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Construction and Expression of DNA Vaccine pIRES-Sj97-Sj14-Sj26 and Its Immunogenicity in Mice*

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Summary: To find a new preventive strategy for the infection of *Schistosoma japonica*, plasmid pIRES-Sj97-Sj14-Sj26 that contains fatty binding protein (Sj14), GST (Sj26) and paramyocin (Sj97) that are expressed on the membrane, was constructed. RT-PCR was used to detect the expression of Sj14 mRNA, Sj26 mRNA and Sj97 mRNA in the Hela cells, the indirect immunofluorescent test was employed for the detection of the expression of trans-membrane Sj26 after the plasmid was transfected into Hela cells. Fifty BALB/c mice were randomly divided into 5 groups and pIRES-Sj97-Sj14-Sj26 plasmid DNA, pIRES-Sj14-Sj26 plasmid DNA, pIRES-Sj26 plasmid DNA, pIRES blank vector and normal saline were respectively injected into the quadriceps muscles of thigh. Eight weeks after the immunization the mice were killed and significantly higher level of IgG was detected in the pIRES-Sj97-Sj14-Sj26 group as compared with the pIRES blank vector, normal saline and pIRES-Sj26 groups ($P< 0.01$) and the pIRES-Sj14-Sj26($P<0.05$). Single splenocyte suspension was prepared to detected the level of IFN-γ by ELISA and the lymphocyte stimulating index (SI) by MTT. SI was significantly higher of in the pIRES-Sj97-Sj14-Sj26 group than in other groups ($P<$ 0.01), while the IFN-γ level was significantly higher the pIRES-Sj97-Sj14-Sj26 group than in pIRES blank vector and normal saline groups ($P<0.01$), but no significant differences were found when compared with pIRES-Sj14-Sj26 and pIRES-Sj26 groups. Flow cytometery showed that the percentages of CD4+ and CD8+ T cells were much higher in the pIRES-Sj97-Sj14-Sj26 group ($P< 0.01$, $P<0.05$). It was concluded that pIRES-Sj97-Sj14-Sj26 vaccine may induce stronger immune response in BALB/c mice.
Key words: *schistosoma japonicum*; Sj14; Sj26; Sj97; DNA vaccine immunization

Infection of *Schistosoma japonicum* is endemic in southern China and Philippines. In terms of morbidity and mortality, it represents an even greater disease burden. With the advent of the recombinant techniques, schistosome muscle proteins and enzymes can be used to develop DNA vaccine.

DNA vaccination offers many potential advantages, including safety, cost-effective production, thermal stability and synthesis of the native-like protein by folding and glycosylation in hosts[1], while the relatively lower-level expression of antigens makes it hard to induce significant immune responses in schistosome, a kind of muticellular organism, with the vaccine encoding only one antigen. More and more candidate antigens for schistosome vaccine were discovered over the past few years, leading to the development of such DNA vaccines from the those encoding only one antigen to the cocktail vaccines and to the immunization with one-antigen DNA vaccine in combination with adjuvant, and even the new type of DNA vaccine which encodes two or more antigens[2−4].

In this study, we chose fatty acid binding protein (Sj14), GST(Sj26) and paramyosin (Sj97) from *Schistosoma japonicum* as target antigens, and used pVAC vector to modify three coding sequences by adding signal peptide upstream and transmembrane sequence downstream, in order to subclone these modified genes into eukaryotic expression vector pIRES. Then its expression in eukaryotic cells and its immunogenicity in mice were examined to elucidate its anti-infection mechanism.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Bacterial Strains and Plasmids *Escherichia coli* (*E. coli*) DH5α was from our laboratory. Plasmid pVAC was provided by the Center for Disease Control and Prevention, Shenzhen, China. Plasmid pIRES was kindly given by Doctor Liao Xiang from the Department of Orthopaedics, Union Hospital, HUST, Wuhan, China.

