THE PROTECTIVE IMMUNITY PRODUCED IN INFECTED C57BL/6 MICE OF A DNA VACCINE ENCODING *SCHISTOSOMA JAPONICUM* CHINESE STRAIN TRIOSE-PHOSPHATE ISOMERASE

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Abstract. The development of a SjCTPI DNA vaccine for *Schistosoma japonicum* and the detection of the immune responses to and the protective efficacy of immunization were performed and challenged in C57BL/6 mice. According to the gene sequence of SjCTPI and murine IL-12, three pairs of primers were designed. The full length cDNA encoding SjCTPI and P35, P40 amplified from pUCl9-SjCTPI and murine IL-12 by PCR were subcloned into an eukaryotic expression vector (pcDNA3.1). Forty-five female C57BL/6 mice were divided into three groups; each mouse of the control group was injected with l00 μg of pcDNA3.1 by i.m. route; the TPI group was injected with l00 μg of pcDNA3.1-SjCTPI; the TPI+IL-12 group was injected with l00 μg of pcDNA3.1-SjCTPI and l00 μg of mixture of pcDNA3.1-P35 and pcDNA3.1-P40. Each mouse was immunized at weeks 1 and 5 and challenged with 45 cercariae of *Schistosoma japonicum* Chinese strain at week 9. The mice were killed and perfused 45 days after challenge; the numbers of recovered worms and hepatic eggs were counted. The expression of SjCTPI in muscle tissue was determined by an immunohistochemical method. Culture of spleen cells showed the production of IL-2, IL-4, IL-10 and IFN-γ with the stimulation of specific antigen before and after challenge. Sera were collected from each group before immunization, before challenge and two weeks post challenge; ELISA and Western-blot tests were performed for detection of anti-rTPI antibodies. The antigen of SjCTPI was expressed in the membrane and plasma of the muscle cells of C57BL/6 mice. The obvious rising of IL-2 in TPI group and TPI+IL-12 group before and after challenge was seen. The anti-rTPI antibody detection with Western-blot showed that ten serum samples from the control group were negative; nine of ten serum samples from the TPI group were weakly positive, eight of ten from the TPI+IL-12 group were weakly positive. The worm and egg reduction rates of TPI group and TPI+IL-12 group were 27.9% and 13.7%, 31.9% and 18.6% respectively in comparison with the pcDNA group. pcDNA3.1-TPI DNA vaccine could confer partial protection against a subsequent challenge of *Schistosoma japonicum* in C57BL/6 mice and might therefore be a potential DNA vaccine.

INTRODUCTION

Wolff *et al* (1990) reported that the i.m. injection of purified recombinant DNA or RNA could provoke foreign gene expression in murine skeletal muscle. Williams *et al* (1991) using a gene gun to bombard murine skin or liver with DNA-coated microparticles, noted the expression of encoded genes in both tissues for up to 14 days. The potential of this technology for the induction of immune responses was first illustrated by Tang *et al* (1992): it was proved that the recombinant DNA could induce not only cellular immunity but also specific antibodies in animal experiments. Animal experiments have been outstandingly useful in research into parasitic diseases, *eg* leishmaniasis, malaria, schistosomiasis. Bergquist and Colley (1998) regarded triose-phosphate isomerase as a potential *Schistosoma mansoni* vaccine candidate. It was proved that *Schistosoma japonicum* Chinese strain triose-phosphate isomerase (SjCTPI) could provide partial protection against *Schistosoma japonicum* infection (Yu *et al*,

1999). In our research, we study the protective immunological efficacy and humoral and cellular immunity of SjCTPI DNA vaccine.

