prep Cell was prepared by pouring 50 ml of 15% acrylamide, and after polymerization, 8 ml of stacking acrylamide. The oncosphere supernatant was loaded and fractions collected into a fraction collector from the time when the dye-front began to appear in the eluate, for a period of 24 h. Samples of representative fractions were analysed by SDS PAGE and silver staining (Blum *et al.* 1987).

Immunoblotting

Separated oncosphere antigens were transferred electrophoretically (Towbin *et al.* 1979) to nitrocellulose paper (0·45 μm, Schleicher & Schull, Dassel, Germany) in a cooled Bio-Rad Transblot Cell with plate electrodes at 50 v for two h in 10 mM carbonate buffer pH 9·0 containing 20% methanol (Dunn 1986). After transfer, nitrocellulose sheets were placed in 0·5% Ponceau S (BDH) in 1% acetic acid for five min, then rinsed with distilled water until protein bands were clearly visible. Molecular weight markers (Pharmacia, Sweden) were marked with pencil. Individual strips of nitrocellulose containing antigen were cut and placed in incubation trays (Schleicher & Schull). Strips were blocked with 5% fat-free milk powder and 0·1% Tween 20 (BDH) dissolved in 20 mM Tris-HCl pH 7·5, 0·5 M NaCl for one h at 37°C. Sheep antisera were diluted 1 : 100 in the blocking buffer. All subsequent steps were performed at room temperature. Strips were incubated in diluted sera overnight on a rocking platform. Strips were then washed with 4 × 10 min changes of 20 mM Tris-HCl pH 7·5 containing 0·5 M NaCl and 0·1% Tween 20. Bound antibody was detected following reaction for four h with peroxidase-conjugated rabbit anti-sheep IgG (Cappel Organon Teknika NV, Turnhout, Belgium) diluted 1 : 1000 in the blocking buffer. The strips were then washed three times with the washing buffer, and then once with the washing buffer without Tween 20. Peroxidase activity was visualized with 4-chloro-1-naphthol, or 3-amino-9-ethyl-carbazole (Sigma).

Sheep

Six-month-old lambs were raised free of infection with larval cestodes, on the Research Farm of Wallaceville Animal Research Centre. For experimentation, they were housed indoors and fed dry rations. Blood for serum was taken from the jugular vein. All experiments with sheep were approved by the Wallaceville Animal Research Centre Animal Ethics Committee.

Serum preparation

For antibody analysis, blood was collected into SST Vacutainers (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA). After centrifuging tubes for 30 min at 1000 ***g***, sera were poured off and stored at −20°C.

For serum containing complement, foetal lambs close to term, or new-born non-suckled lambs were bled with a sterile syringe, and blood was immediately transferred to 50 ml sterile polypropylene tubes. After clotting for two h at ambient temperature, clots were separated from the walls with sterile applicator sticks in a laminar flow cabinet. Tubes were then centrifuged at 1500 ***g*** in a refrigerated centrifuge at 4°C for 30 min. Tubes were stored in ice while sera was removed. Sera was stored in 2 ml aliquots at −70°C until required for cultures.

Affinity purification of antisera

Antibodies specific to *E. granulosus* Oncosphere FTS/SDS PAGE antigens of relative mobilities of 0–20, 20–25, 25–30, 30–36, 36–37 kDa were affinity-purified from nitrocellulose strips containing the above antigens using acid elution (Beall & Mitchell 1986). The carrier protein was 1% foetal lamb serum. Affinity-purified antibodies were washed and then concentrated 10× using a stirred cell and Amicon PM10 membrane.

Necropsy

Lambs were stunned with a captive bolt and exsanguinated. Liver and lungs were removed and sliced as thinly as possible (2 mm for liver, 4 mm for lungs). Each slice was

observed on both sides and also palpated to detect developing cysts. At the necropsy after four months, cyst outer diameter was 1–2 mm; after 6 months 1–5 mm; after 9 months 2–10 mm. Representative lung nodules were sliced to show the interval cavity, which differentiated these lesions from those induced by lungworms (*Muellerius capillaris*).