1.1.2 Enzyme and Reagents Restriction endonuclease was purchased form Takara Biotechnology, China. T4 DNA Ligase and DNA Polymerase were bought from MBI (Lithuania). The 1-Kb DNA marker was from

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Jingmei Co., China. DNA marker Ⅲ and murine anti-26-kD GST monoclonal antibody was from Tiangen Biotech Co., China. Goat-anti-mouse fluorescent antibody labeled with FITC was purchased from Boster Biological Engineering Co, China. Lipofectamine 2000™ was from Invitrogen, USA. Mouse serum IgG ELISA kit was procured from 4adi, USA. IFN-γ ELISA kit was obtained from Endogen, USA. PE anti-mouse CD4, FITC anti-mouse CD3, PE-Cy5 anti-mouse CD8 antibodies were from eBioscience, USA.

1.1.3 Parasites and Experiment Animals Adult worms of the Chinese strain of *S. japonicum* were bought from the Department of Parasitology, Tongji Medical College, HUST, China. 8-week old BALB/c mice were purchased from the Center of Experiment Animals, Tongji Medical College, HUST, Wuhan, China.

1.2 Construction and Preparation of Plasmid pIRES-Sj97-Sj14-Sj26

Total RNA was extracted from adult Chinese *S. japonicum*, DNA of three target genes, Sj14, Sj26 and Sj97, were obtained by RT-PCR with specific primers, and then they were inserted respectively into pVAC vector. The recombinants were designated as pVAC-Sj14, pVAC-Sj26, pVAC-Sj97. Three modified genes IL2ss-Sj14-PLAPsa, IL2ss-Sj26-PLAPsa, IL2ss-Sj97-PLAPsa, which were added IL2 signal peptide at the upstream of the target gene and PLAPsa at the downstream of the target gene, were obtained by PCR by using the three recombinants as templates. IL2ss-Sj14-PLAPsa was digested with *Nhe* Ⅰ and *Mlu* Ⅰ and then inserted into the multiple clone site (MCS) A in pIRES vector; IL2SS-Sj26-PLAPsa was digested with *Sal* Ⅰ and *Not* Ⅰ and then inserted into MCS B in pIRES vector. The recombinant plasmid pIRES- Sj14-Sj26 was constructed. The fragment of IL2ss-Sj14- PLAPsa and IL2ss-Sj26-PLAPsa and the IRES sequence between them were obtained by digesting pIRES-Sj14- Sj26 with *Nhe* Ⅰ and *Not* Ⅰ. This fragment was subsequently inserted into MCS B in a blank pIRES vector because there was Xba Ⅰ, a isocaudarner of *Nhe* Ⅰ, in MCS B. IL2ss-Sj97-PLAPsa was finally inserted into MCS A, and pIRES-Sj97-Sj14-Sj26, the plasmid encoding modified Sj14, Sj26 ,Sj97 was constructed successfully.

The DNA of pIRES blank vector, pIRES-Sj26, pIRES-Sj14-Sj26, pIRES-Sj97-Sj14-Sj26 was extracted and dissolved in sterile normal saline. Purity and concentration of DNA were determined by UV spectrophotometry and agarose gel electrophoresis. Purified plasmid DNA was stored at –20°C.

1.3 Transient Transfection of Hela Cells and Detection with Indirect Immunofluorescence (IFA)

The plasmid pIRES-Sj97-Sj14-Sj26 was transfected into Hela cells with Lipofectamine 2000 (Invitrogen, USA), and 48 h after the transfection, the expressions of IL2ss-Sj14-PLAPsa, IL2ss-Sj26-PLAPsa, IL2ss-Sj97-PLAPsa were detected by RT-PCR. Forward primer 5 '-ccatgttcgtcatggtgtgtgaacc-3', reverse primer 5'- gccagtagaggcagggatgatgttc-3' were used to amplify β-actin with the target length of 251 bp. The transfected Hela cells were stained by an immunofluoresence method using mouse monoclonal antibody of Sj26 and goat anti-mouse IgG fluorescein isothiocynanate conjugate (FITC). Fluorescence was observed under a fluorescence microscope.