MATERIALS AND METHODS

The enzymes used were: *Taq* DNA polymerase; restrictive enzymes Xhol and BamH1; T4 DNA polymerase; T4 DNA ligase (Promega). The antigens were: *Schistosoma japonicum* rTPI; *Schistosoma japonicum* adult worm antigen (SWAP); *Schistosoma mansoni* multiple antigen peptide 4 (Sm-MAP4). The rTPI and SWAP were prepared by our laboratory (Jiangsu Institute). The Sm-MAP4 was provided by a member of the study team, DA Harn, who also supplied the eukaryotic expression vector pcDNA3.1, pcDNA1.1-IL-12 and host bacteria XL-1 blue. A QIAGEN-2500 (Qiagen Co, Germany) plasmid preparation kit was used.

Female C57BL/6 mice (14-16 g, 5-6 week-old) were purchased from Shanghai Experimental Animal Center of the Chinese Academy. Cercariae was provided by the snail laboratory of Jiangsu Institute of Parasitic Diseases.

Construction of SjCTPI DNA vaccine

A pair of primers, P1 and P2, was designed according to the TPI DNA sequence of *Schistosoma japonicum* Chinese strain: P1: 5′-GCGGATCCATGTCTGGTTCTCGGA-3′; P2: 5′-GCCTCGAGTTATTGTCTAGCTTTAC-3′.

The restriction site of BamH1 was added at the 5′-end of primer Pl; the Xhol site was added at the 5′-end of primer P2, the primers were synthesized by Shanghai Sangon Biological Engineering Co (Shanghai, China).

Amplification of the SjCTPI gene was as followed: P1 (20 μM) 2 μl, P2 (20 μM) 2 μl, 10xPCR buffer 10 μl, 4xdNTP (2.5 mM) 10 μl, DNA template (pUC19-SjCTPI, 1 mg/ml) 2 μl, and *Taq* DNA polymerase (4 u/μl) 2 μl were mixed; 50 μl of paraffin oil were added to the surface of the mixture. PCR was performed at 94ºC for 1 minute; at 55ºC for 1 minute; and at 72ºC for 2 minutes for 35 cycles, followed by 72ºC for 10 minutes. The amplified fragments were characterized by 1% agarose gel electrophoresis.

The PCR product gave a single DNA band during agarose gel electrophoresis; its molecular weight was similar to that of the predicted TPI gene fragment, the PCR fragment was then purified by a direct purification method using a Wizard PCR preps Purification system (Promega) according to the manufacturer's instructions.

The purified SjCTPI DNA fragment and the eukaryotic expression vector pcDNA3.1 were digested by the restriction enzymes BamHl and Xhol respectively. The products were purified with the Wizard DNA cleanup system (Promega) according to the manufacturer's instructions. 1.5 μl of the purified and digested SjCTPI DNA fragment, 6.5 μl of enkaryotic digested expression vector pcDNA 3.1, 1 μl 10xligation buffer, and 1 μl of T4 DNA ligase (Shanghai Sangon Biological Engineering Co) were placed in a 0.5 ml sterile microcentrifuge tube, mixed, and incubated at 22ºC for 2 hours. The competent XL-1 blue cells were prepared. Two hundred microliters of competent XL-1 blue cells and 10 μl of SjCTPI DNA fragment-vector ligated products were mixed in a 0.5 ml sterile tube, placed on ice for 30 minutes, and heat shocked at 42ºC for 2 minutes; 0.5 ml of pre-warmed LB medium at 37ºC was added to the tube. Following incubation with slow shaking at 37ºC for 30 minutes, 200 μl of the incubated product was inoculated on a LB culture plate with 50 μg/ml ampicillin and incubated at 37ºC overnight. The plasmid DNA containing the recombinants in the single colony was extracted by fast plasmid identification using the glass milk method. A single colony containing the recombinants was placed into 3 ml medium and incubated at 37ºC overnight; the recombinants were purified with the Wizard Minipreps DNA purification system (Promega). The recombinant was digested by restrictive enzymes; the digested product was then analyzed by 1% agarose gel electrophoresis. The recombinant plasmid was then sent to the Beijing

SBS Biotechnology Co for DNA sequencing.

