VACCINATION AGAINST HYDATIDOSIS: ANTICIPATING THE POTENTIAL FOR ANTIGENIC VARIATION

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Abstract. A vaccine based on the recombinant EG95 antigen has been found to be effective in protecting intermediate hosts against infection with hydatid cysts caused by *Echinococcus granulosus*. The vaccine typically induces more than 90% protection in both experimental challenge trials and field trials. Commercial-scale manufacturing of the vaccine has been undertaken in New Zealand as well as in China where large scale field trials are currently in progress, representing the first practical application of the vaccine. The EG95 antigen is a single recombinant protein originating from the parasite's oncosphere life cycle stage. The antigen is relatively small, being 16.5kDa, and available evidence suggests that the host protective component(s) are associated with conformational epitopes. Practical application of the vaccine has the potential to select for parasites that are antigenic variants and allow such parasites to escape the protective effects of the vaccine. While there is no evidence to date to suggest the existence of such vaccine-insusceptible variants, investigations have begun to determine the extent to which the EG95 gene varies between known genetic strains of *E. granulosus*. Complicating these investigations, DNA sequence analyses have identified EG95 as belonging to a family of at least six, closely related and expressed genes within the G1 strain parasites from which the EG95 mRNA was originally obtained. Further sequencing of the gene flanking sequences has indicated that the gene family has arisen from a relatively recent series of gene duplications involving gene segments of as yet unknown lengths but extending beyond the EG95-related sequence. Investigations to date have failed to identify sequence variation in the EG95 gene family in G1 strain parasites. However, preliminary analysis of a G6/G7 strain isolate has revealed significant EG95 sequence variation which will need to be investigated further in relation to the influence that this may have on protection afforded by the vaccine against parasites of this genotype.

INTRODUCTION

An effective vaccine has been developed to assist with the control of transmission of the medically important zoonotic parasite *Echinococcus granulosus,* the etiological agent for cystic hydatid disease (Lightowlers *et al*, 1996, 1999; Heath *et al*, 2003). The vaccine has been developed principally for use in livestock species that play an important role in the transmission of the parasite to domestic dogs and subsequently, to humans.

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Practical use of vaccines has the potential to select for genetic variants of a pathogen which differ in their antigenicity and allow the pathogen to escape vaccine-induced immune responses. While this potential is well recognized, it has not as yet had a major impact on the effectiveness of the many current vaccines against infectious diseases in humans or animals (MacLean, 1995, 1998). Nevertheless, immune selection of genetic mutants is known to occur, for example in hepatitis B virus infections in response to anti-viral immune responses and vaccine-induced immune responses (Carman *et al*, 1990, 1996, reviewed by Torresi, 2002; Locarnini *et al*, 2003) and in *Bordetella pertussis* (van der Zee *et al*, 1996). Application of selection pressure by other means also selects for variants in which the selective force is mitigated; for example, in response to insecticide use (Forrester *et al*, 1993; Chalfant, 1995; Prabhaker *et al*, 1998). Perhaps because so few vaccines have ever been developed against parasitic organisms, there are no data available about the selection of vaccine-insusceptible genetic variants. However, there is an analogous phenomenon in parasitology with the

appearance of anthelmintic resistance (Geerts and Gryseels, 2000).

The potential impact of vaccine-escape mutants is being considered increasingly in the development of new vaccines (for example, see Kaslow, 1997; Magiafoglou *et al*, 2003). The EG95 hydatid vaccine is being used in large-scale field trials in China (Heath *et al*, 2003), preceding what is expected to be the first practical application of a non-living vaccine to prevent infection with a helminth or protozoan parasite. This being the case, it is timely to consider what information is available concerning the potential for the EG95 vaccine to select for vaccine-escape mutants and what strategies might be used to mitigate against the impact of the appearance of such mutants.

