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A recombinant plasmid containing CpG motifs as a novel vaccine adjuvant for immune protection against herpes simplex virus 2

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Abstract. The aim of the present study was to evaluate the efficacy of a herpes simplex virus type 2 (HSV-2) DNA vaccine co-immunized with a plasmid adjuvant containing CpG motifs. A novel eukaryotic expression plasmid vector containing kanamycin resistance gene (pcDNA3Kan) was acquired from pET-28a(+) and pcDNA3 plasmids. A gene encoding full length HSV-2 glycoprotein D (gD) was amplified from the pcDNA3-gD plasmid, which was cloned into pcDNA3Kan resulting in the construction of the recombinant plasmid pcDNA3Kan-gD (pgD). A DNA segment containing 8 CpG motifs was synthesized, and cloned into pcDNA3Kan, resulting in the recombinant plasmid pcDNA3Kan-CpG (pCpG). Mice were co-inoculated with pgD (used as a DNA vaccine) and pCpG (used as an adjuvant) by bilateral intramuscular injection. Mice inoculated with pgD+pCpG showed higher titers of antibodies than those inoculated with the DNA vaccine alone ($P<0.05$). In addition, mice inoculated with pgD+pCpG showed the highest percentage of $CD4^+$ T cells in the blood of all the groups ($P<0.05$). Thus, the present study demonstrated that pCpG could stimulate the HSV-2 DNA vaccine to induce a stronger cell-mediated immune response than the DNA vaccine alone. The aim of the present study was to evaluate the efficacy of a HSV-2 DNA vaccine (pgD) co-immunized with a plasmid adjuvant containing CpG motifs (pCpG). Whether the pCpG would be able to stimulate the pgD to induce a stronger immune response compared with pgD alone.

Introduction

Herpes simplex virus type 2 (HSV-2) infection is a common infectious disease in humans. HSV-2 generally causes genital infections. The standard treatment of genital herpes is dependent on guanosine analogues. Despite the efficacy of the treatment, there is still no cure to prevent recurrence. Over the last few decades, considerable efforts have been made to develop a vaccine against genital herpes. Several candidate vaccines have been investigated experimentally in different genital HSV model systems. It is considered that inoculation with a HSV vaccine to promote an immune reaction against HSV is an ideal method to prevent and treat HSV infection.

Previous studies have demonstrated that humoral (1,2) and cellular immune responses (3) are responsible for protective immunity against HSV infection. During viral infection, neutralizing antibodies can inactivate free viral particles, but are unable to inhibit intracellular infection. Furthermore, results indicated that antibodies at the site of mucosal infection were inadequate to prevent invasion (4), which indicated cellular immunity as the main factor involved in the control of HSV infection (5,6). Traditional candidate vaccines such as those containing live attenuated or killed viruses have been shown to confer protective immunity; however, due to safety concerns, the application of these vaccines has been precluded in humans only a few have been assessed in clinical trials (7). HSV recombinant glycoprotein vaccines and subunit vaccines have shown the capacity to stimulate antigen-specific immune responses. However, they were not able to induce efficient cell-mediated immunity, and displayed poor protective immunity in animal models (8). DNA vaccines are the third generation of vaccines following vaccines containing whole pathogen bodies and recombinative protein by gene engineering. DNA vaccines have characteristics of the safety of recombinative sub-unit vaccines and the efficiency of live pathogen vaccines. They can induce humoral and cellular immune responses.

CpG oligodeoxynucleotide (ODN) is a synthetic ODN containing unmethylated cytidine-phosphate-guanosine with appropriate flanking regions (CpG motif). Several recent studies have demonstrated the potent adjuvant activity of CpG ODN in the induction of systemic and mucosal immune responses (9,10). In particular, animal challenge models showed that protective immunity can be accelerated and enhanced by co-administering CpG DNA with vaccines (11). Ongoing clinical studies indicate that CpG ODNs are safe and well-tolerated when administered as adjuvants to humans, and in certain cases they have been shown to increase vaccine-induced immune responses (11).

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In the present study, a novel eukaryotic expression plasmid vector was constructed containing the kan[r] gene from pET-28a(+) and pcDNA3 plasmids. A gene encoding full length HSV-2 gD was cloned into the eukaryotic expression plasmid vector (pgD). A DNA segment containing 8 CpG motifs was also synthesized and cloned into a eukaryotic expression plasmid vector (pCpG). Mice were co-inoculated with pgD and pCpG by bilateral intramuscular injection into the rear leg and the immune response was observed.

