Assessment of protective immune responses against hydatid disease in sheep by immunization with synthetic peptide antigens

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SUMMARY

Four synthetic peptides which comprise the immunodominant linear epitopes of the EG95 recombinant protein, were investigated for their ability to induce host-protective immunity against *Echinococcus granulosus* in sheep. Sheep were immunized with either free peptide or peptide conjugated to diphtheria toxoid and challenge infected with *E. granulosus* eggs. All of the peptides elicited specific antibody, but these did not kill the parasite in *in vitro* culture assays, nor did the peptides induce protection against challenge infection. In contrast, anti-EG95 antibodies affinity purified against each of the 4 peptides were lethal to the parasite in *in vitro* culture. These affinity-purified antibodies were shown to contain specific antibody to both peptide and EG95. In *in vitro* inhibition assays, the peptides did not diminish anti-EG95 antibody binding to EG95 or parasite lysis in oncosphere killing assays. These results suggest that the fine specificities of antibodies raised against the recombinant protein are different to those raised against the peptide immunogens and that the majority of the antibody induced by vaccination with EG95 is raised against conformational determinants.

Key words: synthetic peptide, *Echinococcus granulosus*, hydatid, vaccine, epitope, antibody.

INTRODUCTION

The development of effective vaccines against medically and economically important diseases remains a high priority in parasitology. A number of recombinant vaccines against parasitic infections have been described over the last decade since publication of the first defined antigen vaccine against a parasitic disease. These vaccines rely on expression of parasite antigens in either prokaryotic or eukaryotic cells. Practical application of recombinant vaccines requires large-scale production which is often hampered by levels of expression, solubility of the antigen in non-denaturing conditions and degradation of the expressed protein.

Advances in peptide synthesis technology have enabled peptides to be considered as a more cost-effective option than recombinant proteins (Meloen, 1997; Saul & Geysen, 1990). A large quantity of peptide can be produced which is chemically defined and stable (Brown, 1990). Peptides may also be advantageous in that epitopes which elicit unwanted or harmful side-effects can be eliminated (Roitt, 1989).

EG95 is a recombinant antigen which has been used effectively as a vaccine against hydatid disease in sheep. The vaccine protein was identified as a host-protective antigen in the oncosphere and the recombinant antigen consists of a 16·5 kDa protein which has been expressed as a fusion with glutathione-S-transferase (EG95–GST). Vaccination of sheep twice with EG95–GST has been shown to elicit 96–99% protection against experimental challenge infection with *E. granulosus* (Lightowlers *et al.* 1996, 1999).

Protection against infection with *E. granulosus* which is induced by the EG95 vaccine is strongly associated with humoral immune responses. High levels of EG95-specific IgG antibody are elicited following vaccination (Woollard *et al.* 1998) and these antibodies are able to kill the parasite in *in vitro* culture (Lightowlers *et al.* 1996). In addition, immunity is transferred from vaccinated ewes to their new-born young, implying that IgG_1 antibodies are important in vaccine-induced immunity (D. Heath, unpublished observations).

The host-protective epitope(s) of the native antigen homologue of EG95 appears to be resistant to denaturation because solubilization of this protein in SDS diminishes but does not abrogate its ability to induce protection (Heath & Lawrence, 1996). This suggests that the host-protective epitope(s) may be linear and peptide regions of EG95 which contain strong antibody-binding sites could be potential vaccine candidates.

Linear regions of the EG95 recombinant which display prominent antibody binding have been

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determined in epitope mapping experiments (Woollard *et al*. 1998). Four immunodominant regions were identified which consisted of 2 sequences of 20 amino acids and 2 sequences of 14 amino acids. In this study we determine if peptide regions of the EG95 recombinant can elicit host-protective responses and if they elicit antibodies which are lethal to the parasite *in vitro*.

MATERIALS AND METHODS

Preparation of peptide and EG95–GST

Four peptides were synthesized by Chiron Technologies Pty Ltd (Clayton, Victoria, Australia) as follows: TETPLRKHFNLTPVC (peptide 6), SLKAVNPSDPLVYKRQTAKFC (peptide 12/13), DIETPRAGKKESTVMTSGSAC (peptide 21/22) and SALTSAIAGFVFSC (peptide 24). The peptides corresponded to 4 antigenic regions identified by Woollard *et al*. (1998) with an additional carboxy terminal cysteine residue added to peptides 6, 12/13 and 21/22 to facilitate conjugation to a carrier protein. The peptides were synthesized on polyethylene pins (Valerio *et al*. 1993) and then purified to $\geqslant 95\%$ purity using reverse-phase chromatography. A sample of each peptide was conjugated to diphtheria toxoid (DT) by coupling the peptide via the carboxy terminal cysteine to maleimido activated diphtheria toxoid (Lee *et al*. 1980). A substrate for affinity purification of antisera was prepared by coupling of peptide 6, 12/13, 21/22 or 24 to thiopropyl Sepharose 6B (Amersham Pharmacia Biotech, Sweden). A control affinity column was also made by conjugation of cysteine residues to the thiopropyl Sepharose gel.

EG95–GST was prepared as described by Lightowlers *et al*. (1996). Soluble EG95–GST was affinity purified from *Escherichia coli* proteins using glutathione–agarose beads (sulphur linkage, Sigma, USA (Smith & Johnson, 1988)).

