SCIENCE CHINA
Life Sciences

• RESEARCH PAPER •

February 2013 Vol.56 No.2: 174–180
doi: 10.1007/s11427-012-4434-4

Assessment of the immune responses to *Treponema pallidum* Gpd DNA vaccine adjuvanted with IL-2 and chitosan nanoparticles before and after *Treponema pallidum* challenge in rabbits

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Received September 24, 2012; accepted December 7, 2012; published online January 17, 2013

Syphilis is a multistage, sexually transmitted disease caused by the spirochete, *Treponema pallidum* (Tp). A significantly high incidence of syphilis has been reported in several countries, including China, and there is an urgent need for the development of efficacious vaccines against syphilis. DNA vaccines are a major breakthrough in the field of vaccination with several advantages over traditional vaccines. Animal model studies of Tp DNA vaccines have not been reported elsewhere but our previous reports describe the development of a single-gene Tp DNA vaccine and preclinical immunization study. In this study, chitosan (CS) nanoparticles were used as a vector and an interleukin-2 expression plasmid (pIL-2) as an adjuvant to enhance a TpGpd DNA vaccine candidate (pTpGpd) in a rabbit Tp skin challenge model. At week 8 after the first immunization, three rabbits from each group were used to determine cytokine measurements and spleen lymphocyte proliferation assay. pTpGpd in combination with pIL-2 wrapped with CS led to the greatest enhancement of anti-TpGpd antibodies and T-cell proliferation. During infection, levels of anti-TpGpd antibodies and T-cell proliferation were measured. Both the serum special IgG and IL-2, interferon-γ were significantly increased by the co-injection of the IL-2 plasmid compared with the injection of TpGpd DNA alone (P<0.05). Furthermore, IL-2 plasmid coinjection efficiently enhanced the antigen-specific lymphocyte proliferation response. Additionally, the ratios of positive skin lesions and ulcer lesions in groups immunized with pTpGpd were significantly lower than those of the pIL-2, CS or pIL-2 mixed with CS control groups (P<0.001). CS vectored and pIL-2 adjuvanted pTpGpd immunized animals exhibited the lowest rates of positive skin tests (8.33%) and ulcer lesions (4.17%) and the fastest recovery (42 d). These experiments indicate that co-injection of a pIL-2 plasmid with pTpGpd DNA vaccine wrapped with CS can significantly strengthen the long-term stability of immune response during infection, efficiently improve the protective effect against *T. pallidum* spirochetes infection and attenuate syphilitic lesion development.

***Treponema pallidum* (Tp), membrane protein, Gpd, CS, IL-2, nucleic acid vaccine**

Citation: Zhao F J, Zhang X H, Liu S Q, et al. Assessment of the immune responses to *Treponema pallidum* Gpd DNA vaccine adjuvanted with IL-2 and chitosan nanoparticles before and after *Treponema pallidum* challenge in rabbits. Sci China Life Sci, 2013, 56: 174–180, doi: 10.1007/s11427-012-4434-4

Transduction efficiency affects DNA vaccine delivery in the immunization process and has a large bearing on dosage. Chitosan (CS) is a natural polysaccharide derived from the deacetylation of chitin. Given the high number of positively

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charged amino acids, CS possesses unique polycationic characteristics and easily combines with DNA. CS protects the DNA from intracellular nuclease degradation and associates with the cell membrane for easier transport into the cell. Exogenous gene transfection efficiency and expression of DNA in mammalian cells are significantly improved when coadministered with CS [1,2]. As a new, efficient type of gene transduction vectors, nanoparticles are becoming increasingly popular and it has been widely shown that CS nanoparticles effectively enhance the immune effects of DNA vaccines in mice [3–5].

Cytokines play important roles in regulation of the immune responses and inflammation. Intramuscular injection of cytokine expression vectors can significantly regulate the immune response [6–8]. Barouch et al. [9] co-injected an interleukin (IL)-2 expression plasmid together with HIV-1 DNA vaccines into rhesus macaques and found that IL-2 induced much higher levels of antibody and cytotoxic lymphocyte (CTL) activity, when compared with the DNA vaccine alone.