Materials and methods

Ethics statement. The study was approved by the Ethics Committee of Zhejiang Academy of Medical Sciences (Hangzhou, China).

Mice. Female Balb/c mice (n=48; weight, 20±2 g; age, ~7 weeks) were provided and bred by the Experimental Animal Center, Zhejiang Academy of Medical Sciences and maintained in a pathogen-free animal facility. Balb/c mice were maintained at 20±2˚C, humidity 55±5% with a 12-h dark:light cycle. They were given food pellets (Zhejiang Academy of Medical Sciences, Hangzhou, China) and water *ad libitum*. Adequate measures were taken to minimize animal discomfort.

Virus. HSV-2 strain *Sav*, obtained from the National Institute for Viral Disease Control and Prevention (Beijing, China), was grown in Vero cells (Institute of Biochemistry and Cell Biology, Shanghai, China) and was stored at -80˚C. Virus was routinely prepared by infection of almost confluent Vero cells (Institute of Biochemistry and Cell Biology) with a multiplicity of infection of 0.1 at 37˚C in a small volume of high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) without serum. After 1 hr, virus inoculum was removed and cultures were re-fed with high glucose DMEM. Incubation was continued until cytopathic effect was extensive; usually for 24-48 hr. Before use, the virus particles were released from the cells by freezing and thawing cycles and cellular debris was removed by centrifugation (640 x g for 10 min at 4˚C). The method of titration was a plaque assay in Vero cells and results were expressed as PFU/ml (12).

Bacterial strains and plasmids. *E. coli* DH5a and *E. coli* Tg1 (Beijing ComWin Biotech Co., Ltd., Beijing, China) were used as hosts during the cloning experiments and for propagation of the plasmids. Bacterial strains were grown at 37˚C in Luria Bertani (LB) media, supplemented with ampicillin or kanamycin when required. pCDNA3 (Invitrogen; Thermo Fisher Scientific Inc.), pET-28a(+) (Novagen, EMD Millipore, Billerica, MA, USA) and pcDNA3-gD (HSV-2 glycoprotein D gene was inserted) plasmids [pcDNA3-gD (HSV-2 glycoprotein D gene was inserted)] were constructed by the Institute of Bioengineering, Zhejiang Academy of Medical Sciences (13). Plasmids were amplified by *E. coli* DH5a, purified by the pure plasmid mini kit (Beijing ComWin Biotech Co., Ltd., Beijing, China), and sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Constructing a new eukaryotic expression plasmid vector (pcDNA3Kan) containing the kan[r] gene from plasmid pET-28a(+) and pcDNA3. The kan[r] gene was amplified from the pET-28a(+) plasmid by polymerase chain reaction according to standard protocol (14) using the following primers: Forward, 5'-GCC**CTTAAG**ATGAGCCATATTCAACGG-3' (bold section indicates restriction enzyme site of *Afl*II) and reverse, 5'-AGT**CCGCGG**TTAGAAAAACTCATCGAG-3' (bold section indicates restriction enzyme site of *Sac*II). The whole sequence of the pcDNA3 plasmid except the Amp[+] gene was amplified from pcDNA3 by PCR according to standard protocols using the following primers: Forward, 5'-GCG**GCT TAAG**ACTCTTCCTTTTTCAAT-3' (bold section indicates restriction enzyme site of *Afl*II) and reverse, 5'-ATA**CCG CGG**CTGTCAGACCAAGTTTAC-3' (bold section indicates restriction enzyme site of *Sac*II). The two PCR products that were digested with *Afl*II and *Sac*II were sealed together by T4 DNA ligase and transformed into *E. coli* DH5a. After selection with kanamycin, a new eukaryotic expression plasmid vector, pcDNA3Kan, was obtained (Fig. 1). The new eukaryotic expression plasmid vector pcDNA3Kan was identified by restriction enzyme *Bgl*II/*Xho*I or *Ssp*I digestion analysis.

