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Short communication

Schistosoma japonicum: THE DESIGN AND EXPERIMENTAL EVALUATION OF A MULTIVALENT DNA VACCINE

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Abstract: The aim of this study was to construct and evaluate the immunity efficacy of the DNA multivalent vaccine pVIVO$_2$SjFABP-23. The vaccine was constructed and produced as follows. Forty BALB/c mice were divided into four groups designated pVIVO$_2$, pVIVO$_2$Sj23, pVIVO$_2$SjFABP and pVIVO$_2$SjFABP-23. Each mouse was immunized with 100 μg of the corresponding plasmid DNA by intramuscular injection. 28 days post-vaccination, the mice were challenged with *S. japonicum* cercariae, and the worm and egg burdens were determined 42 days post-challenge. Serum samples were collected from all the mice before and after vaccination and at the end of the experiment, and used for antibody detection. The IFN-γ and IL-4 levels were quantified in the supernatants of specifically stimulated spleen cells. The number of worms was reduced by 52%, 40% and 42% in mice respectively immunized with pVIVO$_2$SjFABP-23, pVIVO$_2$Sj23 or pVIVO$_2$SjFABP. A respective 61%, 38% and 39% egg reduction was determined relative to those mice that only received the empty pVIVO$_2$ plasmid. pVIVO$_2$SjFABP-23 immunization increased IgG levels against SWAP and SEA. Increased IFN-γ levels were detected in the supernatant of specific stimulated spleen cells from mice

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Abbreviations used: FABP – fatty acid binding protein; SEA – soluble egg antigen; Sj – *Schistosoma japonicum*; SOE – splicing by overlapping extension; SWAP – soluble adult worm antigens; WHO – world health organization; 23 – 23 kDa membrane protein

immunized with the 3 different constructs. The multivalent DNA vaccine developed induced higher levels of protection than the two monovalent tested vaccines.

Key words: DNA vaccine, *Schistosoma japonicum*, Sj23, SjFABP, Protective efficacy

INTRODUCTION

Schistosomiasis is a parasitic disease with a chronic debilitating character, occurring in tropical and subtropical environments. Although a lot of effort has been made to control its impact on human beings, it remains a major public health problem in Afria, Asia and some regions of South America [1]. It ranks second only to malaria in terms of the extent of its endemic areas and the number of people infected, currently 200 million in 74 countries [2]. It is estimated that 120 million people are symptomatic and 20 million people have severe schistosomiasis [3]. The annual mortality rates in sub-Saharan Africa are predicted to be 150,000 and 130,000, respectively due to non-functioning kidneys and haematemesis, the effects of long-term heavy *S. haematobium* and *S. mansoni* infection [4]. In China, up to 1.52 million people are infected with *S. japonicum*, and about half of them have clinical symptoms. The majority of the infected people (86%) live in the warm, humid and rainy area of southern China, mainly in the five provinces of Hunan, Hubei, Jiangxi, Anhui and Jiangsu [5], where disastrous flood events frequently occur.

The control of Asian schistosomiasis has relied primarily on snail eradication and large-scale chemotherapy using the highly effective drug, praziquantel. A study demonstrated that appropriate drug treatment reduces worm burden and decreases the intensity of the infection and its prevalence. Therefore, chemotherapy is an important basis for schistosomiasis control. However, the costs of diagnosis and treatment are high. Reinfection occurs rapidly, treatment must be repeated at frequent intervals, and there is the potential for drug resistance to develop. In addition, praziquantel has a limited effect on already developed hepatosplenic lesions, thus leaving a large part of the affected population untreated. As chemotherapy alone can barely reduce the transmission of the disease, it is necessary to develop another form of control for schistosomiasis.

DNA vaccination offers many potential advantages, including cost-effective production, thermal stability and the ability to induce a wide variety of immune responses including the induction of cytotoxic T-lymphocytes [6, 7]. To date, DNA vaccination has been explored for a variety of parasites, including helminthic infection [8], and significant reductions in parasite burdens have been demonstrated in animal models of some of these infections [9, 10]. These studies suggest that the development of a safe and effective DNA vaccine is feasible.

