Induction of protective T-helper 1 immune responses against *Echinococcus granulosus* in mice by a multi-T-cell epitope antigen based on five proteins

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In this study, we designed an experiment to predict a potential immunodominant T-cell epitope and evaluate the protectivity of this antigen in immunised mice. The T-cell epitopes of the candidate proteins (EgGST, EgA31, Eg95, EgTrp and P14-3-3) were detected using available web-based databases. The synthesised DNA was subcloned into the pET41a+ vector and expressed in Escherichia coli *as a fusion to glutathione-S-transferase protein (GST). The resulting chimeric protein was then purified by affinity chromatography. Twenty female C57BL/6 mice were immunised with the antigen emulsified in Freund's adjuvant. Mouse splenocytes were then cultured in Dulbecco's Modified Eagle's Medium in the presence of the antigen. The production of interferon-γ was significantly higher in the immunised mice than in the control mice (> 1,300 pg/mL), but interleukin (IL)-10 and IL-4 production was not statistically different between the two groups. In a challenge study in which mice were infected with 500 live protoscolices, a high protectivity level (99.6%) was demonstrated in immunised BALB/C mice compared to the findings in the control groups [GST and adjuvant (Adj)]. These results demonstrate the successful application of the predicted T-cell epitope in designing a vaccine against* Echinococcus granulosus *in a mouse model.*

Key words: multi-epitope - vaccine - EgGST - Eg95 - EgA31 - EgTrp

Echinococcus granulosus is the causative agent of hydatidosis, one of the most prevalent helminthic diseases in humans. *E. granulosus* is a cestode that requires two hosts to complete its life cycle. A wide spectrum of mammals, including sheep and humans, can serve as intermediate hosts for the parasite, as its larvae can create hydatid cysts in these hosts. The adult form of these worms develops in the small intestine of animals belonging to the family Canidae. Several reports regarding the economic losses and health problems caused by this zoonosis have been published (Thompson 1995). Accordingly, many attempts have been made to design a vaccine against *E. granulosus*.

For this purpose, various recombinant proteins have been expressed and analysed as immunological indicators or vaccine candidates. EgA31, which belongs to the paramyosin family and EgTrp, which shares high similarity with the tropomyosin protein, have been evaluated as two protective antigens in dogs (Fu et al. 1999). These antigens are particularly interesting because both are expressed at the protoscolex stage (Fu et al. 1999). In a previous study, Eg95 induced a significant immune response in sheep (Woollard et al. 2000). Other reports discussed the roles of P14-3-3 (Siles-Lucas et al. 2008), recombinant myophilin (Sun et al. 2011) and phosphoglucose isomerase as protective antigens. However, none of these proteins has been found to induce relevant protectivity against *E. granulosus* in its definitive host. The present study was undertaken to design a chimeric protein consisting of a multi-epitope antigen based on the five major antigens of *E. granulosus*: EgA31, EgTrp, EgGST, Eg95 and P14-3-3. This chimeric protein was designated ChMEA.

MATERIALS AND METHODS

T-cell epitope prediction and construction of the chimeric protein - The sequences of the EgTrp, EgA31, P14-3-3 and EgGST antigens were analysed by the Propred and IEDB servers and potential T-cell epitopes were predicted. The server helped to identify allele-specific binding regions in the candidate antigens. Eighteen peptides [14 peptides from the EgTrp, EgGST, EgA31 and P14-3-3 proteins and an additional 4 linear epitopes from the EG95 protein that had been previously mapped (Woollard et al. 1998)] were combined for the construction of the synthetic DNA. The 15 amino acids (EAAAKEAAAKEAAAK) between the glutathione-S-transferase protein (GST) tag of the pET41a+ expression vector and the chimeric antigen were regarded as a linker. The predicted peptides were assembled with a furin linker (RVKR) between the two epitopes. A partial HIV Tat sequence (AAARKKRRQRRR) was added as a trans-membrane domain in the C-terminal region of the protein. All of the rare

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codons of the multi-epitope DNA were optimised according to the *Escherichia coli* codon usage table available in the National Centre for Biotechnology Information database. The synthetic gene was 2,112 base pairs in length and it was subcloned into the *Eco*RI and *Xho*I restriction sites of the pET41a+ expression vector.