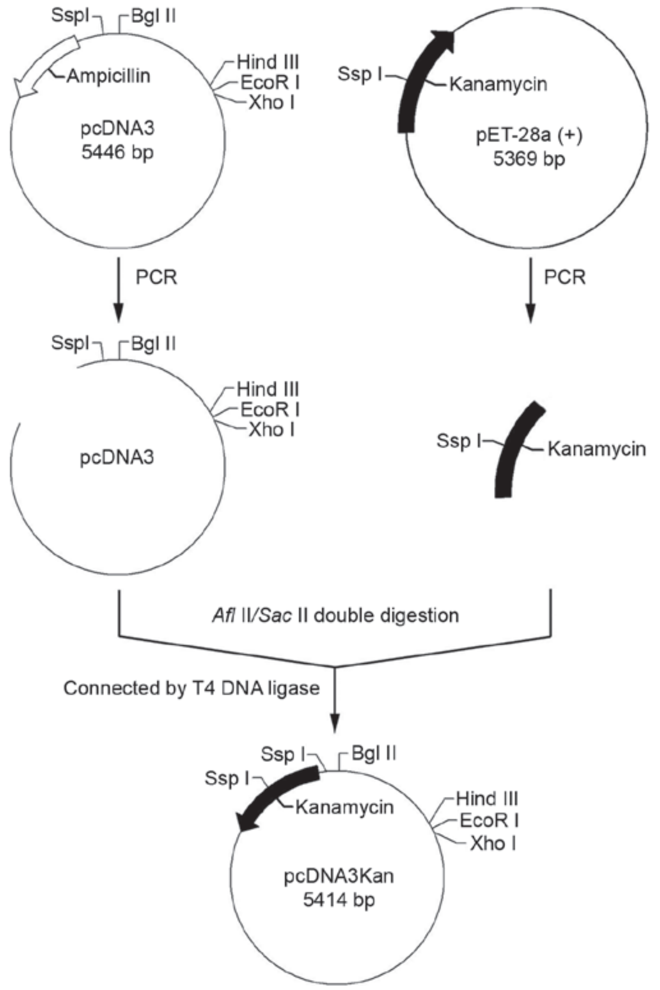

Figure 1. Structure and formation of eukaryotic expression plasmid vector pcDNA3Kan.

Cloning of gD into the eukaryotic expression vector pcDNA3Kan. A gene encoding full length HSV-2 gD was amplified from the pcDNA3-gD plasmid by polymerase

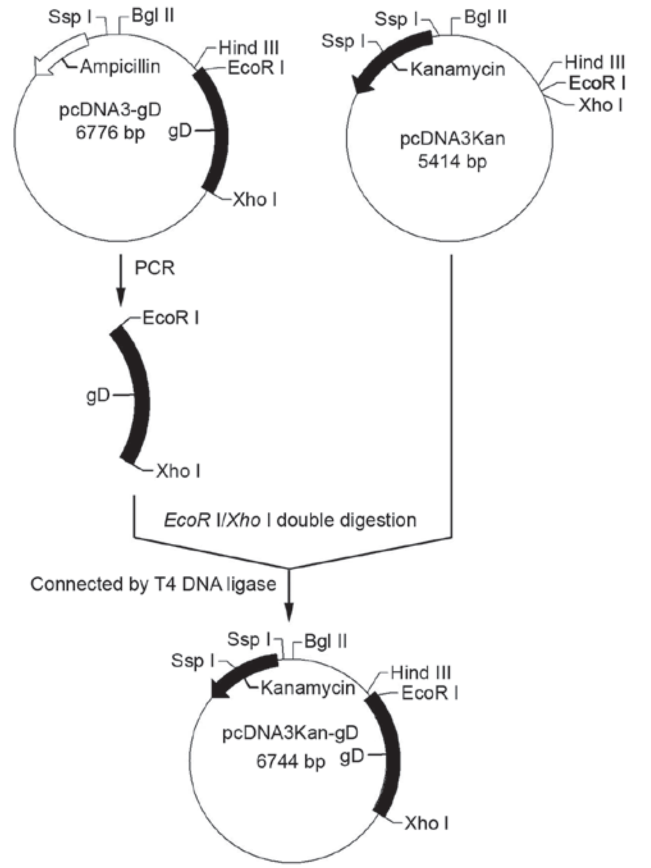

Figure 2. Structure and formation of eukaryotic expression plasmid pcDNA3Kan-gD (pgD).