Therefore, based on preliminary protection studies of New Zealand rabbits immunized with recombinant pcDNA3.1/Tp92 and pcDNA3.1/IL-2 (pIL-2) [10], the current study investigated the additional protective effects of using CS for vaccination. In this study, a *Treponema pallidum* (Tp) membrane protein Gpd expression plasmid, pcDNA3.1/TpGpd (pTpGpd), was coadministered with a cytokine adjuvant, pIL-2, and CS nanoparticles. This study aims to explore the pTpGpd DNA vaccine-induced immune response before and after Tp infection in rabbit skin, with the use of the targeting and slow release of CS nanoparticles and adjuvant effect of pIL-2. Further, this study evaluated the adjuvant effect of CS nanoparticles and pIL-2 for the eventual establishment of a highly active anti-Tp response in animal models with the hope for a human syphilis vaccine.

1 Materials and methods

1.1 Rabbits

Adult New Zealand White rabbits were obtained from the Department of Laboratory Animals, University of South China (Hengyang, China). All animal experiments were approved by the governing Animal Welfare Committee and conducted in accordance with the regulations of the institution. Rabbits were individually housed at 18–20°C and given antibiotic-free food and water.

1.2 Bacteria strains, DNA plasmids and reagents

The Tp Nichols strain was a gift from Dr. Gu WeiMing (Shanghai Skin Disease and Sexual Disease Hospital) and was stored at the Pathogenic Biology Institute at University of South China (Hengyang, China). *Escherichia coli* JM109 and syphilis standard serum were obtained from the Pathogenic Biology Institute (Hengyang, China). The two DNA plasmids pTpGpd, encoding the TpGpd protein, and pIL-2, the IL-2 expression plasmid, were constructed and identified in our laboratory, as described previously [10,11]. HRP-labeled goat anti-human/rabbit IgG secondary antibody was purchased from Invitrogen (Shanghai, China). Rabbit interferon (IFN)-γ and IL-2 enzyme-linked immunosorbent assay (ELISA) detection kits were purchased from Shanghai Jianglai Biological Technology (Shanghai, China). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from the HyClone Company (Logan City, Utah, USA). CS solutions (pH 5.5) were purchased from Hebei Taijihuan Nanoproducts (Qinhuangdao, China). DNA-CS mixtures were prepared by combining CS solution (pH 5.5) and plasmid DNAs at 60°C with a mass ratio of 3:10, followed by vortexing for 5 min.

1.3 Immunization protocol

As described in previous studies [10], rabbits were divided into seven groups, with 18 rabbits in each group. DNA vaccine groups: (i) pTpGpd (100 μg), (ii) pTpGpd (100 μg) coated with CS (30 μg), (iii) pTpGpd (100 μg) and pIL-2 (100 μg), (iv) pIL-2 (100 μg) and pTpGpd (100 μg) coated with CS (30 μg) were inoculated in the left hind leg quadriceps of rabbits. Controls included pIL-2 coated with CS, pIL-2 and CS. The complete regimen included three immunizations administered once every two weeks. Two days before injections, 100 μL of 0.25% bupivacaine was injected into the inoculation sites. At week 8 after the first immunization, three rabbits from each group were used to determine cytokine measurements and splenocyte proliferation assay.

1.4 Infection and rabbit lesion and ulcer scoring

At week 10 after the first immunization, 15 rabbits in each group were challenged subcutaneously with the Tp Nichols strain at eight sites (10^5 bacteria/site) in the back. Skin lesions were observed and measured at the injection sites every three days for 60 days. Lesion spirals were investigated by dark-field (DF) and silver staining methods at day 21 post-Tp challenge. The ratio of the number of DF-positive lesions to the total number of the lesions was calculated for each group. The ratio of the number of ulcerative lesions to the total number of the lesions was also calculated for each group.