ANTIGENIC COMPLEXITY OF THE EG95 ANTIGEN

The probability of a vaccine selecting for pathogens capable of escaping the vaccine-induced immune responses could be expected to be influenced by the complexity of the vaccine antigen(s). The greater the number of host-protective epitopes, the lower the probability that a genetic variant would be selected which was resistant to the vaccine because changes had rendered it insusceptible for all protective epitopes. In the case of the hydatid vaccine, the EG95 antigen is a single, relatively small recombinant protein of about 16.5kDa (Lightowlers *et al*, 1996). Detailed investigations have been undertaken to determine the epitope complexity of the protein (Woollard *et al*, 1998, 1999, 2000a,b). Sheep immunized with the vaccine produce specific antibodies that react with numerous linear peptide epitopes on the EG95 polypeptide. However, despite these antibodies reacting prominently with the corresponding parasite-derived protein in Western blots, none of these epitopes appears to be associated with the host-protective responses (Woollard *et al*, 2000a,b). Strong evidence has been obtained to indicate that the host-protective epitope(s) are immunodominant and are associated with the tertiary conformational structure of the polypeptide (Woollard *et al*, 2000b). No information is available to indicate whether there is one or more host-protective epitope. These data would suggest that EG95 may be very simple antigenically in relation to the protective immune responses induced by the vaccine. On-going investigations using phage-displayed peptide mimotopes corresponding to EG95 epitopes (Lightowlers *et al*, 2003) may shed light on the nature and number of host-protective conformational epitopes.

GENETIC VARIABILITY IN EG95: DEFINITION OF A GENE FAMILY

In anticipation of the potential importance of genetic diversity in *E. granulosus* with respect to EG95 and its long-term effectiveness as a vaccine, investigations were initiated to determine the level of existing genetic variability in the parasite in relation to this protein. The intended strategy was to design PCR primers specific for EG95 for use either in RTPCR with parasite mRNA or in PCR with genomic DNA to amplify the gene from individual parasite isolates. However, initial investigations of the *E. granulosus* genome in Southern blots probed with EG95 cDNA revealed that the gene was a member of a family of closely related genes. This gene family was characterized extensively in the parasite isolate from which the original EG95 mRNA was originally cloned (corresponding to the G1 or common sheep strain). EG95 was revealed to belong to a family of (at least) seven genes, six expressing proteins closely related to EG95 and one pseudogene (Chow *et al*, 2001). Gene structure was revealed to be highly conserved for all members of the gene family (Fig 1), suggesting that the family had arisen by gene duplication events relatively recently in the parasite's evolution.

Sequence analyses of the EG95 gene family have indicated that four of the *eg95* gene family members, designated *eg95-1* to *eg95-4,* express an identical protein (Table 1), and that these genes are transcribed in an identical, stage-specific pattern (Chow *et al*, 2001). The other two expressed genes, designated *eg95-5* and *eg95-6,* express proteins which are substantially more similar to each other than they are to EG95. A vaccine trial has been undertaken with the protein expressed by the *eg95-6* gene according to a schedule which reliably induces strong immunity using EG95. However, sheep vaccinated with the EG95-6 protein were not protected against a challenge infection with *E. granulosus* eggs. Antibodies raised by this protein, being closely related to EG95-1 but lacking the capacity to induce host-protective responses, may be useful in delineating the protective epitope(s) of EG95-1.

INTRA-STRAIN VARIABILITY IN EG95

Few investigations have been undertaken to date into variability in *E. granulosus* with respect to the EG95 gene family. Initial studies on different G1 strain isolates of *E. granulosus* have identified three banding patterns in Southern blots of genomic DNA probed with the *eg95-1* cDNA (Chow, *et al*, 2004; Fig 2, Panel