Construction of pcDNA3.1-P35

A pair of primers P3 and P4 designed according to the cDNA of murine IL-12 was used to amplify the IL-12 subunit gene P35. P3: 5′-GCGGATCCTCCTGGGAAAGTCTG CCG-3′; P4: 5′-GCCTCGAGTCCTATCTGT GTGTGAGG-3′. The restriction site BamHl was added to the 5′-end of primer P3, the Xhol site was added to the 5′-end of primer P4. The primers were synthesized by the Shanghai Sangon Biological Engineering Co. The cloning of the P35 gene into pcDNA3.1 was by the method of cloning SjCTPI (above).

Construction of pcDNA3.1-P40

A pair of primers P5 and P6 designed according to the cDNA of murine IL-12 were used to amplify the IL-12 subunit gene P40. P5: 5′-GCGGTCCGCACATCAGACCAG-3′; P6: 5′-GCCTCGAGCAACGTTGCATCCTAG-3′. The restriction site BamHl was at the 5′-end of primer P5; the Xhol site was added to the 5′-end of primer P4. The primers were synthesized by the Shanghai Sangon Biological Engineering Co. The cloning of the P40 gene into pcDNA3.1 was by the method given above.

Large-scale preparation of pcDNA3.1-SjCTPI, pcDNA3.1-P35 and pcDNA3.1-P40

Plasmid DNA of pcDNA3.1-SjCTPI, pcDNA3.1-P35 and pcDNA3.1-P40 for intramuscular injection was prepared according to the manufacturer's instructions using Qiagen plasmid Mega kits (Qiagen Co, Germany) and redissolved in 0.9% NaCl. The concentration of plasmid DNA was detected by a nucleic acid and protein analyser (Bio-rad). The method of preparation of the pcDNA3.1 was as given above.

Protective trails with C57BL/6 mice

Forty-five female C57BL/6 mice were divided into three groups of 15. The quadriceps muscle of the control group mice was injected with 100 μg pcDNA3.1 (50 μg per leg). In the TPI group each mouse was immunized with 100 μg pcDNA3.1-SjCTPI. In the TPI+IL-12 group each mouse was injected with 100 μg pcDNA3.1-TPI (left leg) and l00 μg of the mixture of pcDNA3.1-P35 and pcDNA3.1-P40 (right leg); boosting was performed four weeks later with the immunization methods and doses as given above.

Each mouse was challenged with 45 *Schistosoma japonicum* cercariae by the abdomen penetration method four weeks after boosting. Forty-five days post-challenge, mice were sacrificed and the numbers of worms and liver eggs were counted. The worm and egg reduction rates were calculated with the following formulae: the worm reduction rate (%) is the mean number of adult worms per mouse of the control group minus the mean number of adult worms per mouse of the experimental group divided by the mean number of adult worms of the control group; the egg reduction rate is the mean number of eggs per mouse of the control group minus the mean number of eggs per mouse of the experimental group divided by the mean number of eggs per mouse of the control group.

Expression of SjCTPI in quadriceps muscle cells of C57BL/6 mice

Two mice from each group were immunized again ten days after priming; these mice were killed four days later and frozen slices were made of the quadriceps muscle. The frozen muscle tissue slices were fixed with cold acetone for 10 minutes, washed in 0.01 M PBS (pH 7.6) three times, treated with 0.3% hydrogen peroxide methanol for 30 minutes, washed three times with 0.01 M PBS, incubated in normal sheep serum (diluted 1:10 in 0.01 M PBS) for 10 minutes, incubated with the mouse serum for 24 hours at 4ºC, washed three times with 0.01 M PBS, incubated in sheep-anti-mouse IgG-HRP (diluted 1:25 in 0.01 M PBS) for 1 hour at 37ºC, washed and incubated with the hydrogen peroxide/DAB mixture for 5 minutes, and finally washed several times in distilled water to stop the reaction. The results were observed with a microscope.