1.5 Analysis of TpGpd-specific antibody responses by ELISA during immunity and infection

To evaluate anti-Gpd humoral responses during infection, 1 mL of blood was collected from immunized rabbit ear

veins for each group (n=18, 18, 18, 18, 18, 15, 12, 9, 9, 9, 9, and 6) at corresponding times (weeks 0, 2, 4, 6, 8, 10, 12, 14, 20, 24, 28, and 32) after the first immunization, followed by centrifugation for isolation of serum. Each well was coated with 100 μL purified recombinant Gpd protein (50 μg mL^{-1}) overnight at 4°C and incubated with 100 μL of serial two-fold dilutions of sera for 60 min at 37°C, before the addition of 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1∶2000) for 60 min at 37°C and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution. Each sample was tested in triplicate and the absorbance was measured at 450 nm using an automatic ELISA reader (Multiskan MK-3, Finland). Antibody titers were calculated as the highest dilution that gave a positive reading. The cutoff value was set as twice the mean absorbance of sera from the negative control.

1.6 Preparation of spleen cell suspensions

Spleen cells were obtained from three rabbits from each group of infected rabbits (n=18, 15, 12, 9, 6 and 3) at corresponding times (weeks 8, 10, 12, 14, 30, and 34), as previously described [10]. Briefly, rabbits were euthanized by the air embolism method. Partial spleens were taken and incubated in 5 mL of cold D-Hank's solution. Spleen tissues were ground through copper grids (100- to 200-mesh) to obtain spleen cell suspensions. After centrifugation for 10 min at 2100×g, supernatants were discarded and 10 mL of red blood cell lysis buffer (139.6 mmol L^{-1} NH_4Cl, 16.96 mmol L^{-1} Tris; pH 7.2) was added, followed by incubation at 37°C for 10 min. After centrifugation for 10 min at 2100×g, supernatants were discarded and the cells resuspended in RPMI-1640 medium containing 10% FBS.

1.7 Detection of cytokines

IL-2 and IFN-γ levels of spleen cells were determined according to the instructions of the cytokine ELISA detection kit. Briefly, spleen cells were added into 96-well plates with 6×10^5 cells in each well, followed by incubation at 37°C for 72 h. Purified protein was used as the stimulating antigen (20 μg mL^{-1}). Samples with concanavalin A (ConA, 10 μg mL^{-1}, Sigma) as the stimulating antigen were used as positive controls. Samples without stimulating antigens were used as negative controls. Supernatants harvested from cultured cells were used immediately or stored at −80°C.

1.8 Spleen lymphocyte proliferation assays

Spleen lymphocyte proliferative response was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method, as previously described [10]. Briefly, spleen cells were seeded at 4×10^6 cells mL^{-1} in triplicate in 96-well plates and cultured in RPMI-1640 medium (HyClone) with 10% heat-inactivated FBS (HyClone), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2 mmol L^{-1} L-glutamine at 37°C and 5% CO_2 with or without antigen stimulation. Purified protein was used as stimulating antigen (10 μg mL^{-1}). Samples with ConA (5 μg mL^{-1}, Sigma) as stimulating antigen was set as positive controls. Samples without stimulating antigens were used as negative controls. Following incubation for 68 h, 20 μL MTT (Sigma) was added to each well; plates were then incubated for 4 h. At the end of the incubation, plates were centrifuged at 1500×g for 8 min and the supernatants in each well were removed. One hundred microliters of dimethylsulfoxide (DMSO) was then added into each well to solubilize the formazan precipitates for 10 min under oscillation. The stimulation index (SI) was determined at a wavelength of 570 nm, calculated as the ratio of A_{570} of stimulated proliferation to that of unstimulated proliferation.

1.9 Statistical analyses

The experimental data, including the absorbance values at 450 nm of antibodies in sera, levels of IL-2 and IFN-γ secreted by spleen cells, and SI of spleen cells were given as mean±standard error (SE). Statistical software (SPSS, version 13.0) was used for a two-way analysis of variance with a Bonferroni post-hoc test, P<0.05 indicated a significant difference. The ratio of the number of DF-positive lesions and ulcerative lesions to the total number of the lesions were calculated for each group, which was used for χ^2 tests. P<0.05 indicated a significant difference.