Although a number of studies on schistosomiasis have reported success in using DNA constructs to induce encoded antigen-specific responses [11, 12], there has

been limited efficacy in inducing protective immunity against *S. japonicum*. One group [13] achieved some encouraging results using a DNA cocktail in mice. The cocktail vaccine, consisting of four DNA plasmids encoding four different *S. japonicum* antigens, Sj62, Sj28, Sj23 and Sj14-3-3, induced significant resistance against *S. japonicum* cercarial challenge in two out of three experiments. Nowadays, vaccine development remains an important long-term goal in the control of schistosomiasis.

SjFABP (fatty acid binding protein) and Sj23 (23 kDa membrane protein) have been recognized by WHO as protective antigens in hosts infected by *S. japonicum*, and are promising anti-schistosome vaccine candidates currently under investigation. It was found that the worm reduction rate in SjFABP [14] and Sj23 [15] DNA immunized mice was respectively 23.60% and 18.24%, and the egg reduction rate was 59.36% and 33.93%. In this study, these two antigens were chosen to construct a multivalent DNA vaccine, SjFABP-23, via SOE (splicing by overlapping extension) to further increase the immunity efficacy of the DNA vaccine. Its protective capacity and the immune response elicited by vaccination were determined.

MATERIALS AND METHODS

Plasmids and other materials

The plasmid used in these experiments was $pVIVO_2$/mcs (Invivogen). This plasmid was used to clone the two genes of interest separately or combined. The pGEM-T vector was from Promega. The host strain, *Escherichia coli* GT110 was maintained in our laboratory. Two $pVIVO_2$ vectors containing the Sj23- or the SjFABP-coding gene were from the Tongji Medical College of our university. The enzymes for DNA manipulation were from Promega. The PCR cocktail reagents were purchased either from Shanghai Sangon Biological Engineering Technology & Service or from Jingmei Biotech Co. Ltd. Experimental mice were obtained from the Wuhan Institute of Biological Products, China. All the chemicals used were of analytical grade.

Construction of the multivalent vaccine $pVIVO_2$SjFABP-23

Primer design

Specific primers were designed to amplify gene fragments encoding SjFABP and Sj23 using DNA GenBank sequence information [accession No. AF 000369 (SjFABP) and M63706 (Sj23)] as follows:

upstream primer of SjFABP: 5'-CGGGATCCGCCACC**ATG**GAAAATGGAAACTAA GCGAAT-3'; downstream primer of SjFABP: 5'-CGCCAT(GCTGCCGCCACCGCCG CTTCCGCCACCGCCGCTTCCACCGCCACC)CAATCGTTTGTAATTCCGAATAG-3'; upstream primer of Sj23: 5'- CGATTG(GGTGGCGGTGGAAGCGGCGGTGGCGG AAGCGGCGGTGGCGGCAGC)**ATG**GCGACTTTGGGTACTG-3'; downstream primer of Sj23: 5'-GCGAATTC**TTA**ACATTCTGATAATCATGTATTCGTT-3'.

The upstream primer of SjFABP and downstream primer of Sj23 respectively incorporated the restriction sites BamH I and EcoR I (underlined). The upstream primers of SjFABP and Sj23 contained a start codon (in bold), and the downstream primer of Sj23 contained a stop codon (in bold). In addition, a kozark sequence (GCCACC) was inserted into the upstream primer of SjFABP between the restriction site of BamH I and the start codon to enhance the expression of the gene. In order to construct the fusion gene, a linker sequence was designed containing a hydrophobic multiple peptide sequence $(Gly_4Ser)_3$ coding 15 amino acids. Both the downstream primer of SjFABP and the upstream primer of Sj23 included this linker sequence, which was complementary. The primers were synthesized by Shanghai HuaNuo Bio Technology Co. Ltd.