Dynamics of the development of immunity

Immunizations consisted of 12 000 activated oncospheres, injected subcutaneously into a different site on the animal each time, so that development of subcutaneous cysts at the primary and other sites could be monitored. Challenges of 2000 *E. granulosus* eggs were administered orally. Three lambs were immunized on day 0 and challenged on day 7 (Group 2). Three controls were also challenged on day 7 (Group 1). Three lambs were immunized on day 0 and again on day 7, and challenged on day 14 (Group 3). Three lambs were immunized on day 0, day 7 and day 14, and challenged on day 21 (Group 4). Two lambs received no immunization or challenge. Serum was collected aseptically from all lambs on days 0, 4, 7, 11, 14, 18, 21, 32 and 45. All lambs were necropsied six months after the beginning of the experiment. Development of the antibody profile on immunoblots was compared with the development of resistance, as revealed by oral challenge, by *in vitro* culture of oncospheres in sequentially collected sera, and by the subcutaneous development of cysts at injection sites.

The effect of SDS and urea on protection induced by FTS oncospheres

Lambs receiving oncosphere antigen each received the equivalent of 30 000 oncospheres on each of two occasions 14 days apart. The antigens, or buffer only for the controls, were homogenized with an equal volume of STM (Span Tween Markol; Bokhout *et al*. 1981) using a Sorvall Omnimixer so that each lamb received a 2 ml volume on each occasion. The injection was divided equally between subcutaneous and intramuscular sites. Four groups of five lambs were immunized, as shown in Table 2. The FTS, FTS/urea and FTS/SDS antigens were diluted as necessary in 20 mM Tris-HCl pH 8·0. Two weeks after the second injection, lambs were bled and then orally challenged with 2000 freshly-collected *E. granulosus* eggs. Lambs were necropsied four months after challenge.

Affinity depletion of antibody with oncospheres

Three lambs from the first experiment had been immunized with oncospheres three times, and then challenged orally. Two weeks after challenge 10 ml of blood was collected aseptically and the sera pooled from the three animals. Two separate aliquots of 1 ml of pooled serum were inactivated at 56°C for 30 min. One million activated oncospheres in 1 ml of NCTC 135 and 1 ml of foetal lamb serum were obtained from five million eggs after hatching and activation. The oncospheres were placed in 1 ml of the inactivated serum, and the resulting 3 ml culture incubated in a CO_2 incubator for 4 h. The oncospheres were then centrifuged at 1000 ***g*** for five min and the supernatant withdrawn. The second aliquot of inactivated serum was placed on the oncospheres, together with 1 ml of NCTC 135 and 1 ml of foetal lamb serum and the oncospheres incubated for a further 20 h.

The starting sera and the two absorbed sera were immunoblotted against oncosphere FTS after SDS-PAGE.

In vitro culture of oncospheres in affinity-purified antibodies

Cultures were established as described above, with appropriate controls. At 24 h, the proportion of oncospheres beginning to develop into metacestodes (pear-shaped organisms, with a halo of microvilli), was compared with the proportion that were dead (round and swollen, with hooklet tips protruding, and no microvilli). At day nine of culture, the total number of developing cysts was assessed for each culture well (organisms with a central cavity and one or two outer layers of refractory material).

Immunization of lambs with an extract of 34 day old *E. granulosus* worms

Ten lambs were immunized with Immature worm ext, five with the Oncosphere FTS and ten adjuvant-only lambs served as controls. The immunization procedure and challenge was the same as in the experiment analysing the effect of SDS and urea, except that lambs received 1 ml of the immature worm antigen on each occasion. Serum was collected at the time of challenge, and examined for *in vitro* killing capacity, and by immunoblots.

Immunization with fractions of oncospheres collected using preparative SDS PAGE

Fractions were bulked according to the immunoblot results of previous experiments as follows. F1–42 (<20 kDa); F57–72 (23 + 25 kDa); F82–92 (30 kDa); F102–132 (34 kDa); F142–162 (40 kDa); F167–172 (43 kDa); F197–F400 (>43 kDa). Fractions were concentrated using an Amicon stirred cell and Amicon UM-3 membrane to approximately

Table 1 Numbers of *E. granulosus* cysts establishing after an oral challenge of 2000 *E. granulosus* eggs in lambs immunized subcutaneously with *E. granulosus* oncospheres

Treatment	Sheep no.	Development of subcutaneous cyst mass at: site 1	2	3	Total cysts in liver and lungs
Challenged Day 7	1	ni	ni	ni	696
(Group 1)	2	ni	ni	ni	440
	3	ni	ni	ni	545
Immunized Day 0	1	+	ni	ni	0
Challenged Day 7	2	+	ni	ni	0
(Group 2)	3	+	ni	ni	0
Immunized Day 0 & Day 7	1	+	−	ni	0
Challenged Day 14	2	+	−	ni	0
(Group 3)	3	+	−	ni	0
Immunized Days 0, 7 & 14	1	+	−	−	0
Challenged Day 21	2	+	+	−	0
(Group 4)	3	+	−	−	0
No immunization	1	−	−	−	0
No Challenge	2	−	−	−	0

ni = no injection.