2 Results

2.1 Expression of Gpd or IL-2-encoding gene in HeLa cells

The plasmids, pTpGpd and pIL-2, were transfected into HeLa cells. After 48 h, the total proteins were harvested. Transient expression of TpGpd and IL-2 were examined by immunoblotting. The present study confirmed that cells transfected with these plasmids expressed the corresponding proteins, based on the predicted molecular masses for Gpd (~41 kD) and IL-2 (~22 kD) (data not shown), as previously described [10,11].

2.2 TpGpd antibody levels increased when DNA vaccines were coated with CS or co-inoculated with pIL-2 during immunization and infection

At weeks 0, 2, 4, 6, 8, 10 (Tp challenge), 12, 14, 20, 24, 28, and 32 after the first immunization, blood was obtained from rabbits and the anti-TpGpd special antibody levels were determined by ELISA. The ELISA results indicated that pTpGpd induced an increase in the anti-TpGpd antibody

levels from week 2, when compared with the pIL-2 coated with CS, pIL-2 alone or CS immunized rabbits (Figure 1). CS vectoring (0.87, 1.07 and 1.18 at weeks 4, 6 and 8, respectively) and coadministration of pIL-2 (1.37, 1.66 and 1.69 at weeks 4, 6 and 8, respectively) adjuvanted pTpGpd (0.54, 0.82 and 0.84 at weeks 4, 6 and 8, respectively) as evidenced by the week 4 ,6 and 8 antibody titers (P<0.05). The combination of CS vectoring and pIL-2 resulted in the highest anti-TpGpd antibody levels achieved (1.46, 1.75 and 1.78 at weeks 4, 6 and 8 respectively, P<0.05). However, there was no significant difference (P>0.05) observed in rabbits when administered with pTpGpd coated with CS (0.87, 1.07 and 1.18 at week 4, 6 and 8, respectively) compared with the pTpGpd alone (0.54, 0.82 and 0.84 at weeks 4, 6 and 8, respectively).

During the infection period (weeks 10–32), vaccine groups (CS+pTpGpd+pIL-2 group, pTpGpd +pIL-2 group and the CS+ pTpGpd group) maintained stable and significantly higher levels of anti-TpGpd IgG antibodies compared with the respective negative controls (pIL-2+CS, pIL-2 alone and CS groups; P<0.05) (Figure 2).

2.3 The measurements of cytokine levels of IL-2 and IFN-γ

Rabbit spleen cells were collected and cultured at week 8. The IL-2 and IFN-γ levels in the supernatants harvested from the cultured cells were determined by ELISA. As shown in Figure 3, pTpGpd induced a significant increase in