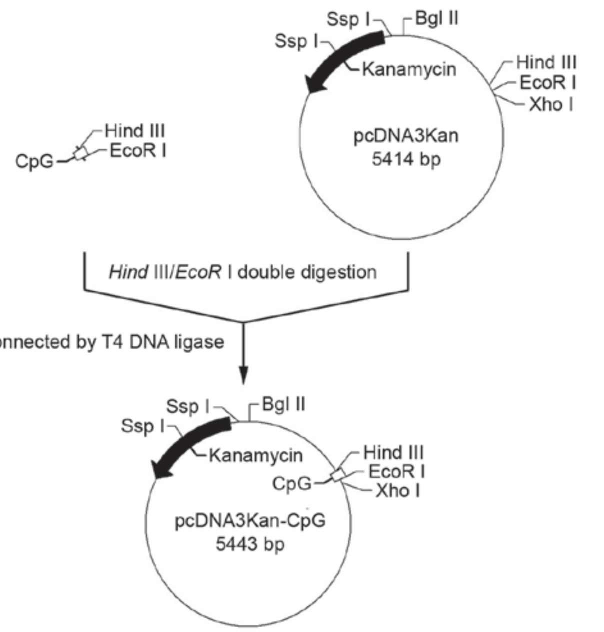

Figure 3. Structure and formation of eukaryotic expression plasmid vector pcDNA3Kan-CpG (pCpG).

chain reaction using the following primers: Forward, 5'-ATC **GAATTC**AACCACTAGTCGCCG-3' (bold section indicatesrestriction enzyme site of *Eco*RI) and reverse, 5'-CG**C TCGAG**ACTCCCTTTATGC-3' (bold section indicates

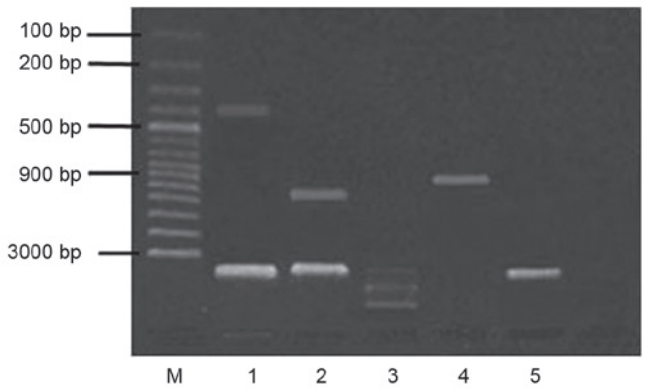

Figure 4. Restriction pattern of the recombinant plasmid vector pcDNA3Kan. The recombinant plasmid vector pcDNA3Kan was detected by restriction enzyme analysis and polymerase chain reaction. M, DNA Marker; lane 1, pcDNA3Kan/*SspI* (~380 bp); lane 2, pcDNA3Kan/*Bgl*II and *Xho*I (~960 bp); lane 3, pcDNA3Kan; lane 4, the polymerase chain reaction product of Kan^r gene (~810 bp); and lane 5, the PCR product of the longer segment in pcDNA3Kan (~4,580 bp).

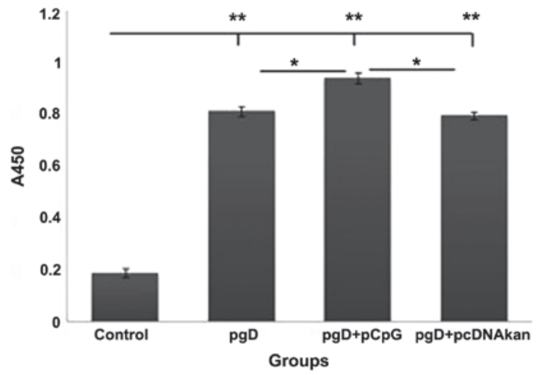

Figure 5. Serum anti-HSV-2-gD specific total IgG exhibited different levels in various groups after inoculation. Mice showed IgG level changes at week 8 post-inoculation. The IgG levels were significantly increased in the pgD+pCpG group compared with other groups. *P<0.05, **P<0.001.

restriction enzyme site of *Xho*I). The PCR product and plasmid pcDNA3Kan were digested with *Eco*RI and *Xho*I. The two DNA strands were then joined at their sticky ends and were sealed together by T4 DNA ligase, to form the recombinant plasmid pcDNA3Kan-gD (pgD) (Fig. 2). The plasmids pgD and pCpG was sequenced by Sangon Biotech (Shanghai) Co. Ltd.