Vaccination, infection and post-mortem examination of sheep

Two experimental vaccine trials were performed. In Trial 1, 50 Dorset/Merino cross lambs, 6–12 months of age, were immunized with either DT conjugated peptide, free peptide or EG95–GST, challenge infected with *E. granulosus* eggs and then examined at post-mortem, 12 months after infection. In Trial 2, 39 Merino/Border Leister first cross lambs were immunized with either EG95, DT-conjugated peptide alone or all 4 DT-peptide conjugates combined. In this trial, antisera were tested *in vitro* for their ability to kill *E. granulosus* oncospheres (see below). Sheep immunized with EG95 in Trials 1 and 2 received 2 immunizations with 50 μg of EG95–GST and 1 mg of Quil A (Superfos, Denmark), subcutaneously, 1 month apart. Sheep immunized with individual peptides in Trials 1 and 2 were given 2 immunizations, 1 month apart, intramuscularly in the right hind leg using 50 nmol of peptide at each injection. In Trial 2, sheep immunized with the combination of all 4 peptides were immunized with either all 4 DT-conjugated peptides in the right hind leg (same injection site) or the 4 DT-peptides in different injection sites. For this group, peptides 6 and 24 were given intramuscularly in the caudal humeral region of the left and right front legs, respectively, peptides 12/13 and 21/22 were given intramuscularly in the caudal thigh region of the left and right hind legs, respectively. Sheep immunized with combined peptides were given 2 immunizations with each peptide at 50 nmol per dose, 1 month apart. The peptides were dissolved in distilled deionized water and then emulsified in Freund's adjuvant (Difco, USA) at a ratio of 1:2 of dissolved peptide to oil adjuvant (final volume 1 ml per injection). The initial immunization was with complete Freund's adjuvant and Freund's incomplete was used for the second immunization. Serum samples were obtained at first and second immunizations, 2 weeks after the first immunization, 3 and 2 weeks after the second immunization (for Trials 1 and 2, respectively) and at 2-month intervals after the second immunization.

In Trial 1, sheep were challenged with viable *E. granulosus* eggs 6 weeks after the second vaccination. The eggs were prepared as described by Heath & Lawrence (1976). Each sheep was given 1000 eggs intra-rumenally as described by Lightowlers *et al*. (1999). The sheep were killed 12 months after challenge infection and the liver, lungs, heart, spleen, kidneys and omentum were examined for the presence of hydatid cysts. The livers were sliced at approximately 3 mm intervals. The lungs were sliced at approximately 5 mm intervals and palpated. Hydatid cysts were regarded as viable if they contained a cavity and clear cyst fluid, confirmed where necessary by histological examination.

Enzyme-linked immunosorbent assays (ELISAs)

Antisera raised against peptide were assessed for reactivity to homologous peptide. Antisera raised against EG95 as well as affinity-purified anti-EG95 antibodies were assessed for EG95 and peptide specific antibody. Antisera raised against EG95–GST were assessed for EG95-specific antibodies using an EG95–maltose binding protein fusion (EG95–MBP) as antigen (Maina *et al*. 1988; Woollard *et al*. 1998). EG95–MBP or EG95 peptide were bound to 96-well microtitre plates (Maxi Sorp, Nunc, Denmark) by incubating 0·2 or 0·1 μg of the appropriate antigen, respectively, per well in 100 μl of 50mM carbonate buffer (pH 9·6), overnight at

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4 °C. The plates were post-coated with 100 μl per well of phosphate-buffered saline (PBS, pH 7·2) containing 2% (w/v) bovine serum albumin (BSA, Sigma, USA) for 1 h at room temperature. After 4 washes with PBS containing 0·05% Tween 20 (PBST), 100 μl of serial dilutions of serum samples were added to the wells for 1 h at room temperature. Plates were again washed 4 times with PBST. For the total IgG assay, 100 μl of a 1/1000 dilution of a monoclonal anti-cow/sheep IgG–HRP conjugate (Silenus, Australia) diluted in PBST containing 2% (w/v) BSA was added for 1 h at room temperature followed by the addition of substrate as described below. IgG_1 and IgG_2 antibodies were measured by the addition of 100 μl per well of a pre-determined concentration of an anti-isotype monoclonal antibody specific for each antibody class for 1 h. Monoclonal antibodies specific for ovine IgG_1 and IgG_2 were obtained from Dr Ken Beh. Plates were washed and 100 μl per well of a 1/1500 dilution of sheep anti-mouse IgG–HRP conjugate (Silenus) added for a further 1 h incubation at room temperature. For both assay types, the plates were washed 6 times with PBST before the addition of 100 μl of tetramethyl benzidine (TMB) substrate to each well for 30 min at 25 °C. The reaction of substrate with HRP was stopped by the addition of 50 μl of 0·5 M H_2SO_4 per well and the absorbance measured at 450 nm using a Dynatech 4500 plate reader.

Antibody titres for each serum sample were calculated at an optical density (OD) of 1·0 and expressed as the reciprocal of the serum dilution. On each ELISA plate, a sheep serum raised against EG95–GST was titrated as a positive control and any plates on which the titre of this positive serum differed from the mean by more than 20% were considered invalid and the samples re-assayed.

Epitope mapping with overlapping peptides

Twenty-five peptides were synthesized (Chiron Technologies Pty Ltd), each consisting of a 14 amino acid sequence identical to a particular region of the EG95 antigen. The sequence of the peptides overlapped that of the adjacent one by 8 amino acids. The peptides were biotinylated at their amino terminus and included a spacer sequence as follows: biotin–SGSG–EG95 peptide. Dried peptide was dissolved in 200 μl of dimethyl sulphoxide and stored at −20 °C. Prior to immunoassay, the peptides were diluted 1:1000 in PBST containing 0·1% (w/v) sodium azide.

Microtitre plates (Maxi Sorp, Nunc) were coated with 100 μl of streptavidin (Sigma) at 5 μg/ml and left overnight uncovered in a 37 °C incubator and allowed to evaporate to dryness. Non-specific binding was blocked by dispensing 100 μl per well of PBS containing 2% (w/v) BSA and incubating for 1 h at room temperature. After washing the plates 4 times with PBST, 100 μl of diluted peptide was added and incubated for 1 h. Each peptide was placed in a separate microtitre plate well. The protocol used for the remainder of the assay was identical to the ELISA protocol described above.