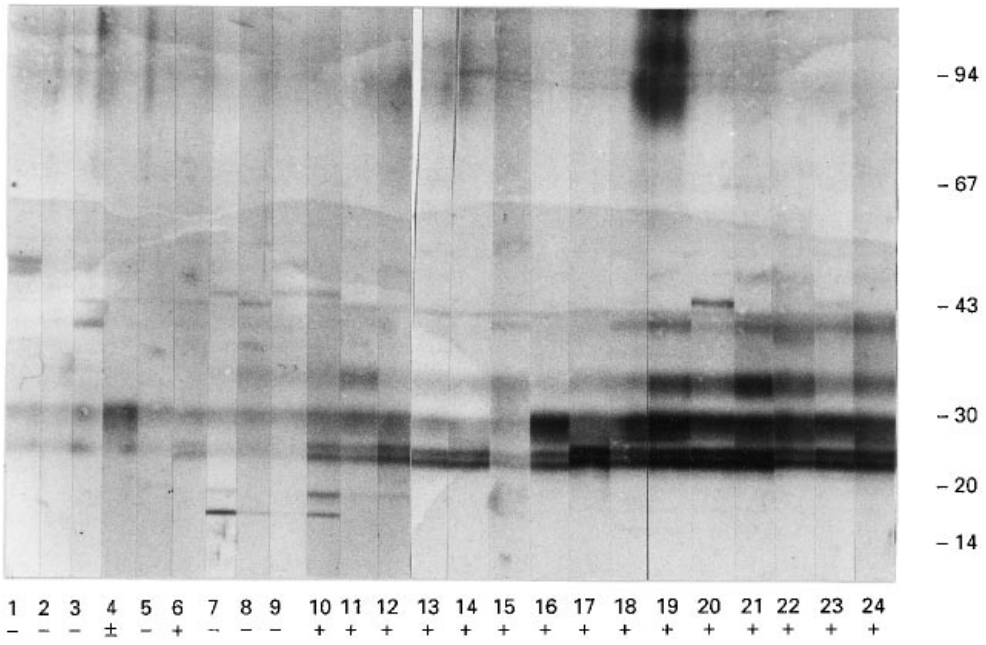

Figure 1 Immunoblotting of *Echinococcus granulosus* Oncosphere FTS/SDS probed with pre- and post-challenge sera from lambs challenged with *E. granulosus* eggs orally, or immunized once, twice, or three times with *E. granulosus* oncospheres prior to challenge. The results of the oncosphere-killing test on these sera is shown below respective lanes as: −, no killing; ±, some killing and immune precipitates on developing cysts; +, all oncospheres killed. Group 1: lambs received no immunization and were challenged on Day 7. Lanes 1–3, prechallenge, Lanes 4–6, 28 Days post-challenge. Group 2: lambs received an immunization at Day 0 and were challenged at Day 7. Lanes 7–9, pre-challenge, Lanes 10–12, 28 Days post-challenge. Group 3: lambs received immunizations at Day 0 and Day 7, and were challenged at Day 14. Lanes 13–15, pre-challenge, Lanes 16–18, 21 Days post-challenge. Group 4: lambs received immunizations on Day 0, Day 7, and Day 14, and were challenged at Day 21. Lanes 19–21 pre-challenge, Lanes 22–24, 14 Days post-challenge. Relative mobilities of proteins were estimated from Pharmacia Low Molecular Weight Markers run on the same gel.

Table 2 Numbers of *E. granulosus* cysts establishing in the livers and lungs of lambs immunized with various extracts of *E. granulosus* oncospheres, following challenge with 2000 *E. granulosus* eggs

Treatment	Sheep no.	Number of cysts
Adjuvant only	1	159
	2	137
	3	230
	4	70
	5	93
Oncos/FTS	1	0
	2	0
	3	0
	4	0
	5	0
Oncos/FTS/urea	1	0
	2	0
	3	0
	4	0
	5	0
Oncos/FTS/SDS	1	88
	2	63
	3	38
	4	107
	5	44

10 ml. They were then centrifuged at 1000 ***g*** for 30 min at 1°C and the supernatants were dialysed against four changes of 20 mM Tris buffered saline (pH 7·5) containing the cocktail of enzyme inhibitors at 1 : 100 of the previously described concentration, at 4°C over 3 days to remove most of the SDS.