Expression of chimeric antigen - The pET41-ChMEA plasmid encoding a fusion protein of 704 amino acids (81 kDa) was transformed into *E. coli* strain BL21 (DE3). A single colony was transferred into 5 mL of 2YT medium and the culture was then incubated on a platform shaker at 37ºC until an optical density of 0.8 was reached. The chimeric protein was induced by incubation with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 22ºC.

Western blotting analysis - Expression of the chimeric protein was visualised by Western blotting using a goat polyclonal anti-GST antibody conjugated to horseradish peroxidase (HRP) (GE Healthcare Life Sciences). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% polyacrylamide gel. The proteins were transferred onto a polyvinylidene difluoride membrane, which was incubated for 2 h in blocking buffer (1% bovine serum albumin). The membrane was incubated with the HRP-conjugated anti-GST antibody at a dilution of 1:5,000 for 1 h and then washed twice with phosphate-buffered saline (PBS)/Tween. The blots were developed using 4CN and H_2O_2.

Purification of the chimeric protein - Following IPTG induction of the bacterial culture, the cell pellet was resuspended in binding buffer (1X PBS) and the cells were disrupted by six cycles of sonication, with each cycle consisting of 1 min of sonication followed by 1 min of no sonication. Phenylmethanesulfonyl fluoride was then added to a final concentration of 1 mM. Following treatment with 2% Triton X-100 for 1 h on ice, the suspension was centrifuged at 10,000 *g* for 20 min. Then, a 500-µL sample of 50% glutathione beads was added to the supernatant, which was subsequently incubated for 2 h at 4ºC. The glutathione beads were then washed twice with 2 mL of binding buffer. Finally, the fusion protein was eluted by the addition of 50 mM reduced glutathione.

Enzyme assay using 1-chloro-2, 4-dinitrobenzene (CDNB) - The chimeric protein (GST-ChMEA) was detected by assessing its enzymatic activity via incubation with the substrate CDNB. The GST-mediated reaction of CDNB with glutathione produces a conjugate that was measured by the absorbance at 340 nm using an Ultrospec 2000 UV-visible spectrophotometer (Pharmacia Biotech). The absorbance was measured at 340 nm at 1 min intervals for 10 min by first blanking the spectrophotometer.

Immunisation of C57BL/6 and BALB/C mice - Trial I: 20 female C57BL/6 mice (7-8 weeks of age) (provided by the Experimental Animal Centre at the Razi Vaccine and Serum Research Institute, Iran) were immunised by subcutaneous injection with 50 µg of antigen emulsified in Freund's complete/incomplete adjuvant in the three sessions every second week (day 0, day 14, day 28). Trial II: this trial included female BALB/C mice (7-8 weeks of age, provided by the same source as mentioned in Trial I) that were divided into four groups of five each including one main group (GST-ChMEA) and three control groups [PBS, adjuvant (Adj) and GST]. The PBS group was injected with phosphate buffer only. The Adj group of mice was subcutaneously injected with PBS + Freund's incomplete adjuvant, the GST group was injected with 50 µg of GST and the GST-ChMEA group was immunised with 50 µg of GST-ChMEA. All antigens were injected subcutaneously three times after being emulsified in Freund's incomplete adjuvant at 14-day intervals.

The national guideline of the Razi Institute was followed throughout the experiments that involved laboratory animals by minimising the suffering to which the animals were exposed.