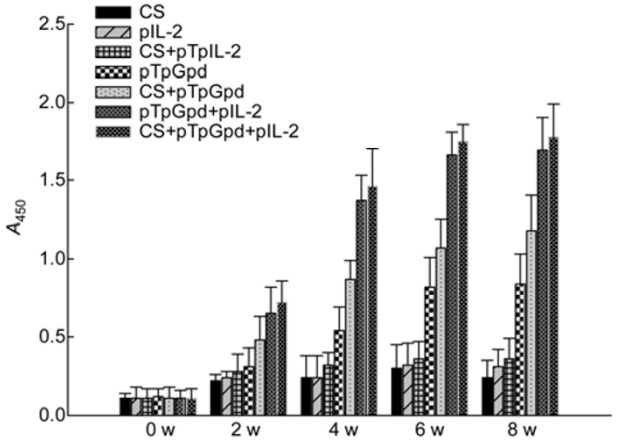

Figure 1 Absorbance values indicating IgG antibody levels induced by TpGpd vaccines adjuvanted with pIL-2 adsorbed to CS during immunization. Groups of rabbits (n=18) were immunized with DNA vaccines pTpGpd (100 μg), pTpGpd coated with CS (30 μg) (CS+pTpGpd), pTpGpd and pIL-2 (100 μg) (pTpGpd+pIL-2), pTpGpd and pIL-2 coated with CS(CS+pTpGpd+pIL-2) at 0, 2 and 4 weeks, pIL-2 adsorbed to CS-immunized rabbits(CS+pIL-2), pIL-2 alone and CS-immunized rabbits (CS) were used as negative controls. TpGpd DNA vaccines adsorbed to CS were inoculated into the left hind leg quadriceps of rabbits Blood was drawn from ear veins at 0, 2, 4, 6, and 8 weeks post-immunization. The anti-TpGpd antibody levels from sera were determined by indirect ELISA. A_{450}, optical density at 450 nm.

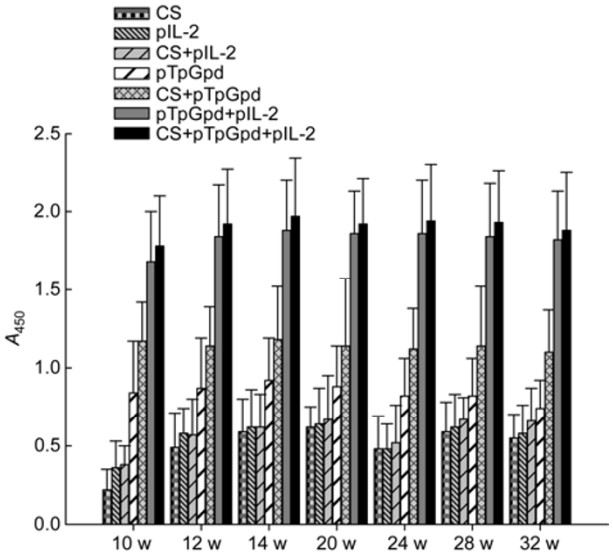

Figure 2 Absorbance values indicating IgG antibody levels induced by TpGpd vaccines adjuvanted with pIL-2 wrapped with CS during infection. The groups of rabbits are as in Figure 1. At week 10 after the first immunization, 15 of 18 rabbits in each group were challenged intradermally at eight sites on the back with 10^5 *T. pallidum* (Nichols) spirochetes. Blood was drawn from ear veins at 10 (Tp challenge), 12, 14, 20, 24, 28 and 32 weeks post-immunization. The anti-TpGpd antibody levels from sera were determined by indirect ELISA. A_{450}, optical density at 450 nm.

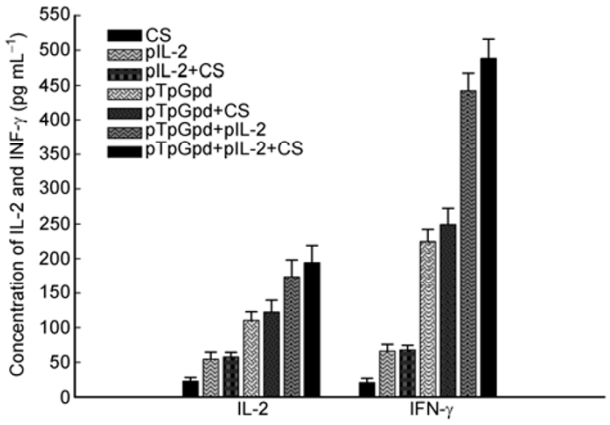

Figure 3 Levels of IL-2 and IFN-γ secreted by spleen cells in rabbits with TpGpd stimulations. The groups of rabbits are as in Figure 1. Two weeks after the final immunization (week 8), rabbits (three per group) were sacrificed and the splenocytes isolated. Splenocytes were then stimulated with Gpd at 20 μg mL^{-1} for 3 d. The experiment was repeated with similar results; values are shown as means±SE.

the IL-2 (110.3) and IFN-γ (224.8) levels, when compared with the IL-2 and IFN-γ levels of the pIL-2+CS (IL-2, 57.6; IFN-γ, 67.7), pIL-2 (IL-2, 54.4; IFN-γ, 64.2) or CS (IL-2, 22.8; IFN-γ, 20.7) control groups, respectively. Administration with pTpGpd+CS did not significantly affect the IL-2 (pTpGpd+CS, 122.2; pTpGpd, 110.3) and IFN-γ levels (pTpGpd+CS, 249.4; pTpGpd, 224.8) induced by TpGpd, while pTpGpd+pIL-2 resulted in an increase of IL-2

(pTpGpd+pIL-2, 173.4; pTpGpd, 110.3; $P<0.05$) and IFN-γ level (pTpGpd+pIL-2, 442.3; pTpGpd, 224.8; $P<0.05$) induced by TpGpd.