1.4 Immunization Schedule

Fifty 8-week old male BABL/c mice (weighing 18–22 g) were randomly divided into 5 groups, with 10 mice in each group. Group A were treated with normal saline, group B with blank pIRES vector, group C with pIRES-Sj26, group D with pIRES-Sj14-Sj26 and group E with pIRES-Sj97-Sj14-Sj26. Each group was vaccinated at week 0, 3 and 5 by injection of 100 μg of plasmid DNA into each quadriceps (at 100 μL/mouse). Four weeks after the final DNA vaccination, all the mice were executed.

1.5 Detection of Immunogenicity Index of Vaccinated Mice

1.5.1 Detection of Total IgG in Serum Blood (1 mL) was collected from eye balls of each mice before it was executed, and serum was extracted by centrifugation. ELISA was conducted by using a total IgG ELISA kit according to the manufacturer's protocol. Absorbance (*A* value) was determined at 450 nm, and results were calculated according to the standard curve.

1.5.2 Proliferation of Lymphocytes and Quantification of IFN-γ from Stimulated Splenocytes Single cell suspensions of spleen cells from 50 mice (2×10^6 cells/mL in 96-well plates) were incubated in the presence of Con A. Three experiment wells and 3 control wells were set for each mouse. Following stimulation for 48 h, culture supernatant was removed from each well and that from the stimulating wells was stored at–70°C for subsequent analysis. MTT was added into each well and cultured for 6 h, and then DMSO was added and incubated at 37°C for another 20 min. The absorbance (A value) was determined at 570 nm, and stimulating index (SI) of each mice was calculated (SI= *A* value in the experimental well/*A* value in the control wells). IFN-γ level were determined by ELISA kit by using the supernatant previously stored. *A* values were determined at 492 nm, and results were calculated according to the standard curve.

1.5.3 Detection of the Subgroups of T Cells Single cell suspensions of spleen cells from 50 mice were prepared at the concentration of 1×10^8 cells/tube. Each tube was added PE anti-mouse CD4, FITC anti-mouse CD3, PE-Cy5 anti-mouse CD8, incubated at 4°C for 20 min washed by PBS, and finally detected by flow cytometry (FCM). The percentages of CD4+ cells and CD8+ cells and the ratio between them were calculated.

1.6 Statistic Analysis

Student's *t*-test was used for comparison of data.

2 RESULTS

2.1 Construction of pIRES-Sj97-Sj14-Sj26

The target genes Sj14, Sj26, Sj97 were subcloned respectively into pVAC to add IL2ss upstream and PLAPsa downstream. The modified genes IL2ss-Sj14-PLAPsa, IL2ss-Sj26-PLAPsa, IL2ss-Sj97- PLAPsa were obtained by PCR with specific primers. Each modified gene was about 200 bp (IL2ss was 63 bp, PLAPsa 105 bp, multi clone site about 20 bp) longer than the original target gene. As a result, IL2ss-Sj14-PLAPsa was

about 600 bp, IL2ss-Sj26-PLAPsa about 860 bp and IL2ss-Sj97-PLAPsa about 2.8 Kb. PCR amplification and restriction enzyme splicing were used to identify the subclone of each gene (fig. 1). Sequencing of three modified gene showed there was no mutation in amino acid it encoded.