Culture of spleen cells and detection of cytokines

Two mice from each group were killed two weeks before and after challenge; spleens were removed and single cell suspensions were prepared; Boyle's solution was added for lysis of RBC, and the mixture was centrifuged and the supernatant discarded; 5 ml of RPMI medium was added to re-suspend spleen cells and the mixture was centrifuged and the supernatant discarded. After 2 ml of RPMI complete medium (containing 20% FBS) was added, spleen cells were counted and diluted to $6x10^6$/ml, $6x10^5$ (100 μl) of spleen cells were stimulated with 100 μl ConA (10 μg/ml), rTPI (40 μg/ml), SWAP (40 μg/ml), Sm-MAP4 (40 μg/ml) and cultured for a total of 96 hours, cytokines including IL-2, IL-4, IL-10 and IFN-γ, were measured with ELISA detection kits (Jingmei Biotech Co).

Detection of anti-SjCTPI antibodies

Mice sera were collected from each group before immunization, and before challenge and two weeks post-challenge. ELISA plates were coated with rTPI (20 μg/ml) and SWAP (20 μg/ml) overnight at 4ºC, blocked with 1% BSA-PBST for 1 hour at 37ºC, washed three times with PBST, incubated with serum diluted 1:50 with PBST for 1 hour at 37ºC; plates were washed three times with PBST and incubated with sheep anti-mouse IgG-HRP (1:1,000) for 1 hour at 37ºC; TMB was added for 5 minutes, then 2M H_2S0_4 was added to stop the reaction; OD value was read at 450 nm.

Ten serum samples from the day before challenge and five sera from two weeks post challenge from each group were randomly selected for Western-blot assay. Ten percent SDS-PAGE gel was prepared. Two hundred microliters rTPI (1.87 mg/ml) and 200 μl lxSDS-loading buffer were mixed and heated at 100ºC for 5 minutes. The rTPI sample was loaded, after running SDS-PAGE and transferred to the NC membrane; the transferred NC membrane was blocked with 0.3% PBST, and cut it into strip; the strips were incubated with each serum for 2 hours at 37ºC, washed with 0.3% PBST for 30 minutes and then a substrate DAB was added; the reaction was stopped with water.

RESULTS

Construction and preparation of pcDNA3.1-SjCTPI, pcDNA3.1-P35 and pcDNA3.1-P40

The PCR fragments of SjCTPI, P35 and P40 were subcloned into the eukaryotic expression vector of pcDNA3.1. Selected positive colony was added to 3 ml LB media, and cultured overnight at 37°C; the recombinants were purified with Wizard Minipreps DNA purification system, and analyzed by digestion with restrictive enzymes of BamH1 and Xho1; the digested products were analyzed by 1% agarose gel electrophoresis.

The molecular weight of the target fragments was the same as that of the PCR fragments (the molecular weight of TPI, P40 and P35 were about 750 bp,1,100 bp and 800 bp respectively, Fig 1). The results of DNA sequencing showed that the DNA sequences of the cloned genes were the same as the DNA sequence of SjCTPI, and subunit P35 and P40 of murine IL-12 respectively.

The concentration of pcDNA3.1-SjCTPI prepared with Qiagen plasmid Mega Kit was 3.10 mg/ml (OD260/280) (1.87); the concentration of pcDNA3.1-P35 was 3.11 mg/ml (0D260/280 1.9); pcDNA3.1- P40 2.3 mg/ml (OD260/280 1.92).

Detection of cytokines

Obvious increases of IFN-γ. IL-4 and IL-10 in spleen cells culture two weeks before and post challenge were not seen, although there were differences in the level of IL-2 before and after challenge among the three groups. Before challenge, IL-2 of pcDNA group and TPI group could not be detected, but the concentrations of IL-2 in TPI+IL-12 group stimulated with SWAP and Sm-MAP4 were 35.49 ng/ml. After challenge, IL-2 in pcDNA group could not be detected; the TPI group with stimulation of various stimulates had an ob-