2.4 T-cell proliferation is stimulated by pTpGpd and pIL-2 during immunization and infection

At weeks 8, 10 (Tp challenge), 12, 14, 30, and 32 after the first immunization, rabbit spleen cells were collected and proliferation of T cells were determined by the MTT method. As shown in Table 1, pTpGpd induced significant proliferation of T cells, when compared with the levels of the pIL-2 coated with CS, pIL-2 and CS (30 μg) control groups, respectively ($P<0.05$). pTpGpd+CS alone did not significantly affect the stimulation induced by TpGpd at week 8 (pTpGpd+CS, 3.34; pTpGpd, 2.88; $P>0.05$), while pTpGpd+pIL-2 resulted in increased stimulation induced by TpGpd (pTpGpd+pIL-2, 4.52; pTpGpd, 2.88; $P<0.05$). Combination of CS vectored pTpGpd and pIL-2 led to the most elevated T-cell proliferation stimulation level (4.96, $P<0.05$). During the infection period (0–168 d), all vaccine groups maintained stable and significantly higher levels of T-cell proliferation stimulation compared with the respective negative controls (pIL-2+CS, pIL-2 alone and CS groups; $P<0.05$).

2.5 Determination of the protective effect of pTpGpd based DNA vaccines from Tp infection

At week 10 after the first immunization, 15 of the 18 rabbits in each group were challenged with the Tp Nichols strain. Skin lesions were observed and measured in the infected sites every three days. Lesion spirals were investigated by dark-field and silver staining methods 21 days after Tp infection. As shown in Table 2, ratios of positive skin lesions and ulcer lesions in groups immunized with pTpGpd were significantly lower than those of the pIL-2+CS, pIL-2, or CS groups ($P<0.001$), demonstrating that pTpGpd elicits significant protection against the Tp challenge. Co-administration of pIL-2 with pTpGpd conferred superior protection (the positive skin lesions and ulcer lesions observed were 11.67% and 10.00%, respectively) to CS vectored pTpGpd (the positive skin lesions and ulcer lesions observed were 25.00% and 40.00%, respectively). In addition, the presence of both pIL-2 and CS with pTpGpd led to the greatest level of protection whereby the positive skin lesions and ulcer lesions observed were 8.33% and 4.17%, respectively ($P<0.05$).

The lesions in rabbits immunized with CS+pTpGpd+pIL-2 were the smallest, least indurated, and quickest to heal (42 d) (Figure 4); intermediate results were seen with the lesions on rabbits immunized with CS+pTpGpd or pTpGpd+

Table 1 Stimulation index (SI) of spleen cells at corresponding times during immunization and infection[a)]

Group	Days (weeks)					
	(8 w)[b] (n=3)[c]	0 d[a] (10 w) (n=3)	14 d (12 w) (n=3)	28 d (14 w) (n=3)	140 d (30 w) (n=3)	168 d (32 w) (n=3)
CS	0.65±0.28[d]	0.57±0.23	1.21±0.42	1.44±0.45	1.23±0.45	1.52±0.37
pIL-2	0.85±0.24	0.83±0.33	1.22±0.44	1.56±0.54	1.27±0.46	1.54±0.37
pIL-2+CS	0.84±0.36	0.88±0.32	1.28±0.43	1.68±0.57	1.32±0.36	1.58±0.44
pTpGpd	2.88±0.21	2.84±0.32	3.14±0.46	3.47±0.52	3.24±0.34	3.32±0.44
pTpGpd+CS	3.34±0.46	3.28±0.55	3.58±0.63	3.88±0.38	3.62±0.44	3.60±0.47
pTpGpd+pIL-2	4.52±0.47	4.54±0.56	4.84±0.49	4.98±0.65	4.73±0.38	4.68±0.47
pTpGpd+pIL-2+CS	4.86±0.38	4.82±0.46	4.97±0.53	5.17±0.55	4.92±0.47	4.88±0.62