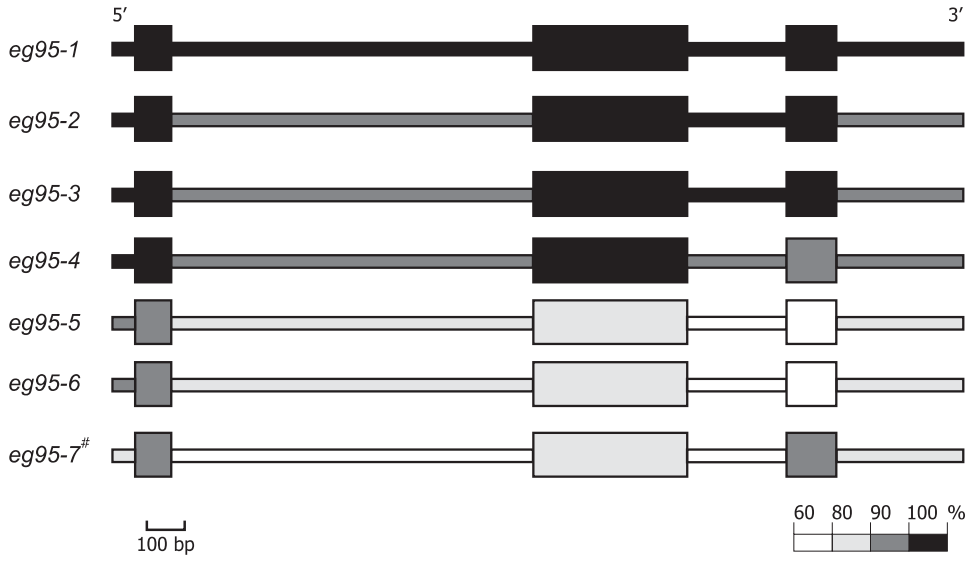

Fig 1- Diagrammatic representation of the gene structure and percent-nucleotide homology the EG95 family of genes in *E. granulosus* (after Chow *et al*, 2001, with permission). Seven genes have been characterized corresponding to each of the EG95-related gene fragments evident in Southern blots of *E. granulosus* genomic DNA probed with EG95 cDNA. Six genes were identified expressing proteins in the parasite oncosphere life cycle stage plus one pseudogene.

Table 1

Pairwise comparisons of nucleotide differences (italics) among *eg95* gene family members and the number of amino acid differences (normal font) between the predicted proteins expressed by these genes. The vaccine antigen EG95 is expressed by gene *eg95-1*. Numerical values represent the number of differences between each comparative pair. Amino acid sequence comparisons are not included for the protein encoded by *eg95-7* because it is believed to be a pseudogene.(GenBank® accession numbers: AFl34378, AFl99347, AFl99348, AFl99349, AFl99350, AFl99351, AFl99352, AFl99353, and AFl99354; Chow *et al*, 2001).

	eg95-1	*eg95-2*	*eg95-3*	*eg95-4*	*eg95-5*	*eg95-6*	*eg95-7*
eg95-1		*11*	*13*	*20*	*282*	*285*	*338*
eg95-2	0		*14*	*17*	*281*	*284*	*341*
eg95-3	0	0		*17*	*281*	*283*	*338*
eg95-4	0	0	0		*281*	*283*	*339*
eg95-5	39	39	39	39		*14*	*254*
eg95-6	38	38	38	38	2		*259*
eg95-7	-	-	-	-	-	-	

I). The apparent absence of particular DNA fragments corresponding to the six characterized genes comprising the *eg95* gene family suggests the possible absence of the genes associated with these fragments. However, homology between these gene family members extends into flanking sequences such that the positions of up and down-stream restriction sites are largely conserved. Hence, Chow *et al* (2001) observed conservation of a particular *Eco*RI restriction site downstream in seven members of the gene family and conservation of an upstream restriction site in three members, leading to these three genes having an identical DNA fragment size in Southern blots with *Eco*RI digested genomic DNA. The extent of conservation in the flanking sequences is unknown at this time, however, some investigations have been