Construction and preparation of recombinant plasmid pVIVO$_2$ SjFABP-23

The recombinant plasmid bearing the fusion gene fragment of SjFABP and Sj23 was created via SOE and double PCR. PCR amplification of a single gene was performed in a final reaction volume of 50 µl, containing PCR cocktail reagents consisting of 10 × PCR buffer, $MgCl_2$, deoxynucleotide triphosphate, Taq polymerase, the relevant template (pVIVO$_2$SjFABP or p VIVO$_2$Sj23), and the appropriate primers. The PCR conditions were initial denaturation for 5 min at 95ºC, 35 cycles of denaturing at 94ºC for 1 min, annealing at 45ºC for 1 min and extension at 72ºC for 0.5 min, followed by extension at 72ºC for 10 min, and then the temperature was reduced to and maintained at 4ºC. The resulting PCR products were separated by electrophoresis, and extracted with a gel extraction kit (Shenergy Biocolor, China).

The second PCR was carried out with the first PCR products as templates, no primers, and at 95ºC for 5 min, followed by 5 cycles at 94ºC (1 min), 50ºC (1 min), and 72ºC (1 min). After extension at 72ºC, the reaction was paused. The upstream primer of SjFABP and downstream primer of Sj23 were added into the reaction system. The reaction continued with 35 cycles of denaturing at 94ºC for 1 min, annealing at 45ºC for 1 min and extension at 72ºC for 1 min, followed by extension at 72ºC for 10 min.

After analysis, extraction and purification, the fusion gene fragment SjFABP-23 was obtained and cloned into the pGEM-T vector in *E. coli* strain GT110. The correct sequence of the SjFABP-23 construct was confirmed. Then, the SjFABP-23 fragment was excised using the restriction endonucleases BamH I and EcoR I, cloned in the corresponding restriction sites of pVIVO$_2$, and transformed into *E. coli* strain GT110-competent cells. The transformant was cultured in Luria-Bertani broth supplemented with 50 mg/l Hygromycin B. The concentration of the plasmid DNA was determined by optical density measurements. The plasmid was dissolved in sterile saline solution at 2 mg/ml.

Vaccination of BALB/c mice with DNA vaccines and infection challenge

Forty 6- to 8-week old male BALB/c mice were randomly divided into four groups of 10 mice each. The mice were i.m. immunized with the different

constructs in sterile normal saline in the hamstring muscles of their hind legs as follows: (1) $pVIVO_2$ (control group): 100 μg of $pVIVO_2$ per mouse; (2) $pVIVO_2Sj23$: 100 μg of $pVIVO_2Sj23$ per mouse; (3) $pVIVO_2SjFABP$: 100μg of $pVIVO_2SjFABP$ per mouse; (4) $pVIVO_2SjFABP$-23: 100 μg of $pVIVO_2SjFABP$-23 per mouse. Four weeks after vaccination, each mouse was challenged with 40 ± 2 *S. japonicum* cercariae by direct skin penetration on the abdomen, as previously described [16]. Six weeks after the challenge, the mice were killed. The worms were recovered by perfusion and counted using the cover glass method, as described elsewhere [16]. The worm reduction rate in the vaccinated mice was calculated as follows:

worm reduction rate (%) = [(mean number of recovered worms in the control group - mean number of recovered worms in vaccinated group)/mean number of recovered worms in control group] × 100%.

At the end of the perfusion, the livers, spleens and intestines were collected from all of the mice. Eggs were recovered from these tissues and counted as previously described by Zhou *et al.* [17]. The egg reduction rate was calculated as follows:

Egg reduction rate (%) = [(average number of eggs/g liver tissue in control group - average number of eggs/g liver tissue in vaccinated group)/average number of eggs/g liver tissue in control group] × 100%.