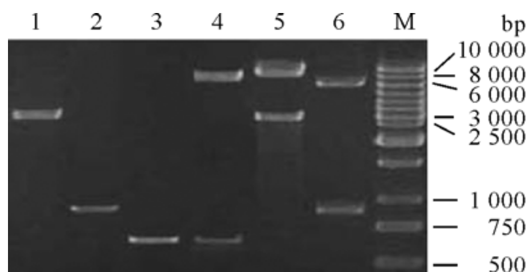

Fig. 1 Restriction enzymes maps and PCR amplification of recombinant plasmid pIRES-Sj97-Sj14-Sj26
1: PCR amplification of IL-2ss-Sj97- PLAPsa;
2: PCR amplification of IL-2ss-Sj26-PLAPsa;
3: PCR amplification of IL-2ss-Sj14-PLAPs;
4: pIRES- Sj14-Sj26 (*Nhe* Ⅰ/*Mlu* Ⅰ, small fragment is IL-2ss-Sj14-PLAPsa);
5: pIRES-Sj97-Sj14-Sj26 (*Nhe* Ⅰ/*Xho* Ⅰ, the small fragment is IL-2ss-Sj97-PLAPsa);
6: pIRES-Sj26 (*Sal* Ⅰ/*Not* Ⅰ and the small fragment is IL-2ss-Sj26-PLAPsa); M: 1 Kb DNA ladder

2.2 Expression of pIRES-Sj97-Sj14-Sj26 in Hela Cells

The expression of the three fragments IL2ss-Sj14-PLAPsa, IL2ss-Sj26-PLAPsa, IL2ss-Sj97- PLAPsa in Hela cells was detected by RT-PCR, with RNA extracted from the transfected Hela cells acting as template (fig. 2). Green fluorescence was detect on the membrane of Hela cells by indirect immunofluorescence, proving the trans-membrane expression of Sj26. By contrast, Sj26 protein was not found in the blank cells and the cells transfected with the empty plasmid pIRES.

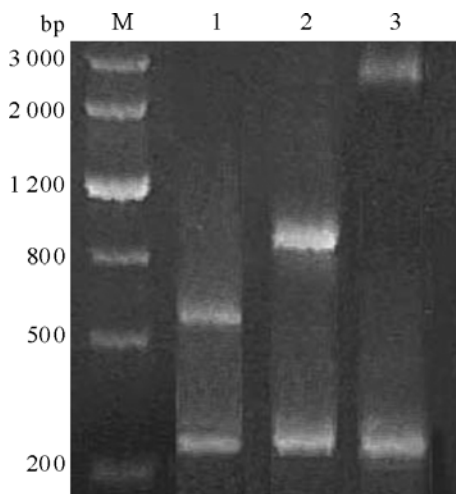

Fig. 2 RT-PCR amplification of recombinant plasmid pIRES-Sj97-Sj14-Sj26 RT-PCR
M: Marker Ⅲ; 1: RT-PCR product of IL-2ss-Sj14-PLAPsa and β-actin; 2: RT-PCR product of IL-2ss-Sj26-PLAPsa and β-actin; 3: RT-PCR product of IL-2ss-Sj97-PLAPsa and β-actin

2.3 Immunogenicity Index of Vaccinated Mice

2.3.1 Detection of Total IgG in Serum ELISA assays were performed to quantitatively determine the total IgG in serum. The *A* values were obtained, the standard curve was drawn and the amount of IgG was calculated (table 1). Significantly higher IgG was detected in the mice vaccinated with pIRES-Sj97-Sj14-Sj26 than in mice vaccinated with normal saline, pIRES blank vector and pIRES-Sj26 ($P<0.01$). Moreover, IgG was significantly increased in the pIRES-Sj97-Sj14-Sj26 group when compared to mice vaccinated with pIRES-Sj14-Sj26 ($P<0.05$).