Affinity purification of antisera

Antisera were affinity purified using peptide conjugated to thiopropyl-Sepharose 6B gel in a plastic 12 ml syringe. Each syringe contained 2 mg of peptide conjugated to 2 ml of packed gel. The control column consisted of 10·6 mg of cysteine coupled to 2 ml of thiopropyl Sepharose gel. Antisera were diluted in PBS at a ratio of 1:2 and 1:5 for anti-peptide and anti-EG95 antisera, respectively. The syringes containing diluted antisera and affinity gel were placed on a rotating wheel for 1 h at room temperature after which the supernatant was discarded and the gel washed 5 times with PBS and 3 times with saline (0·15 M). Bound antibody was eluted in 10 ml of 0·1 M glycine/HCl (pH 2·5). The eluate was collected into a pre-determined neutralizing volume of 0·1 M NaOH. The gel was washed briefly with a further 10 ml of PBS and this added to the first eluate. The pH of the eluate was adjusted to pH 7 if necessary.

The affinity-purified antibody solutions were concentrated from approximately 15 ml to 1 ml by centrifugation at 1850 ***g*** for 15 min using Millipore Ultrafree-15 centrifugal filter devices with a 50000 molecular weight cut off membrane (Millipore Corporation, USA).

In vitro *oncosphere killing assays*

The technique used for *in vitro* oncosphere killing assays was adapted from that described by Heath & Lawrence (1996). Hydatid eggs obtained from infected dogs (as described by Heath & Lawrence, 1976), were suspended in PBS in 50 ml polypropylene tubes, centrifuged at 1000 ***g*** for 5 min and resuspended in PBS. The quantity of eggs in each sample was determined by counting the number in 10 μl on both sides of a haemocytometer slide (Improved Neubauer). After repeat centrifugation, the eggs were suspended in a small volume of PBS and transferred to 15 ml centrifuge tubes and incubated in a solution of 1% pepsin (BDH, England) and 1% concentrated HCl made up in 0·85% saline for 1 h at 30 °C. After centrifugation, the pepsin solution was decanted and the eggs incubated for 30 min in a filter sterilized solution of 1% pancreatin (Sigma), 1% $NaHCO_3$ and 5% sheep bile made up in distilled deionized water. The number of activated oncospheres was counted. Where less than 10% of the eggs had activated, they were incubated for a further 30 min in a fresh solution of pancreatin, $NaHCO_3$ and bile. Activated

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oncospheres were pelleted by centrifugation (1000 ***g***, 5 min) and the tubes were filled with a sterile solution of warmed Percoll (Amersham Pharmacia Biotech, Sweden) diluted aseptically 9:1 (v/v) with 10 × concentrated NCTC135 (Gibco, BRL Life Technologies Inc., Gaithersberg, Maryland, USA). The NCTC135 was supplemented with 300 μg/ml of cysteine hydrochloride (Sigma), 100 μg/ml streptomycin sulphate (Sigma), 50 μg/ml of gentamicin sulphate (Serva, Heidelberg, Germany) and 100 U/ml of penicillin G (Sigma). The tube contents were mixed by vigorous shaking and centrifuged for 15 min at 1000 ***g***. The supernatant containing activated oncospheres was carefully decanted into a clean tube and the pellet containing the embryophoric blocks discarded. The supernatant was divided into 3 between 3 sterile 15 ml tubes and NCTC135 was added to make the volume in each tube 15 ml. The tubes were shaken and then centrifuged at 1000 ***g*** for 15 min and the supernatant discarded. The tubes were filled with fresh culture medium and the numbers of activated oncospheres were counted in duplicate as per the determination of the number of eggs. NCTC135 was added to give 50 oncospheres per 100 μl of medium. The antifungal agent, Nystatin (Sigma) was added to give 100 units per ml. This solution was centrifuged to remove crystals before addition to the culture plates.

The culture plates were set up as follows. One hundred and fifty μl of sterile test sera were added per well of a 96-well culture plate (low absorption, Nunc). A further 100 μl of NCTC135 containing a minimum of 50 activated oncospheres was added per well. Serum from sheep known to be free from exposure to *E. granulosus* was used in the cultures at 50 μl per well as a supplementary source of complement. For each test serum, oncosphere cultures were set up in duplicate.

The culture plates were placed in a humidified, 37 °C incubator containing 5 % CO_2. After 10 days the number of living oncospheres was counted in each well using an inverted microscope.

Inhibition ELISA and inhibition of in vitro *oncosphere lysis*

A serially diluted sheep anti-EG95 antiserum was pre-incubated with EG95 or peptide 6, 12/13, 21/22 and/or 24 for 1 h and subsequently assayed for reactivity with EG95 in ELISA and for oncosphere killing ability *in vitro*. EG95–GST, EG95–MBP, GST and MBP were used at a concentration of 5 μg per ml of antiserum. Peptide antigens were used at a concentration of 10 μg per ml of antiserum. The amount of peptide used was pre-determined as the lowest concentration at which maximal inhibition of anti-peptide antibody reactivity with peptide was observed in ELISA.

RESULTS

Antibody response to peptide and EG95

Trial 1. No anti-peptide or anti-EG95 antibodies were detected in the sera of sheep prior to immunization with peptide or EG95. All sheep vaccinated with peptide developed detectable titres of specific IgG_1 and IgG_2 antibody. Maximum IgG_1 and IgG_2 antibody titres for sheep immunized with DT-conjugated peptide ranged from 10200 to 460000 and 5240 to 361000, respectively. The corresponding values for unconjugated peptide were 10552 to 65000 and 2320 to 36250, respectively. Antibody titres at post-mortem (59 weeks after the second immunization) ranged from 466 to 9260 and 0 to 2274 for IgG_1 and IgG_2, respectively. Peak IgG_1 and IgG_2 antibody titres for sheep immunized with EG95 were 252000 and 264000, respectively.