The 12 ml of each concentrated fraction combined with 10 ml of STM adjuvant was divided into ten injections of approximately 2 ml. Five ten-month-old lambs received the antigen from each fraction, half the injection being subcutaneous and half intramuscular on the left hand side. For the second injection, one month later, the procedure was repeated on the right hand side. However, the second injection also contained 2 mg of killed *Mycobacterium phlei* per injection. Control groups of five received adjuvant only or a mix of equal amounts of all the fractions (1 ml of each fraction at each time, equals 7 mls plus adjuvant divided into five lambs each time). Oncosphere killing tests were carried out on serum collected from each group of lambs at the time of challenge, which was one month after the second injection. Lambs were challenged with an oral dose of 1000 freshly-collected well-developed *E. granulosus* eggs, and were necropsied nine months later.

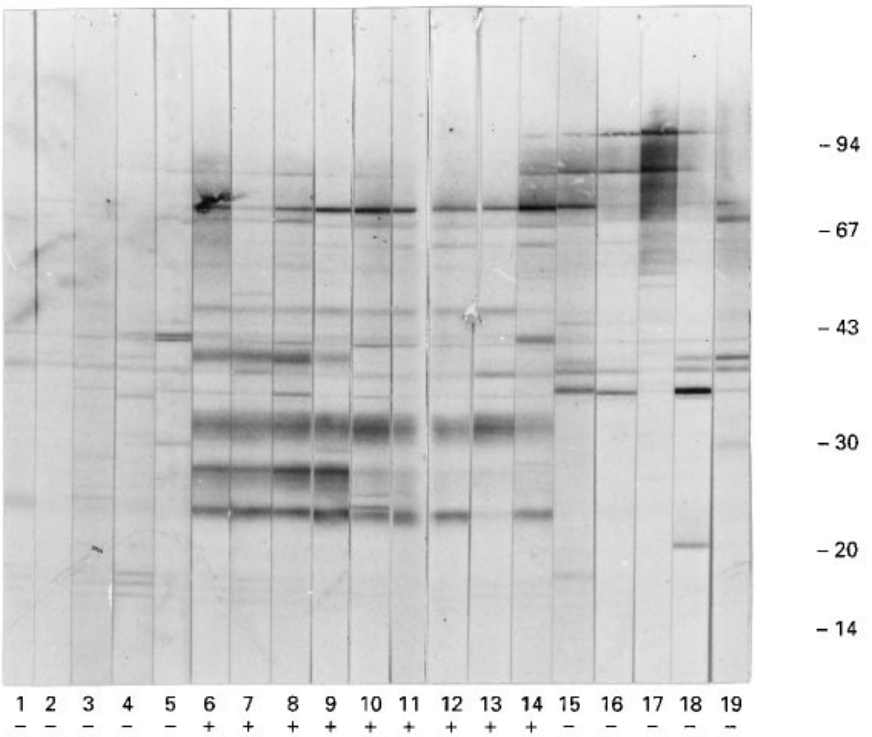

Figure 2 Immunoblots of *E. granulosus* Oncosphere FTS/SDS probed with pre-challenge sera from lambs receiving Adjuvant only (Lanes 1–5), or Oncosphere FTS (Lanes 6–9), or Oncosphere FTS/urea (Lanes 10–14), or Oncosphere FTS/SDS (Lanes 15–19). Relative mobilities of proteins were estimated from Pharmacia Low Molecular Weight Markers. The results of the oncosphere-killing test on these sera is shown below respective lanes as: −, no killing; ±, some killing and immune precipitates on developing cysts; +, all oncospherse killed.

RESULTS

Dynamics of the development of immunity

Table 1 shows that 7 days after a primary immunization with oncospheres s.c., the lambs resisted the development of cysts from a second injection and the development of cysts from an oral challenge with *E. granulosus* eggs.

Figure 1 shows that the first antibodies to develop after immunization with oncospheres were directed against antigens of molecular weight 23 and 25 kDa (Lanes 13, 14, 15). This was followed by antibodies to antigen at 30 kDa, (Lanes 19 to 21) and then antigens at 34 and 40 kDa. The *in vitro* results confirm the rapid onset of solid immunity in that oncosphere-killing antibodies were present in prechallenge sera at Day 14 after two immunizations at Day 0 and Day 7. (Lanes 13–15).