Table 1 Comparison of total serum IgG in mice from each group (*n*=10)

Groups	IgG (mg/mL)
A: Normal saline	1.22±0.20
B: pIRES blank vector	1.48±0.36
C: pIRES-Sj26	3.32±0.51
D: pIRES-Sj14-Sj26	5.11±0.62
E: Pires-Sj97-Sj14-Sj26	5.62±0.64$^{*\#}$

$^{*}P<0.01$ as compared with groups A, B, C;
$^{\#}P<0.05$ as compared with group D

2.3.2 Detection of Stimulating Index (SI) of Lymphocytes and IFN-γ Production Table 2 shows that the lymphocytes from the spleen of mice vaccinated with pIRES-Sj97-Sj14-Sj26 could be stimulated to produce significantly higher proliferating activity (as reflected by SI) than mice in the other four groups ($P<0.01$). The level of IFN-γ in pIRES-Sj97-Sj14-Sj26 group was significantly higher than in normal saline group and pIRES blank vector group ($P<0.01$). No significant differences were found between the pIRES-Sj97-Sj14-Sj26 group pIRES-Sj26 group and between the pIRES-Sj97-Sj14-Sj26 group and pIRES-Sj14-Sj26 group ($P>0.05$).

Table 2 Comparison of SI and IFN-γ in the culture supernatant of spleen lymphocytes in mice from each research group (*n*=10)

Groups	SI	IFN-γ (pg/mL)
A: Normal saline	1.175±0. 074	24.65±6.10
B: pIRES blank vector	1.221±0.092	62.04±21. 44
C: pIRES-Sj26	1,446±0.278	215.07±50. 26
D: pIRES-Sj14-Sj26	1,746±0.200	217.74±50. 41
E:pIRES-Sj97-Sj14-Sj26	2,245±0.287*	228.95±52. 94$^{*\#}$

SI: $^{*}P<0.01$ as compared with group A, B, C, D;
IFN-γ: $^{*}P<0.01$ as compared with groups A, B;
$^{\#}P>0.05$ as compared with group C, D

2.3.3 Detection of CD4+T Cells and CD8+ T Cells in the Spleen of Mice The suspensions of the immunofluorescence-stained spleen lymphocytes were examined by flow cytometry. The results showed that the percentages of CD4+ T cells in pIRES-Sj97-Sj14-Sj26 group were significantly higher than in the normal saline group ($P<0.01$) and other three groups ($P<0.05$) (table 3). The percentages of CD8+ T cells in pIRES-Sj97-Sj14-Sj26 group was significantly higher than in the normal saline group, pIRES blank vector group ($P<0.01$) and the pIRES-Sj26 pIRES-Sj14-Sj26

group (P<0.05).

Table 3 Comparison of the percentage of CD4+, CD8+T cells in each group (n=10)

Groups	$CD3^+ CD4^+$(%)	$CD3^+ CD8^+$(%)
A: Normal saline	25.24±4.23	8.23±0.56
B: pIRES blank vector	32.30±2.66	9.81±1.52
C: pIRES-Sj26	32.70±4.61	12.51±0.37
D: pIRES-Sj14-Sj26	35.16±4.01	13.27±4.00
E: pIRES-Sj97-Sj14-Sj26	40.53±9.01*#	16.16±3.16*#

Percentage of $CD4^+$: *P<0.01 as compared with group A; #P<0.05 as compared with group B, C, D;
Percentage of $CD8^+$: *P<0.01 as compared with groups A, B; #P<0.05 as compared with groups C, D

3 DISCUSSION

Because *Schistosoma japonicum* has a complicated life cycle and tissue structure, vaccination in its hosts with only one antigen is not able to induce sufficient protection. Two or more than two target antigens are needed in order to induce adequate immunization against the disease[5]. Schistosomes are unable to synthesize long-chain fatty acids and sterols *de novo* and appear to utilize cytoplasmic and plasmalemmal FABP in the uptake, transport and compartmentalization of host-derived fatty acids. Fatty acid-binding protein (Sj14) plays a vital role in the physiology and survival of the parasite[6]. Glutathione S-tranferase (Sj26) localized within the parenchymal region of the male parasite and in the parenchymal cells between the vitelline glands in the female worms, can catalyze the detoxification by thioconjugation of lipopholic molecules and have demonstrated a remarkable effect directed against the fecundity of adult worms, and is able to prevent, on a long-term basis, the development of serious disease and to significantly decrease parasite transmission. Paramyosin is a myofibrillar protein found exclusively invertebrates. It is located in the muscle layer of *cercariae*, lung-stage schistosoma and adult worms. Because of its strong effect on the fecundity of adult worms and the ability to develop varian follicles in schistosoma, it has been attracting the attention of researchers as a most promising vaccine candidate[7]. In this study, we chose the above-mentioned three antigen genes as the target genes to induce different types of immunization at different life stages of the worm, in a hope to achieve strengthened protective effect.