Trial 2. Sheep immunized with EG95 had a specific IgG antibody titre of 74667 at 2 weeks after the second immunization. Anti-peptide antibody titres for sheep immunized with individual peptide and combined peptide are shown in Fig. 1. All sheep immunized with peptide produced detectable anti-peptide antibodies. IgG antibody titres at 2 weeks after the second vaccination for sheep immunized with individual peptide ranged from 3775 to 161250. The corresponding titres for sheep immunized with combined peptide in the same and different sites were 182 to 64600 and 608 to 46100, respectively. The titres recorded for sheep immunized with individual peptide were significantly higher than the corresponding peptide titres in sheep immunized with combined peptide ($P < 0{\cdot}05$, Mann–Whitney *U*-test), except for titres to peptide 12/13 given individually and combined in the same site, which were equivalent. Antibody titres to peptides 6, 12/13 and 21/22, recorded in sheep immunized with combined peptides, ranged from 17 to 56 % of the corresponding titre measured in sheep immunized with individual peptide. Antibody titres for peptide 24 measured in sheep immunized with combined peptides were lower than those recorded in sheep given peptide 24 alone (5 and 16 % for sheep immunized in the same and different sites, respectively).

The specificities of antibody raised against individual peptide and combined peptide were determined by reacting the antisera with a series of 25 overlapping 14mer peptides spanning the EG95 sequence. Antisera from sheep vaccinated with all 4 peptides in the same and different injection sites reacted to the homologous peptide sequences as well as to the overlapping flanking peptides (data not shown). Sheep vaccinated with peptides 6, 12/13, 21/22 or 24 alone also displayed reactivity to homologous peptide sequences in addition to the flanking peptides (data not shown).

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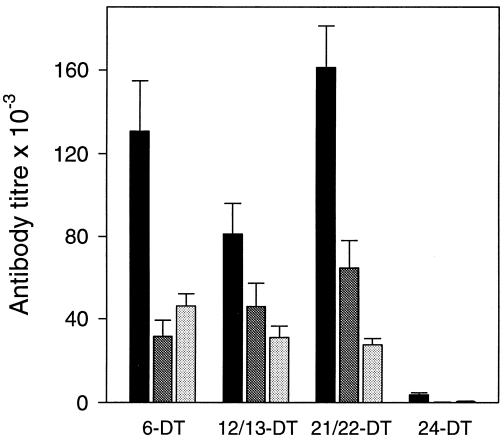

Fig. 1. IgG antibody titres of sheep immunized with peptides 6, 12/13, 21/22 or 24 conjugated to diphtheria toxoid (DT) at 2 weeks after a secondary immunization measured against homologous peptide. Solid bars show response of sheep immunized with individual peptide while dark shaded and light shaded bars show response of sheep immunized with all 4 peptides injected in the same site or different sites, respectively.

ELISAs with affinity-purified anti-EG95 antibodies

Affinity-purified anti-EG95 antibodies were assayed for reactivity to both peptide and EG95 (Fig. 2). For all 5 sheep immunized with EG95, antibodies affinity purified on peptides 12/13, 21/22 and 24 were reactive with EG95 in ELISA. A similar pattern was observed for the reactivities of these antibodies with peptide, except that 1 of the affinity-purified eluates to peptide 24 did not have detectable reactivity (no. 48). These titres ranged from 1000 to 4300 and 0 to 2900 for antibodies specific for EG95 and peptide, respectively. By comparison, the titres of anti-EG95 antibody from whole serum ranged from 0 to 12500 for reactivity against peptides 12/13, 21/22 and 24 (Fig. 2). Three of the 5 samples affinity purified to peptide 6, were reactive with EG95, but none showed detectable reactivity with peptide 6. Anti-EG95 antibody affinity purified on peptide 6 did not have detectable reactivity to peptides 12/13, 21/22 or 24 (data not shown). The same pattern was also seen for antisera affinity purified to peptides 12/13 and 21/22. Antisera affinity purified to peptide 24 reacted with peptide 24 as well as peptide 21/22. The unbound fraction of these affinity purifications had no detectable reactivity with the peptide to which it was purified but had equivalent reactivity to EG95 compared to whole serum (data not shown). The anti-EG95 antibodies which were reacted with the control affinity-purification column had no detectable reactivity with EG95 or peptide (data not shown).

Necropsy results

Sheep were examined for hydatid cysts at post-mortem and the numbers of viable cysts detected are