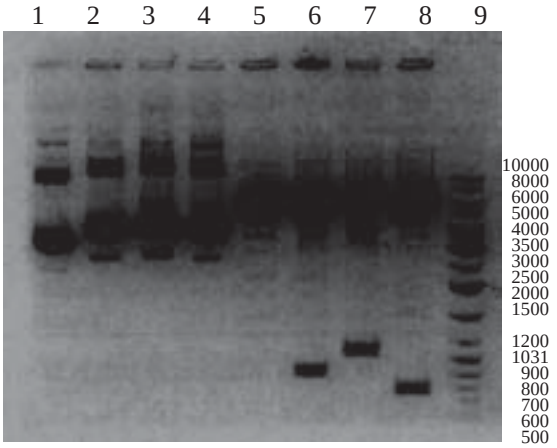

Fig 1–Lane 1: plasmid pcDNA3.1; Lane 2: recombinant plasmid of pcDNA3.1-P35; Lane 3: recombinant plasmid of pcDNA3.1-P40; Lane 4: recombinant plasmid of pcDNA3.1 -SjCTPI; Lane 5: Restriction analysis of pcDNA3.1; Lane 6: Restriction analysis of pcDNA3.1-P35; Lane 7: Restriction analysis of pcDNA3.1-P40; Lane 8: Restriction analysis of pcDNA3. 1-SjCTPI; Lane 9: DNA standard marker.

vious increasing IL-2; the concentrations of IL-2 in TPI group stimulated with SWAP, Sm-MAP4 and rTPI were 93.61 ng/ml, 116.86 ng/ml and 151.73 ng/ml respectively; IL-2 in the TPI+IL-12 group was not detected.

Detection of anti-SjCTPI antibody

The ELISA assay showed that besides a slight titer of anti-SjCTPI antibody in several mice in TPI+IL-12 group, anti-SjCTPI antibody was negative in the three groups. There was no difference between the three groups two weeks post challenge, although anti-rTPI antibody was positive. Western-blot assays revealed that the serum samples in the pcDNA group before challenge were negative; nine of ten sera samples from TPI group were weakly positive; eight of ten from TPI+IL-12 group were weakly positive.

Expression of SjCTPI in quadriceps muscle of C57BL/6 mice

The results of immunohistochemistry showed that there was no specific brown staining in the quadriceps muscle cells from the pcDNA group; in the membrane and plasma of muscle cells from the TPI and TPI+IL-12 groups, specific obvious brown staining could be observed.

Table 1
Comparison of the number of worms recovered in the three groups of mice.

Groups	No. of mice	Worm reduction rate (%)
TPI	10	27.9
TPI+IL-12	12	31.9
pcDNA	9	-

Protective imunological efficacy

The collection and calculation of worms after portal perfusion six weeks post cercarial challenge gave worm reduction rates of 27.9% (TPI group) and 31.9% (TPI+IL-12 group) (Table 1).

The calculation of the mean number of eggs per mouse according to the total number of eggs gave egg reduction rates of 13.7% (TPI group) and 18.6% (TPL+IL-12 group) in comparison with the pcDNA group (Table 2).

DISCUSSION

Nucleic acid vaccine technology provides a new approach for the prevention of infectious diseases; the more obvious advantages of DNA vaccines appear to be purity, physiochemical stability, simplicity of preparation of plasmid DNA; the vaccines are also comparatively inexpensive. DNA vaccines against schistosomiasis are being developed; the candidate vaccines reported include the *Schistosoma japonicum* 23 kDa antigen (Sj23), the *Schistosoma japonicum* 32 kDa antigen (Sj32), glyceraldehyde-3-phophate dehydrogenase, paramyosin, 14 kDa fatty acid binding-protein, the 22 kDa tegumental membrane-associated

Table 2
Influence of immunization on egg output of adult worms in mice.

Groups	No. of mice	Mean no. of eggs per mouse	Egg reduction rate (%)
TPI	10	125,440±22,827	13.7
TPI+IL-12	12	115,829±39,711	18.6
pcDNA	9	141,736±13,181	-

antigen, 26 and 28 kDa glutathione-s-transferases, cathepsin B and calreticulin (Xie *et al*, 1998; Waine *et al*, l999a,b; Yi *et al*, 2000).