Effect of SDS and urea

Table 2 shows that the protective epitopes are denatured when injected in the presence of SDS, but not when injected in the presence of 3 M urea. Figure 2 shows that antibodies associated with full resistance to challenge occur at 23, 25 and 34 kDa. The *in vitro* oncosphere killing by respective sera matched the necropsy results.

Affinity depletion of antibody with oncospheres

The hyperimmune starting sera recognized antigen bands at 23, 25, 27, 28, 30, 34, 40, 50 kDa and a few others (Figure 3) in the 67–75 kDa region. The first absorption partially depleted most bands, and completely depleted the 23, 25 kDa region. The second absorption appeared to only partially deplete the 23, 25 kDa region.

In vitro culture in affinity-purified antibodies

Figure 4 shows that the lethal effect of antibodies is associated with the 23–25 kD region, and the 30, 34, 40, 43 and 50 kDa region, as revealed by oncosphere-killing *in vitro* in sera containing antibodies to these regions.

Immune status of lambs injected with an extract of 34 day old worms

As shown in Table 3 and Figure 5a and 5b there was no protection induced by immunization with 34-day-old worms containing immature eggs (Immature worm ext), and the immunoblots of sera from these lambs, compared to sera from lambs immunized with Oncosphere FTS (and which were immune to challenge), showed that each set of sera recognized different specificities within the extracts of immature worms or mature oncospheres.

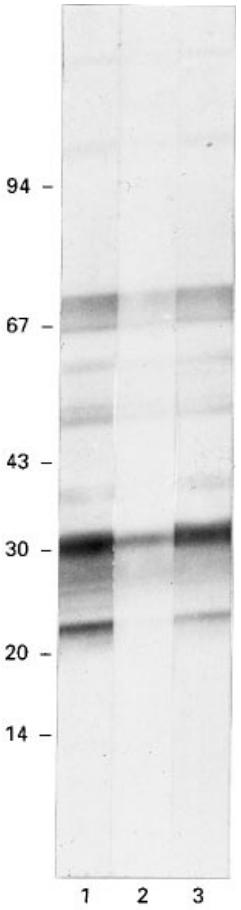

Figure 3 The effect of absorbing a serum sample from a sheep hyperimmune to *E. granulosus* oncospheres, and which was refractory to oral challenge with 2000 *E. granulosus* eggs. The absorbent was one million activated oncospheres of *E. granulosus.* Lane 1 shows the reaction of the hyperimmune serum with the immunoblotted profiles of *E. granulosus* oncospheres (Oncosphere FTS/SDS). Lane 2 shows the antibodies remaining after four h absorbance of half of the serum. Lane 3 shows the absorbance from the other half of the serum, into which the oncospheres were transferred at four h and remained for a further 20 h.

Immunization with fractions of oncospheres from preparative SDS/PAGE

Some oncosphere-killing was observed in the group receiving the mix of all fractions, and complete oncosphere lysis (100%) occurred in the group receiving the 23 + 25 kDa molecules (Table 4). No killing occurred in any other group. Immunoblots against oncosphere FTS of antisera used in the oncosphere-killing test showed recognition of the 23 + 25