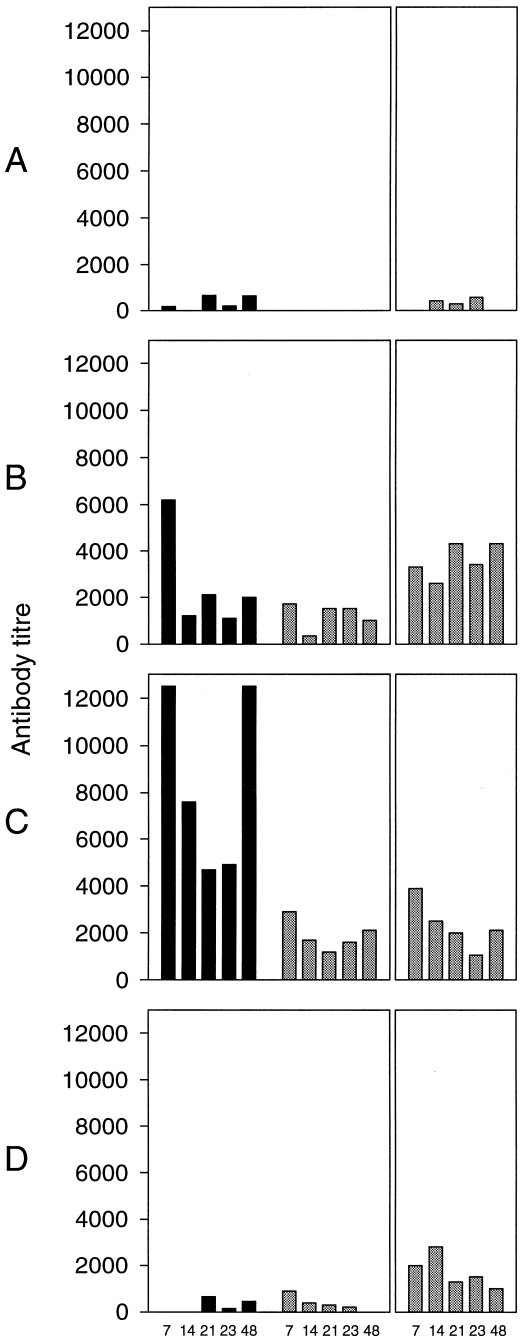

Fig. 2. Antibody titres of individual sheep (numbers 7, 14, 21, 23, 48) immunized with EG95. The panels on the left-hand side represent anti-EG95 antibody reactivity with peptides 6 (A), 12/13 (B), 21/22 (C) or 24 (D) and those on the right-hand side show antibody reactivity to the EG95 recombinant protein. Solid bars represent reactivity of whole serum whereas shaded bars represent binding of antibody affinity purified against peptides 6, 12/13, 21/22 or 24 and reacted against the corresponding peptide or EG95.

shown in Table 1. One sheep in each of 3 different groups died during the 12 months between challenge infection and necropsy from factors unrelated to the experimental treatment. Sheep immunized with EG95 were immune from infection compared with non-vaccinated animals ($P < 0{\cdot}01$, Mann–Whitney

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Table 1. Vaccination of sheep against *Echinococcus granulosus* with EG95 recombinant protein and EG95 peptides

(Sheep received 2 immunizations with EG95 or DT conjugated or unconjugated peptide. After the second immunization, each animal was infected with *E. granulosus* eggs and the number of viable hydatid cysts was assessed 12 months later. The level of protection was expressed as a percentage of the mean number of cysts in the non-vaccinated control animals for those groups showing a statistically significant reduction in cyst numbers compared with controls.)

Antigen group	Number of cysts in individual sheep	Mean	Protection (%)
Non-vaccinated control	22, 90, 164, 186	115·5 ± 37·3	—
EG95	0, 0, 0, 0, 1	0·2 ± 0·2	99·8
Peptide 6-DT	12, 47, 86, 124, 255	104·8 ± 42·0	Nil
Peptide 6	22,32, 65, 73	48·0 ± 12·4	Nil
Peptide 12/13-DT	0, 31, 106, 118, 153	81·6 ± 28·5	Nil
Peptide 12/13	88, 106, 114, 165	118·3 ± 16·5	Nil
Peptide 21/22-DT	28, 43, 60, 117, 172	84·0 ± 26·7	Nil
Peptide 21/22	40, 86, 117, 141, 290	138·8 ± 42·3	Nil
Peptide 24-DT	22, 61, 140, 166	97·3 ± 33·6	Nil
Peptide 24	63, 112, 131, 172, 274	150·4 ± 35·5	Nil

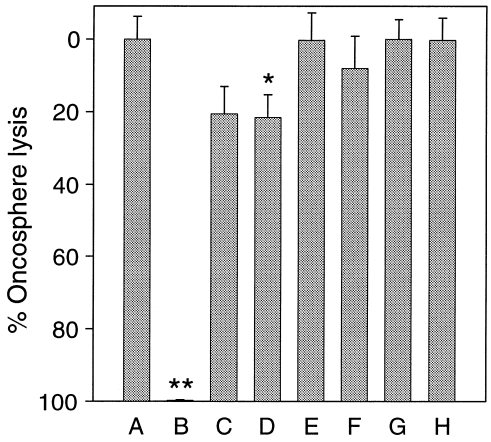

Fig. 3. *In vitro* oncosphere lysis with sera from non-vaccinated sheep (A), sheep immunized with EG95 (B) or with peptides (C–H). Peptide vaccines were peptides 6, 12/13, 21/22 and 24 combined in the same injection site (C) or different injection sites (D) or peptide 6 (E), peptide 12/13 (F), peptide 21/22 (G) or peptide 24 (H) alone. Bars denoted with * and ** indicate significance of $P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively (Mann–Whitney *U*-test).

U-test). Only 1 animal out of the 5 that were vaccinated with EG95 had a single viable cyst. None of the groups immunized with peptide, conjugated or unconjugated, were significantly protected. Of the total numbers of cysts, 65% were found in the lung and 35% were in the liver.

In vitro *oncosphere killing assays with anti-peptide antibody*

Antisera from sheep immunized with EG95 and peptide were used in *in vitro* oncosphere killing assays. The results for antisera from sheep immunized with each of the 4 DT conjugated peptides on their own as well as antisera from sheep immunized with all 4 peptides combined are shown in Fig. 3. Also shown are data for non-vaccinated sheep and sheep immunized with EG95. None of the antisera from sheep immunized with DT conjugated peptides 6, 12/13, 21/22 or 24, individually, elicited significant lysis of the oncospheres compared to the control sera. Antisera from sheep immunized with unconjugated peptide were also non-lethal (data not shown). Antisera from sheep immunized with the peptides given in combination in the same site and different sites elicited lysis of 20·5 and 21·5%, respectively, compared to the control sera. This lysis was only significant for the antisera from sheep immunized with all 4 peptides given in different injection sites ($P < 0{\cdot}05$). Antibodies to EG95 elicited lysis of 99·8% ($P < 0{\cdot}01$).

In vitro *oncosphere killing assays with affinity-purified antibody*

Antibodies from antisera to EG95 which were affinity purified on peptides 6, 12/13, 21/22 or 24 were tested in *in vitro* parasite culture assays. Oncospheres cultured in the presence of antibodies affinity purified on peptides 6, 12/13, 21/22 and 24 were significantly reduced in number compared to control cultures using the column eluates from sera of control non-vaccinated sheep (Fig. 4; peptides 6 and 12/13, $P < 0{\cdot}05$; peptides 21/22 and 24, $P < 0{\cdot}01$). The unbound fractions from these affinity purifications were also highly lethal, with lysis ranging from 93 to 97·7% of control sera treated in the same manner. Anti-peptide antibodies affinity purified to homologous peptide did not cause oncosphere lysis in *in vitro* culture (data not shown).