Antibody detection

For antibody detection, serum samples were collected from the tail veins of all of the mice before and after vaccination and after infection challenge. ELISA was performed using 96-well flat-bottomed plates (Nunc). The ELISA plates were coated with soluble adult worm antigens (SWAP) and schistosome egg antigen (SEA) at 1 μg per well. A horseradish peroxidase labeled affinity-purified goat anti-mice IgG conjugate (Sigma) was added as a second antibody at a dilution of 1:100 in PBS. O-phenylenediamine was used as the substrate at a concentration of 0.4 mg/ml. The optical density values (OD) were measured on an automated model 450 microplate reader (Bio-Rad) at 492 nm.

Lymphocyte culture and cytokine ELISA

Six weeks after infection, spleen cells were cultured in 10% FCS/RPMI-1640 upon stimulation with 5 μg/ml concanavalin A (Con A) or 10 μg/ml soluble egg antigen (SEA) at 37ºC in 5% CO_2. The supernatant fluids were collected at 72 h, and IL-4 assays were performed and IFN-γ levels were determined by ELISA according to the manufacturer's instructions (R & D Systems).

Statistical analyses

Analysis of variance (ANOVA) was used in the study. $P < 0.05$ was considered significant.

RESULTS

Protective efficacy of the multivalent DNA vaccine pVIVO2SjFABP-23

As shown in Tab. 1, mice immunized with the new construct induced a significantly higher level of protection against the experimental schistosomiasis in terms of the number of worms and the number of eggs.

Tab. 1. The multivalent DNA vaccine effectively reduced the number of worms in the experimentally infected mice.

Group	No. of worms	Worm reduction rate	*P*
$pVIVO_2$ group	29.25 ± 7.13		
$pVIVO_2Sj23$ group	18.10 ± 5.32	38.10%	*< 0.05
$pVIVO_2SjFABP$ group	17.7 ± 5.79	39.50%	*< 0.05
$pVIVO_2SjFABP$-23 group	14.00 ± 3.46	52.10%	*,[1]< 0.05

* vs. $pVIVO_2$ group; [1] vs. $pVIVO_2Sj23$ or $pVIVO_2SjFABP$ group. Each group contained ten mice

Tab. 2. The multivalent DNA vaccine effectively reduced the number of eggs in the experimentally infected mice.

Group	EPG	Egg reduction rate	*P*
$pVIVO_2$ group	108720 ± 452230.53		
$pVIVO_2Sj23$ group	64960 ± 15973.53	40.25%	*< 0.05
$PVIVO_2SjFABP$ group	62320 ± 40168	42.68%	*< 0.05
p $VIVO_2SjFABP$-23 group	42480 ± 31330.81	60.93%	*,[1]< 0.05

* vs. $pVIVO_2$ group; [1] vs. $pVIVO_2Sj23$ or $pVIVO_2SjFABP$ group. Each group contained ten mice.

Antibody and cytokine responses

The total IgG antibody response was determined by ELISA before and after vaccination and challenge. As shown in Fig. 1, a significant similar increase in the IgG antibody level was elicited 28 days after $pVIVO_2SjFABP$ and $pVIVO_2SjFABP$-23 vaccinaton. The total IgG values rose significantly in all the groups ($P < 0.05$) after challenge.

The levels of both IFN-γ (Fig. 2) and IL-4 (Fig. 3) increased after ConA stimulation to a higher extent than after SEA stimulation. The splenocytes of $pVIVO_2SjFABP$-23 vaccinated mice showed higher levels of IFN-γ upon stimulation with ConA or SEA ($P < 0.05$) than the controls, while the IL-4 levels were significantly lower ($P < 0.05$). No significant differences in IL-4 levels were observed between the $pVIVO_2$ and $pVIVO_2Sj23$ groups upon stimulation either with ConA or with SEA.