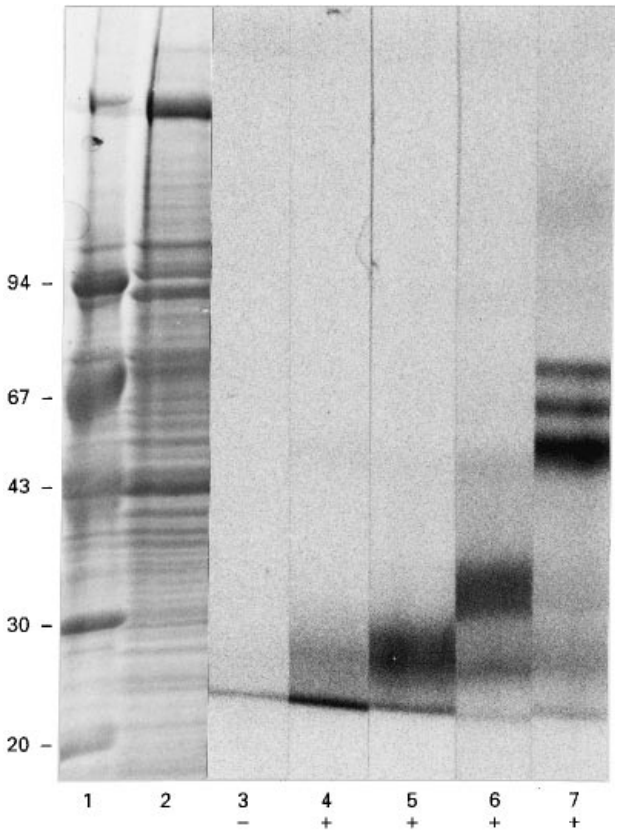

Figure 4 Immunoblotting of Oncosphere FTS/SDS with affinity-purified antibodies to various fractions of Oncosphere FTS/SDS transferred to nitrocellulose. Lane 1; Pharmacia Low Molecular Weight Markers silver-stained. Lane 2; profile of *E. granulosus* Oncosphere FTS, SDS/PAGE, silver-stained. Lane 3; 0–20 kDa; Lane 4; 20–25 kDa. Lane 5; 25–30 kDa. Lane 6; 30–36 kDa; Lane 7; 36–67 kDa. The results of the oncosphere-killing test on these sera is shown below respective lanes as: −, no killing; ±, some killing and immune precipitates on developing cysts; +, all oncospheres killed.

bands by pooled group 23 + 25 kDa and recognition of the 23, 25, 30 and 40 kDa molecules by the group receiving the mix of fractions (data not shown). Only the 23 + 25 kDa group had numbers of cysts significantly lower than controls (mean of 9 *vs* 133; $P < 0{\cdot}01$).

DISCUSSION

The procedure for identifying protective molecules from oncospheres followed, to some extent, that described by Harrison *et al.* (1993) for the protective molecules in *Taenia ovis* oncospheres. The Oncosphere FTS immunoblot profile indicates the involvement of antigens of 23, 25, 30, 34 and 40 kDa in the protective response to *E. granulosus* infection. The rapid appearance of antibodies to the 23, 25 kDa antigens, and their association with the early onset of

Table 3 The number of *E. granulosus* cysts establishing in the livers and lungs of untreated control lambs, or in lambs immunized with Immature worm ext or Oncosphere FTS, and then challenged orally with 2000 *E. granulosus* eggs

Number of *E. granulosus* cysts in liver and lungs		
Adjuvant controls	Immat/worm ext	Oncosphere FTS
253	104	1
303	121	0
450	228	0
335	94	0
85	267	0
273	179	–
285	192	–
32	182	–
107	197	–
204	482	–

protection, and with *in vitro* protection by affinity-purified antibodies containing these fractions, indicates that these antigens contain protective epitopes. The absorption of the 23, 25 kDa bands by activated oncosphere suggests that these molecules occur on the surface of the oncosphere and thus are a prime target for antibody-dependent complement-mediated lysis of the plasma membrane. The plasma membrane can be damaged by antibody and complement (Heath *et al.* 1994). The material contributing to the expansion of the plasma membrane after activation was apparently stored as granules in large cells within the oncosphere, and was elaborated to the plasma membrane via ducts after activation (Harris *et al.* 1989). Nevertheless, in the profile of oncosphere antigens shown by a silver-stain of a gel containing antigens separated by SDS-PAGE (Figure 4, Lane 2) the 23, 25 kDa fractions do not appear to be a major source of antigen. They were, however, immunodominant, and were probably the first molecules from the oncosphere surface taken up by antigen-presenting cells after injection of live oncospheres. When the Oncosphere FTS was injected together with adjuvant, a number of other molecules was also recognized.

While they were denatured and rendered inactive by the SDS treatment used here, the epitopes were able to combine with protective antisera after transfer to nitrocellulose. Further, the antibodies to these antigens when prepared by affinity-purification were able to lyse oncospheres *in vitro* in the presence of a source of complement. It is interesting that all the affinity-purified fractions except that from <20 kDa contained antibodies capable of lysing oncospheres. It is probable that some antibody to the 23, 25 kDa antigen was co-purified with antibody to the other fractions.