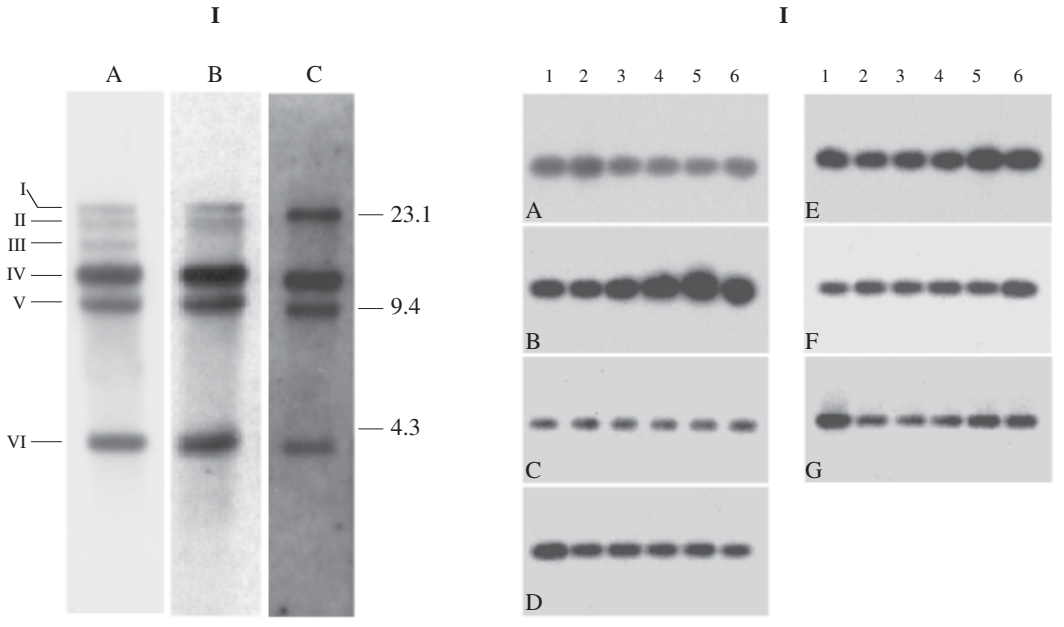

Fig 2- Analyses of eg95 gene family members representing different *E. granulosus* G1 strain isolates (Chow, unpublished observations). Panel I. Southern blot hybridization analysis of genomic DNA restriction digested with *Eco*RI and probed with *eg95-1* cDNA; lane A genomic DNA pooled from adult worms of a New Zealand *E. granulosus* isolate; lane B genomic DNA from protoscoleces obtained from an individual hydatid cyst from a naturally infected Australian sheep; lane C genomic DNA from protoscoleces derived from an individual hydatid cyst collected from a naturally infected sheep in Australia but from a different property to that from which the sample used in lane B was obtained. The six bands showing homology to *eg*95 cDNA are marked on the left and numbered I – VI. Positions of molecular size markers (kb) are denoted on the right. Panel II. PCR products obtained with gene-specific primers representing the seven members of the *eg95* gene family: A; *eg*95-1, B; *eg*95-2, C; *eg*95-3, D; *eg*95-4, E; *eg*95-5, F; *eg*95-6, G; *eg*95-7, in reactions with genomic DNA from different individual hydatid cysts presenting Southern blot hybridization patterns as shown in Panel I lane B (lanes 1 to 3) or presenting Southern blot hybridization patterns as shown in Panel I lane C (lanes 4 to 6), PCR reaction products indicated that, despite the variations seen in Southern blot hybridization patterns among these different isolates, all of the parasites investigated here contained each of the seven known *eg95* gene family members.

undertaken and have confirmed high levels of sequence conservation in some of the family members in the sequence 3´ to the *eg95* gene. Sequence extending 5´ from the *eg95* gene show high levels of homology between the genes *eg95-1,2,3* and *4,* but a lower level of homology between these genes and the sequence upstream from *eg95-5* or *6.* Particularly in relation to the genes *eg95-1,2,3* and *4,* it might be predicted that even if the sequence variation between different *E. granulosus* isolates were only minimal, if the variation affected an up- or down-stream *Eco*RI site, this would most likely lead to the fragment changing position in Southern blots to one corresponding to, rather than being different from, one of the other bands representing another gene family member. In the case of the isolates represented in Fig 2, Panel I, this does seem to be the appropriate interpretation for the differences in the Southern blot patterns because subsequent gene-specific PCR reactions identified the presence of all seven *eg95* gene family members in all of the parasites examined, irrespective of the pattern seen in Southern blots (Fig 2, panel II). With respect to the DNA sequence of the *eg95-1* gene in different G1-strain isolates of *E. granulosus,* no sequence variation has been observed to date in parasites derived from Australia, New Zealand, Argentina, China or Kenya (Chow, unpublished observations).

Zhang *et al* (2003) described the results of their investigations of the genetic heterogeneity of the *eg95* gene family in *E. granulosus* from the Xinjiang region in northwest China where hydatid disease is hyperendemic. They identified a number of PCR

products in which the gene sequence varied slightly from that of *eg95-1* and concluded that a high degree of sequence conservation predicts that the vaccine will continue to be effective in China and elsewhere. However, it is difficult to interpret the data obtained by these authors. The high level of conservation in the DNA sequence between each of the seven members of the *eg95* gene family necessitates careful selection of PCR primers in order to be confident that the products obtained are from the specific gene of interest. The primers used by Zhang *et al* (2003) would be predicted to amplify products form all seven of the known *eg95* gene family members. In addition, PCR amplification of templates containing more than one, closely related, target sequence is well known to induce chimeric or recombination product artefacts arising from incompletely copied DNA products acting as primers together with a similar, but not identical, complimentary template (Wang and Wang, 1996; Judo *et al*, 1998; Zylstra *et al*, 1998).