Constructing a new DNA vaccine adjuvant pcDNA3Kan-CpG containing CpG motifs. Two ssDNA segments containing 8 CpG motifs (the sequence of CpG motif was according to ODN 1826): Forward, 5'-**AGCTT** TCCAT GACGTT CCT GACGTT CCT GACGTT CCT GACGTT CCT GACGTT CCTC GTCGTT TT GTCGTT TT GTCGTT G-3' (bold section indicates restriction enzyme site of *Hin*dIII; underline section indicates CpG motif) and reverse, 5'-**AATTC** AACGAC AA AACGAC AA AACGAC GAGG AACGTC AGG AACGTC AGG AACGTC AGG AACGTC AGG AACGTC ATGGA A-3' (bold section indicates restriction enzyme site of *Eco*RI; underline section indicates CpG motif) were synthesized. The two ssDNA segments were integrated

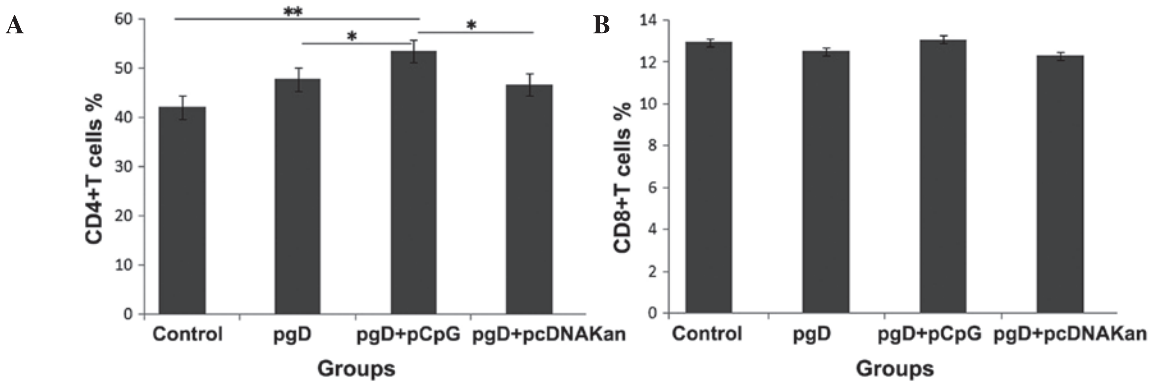

Figure 6. Flow cytometry detection of $CD4^+$ and $CD8^+$ T cell subsets in the peripheral blood of mice. (A) The percentage concentration of $CD4^+$ T cells; (B) the percentage concentration of $CD8^+$ T cells. The subset $CD4^+$ T cells significantly increased in the pgD+pCpG group compared with other groups (P<0.05). *P<0.05 and **P<0.001. No significant difference was observed in groups of subset $CD8^+$ T cells.

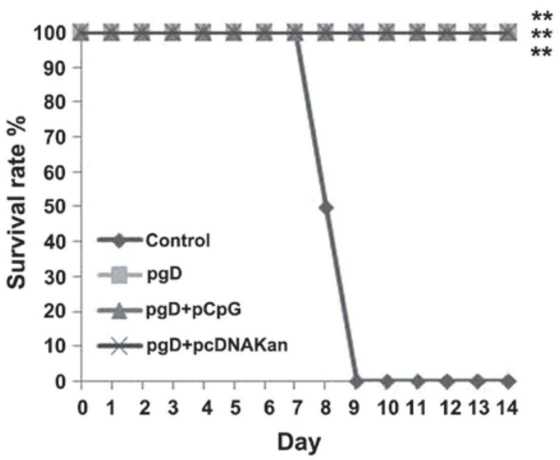

Figure 7. Virus challenge demonstrated the protection of all groups against HSV-2 challenge. **P<0.001, compared with control. Mice were challenged with a lethal dose of HSV-2 strain *Sav*. The pgD, pgD+pCpG and pgD+pcDNA3Kan groups showed complete protection compared with the control group.

into one DNA double-stranded segment and cloned into the pcDNA3Kan eukaryotic expression plasmid vector using *Hin*dIII and *Eco*RI restriction enzymes, to form a recombinant pcDNA3Kan-CpG (pCpG) plasmid (Fig. 3). The plasmid pCpG was then sequenced.