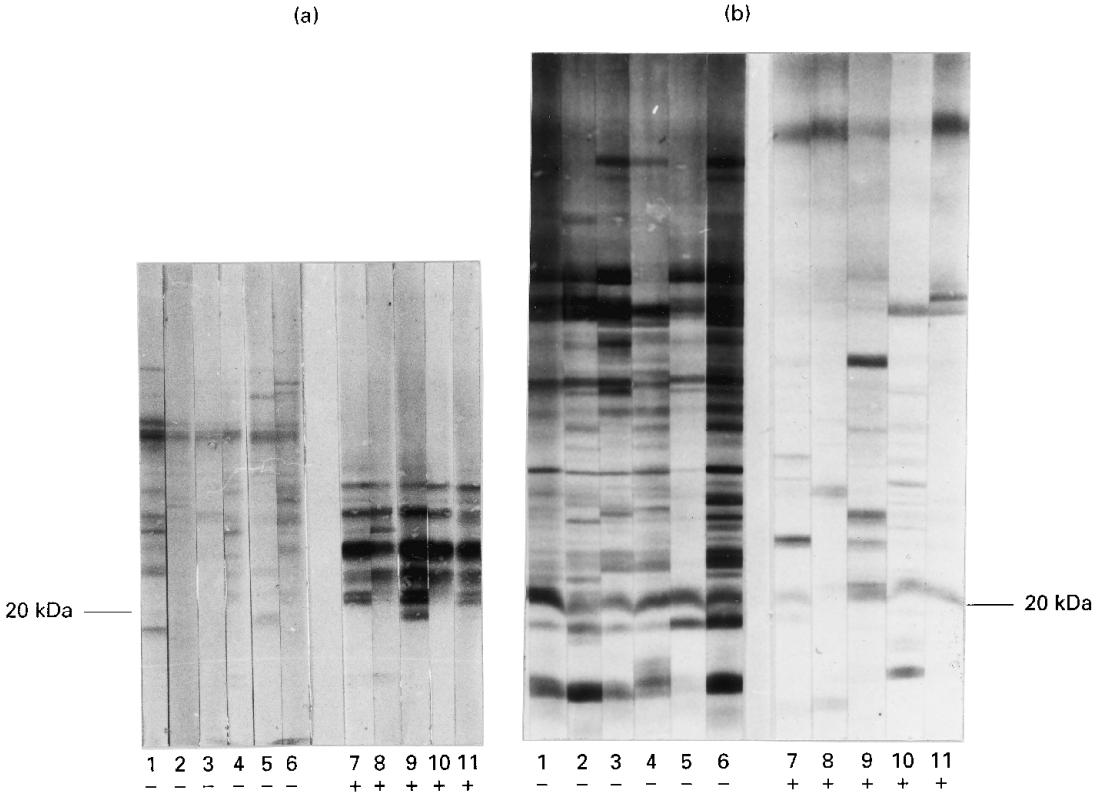

Figure 5 Immunoblots of Oncosphere FTS/SDS (a) or Immature worm EXT/SDS (b) probed with prechallenge sera from lambs immunized with Immature worm ext (not immune to challenge) (Lanes 1–6), or from lambs immunized with Oncosphere FTS (immune to challenge) (Lanes 7–11). The results of the oncosphere-killing test on these sera is shown below respective lanes as: –, no killing; ±, some killing and immune precipitates on developing cysts; +, all oncospheres killed.

The other components of the Oncosphere FTS immunoblot profile, the 30, 34 and 40 kDa bands, appeared later. Our conclusion was that the primary protective antigens of *E. granulosus* occurred as epitopes in proteins of 23, 25 kDa. The 40 kDa molecule appeared to be denatured by treatment with urea (Figure 2), but the urea preparation was still able to protect against challenge infection, suggesting that the presence of the 40 kDa molecule was not absolutely necessary for full protection against challenge to be achieved.

The protection of lambs by the 23, 25 kDa fraction from an SDS preparative gel is evidence that these molecules are not only protective, but also robust, in that they survived treatment with SDS. Perhaps the 30 and 34 kDa molecules are not so robust. The dialysis should have allowed refolding and so the conclusion could be drawn that the 30, 34 and 40 kDa molecules are not protective.

The protective antigens can be produced by isolation from the native *E. granulosus* oncospheres using conventional purification techniques. However, it is recognized that for production of the antigens in commercial quantities, production by synthetic routes is desirable. The cloning and expression of these antigens using recombinant DNA techniques is currently being investigated, and if successful, may lead to the development of a practical vaccine against hydatid disease. The successful cloning and expression of the 23, 25 kDa molecule has recently been reported by Heath & Lightowlers (1993). One molecule, termed Eg95, has induced 97% protection in sheep against a challenge oral-infection with *E. granulosus* eggs.