Sera from EG95-vaccinated sheep and from non-vaccinated controls were incubated with the control affinity purification column and the eluates tested for oncosphere killing ability. No significant lysis was detected with either the anti-EG95 antisera or the sera from non-vaccinated sheep after reaction with the control column.

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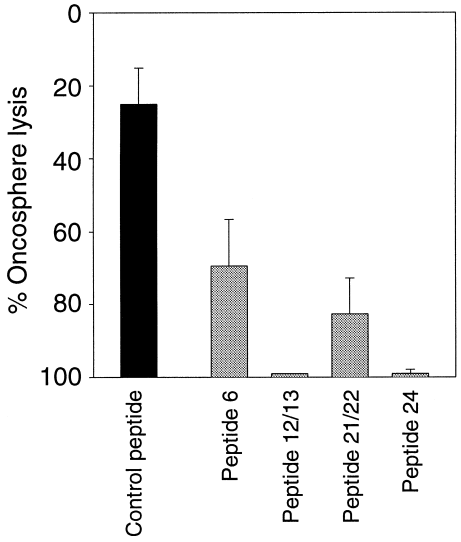

Fig. 4. *In vitro* oncosphere lysis with affinity purified anti-EG95 antibodies. Antisera to EG95 were affinity purified against peptides 6, 12/13, 21/22 and 24. Anti-EG95 antisera were also reacted with the control affinity purification column. Each bar represents the oncosphere lysis with anti-EG95 antibody, affinity purified to a particular peptide as a percentage of the lysis observed with sera from control, non-vaccinated sheep, affinity purified against the same peptide.

Inhibition ELISAs with peptide and EG95 antigens

Antisera raised against EG95 were pre-incubated with peptide 6, 12/13, 21/22 and/or 24 and assayed for reactivity with EG95. None of the peptide antigens on their own or combined, reduced the level of reactivity of anti-EG95 antisera with EG95 in ELISA (Fig. 5A). The EG95 antigens (EG95–GST and EG95–MBP) did inhibit reactivity. The GST and MBP antigens did not effect reactivity of anti-EG95 antibodies with EG95 (Fig. 5A).

Inhibition of anti-EG95 antibody mediated oncosphere lysis with peptide and EG95 antigens

Inhibition assays in *in vitro* oncosphere culture were performed with the same antisera which had been tested in the inhibition ELISA (Fig. 5B). Serially diluted antisera were incubated with and without inhibitor antigens prior to culture of oncospheres. Oncosphere killing was observed at dilutions of anti-EG95 antisera up to 1:2000. Addition of EG95 antigen inhibited oncosphere lysis at all dilutions of the antisera tested, but the addition of peptide antigens on their own or combined had no effect (Fig. 5B).

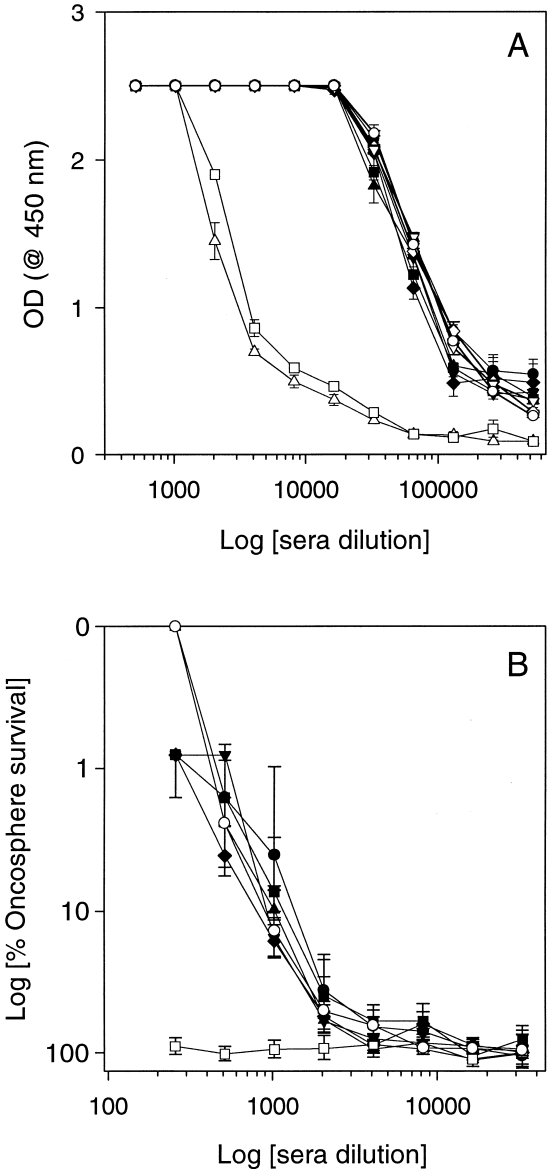

Fig. 5. Inhibition assays using sheep antisera to EG95 pre-incubated with various synthetic peptides or with EG95. (A) Results of inhibition of reactivity measured in ELISA. (B) Inhibition of oncosphere killing in *in vitro* culture. Antisera to EG95 were incubated with EG95–GST (□), EG95–MBP (△), GST (◇), MBP (▽) and peptides 6 (●), 12/13 (■), 21/22 (▲) and 24 (▼) and all 4 peptides combined (◆). Antisera without added antigen (○).

DISCUSSION

The experimental challenge and parasite culture experiments in this study indicate that the 4 peptides of EG95 which contain immunodominant, linear epitopes did not induce protective immune responses. Vaccination of sheep with peptide did not elicit protection from experimental challenge infection. In parasite culture experiments, high titre antisera raised against the 4 peptides had no deleterious effects on the parasite in marked comparison with the lethal effects of antisera to the the EG95 recombinant protein. This occurred despite these peptides eliciting specific antibodies which reacted with both the recombinant protein and the native oncosphere antigen(s) (Woollard, Gauci & Lightowlers, 1999).