a) The groups are as in Figure 1. a, The 0, 14, 28, 140 and 168 days post-infection; b, at week 8, 10, 12, 14, 30 and 32 after the first immunization; c, three rabbits per group were sacrificed and the splenocytes were isolated for test at different times; d, mean±SE.

Table 2 Ratio of DF-positive skin lesion number to the total lesion number and ratio of ulcer lesion number to the total lesion number in each group after Tp challenge[a)]

Group	Rabbit number	Number of DF-positive lesions/total number of lesions (%)	Number of ulcerative lesions/total number of lesions (%)
CS	15	115/120 (95.83)	112/120 (93.33)
pIL-2	15	107/120 (89.17)	104/120 (86.67)
pIL-2 +CS	15	105/120 (87.50)	100/120 (83.33)
pTpGpd	15	30/120 (25.00)	45/120 (37.50)
pTpGpd+CS	15	30/120 (25.00)	48/120 (40.00)
pTpGpd+pIL-2	15	14/120 (11.67)	12/120 (10.00)
pTpGpd+IL-2+CS	15	10/120 (8.33)	5/120 (4.17)

a) The groups are as in Figure 1. DF, dark-field.

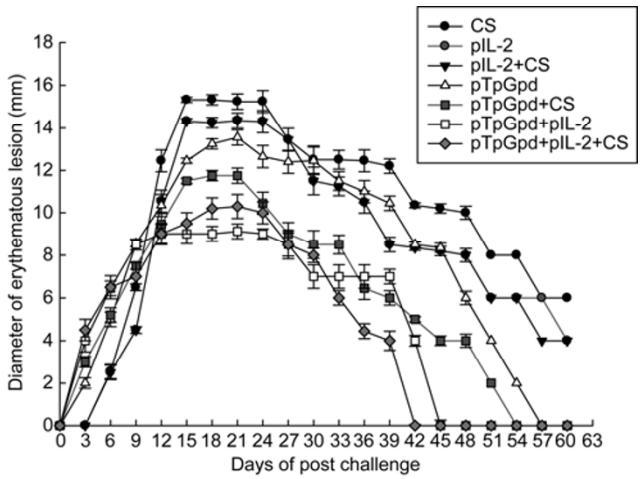

Figure 4 Measurements of erythematous lesions from groups of 15 rabbits infected with *T. pallidum* post-challenge (week 10) at three-day intervals (0–60 d). The groups of rabbits are as in Figure 1. At week 10 after pTpGpd DNA vaccination, 15 of 18 rabbits in each group were challenged intradermally at eight sites on the back with 10^5 *T. pallidum* (Nichols) spirochetes. Each bar represents the mean±SE of diameters of erythematous lesions from 15 rabbits per group.

pIL-2; and the immunized control rabbits had the largest, most ulcerative and indurated lesions that did not heal during the 60-day observation period. Erythema at the challenge sites was apparent on rabbits immunized with DNA vaccines within 2–3 days post-challenge, whereas those immunized with CS+ pIL-2/CS control rabbits did not develop any signs at the challenge sites until approximately 6–9 days after the challenge.

3 Discussion

A significant drawback of DNA vaccines is its nature as a bacterial plasmid, which is able to express its protein product in eukaryotic cells but cannot replicate. The efficiency of a DNA vaccine is limited by the uptake of the plasmid passively into tissue cells or other inflammatory cells. Thus, large doses of plasmid DNA were needed in order to obtain an effective immune response. CS is the only natural polymer of alkaline amino-polysaccharide that is deacetylated from chitin, with good biological absorption and compatibility. A protective effect of different molecular weights and concentrations of chitosan particles has been demonstrated against the destruction of foreign DNA by intracellular lysosomes [12]. Li et al. [1] showed that the CS wrapped gene significantly improved gene transfection efficiency and prolonged the expression of DNA in mammalian cells. Additionally, CS nanoparticles of cytokine genes significantly improved the immune effect in mice through oral or subcutaneous immunization [3–5].