Evaluation of interleukin (IL)-4, IL-10 and interferon (IFN)-γ - Two weeks after the last immunisation, splenocytes from the C57BL/6 mice were cultured in a 24-well plate containing 1.5×10^6 cells/well in the presence of Dulbecco's Modified Eagle's Medium supplemented with 10% foetal calf serum and a penicillin/streptomycin mixture. Fifteen micrograms of the recombinant protein was used to stimulate the splenocytes. Seventy-two hours after stimulation, supernatants were collected and used for cytokine analysis. IL-4, IL-10 and IFN-γ production was measured with an ELISA kit (Quantikine R&D) using an ELISA Reader (BioRad model 680). Each assay was performed in duplicate. The endotoxicity of the purified protein was evaluated by the Limulus amoebocyte lysate assay (Pyrotell Associates, Cape Cod, USA) and rabbit pyrogen tests before cultivation in all of the relevant experiments.

Challenge with protoscolices - In this experiment, 15 immunised BALB/C mice from Trial II were used. Two weeks after the final immunisation, five mice from each group were challenged with live protoscolices (*E. granulosus,* G1 genotype). The viability of the protoscolices was assessed by the eosin (0.1%) staining method. Immunised and control mice were injected intraperitoneally with 500 viable protoscolices suspended in RPMI at a final volume of 500 µL/mice. Three months post-challenge, the mice were examined for the presence of hydatid cysts. The level of protection in mice was determined according to following method: (1 - average number of cysts observed in the test group/average of cysts observed in the control group) × 100 = percentage of protectivity in immunised mice (Dempster et al. 1995).

Statistical analysis - Data obtained from the cytokine assays were analysed by Student's *t* test and $p < 0.05$ was considered statistically significant. SPSS software (version 11.5) was used for statistical analysis.

RESULTS

Eighteen antigenic peptides with appropriate accessibility, hydrophilicity and antigenic propensity were selected from the EgTrp, Eg95, EgGST, EgA31 and P14-3-3 proteins. The peptides with the greatest predicted binding affinity are shown in Table I. The chimeric protein

TABLE I

List of 18 peptides selected from five proteins

Gene	Peptide sequence	Position	Length
EgA31	**V**LLSK**I**KSLEKTAK	87-100	14
EgA31	**M**RIMALEAENERLRISAAEKQ	153-173	21
EgA31	**L**VGADNSKTT**V**QS**I**RNE**M**RG**I**QV**V**QIQL**L**RGGYLDL	242-275	35
EgA31	**L**RKASIQKQK**L**ELKDTIIS	459-488	23
EgTrp	**M**ND**W**LSK**V**KN**I**QTEVDTVQESLQEAISK	43-70	32
EgTrp	**L**R**I**VGNN**M**KS**L**EVSEQES**L**QREESYEE	197-223	31
EgTrp	**LEE**QVKEAKYIA	144-155	12
EgTrp	AERQVSKLQNE	242-252	11
Eg95	TETP**L**RKHFNLTPV	29-42	14
Eg95	SLKA**V**NPSDPLV**Y**KRQTAKF	65-84	20
Eg95	DIETPRAGKKESTVMTSGSA	119-136	20
Eg95	SALTSAIAGFVFS	139-151	13
EgGST	**L**EK**Y**PRLKAYLS	175-185	12
EgGST	**I**DGD**F**KLTQSGAI	64-76	13
EgGST	**L**AY**W**DIRGLAEQ	5-16	12
P14-3-3	**M**AK**M**RNE**L**NNEEANLL	39-54	16
P14-3-3	L**I**QICND**V**LALPVLPI	99-114	16
P14-3-3	**IF**Y**Y**KM**M**GD**Y**YRYSAEVQ	124-141	18

in these peptides, 32 epitopes were identified. Bold residues indicate the primary amino acid of the major histocompatibility complex II epitopes.

was successfully expressed in *E. coli* strain BL21 (DE3) and purified by affinity chromatography using Glutathione Sepharose 4B beads (Fig. 1).

Fourteen days post-immunisation, the sizes of the spleen and mesenteric lymph nodes in the immunised and control mice were measured using a micrometer. As shown in Fig. 2, the spleen and mesenteric lymph nodes from the mice immunised with GST-ChMEA were significantly larger than those from the control mice.