Sera from sheep immunized with all 4 peptides in different injection sites induced a low, but stat-

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istically significant amount of lysis of the parasite in *in vitro* culture, possibly indicating that antibody raised against several regions of the recombinant protein is required to elicit a host-protective response. Furthermore, anti-EG95 antibodies affinity purified on each of the 4 peptides were lethal to the parasite *in vitro*. Taken together, these results suggest that peptides 6, 12/13, 21/22 and 24 are associated with host-protective epitopes. Multiple protective epitopes within a recombinant protein or peptide have been described in bacterial and viral pathogens (Beachey & Seyer, 1986; Dale & Beachey, 1986; Schmidt *et al*. 1988; Grosfeld *et al*. 1991). However, immunization with the peptides either individually or combined did not elicit the degree of oncosphere lysis seen with antisera raised against EG95 nor did incubation of the anti-EG95 antisera with the peptides combined affect oncosphere survival.

Immunization of sheep with the 4 peptides combined elicited antibody which reacted with each peptide region. However, these antibody titres were significantly lower than the corresponding titres for sheep immunized with peptides 6, 12/13, 21/22 or 24 alone. In sheep immunized with the combined peptides, the immunogenicity of peptides 6, 12/13 and 21/22 were similar. Peptide 24 was less immunogenic than the other 3 peptides examined in this study. In contrast to peptides 6, 12/13 and 21/22, peptide 24 has a low hydrophilic index (Mac Vector™, International Biotechnologies) and low probability of being on the surface of the native antigen which are characteristics often associated with peptides of low immunogenicity (Hopp & Woods, 1981; Parker, Guo & Hodges, 1986; Vuento *et al*. 1993; Van Regenmortel & Pellequer, 1994).

The eluate from anti-EG95 antisera incubated with a control affinity matrix did not induce lysis of oncospheres *in vitro*. This indicated that the lysis observed when the peptides were used for affinity purification was not caused by non-specific reactivity of the anti-EG95 antibodies with the affinity purification matrix. The unbound fraction of the affinity purifications against peptide contained no detectable reactivity to the homologous peptide suggesting that these affinity purifications were highly efficient. However, the affinity-purified antibodies were of relatively low titre and, although these eluates were able to induce oncosphere lysis, the antibody titre in ELISA did not correlate directly with the ability of the antibodies to cause lysis of the oncospheres.

The ability of these peptides to affinity purify a specificity of antibody that is lethal to oncospheres and their inability to elicit the same specificity when used as an immunogen, appears contradictory. Yang *et al*. (1998) described analogous results with synthetic peptides derived from a recombinant protein against *Haemophilus influenzae* infection. Differences in the affinity or specificity of the anti-peptide antibodies compared with the anti-EG95 antibodies could account for the failure of the peptides to elicit protective immune responses (Steward *et al*. 1991). Peptides are highly flexible and can adopt a variety of conformations in solution (Marshall, Beusen & Nikiforovich, 1995). In addition, the sequence of each of the EG95 peptides contains a non-protected cysteine residue, allowing dimerization of the peptides by intermolecular disulphide bonds to occur and the formation of peptide–peptide complexes. It is possible that the specificities of antibody elicited by these peptides are different to those induced by the corresponding regions of the recombinant protein due to conformational restriction.

Inhibition assays in ELISA and in *in vitro* oncosphere culture found that the 4 peptides had little or no impact on the reactivity of anti-EG95 antibody with EG95 or on anti-EG95 antibody mediated oncosphere lysis. When antibody raised against the EG95 recombinant protein was assayed against peptides 6, 12/13, 21/22 and 24 and expressed as a percentage of the titre measured using EG95 in ELISA, the proportions for each peptide were 0·11, 0·85, 2·84 and 0·08, respectively. Clearly, most of the antibody response to EG95 is directed against determinants other than those represented on the linear peptides investigated here. Other workers have also found that the titre of antibody raised against peptide antigens may not be reflected in the relative immunogenicity of the same peptide region in sera raised against recombinant proteins (Green *et al*. 1982; Berzins *et al*. 1986; Van Regenmortel, 1989). In the case of anti-EG95 antibody reacting with EG95, it seems that the majority of the antibody response is directed against conformational determinants because the peptides used in these studies contained the immunodominant linear epitopes recognized by antisera raised to the EG95 protein (Woollard *et al*. 1998).

The failure of these peptides to inhibit anti-EG95 antibody mediated oncosphere lysis indicates that there are protective anti-EG95 antibody specificities that do not react with these peptides. The relationship between epitopes expressed as these peptides and the host-protective efficacy of the EG95 vaccine will be further investigated by examination of differences between the fine specificities raised by vaccination with the peptides and recombinant protein, respectively.

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REFERENCES

BEACHEY, E. H. & SEYER, J. M. (1986). Protective and nonprotective epitopes of chemically synthesized peptides of the NH2-terminal region of type 6

streptococcal M protein. *Journal of Immunology* **136**, 2287–2292.

BERZINS, K., PERLMANN, H., WAHLIN, B., CARLSSON, J., WAHLGREN, M., UDOMSANGPETCH, R., BJORKMAN, A., PATARROYO, M. E. & PERLMANN, P. (1986). Rabbit and human antibodies to a repeated amino acid sequence of a *Plasmodium falciparum* antigen, Pf 155, react with the native protein and inhibit merozoite invasion. *Proceedings of the National Academy of Sciences, USA* **83**, 1065–1069.

BROWN, F. J. (1990). The potential of peptides as vaccines. *Seminars in Virology* **1**, 67–74.