pVAC vector has IL2 signal sequence (IL2ss) at the upstream of MCS and PLAPsa at the downstream of MCS. The IL2ss shares some characteristics with signal peptides of other secretory proteins with respect to abundance and positions of hydrophobic amino acids. PLAPsa, a hydrophobic COOH-terminal sequence of 32 residues, which is eliminated during processing of the preprotein, is the C-terminal transmembrane anchoring domain of the placental alkaline phosphatase (PLAP). After Sj14, Sj26, Sj97 were subcloned into pVAC, the expressed protein of the modified gene can be secreted and then anchored on the membrane. It is suggested that transmembrane protein has strong ability to induce immunization than their that intracellular and extracellular counterparts[8]. In pIRES vector there is an internal ribosomal entry site (IRES), which connects two open reading frames, making each product of transcription be translated independently with the participation of ribosome at the same time. During the construction of pIRES-Sj97-Sj14-Sj26, two IRESs are present among three inserted genes which are able to be expressed respectively. Furthermore, pIRES vector has CMV promotor, which can achieve the highly efficient expression of the foreign genes, and SV40 enhancer allows the enhancement of gene expression in a variety of hosts.

Schistosoma DNA vaccination offers a possibility to induce a wide variety of immune responses. The DNA vaccine is taken in by the host cells, transcripted and translated. Some of the peptides are loaded onto the MHC class Ⅰ molecule in the endoplasmic reticulum (ER), and the complex is localized on the surface of the cell and interacts exclusively with CD8+ T cells. By killing infected cells, cytolytic T cells help to control the spread of the worms and eggs. Some proteins are endocytosed, digested in lysosomes, and bound by the class Ⅱ MHC molecule prior to the molecule's migration to the plasma membrane, and the complex interact exclusively with CD4+ T cells, which is activated to become T helper cells. TH1 is responsible for the activation of cell-mediated responses associated with interferon (IFN)-γ, interleukin (IL)-2 and IL-12. TH2 is responsible for the main features of humoral immunity, and can be activated to assist B cells to produce antibody against the infection[9, 10]. In this study, we found that mice vaccinated with pIRES-Sj97-Sj14-Sj26 had significantly higher SI than the other four control groups, indicating that the DNA vaccine can substantially stimulate the activation of the lymphocytes in BALB/c mice. The level of IFN-γ production showed that although pIRES-Sj26, pIRES-Sj14-Sj26, pIRES-Sj97-Sj14-Sj26 have similar effect (P>0.05), the significant differences in IFN-γ production were found between the pIRES-Sj97-Sj14-Sj26 group and the normal saline and pIRES blank vector groups (P<0.01), which demonstrates that all the three vaccines can promote cell-mediated immunity to some extent. The level of total IgG in serum showed that these vaccines are able to activate B cells to different degrees. FCM showed that percentages of both CD4+ and CD8+ T cells in pIRES-Sj97-Sj14-Sj26 group was greatly increased, suggesting that the vaccine can enhance TH cell-mediated immunization and CTL response.

In this research, plasmid pIRES-Sj97-Sj14-Sj26 coding three transmembrane-expressing antigen genes is able to produce better immogenicity in BALB/c mice, both in cell-mediated immunity and humoral immunity.

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