INTER-STRAIN VARIABILITY

E. granulosus is a genetically heterogenous species in which different "strains" of parasite have been recognized for decades as showing different host preferences, morphology, and other characteristics (Thompson and McManus, 2002). The most important genotype, known as the common sheep strain or G1 genotype is responsible for most human infections but there is substantial evidence to indicate that other strains also infect humans. In addition, the host specificities for the various genotypes may not be strictly defined, with sheep being infected with a variety of genotypes and other host species being infected with the G1 parasites. Hence, control of hydatid disease transmission for the purpose of reducing the number of human infections would ideally prevent transmission of all *E. granulosus* genotypes, or at least all of those infecting humans. To date, protection trials have been carried out only against the G1 genotype parasites and it is not known yet whether the vaccine, as it is currently constituted, would be effective in protecting against other recognized genotypes. Investigations have begun into the variability in the *eg95* gene family among parasites belonging to different recognized parasite strains, but these investigations are only in their early stages. Studies have begun only with G6 and G7 strain parasites, however, these preliminary investigations have indicated the existence of an *eg95-1* gene which varies with respect to both nucleotide and predicted amino acid sequence in comparison to *eg95-1* from G1 parasites (Chow, unpublished observations).

IMPLICATIONS FOR VACCINATION

E. granulosus provides an intriguing case for the investigation of the impact of genetic variation in a pathogen in relation to its effects on susceptibility to vaccine-induced immune responses. The available evidence indicates that the EG95 vaccine has its host-protective effect through complement-mediated lysis of the oncosphere or early post-oncosphere metacestode (Lightowlers *et al*, 2000; Woollard *et al*, 2000b; Lightowlers and Gauci, 2001; Heath *et al*, 2003). All of the six expressed *eg95* gene family members are expressed in the oncosphere (Chow *et al*, 2004). While it is the product of *eg95-1* that comprises the vaccine antigen, each of the genes *eg95-2,-3* and *-4* express an identical protein in the oncosphere. Hence, it is anticipated that the EG95 vaccine targets the protein product of (at least) four genes. There are 38 and 39 amino acid differences between the products of *eg95-5* and *eg95-6,* respectively, and *eg95-1,* providing substantial opportunity for differences in antigenicity between these protein groups. It is not known whether immune responses raised against the EG95-1 protein cross react with EG95-5 or EG95-6, however, the available evidence (referred to above) indicates that vaccination with EG95-6 does not induce a protective immune response.

What would be the consequences if an *E. granulosus* isolate were to have a variation in its *eg95-1* gene such that this affected a critical epitope, altering the protein product such that it was not recognized by vaccine-induced immune responses? Possibly, an infection by such parasites may not be prevented by the vaccine. On the other hand, these parasites may continue to express products from *eg95-2, -3* and/or *-4* that remain the same as the vaccine protein. Perhaps the presence in the parasite of protein from any one of these genes which remains immunologically recognized by the protective antibodies induced by EG95-1 vaccination may render the parasite susceptible to the vaccine-induced responses. It could be possible in future to test these hypotheses by effecting gene-specific alterations in the parasite using *in vitro* transfection technology; however at present these techniques are yet to be developed for use in cestodes. Alternatively, identification of genetic variants among the parasite population may provide experiments of nature that will provide some relevant data.

To this time, there is no evidence to suggest that genetic variation affecting the EG95 protein is likely to have a significant impact on the effectiveness of the vaccine in the field. Experimental and/or field trials of the vaccine carried out in New Zealand, Australia,

Argentina, and China with sheep, goats, cattle, and yak have all demonstrated a high level of protection against parasite challenge (Lightowlers *et al*, 1996, 1999; Woollard *et al*, 2000a,b; Heath *et al*, 2003). Nevertheless, continued research into the level of genetic variability that exists in the *eg95* genes, and the development of tools to allow gene-specific investigations of the gene family members in different *E. granulosus* strains and isolates, will assist with investigation of the cause of any vaccine failures, should they occur in future. In addition, this information will assist with developing strategies by which the vaccine could be modified to overcome any problem arising due to antigenic diversity.

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