REFERENCES

Beall J.A. & Mitchell G.F. (1986) Identification of a particular antigen from a parasite cDNA library using antibodies affinity purified from selected portions of Western blots. *Journal of Immunological Methods* **86,** 217–223

Blum H., Beir H & Gross H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8,** 93–99

Table 4 *In vitro* oncosphere-killing activity of pooled group sera collected from lambs at the time of challenge infection with 1000 *E. granulosus* eggs, and the necropsy results presented as numbers of developing cysts in livers and lungs nine months after challenge

Group treatment	Total cysts in liver and lung	Mean and % protection~~	Group treatment	Total cysts in liver and lung	Mean and % protection~~
<20 kD	77		43 kD	57	
	39			67	
	19	57 (57%)		165	69 (48%)
	101			4	
	47			52	
23 + 25 Kd	0		>43 Kd	84	
	29			75	
	10	9 (93%)*""		2	51 (62%)
	2			17	
	4			78	
30 Kd	0		Mix of all fractions	0	
	42			0	
	17	81 (39%)		104	63 (53%)*
	148			133	
	197			79	
34 kD	167		Adjuvant control	27	
	9			137	
	48	87 (35%)		170	133
	85			242	
	126			88	
40 kD	109				
	86				
	156	145 (0%)			
	276				
	99				

* Oncospheres killed *in vitro*; "" Significantly different from controls ($P < 0{\cdot}01$). Statistical analysis was an analysis of variance using log-transformed data on total cysts per lamb $(\log_{10}(x+1))$; ~~% Protection = Control group mean − test group mean/Control group mean × 100/1.

Bokhout B.A., Van Gaalen C. & Van der Heijden Ph.J. (1981) A selected water-in-oil emulsion: Composition and usefulness as an immunological adjuvant. *Veterinary Immunology and Immunopathology* **2,** 491–500

Dunn S.D. (1986) Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. *Analytical Biochemistry* **157,** 144–153

Gemmell M.A. (1966) Immunological responses of the mammalian host against tapeworm infections IV. Species specificity of hexacanth embryo in protecting sheep against *Echinococcus granulosus. Immunology* **11,** 325–335

Hames B.D. & Rickwood D. (1981) *Gel Electrophoresis of Proteins: a Practical Approach.* IRL Press, Oxford.

Harris A., Heath D.D., Lawrence S.B. & Shaw R.J. (1989) *Echinococcus granulosus*: Ultrastructure of epithelial changes during the first 8 days of metacestode development *in vitro. International Journal for Parasitology* **19,** 621–629

Harrison G.B.L., Heath D.D., Dempster R.P., Lawrence S.B., Lightowlers M.W. & Rickard M.D. (1993) Identification of host-protective antigens of *Taenia ovis* oncospheres. *International Journal for Parasitology* **23,** 41–50

Heath D.D. & Smyth J.D. (1970) *In vitro* cultivation of *Echinococcus granulosus, Taenia hydatigena, T. ovis, T. pisiformis* and *T. serialis* from oncosphere to cystic larva. *Parasitology* **61,** 329–343

Heath D.D. & Lawrence S.B. (1976) *Echinococcus granulosus*: development *in vitro* from oncosphere to immature hydatid cyst. *Parasitology* **73,** 417–423

Heath D.D., Parmeter S.N., Osborn P.J. & Lawrence S.B. (1981) Resistance to *Echinococcus granulosus* infection in lambs. *Journal of Parasitology* **67,** 797–799

Heath D.D. & Lawrence S.B. (1991) Daily egg-production of dogs infected with *Echinococcus granulosus. Archivos de la Hidatidosis* **30,** 321–328

Heath D.D. & Lightowlers M.W. (1993) The use of a recombinant antigen to immunise sheep against *Echinococcus granulosus. Proceedings of the International Congress of Hydatidology, Beijing,* October 10–13, 1993

Heath D.D., Holcman B. & Shaw R.J. (1994) *Echinococcus granulosus*: the mechanism of oncosphere lysis by sheep complement and antibody. *International Journal for Parasitology* **24,** 929–935

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* (London) **277,** 680–685

Osborne P.J. & Heath D.D. (1982) Immunity of lambs against *Echinococcus granulosus* using antigens obtained by incubation of oncospheres *in vitro*. *Research in Veterinary Science* **33,** 132–133

Towbin H., Staehelin T. & Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76,** 4350–4354