Cytokine assay - Cytokine levels were evaluated by ELISA. Significantly greater IFN-γ production was observed in mice immunised with the chimeric protein (12.8 ng/mL) (Fig. 3A), but IL-10 and IL-4 levels were not statistically different between the immunised and control adjuvant groups (Fig. 3B, C). In this study, the chimeric protein was found to be highly effective at stimulating T-helper 1 (Th1) responses and cell-mediated immunity in C57Bl/6 mice.

Protective immunity in mice - All of the challenged mice (5 in each group) were euthanised after three months and their internal organs were examined for the presence of hydatid cysts. The number of cysts in the control group was ~50 (in the liver, spleen and kidneys) and the average size of the hydatid cysts was 5 mm. Approximately 99.6% of mice immunised mice with GST-ChMEA + Adj were found to be devoid of cysts (Table II). Examination of the control groups (Adj + PBS and GST + Adj) uncovered obvious parasitic lesions on the surface of the liver, spleen and kidneys in the mice, whereas in the test group (GST-ChMEA), only a single lesion in one mouse was identified. Based on the number of visible cysts, we conclude that the chimeric antigen induced a very high level of protection (99.6-100%) in BALB/C mice (Table II).

DISCUSSION

Over the last two decades, several studies have been conducted to design vaccines against hydatidosis. In these studies, several antigens have been characterised. A potent vaccine based on Eg95 displayed high protectivity in sheep against hydatidosis, but characterisation of the Eg95 gene family members in different strains/isolates of *E. granulosus* revealed substantial nucleotide substitutions encoding amino acid substitutions (Chow et al. 2008) that may influence protein folding (Haag et al. 2009). Another study demonstrated that the Eg95 proteins from a different genotype of *E. granulosus* were unable to bind all of the antibodies raised by animals vaccinated with Eg95-G1 (Alvarez Rojas et al. 2013). Hence, the protectivity of Eg95 in other hosts remains unclear. These observations suggest that Eg95 is not efficient at protecting against different genotypes and isolates of *E. granulosus*. Other *E. granulosus* antigens such as EgA31, EgTrp, EgGST and P14-3-3 have also been found to induce low protectivity against hydatidosis (Fu et al. 1999, Siles-Lucas et al. 2008). Furthermore, linear epitopes from Eg95 that have been mapped previously (Woollard et al. 1998) do not kill the parasite during in vitro culture assays, nor do the peptides induce protection against the challenge infection alone (Woollard et al. 2000). Therefore, the present study considered five antigens (EgA31,

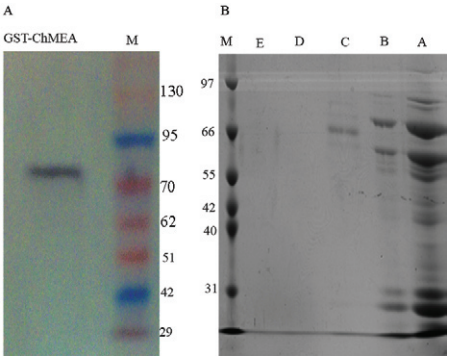

Fig. 1A: western blotting of 81 KDa of chimeric protein by horseradish peroxidase-conjugated anti-glutathione-S-transferase protein (GST) [Lane M: prestained protein molecular weight marker (Vivantis, PR0602); GST-ChMEA: chimeric protein]; B: [Lane M: midrange protein molecular weight marker) Promega, USA); A: supernatant after purification; B: first washing by binding buffer; C: chimeric antigen purified by GST-beads)].

EgTrp, EgGST, P14-3-3 and Eg95) and constructed a model of the T-cell epitope vaccine against hydatidosis.

Epitope prediction and DNA construction - The identification of epitopes capable of binding to multiple major histocompatibility complex (MHC) I and MHC II classes will facilitate the development of new epitope-based vaccines (Doytchinova & Flower 2003). In this study, 18 antigenic positions were found on five candidate proteins using the Predicting Antigenic Peptides software developed by Harvard University and to gather more information about T-cell epitopes, different servers such as MHCPred and ProPred were also utilised. The peptides with the best binding affinity for each allele were investigated and the epitopes that displayed affinities exceeding 50 nM were selected. Forty-six epitopes were found in the selected peptides, which covered all of the MHC II alleles and provided 100% population coverage. Codon optimisation of the synthetic DNA was performed to improve the expression and reduce translational errors in *E. coli* due to the presence of rare codons in the *Echinococcu*s candidate proteins.