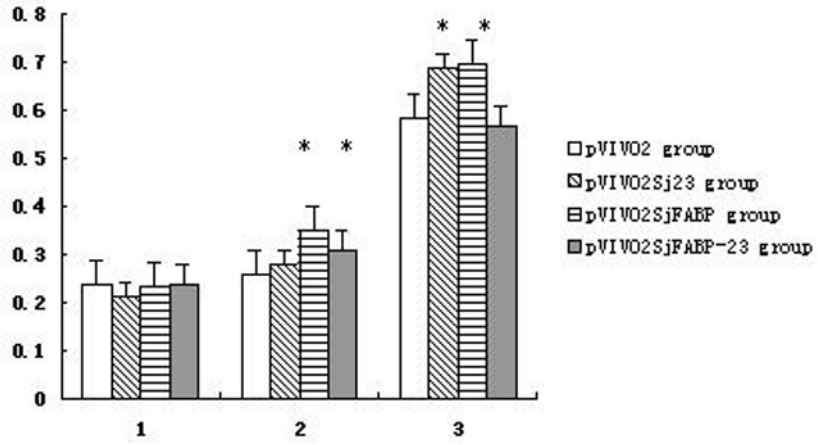

Fig. 1. Optical density (OD) values (+S.D.) in IgG ELISA against SWAP and SEA for serum samples taken before (1) and after vaccination (2), and 6 weeks after infection (3). The asterisks (*) on top of the bars represent the level of statistical significance ($P < 0.05$), compared with the controls.

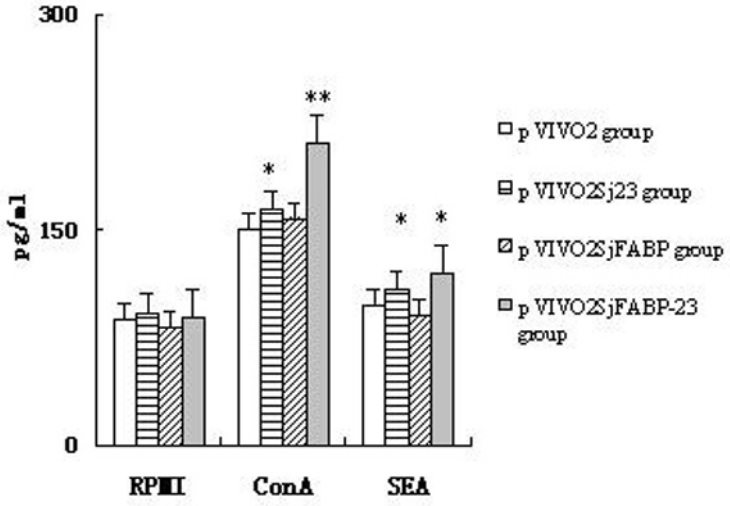

Fig. 2. Cytokine IFN-γ levels in the supernatant of spleen cells after *in vitro* stimulation with ConA or SEA. The asterisks (*) on top of the bars represent the level of statistical significance (**$P < 0.01$; *$P < 0.05$), compared with the controls.

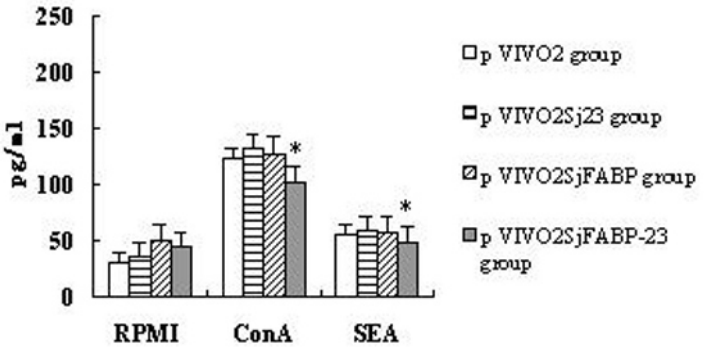

Fig. 3. Cytokine IL-4 levels in the supernatant of spleen cells after *in vitro* stimulation with ConA or SEA. The asterisks (*) on top of the bars represent the level of statistical significance ($P < 0.05$), compared with the controls.

DISCUSSION

Several candidate *S. japonicum* vaccine antigens are currently under study, some of which have yielded promising results. Previous results showed that vaccination with an Sj23 [18-20] or SjFABP [18, 21-23] DNA vaccine induced a significant reduction in worm and egg burdens, and also in the size of egg granulomas, although the protection was only partial.