DALE, J. B & BEACHEY, E. H. (1986). Localization of protective epitopes of the amino terminus of type 5 streptococcal M protein. *Journal of Experimental Medicine* **163**, 1191–1202.

GREEN, N., ALEXANDER, H., OLSON, A., ALEXANDER, S., SHINNICK, T. M., SUTCLIFFE, J. G. & LERNER, R. A. (1982). Immunogenic structure of the influenza virus hemagglutinin. *Cell* **28**, 477–487.

GROSFELD, H., VELAN, B., LEITNER, M., LUSTIG, S., LACHI, B. E., COHEN, S. & SHAFFERMAN, A. (1991). Delineation of protective epitopes on the E2-envelope glycoprotein of Semliki Forest virus. *Vaccine* **9**, 451–456.

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. & LAWRENCE, S. B. (1996). Antigenic polypeptides of *Echinococcus granulosus* oncospheres and definition of protective molecules. *Parasite Immunology* **18**, 347–357.

HOPP, T. P. & WOODS, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proceedings of the National Academy of Sciences, USA* **78**, 3824–3828.

LEE, A. C. J., POWELL, J. E., TREGEAR, G. W., NIALL, H. D. & STEVENS, V. C. (1980). A method for preparing β-hCG COOH peptide-carrier conjugates of predictable composition. *Molecular Immunology* **17**, 749–756.

LIGHTOWLERS, M. W., LAWRENCE, S. B., GAUCI, C.G., YOUNG, J., RALSTON, M., MAAS, D. & HEATH, D. D. (1996). Vaccination against hydatidosis using a defined recombinant antigen. *Parasite Immunology* **18**, 457–462.

LIGHTOWLERS, M. W., JENSEN, O., FERNANDEZ, E., IRIARTE, J. A., WOOLLARD, D.J., GAUCI, C. G., JENKINS, D. J. & HEATH, D. D. (1999). Vaccination trials in Australia and Argentina confirm the effectiveness of the EG95 hydatid vaccine in sheep. *International Journal for Parasitology* **29**, 531–534.

MAINA, C. V., RIGGS, P. D., GRANDEA, A. G. I., SLATKO, B. E., MORAN, L. S., TAGLIAMONTE, J. A., MCREYNOLDS, L. A. & GUAN, C. (1988). A vector to express and purify foreign proteins in *Escherichia coli* by fusion to, and separation from, maltose binding protein. *Gene* **74**, 365–373.

MARSHALL, G. R., BEUSEN, D. D. & NIKIFOROVICH, G. V. (1995). Peptide conformation: stability and dynamics. In *Peptides: Synthesis, Structures and Applications* (ed. Gutte, B.), pp. 193–245. Academic Press, San Diego.

MELOEN, R. H. (1997). Synthetic peptide vaccines. In *Veterinary Vaccinology* (ed. Pastoret, P. -P., Blancou, J., Vannier, P. & Verschueren, C.), pp. 257–308. Elsevier, Netherlands.

PARKER, J. M., GUO, D. & HODGES, R. S. (1986). New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. *Biochemistry* **25**, 5425–5432.

ROITT, I. M. (1989). Basic concepts and new aspects of vaccine development. *Parasitology* **98** (Suppl.), S7–S12.

SAUL, A. J. & GEYSEN, H. M. (1990). Identification of peptides through peptide technology. In *New Generation Vaccines* (ed. Woodrow, G. C. & Levine, M. M.), pp. 117–126. Marcel Dekker, New York.

SCHMIDT, M. A., O'HANLEY, P., LARK, D. & SCHOOLNIK, G. K. (1988). Synthetic peptides corresponding to protective epitopes of *Escherichia coli* digalactoside-binding pilin prevent infection in a murine pyelonephritis model. *Proceedings of the National Academy of Sciences, USA* **85**, 1247–1251.

SMITH, D. B. & JOHNSON, K. S. (1988). Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67**, 31–40.

STEWARD, M. W., STANLEY, C. M., DIMARCHI, R., MULCAHY, G. & DOEL, T. R. (1991). High-affinity antibody induced by immunization with a synthetic peptide is associated with protection of cattle against foot-and-mouth disease. *Immunology* **72**, 99–103.

VALERIO, R. M., BRAY, A. M., CAMPBELL, R. A., DIPASQUALE, A., MARGELLIS, C., RODDA, S. J., GEYSEN, H. M. & MAEJI, N. J. (1993). Multipin peptide synthesis at the micromole scale using 2-hydroxyethyl methacrylate grafted polyethylene supports. *International Journal of Peptide and Protein Research* **42**, 1–9.

VAN REGENMORTEL, M. H. (1989). Structural and functional approaches to the study of protein antigenicity. *Immunology Today* **10**, 266–272.

VAN REGENMORTEL, M. & PELLEQUER, J. (1994). Predicting antigenic determinants in proteins: looking for unidimensional solutions to a three-dimensional problem? *Peptide Research* **7**, 224–228.

VUENTO, M., PAANANEN, K., VIHINEN-RANTA, M. & KURPPA, A. (1993). Characterization of antigenic epitopes of potato virus Y. *Biochimica et Biophysica Acta* **1162**, 155–160.

WOOLLARD, D. J., GAUCI, C. G., HEATH, D. D. & LIGHTOWLERS, M. W. (1998). Epitope specificities and antibody responses to the EG95 hydatid vaccine. *Parasite Immunology* **20**, 535–540.

WOOLLARD, D. J., GAUCI, C. G. & LIGHTOWLERS, M. W. (1999). Synthetic peptides induce antibody against a host-protective antigen of *Echinococcus granulosus*. *Vaccine* **18**, 785–794.

YANG, Y. P., THOMAS, W. R., CHONG, P., LOOSMORE, S. M. & KLEIN, M. H. (1998). A 20-kilodalton N-terminal fragment of the D15 protein contains a protective epitope(s) against *Haemophilus influenzae* type a and type b. *Infection and Immunity* **66**, 3349–3354.