In this study, when the SjCTPI gene was subcloned into the eukaryotic expression vector of pcDNA3.1 (promotor CMV), the worm reduction rate was 27.9% through intramuscular immunization; co-immunization with the encoding IL-12 plasmids gave a 31.9% worm reduction rate, showing that the DNA vaccine of pcDNA3.1-SjCTPI confers partial protection against *Schistosomiasis* in C57BL/6 mice; a worm reduction rate of 27.8% with the peptide vaccine of SjCTPI (IL-12 adjuvant-modified) has also been shown (Rao *et al*, 1996). IL-12, a heterodimeric molecule produced mostly by phagocytes, induces the production of IFN-γ by NK cells and macrophages and promotes the development of Th1 type responses. Co-immunization of encoding IL-12 plasmids will decrease production of specific antibodies, but will concomitantly and dramatically increased induction of antigen-specific CTL (Pasquini *et al*, 1997). In these experiments, the use of adjuvant of IL-12 caused the worm reduction rate to increase from 27.9% to 31.9%, while the egg reduction rate increased from 13.7% to 18.6% ($p<0.05$). It seems that the encoding IL-12 plasmid can promote the immune efficacy of SjCTPI DNA vaccine although the enhancing role was not obvious: further study of the time and dose of immunization with SjCTPI DNA vaccine is needed. The egg reduction rate was lower than the worm reduction rate in these experiments, perhaps because the male reduction rate was higher than the female reduction rate in the TPI group and the TPI+IL-12 group.

In this study, the expression of pcDNA3.1-SjCTPI in the quadriceps muscle of C57BL/6 mice was seen; the immunohistochemical results showed that SjCTPI could be expressed in the membrane and plasma of quadriceps muscle cells of C57BL/6 mice.

SjCTPI DNA vaccine also may directly transfect APC; TPI expressed by APC would be decomposed to a multipeptide of 8-10 amino acids by proteinase in plasma and transferred to the endoplasmic reticulum cavity by TAP of endoplasmic reticulum, formed complex with MHCI, transferred from the Golgi complex to the membrane of APC and recognized by T-cell receptor, so inducing a CTL response against *Schistosoma* infection.

We detected the T-cell response in our study: IFN-γ, IL-4 and IL-10 of the three groups showed no obvious change; we found rising IL-12 in the TPI group and the TPI+IL-12 in varying degrees. IL-2 was secreted by CD_4^+Th1 cells with stimulation of antigen or mitogen; it may have been that the cellular immunity induced by Th1 cell was predominant after immunizing C57BL/6 mice with DNA vaccine of pcDNA3.1-SjCTPI, although we could not detect an obvious change of IFN-γ.

Sera from immunized mice were tested for antigen-specific IgG with ELISA assay and Western-blot assay. Though the results of Western-blot analysis showed that the sera of mice from before challenge had low levels of anti-SjCTPI antibodies, ELISA could not detect anti-SjCTPI antibodies; titers of anti-rTPI

antibodies induced by pcDNA3.1-SjCTPI were very low. According to Waine *et al* (l997) except Sj23, paramyosin, PMY6 and PMYl9 fragments, antigen specific IgG could not be induced with DNA immunization of cDNAs encoding 26 kDa and 28 kDa glutathione-s-transferases, 14 kDa fatty acid-binding protein, glyceraldehyde-3-phosphate dehydrogenase etc. This suggests that cellular immunity may be predominant in the immuno-response mechanism with DNA immunization of *Schistosoma*.

Our study suggests that SjCTPI DNA vaccine could confer partial protection against a subsequent challenge of *Schistosoma japonicum* in C57BL/6 mice and might therefore be a potential DNA vaccine.

ACKNOWLEDGEMENTS

This study received financial support from the UNDP/World Bank/WHO Special program for Research and Training in Tropical Diseases (TDR) (ID:981051) and Department of Health, Jianghsu Province (H9918).

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