The type of immune response induced was also of crucial importance. In mice vaccinated with irradiated cercariae, the induction of protective immunity was essentially dependent on both $CD4^+$ T cells and the production of IFN-γ and specific antibodies [24]. Thus, an optimal vaccination strategy against schistosoma may involve a mixed cellular and humoral response [25]. Zhang *et al.* [13] reported that vaccination using a cocktail DNA vaccine induced an increased IgG-specific response against crude worm antigens.

SjFABP (encoding a 132-amino acid protein of 14.7 kDa) is found within lipid droplets below the subtegumental region of the male worm, and in the vitelline droplets of the vitelline glands of the female worm; these glands provide nutrients to the developing egg [26, 27]. Since schistosomes are unable to synthesize long-chain fatty acids or sterols, and hence are completely dependent on their host for these substances, SjFABP is particularly important to *S. japonicum* for the uptake, transport and compartmentalisation of host-derived fatty acids. It plays a vital role in the physiology and survival of the parasite. Sj23 is an integral membrane protein consisting of four hydrophic trans-membrane domains and a large and a small hydrophilic domain, both thought to be extracellular [28]. The domain structures of Sj23 are strikingly similar to the human membrane proteins ME491, CD37, CD53 and TAPA-1. These similarities suggest a functional role for the schistosome molecules in cellular

proliferation [29]. Both have already been shown to be highly immunogenic in mice [30]. Considering pVIVO$_2$SjFABP-23 DNA vaccination induces a strong Th1 response and an effective protective immunity, it could be a promising approach against schistosomiasis.

We previously reported that DNA vaccination with Sj23 or SjFABP can induce a specific Th1 and IgG response. However, only a low reduction in the number of worms and eggs was induced [19-21, 31, 32]. These results indicate an ineffective specific response elicited by this single vaccine strategy.

On the basis of previous results, we constructed the recombinant plasmid pVIVO$_2$SjFABP-23, and evaluated its protective efficacy and the mechanisms of its activity in BALB/c mice. A 52.14% reduction in worm burden and a 60.93% egg reduction rate showed that the multivalent DNA vaccine pVIVO$_2$SjFABP-23 gave significant protection, which further suggested that it induced stronger protection than the monovalent vaccines pVIVO$_2$Sj23 and pVIVO$_2$SjFABP ($P < 0.05$).

The total IgG antibody responses showed that significant OD values were obtained in the mice immunized with pVIVO$_2$SjFABP-23 ($P < 0.05$) compared with the pVIVO$_2$ group; the increase was also significant compared with the pVIVO$_2$Sj23 group ($P < 0.05$) at pre-challenge. 42 days after challenge, the total IgG values rose in all the groups ($P < 0.05$), but there were no significant differences between the pVIVO$_2$SjFABP-23 group and the pVIVO$_2$ group ($P > 0.05$). Cytokine detection suggested that the splenocytes from mice immunized with pVIVO$_2$ SjFABP-23 released abundant amounts of IFN-γ at levels significantly higher than in mice exposed to pVIVO$_2$Sj23 or pVIVO$_2$SjFABP alone. By contrast, a significant reduction in IL-4 secretion was observed. Thus, we deduce that the pVIVO$_2$SjFABP-23 DNA vaccine may induce a strong Th1 response and suppress Th2 immunity, which was to some extent similar to the results obtained by Zhang *et al.* [13, 33]. Previous studies showed that protection was conferred by Th1 cellular immunity with lymphoid proliferation in the region and mediastinal lymph nodes [34], elevated production of IgG4 and IgG2 antibodies, and inhibition of IgE consistently associated with increased susceptibility to reinfection [35]. The effector function of IgA antibodies appeared to be associated with a decrease in female worm fecundity and egg viability [36]. However, IgG subclass analysis and lymphoproliferation after re-stimulation could not be investigated here due to the limited amount of serum collected. Further study is needed.

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