Early studies indicated that CS nanoparticles could be used as a gene transduction vector and pIL-2 as adjuvant, in order to enhance immune response of pTp92 single-gene DNA vaccine [10]. This previous study showed that pIL-2 gene adjuvant could significantly enhance the immune response effects of pTp92 single-gene DNA vaccine; however, the adjuvant effect of CS nanoparticles was not obvious. Building upon this previous study, the current study further validated the immune response of TpGpd DNA vaccine adjuvanted with IL-2 coated with CS compared with Tp92 DNA vaccine adjuvanted with IL-2 and CS. At the same time, we also assessed the immune response of TpGpd DNA adjuvanted with IL-2 and coated with CS at different periods before and after Tp infect rabbit skin to evaluate protective efficacy of anti-Tp skin infections. It was shown that compared with vaccine group (pTpGpd+pIL-2 combined vaccine group, pTpGpd vaccine group), the vaccine coated with CS nanoparticles group (CS+pTpGpd+pIL-2 combined vaccine group and CS+pTpGpd vaccine group) showed slightly increased TpGpd specific antibody level, splenocyte proliferation level and IL-2 cytokine stimulation level, despite not being statistically significant.

The current study revealed that CS nanoparticles promote the humoral and cellular immunity of the pTpGpd vaccine. This further supports our previous reports [10], indicating that CS nanoparticles increase exogenous genes into the cells, but there was no positive relationship between the number of exogenous genes in the cells and intracellular gene expression [13]. It was shown that aside from the stability of the exogenous gene (including protection from the DNA enzyme degradation *in vivo*), CS is important for efficient translocation to nucleus [14]. Moreover, the size and quantity of electric charge of CS-DNA complexes also contribute to translocation to the nucleus [15]. It has been shown that the bigger the size of transfected DNA, the more difficult translocation to the nucleus is. In this study, we coated a DNA vaccine with CS nanoparticles with an average size around 300–500 nm, and this small size may have led to the successful adjuvant effect.

Given our previous report [16] and results of the pTpGpd+pIL-2 combined vaccine group and pTpGpd vaccine group, a vaccine with (pcD/Gpd-IL-2) or without (pTpGpd+pIL-2) fusion of IL-2 and TpGpdhad was improved immune response during infection. Analysis of skin lesions, ulcer incidence and swelling during Tp skin infection (0–60 d) indicated that the anti-Tp protection of CS+pTpGpd+pIL-2 combined vaccine group showed the best results. However, compared with the vaccine group (pTpGpd+pIL-2 combined vaccine group and the pTpGpd vaccine group) and vaccine wrapped by CS nanoparticles group (CS+pTpGpd+pIL-2 combined vaccine group and CS+pTpGpd vaccine group), CS nanoparticles only slightly promoted the immune response without statistical significance.

In summary, this study demonstrates that all vaccine groups induced humoral and cellular immunity by intramuscular injection. Additionally, the pIL-2 adjuvant significantly enhanced the pTpGpd vaccine to induce the produc-

tion of higher, longer term and stable levels of antibodies and spleen cell proliferation during immunity and infection. However, vaccines coated with CS nanoparticles did not show a significant promotion of the immune response as in other reports. For the protection of skin infection during early stage syphilis infection, the combined regimen of pTpGpd, CS, and pIL-2 achieved the best protection. However, further studies are needed to determine methods for complete protection and to address the insufficiency effect of CS.

This work was supported by the Foundation of Hunan Province Science and Technology Department, China (2010FJ2008, 2012TT2006), the Natural Science Foundation of Hunan Province Science and Technology Department, China (11JJ9023) and the Foundation of Hunan Province Education Department, China (11B107).

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