Expression and purification - To improve protein solubility, a high-density cell population was induced with a low concentration of IPTG (0.1 mM) at a low temperature (22ºC for 4 h). The expression of the chimeric GST fusion protein was evaluated by SDS-PAGE and Western blotting using an HRP-conjugated anti-GST antibody on a nitrocellulose membrane (Fig. 1). The chimeric protein was purified by affinity chromatography using Glutathione Sepharose 4B beads. The enzymatic assay was performed with CDNB to evaluate protein function. The results of this study indicate that the chimeric antigen can maintain its structure in a soluble native form after purification.

Immune response assay - The specific cytokines produced by the splenocytes isolated from the immunised C57BL/6 mice were evaluated by ELISA. IFN-γ is an important immunoregulatory cytokine (Billiau & Matthys 2009) that plays a pivotal role in host protection by exerting anti-proliferative and immunoregulatory activities. It has a wide spectrum of actions, as it induces many cells to produce different cytokines and up-regulates the expression of various membrane proteins, including the class I and II MHC antigens. IFN-γ also influences the development of the Th cell phenotype by inhibiting Th2 differentiation and stimulating Th1 development (Pestka et al. 2004). Previous studies revealed that the sheep hydatid fluid elicits both Th1 and Th2 cell activation in human cystic echinococcosis (Rigano et al. 1995a, b). The induction of a cytokine-related Th2 response leads

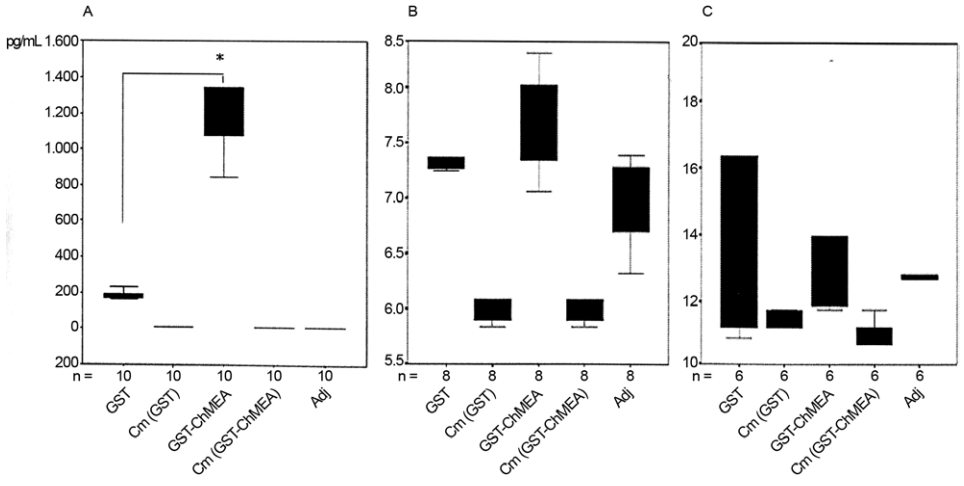

Fig. 2: production of interferon (IFN)-γ, interleukin (IL)-4 and IL-10 by splenocytes isolated from mice immunised with the chimeric protein, as tested by the sandwich ELISA kit (Quantikine R&D). A: level of IFN-γ; B: level of IL-4; C: level of IL-10; Adj: mice immunised with adjuvant; Cm [glutathione-S-transferase protein (GST)]: cytokine levels in the absence of GST; Cm (GST-ChMEA): cytokine levels in the absence of GST-ChMEA; GST: cytokine levels in the presence of GST in mice immunised with the GST protein; GST-ChMEA: cytokine levels in the presence of the GST-ChMEA in mice immunised with the GST-ChMEA protein. Box plot designed by SPSS 11.5. Asterisk means $p < 0.001$.

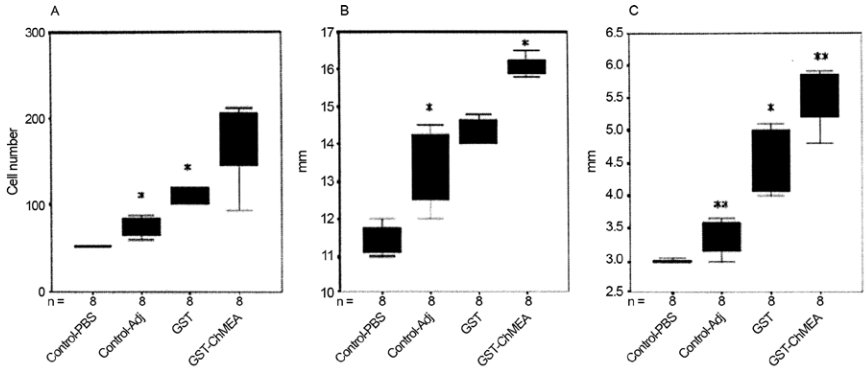

Fig. 3: immunisation of mice with glutathione-S-transferase protein (GST) induced a significant increase in spleen cell number (A) and in spleen size (B) as well as a slight increase in size of mesenteric lymph nodes (C). C-adjuvant (Adj): injected mice with adjuvant; control-phosphate-buffered saline (PBS): injected mice with phosphate buffer; GST: injected mice with GST protein alone; GST-ChMEA: injected mice with the chimeric protein; *: $p < 0.001$; **: $p < 0.006$.

TABLE II

Percentage of protectivity in immunised mice

Groups	Mice (n)	Cysts (n)	Protection levels (%)	p
PBS + Adj	5	50	-	
GST + Adj	5	43	< 14	
GST-ChMEA + Adj	5	0-1	99.6	< 0.001

Adj: adjuvant; GST: glutathione-S-transferase protein; PBS: phosphate-buffered saline.

to susceptibility to hydatid disease, but a Th1 response results in protective immunity against hydatidosis (Rigano et al. 1995a, b, Ortona et al. 2003, Mezioug & Touil-Boukoffa 2009). From an evolutionary perspective, Th2 immunity is a suitable response to metazoan invaders because it helps to control the parasites' growth and restore the damage that they cause (Díaz & Allen 2007). The Th2-type response permits the establishment of infection during secondary infections caused by the spread of protoscolices following the accidental rupture of fertile cysts (Mourglia-Ettlin et al. 2011). Previous studies demonstrated that the native antigen model is not suitable for inducing protective immunity due to an increase in IL-10 levels that results in a Th2 response to different antigens such as EgA31 and EgTrp (Fraize et al. 2005). Increased levels of IFN-γ and IL-4 have been observed in mice immunised with recombinant myophilin (Sun et al. 2011). However, these proteins have the ability to produce a mixed Th1-Th2 response and the presence of a Th2 response may render the host capable of tolerating the presence of the parasite.

In this study, the effects of an epitope-based chimeric antigen (ChMEA) on the expression of cytokines in the splenocytes of immunised mice were evaluated. Surprisingly, the production of IFN-γ was prominently higher in mice immunised with the chimeric protein (> 1,300 pg/mL) than in the control mice. Interestingly, the negligible IL-10 (< 14 pg/mL) and IL4 (< 8 pg/mL) production did not differ statistically between the immunised and control adjuvant groups. The challenge infection results indicate that the chimeric antigen is a potent inducer of the protective Th1 immune response in a mouse model of *E. granulosus* infection (Table II).

Previous studies reported that vaccines that trigger Th1 responses instead of Th2 responses are highly effective against echinococcosis. This study may mark the beginning of a new era in designing vaccines based on particular Th1 epitopes that specifically stimulate the Th1 response and protective immunity.

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