Immunization and sample collection. Forty-eight mice were divided into the following four groups, with 12 in each group: i) Control, inoculated intraperitoneally with 100 μg pcDNA3Kan; ii) pgD, inoculated intraperitoneally with 100 μg pgD; iii) pgD+pCpG, inoculated intraperitoneally with 100 μg pgD and 30 μg pCpG; and iv) pgD+pcDNA3Kan, inoculated intraperitoneally with 100 μg pgD and 30 μg pcDNA3Kan. All groups were inoculated every 3 weeks for 6 weeks. DNA and adjuvant were all dissolved in normal saline with a final volume of 100 μl. Blood samples (0.5 ml per mouse) were collected at day 14 after each inoculation and divided it into two; one for use in flow cytometry and the other was left standing in room temperature. After standing for 1 h, blood sample were divided into upper and lower layers, the upper of which was the serum and was collected for use in enzyme linked immunosorbent assay (ELISA).

ELISA of antibodies. Serum specimens were assessed for IgG antibodies to HSV-2 using an indirect ELISA method. Using recombinant HSV-2 gD protein (produced in the Institute of Bioengineering, Zhejiang Academy of Medical Sciences) according to a previously described protocol by Zhou *et al* (15) and affinity-purified polyclonal goat anti-mouse IgG labeled with horseradish peroxidase (HRP; 1:4,000; cat no. 80U00120; Beijing Dingguochangsheng Biotech Co., Ltd., Beijing, China) as antigen and secondary antibody. Absorbance was determined at 450 nm using a Multiskan MK3 (Thermo Fisher Scientific, Inc.).

$CD4^+$ and $CD8^+$ cell subset detection in peripheral blood by flow cytometry. $CD4^+$ and $CD8^+$ cell subset levels were detected in peripheral blood samples by flow cytometry (16) (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Virus challenge. Karber's method was used to test the lethal dose $(LD)_{50}$ of HSV-2 strain *Sav* in Balb/c mice by intraperitoneal injection. All 4 groups of mice were intraperitoneally injected with 50 LD_{50} (50/50% lethal dose) HSV-2 strain *Sav* at days 21 after the third inoculation. Adverse reactions and the number of fatalities were observed daily in the 4 groups of mice after HSV-2 challenge and the results were recorded. All surviving mice were sacrificed at day 14 after HSV-2 challenge.

Statistical analysis. Statistical differences for antibody titers, T lymphocyte assays and the duration of survival were determined using one-way analysis of variance followed by a Bonferroni correction test using GraphPad Prism (version 4; San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference. All IgG levels and the percentage of $CD4^+$ or $CD8^+$ T cells are presented as the mean ± standard deviation.

Results

Identification of plasmid vector pcDNA3Kan by restriction enzymes. Based on the gene content of pcDNA3Kan, there are two consensus restriction site sequences (*Ssp*I) at ~20 bp upstream of the kan^r insertion site and ~360 bp in the kan^r

gene. pcDNA3Kan treated with *Ssp*I showed a 380 bp fragment in agarose gel electrophoresis (Fig. 4). The pcDNA3Kan digested with *Bgl*II (existed upstream of the kan^r insertion site) and *Xho*I (existed at multiple cloning sites) showed a 960 bp fragment in agarose gel electrophoresis. Thus, the sequences of pcDNA3Kan were interconnected successfully. The *E. coli* Tg1 cells transfected with pcDNA3Kan grew well on LB medium supplemented with 50 μg/ml kanamycin. Therefore, the eukaryotic expression vector containing the kan^r and gD genes was successfully constructed.

Identification of the recombinant plasmids pgD and pCpG by DNA sequencing. The gD gene in the pgD recombinant plasmids was successfully cloned with the correct open reading fragment. The fragment containing 8 CpG motifs in the recombinant plasmid pCpG had the same DNA sequence as that designed.

Enhancement of the anti-HSV-2-gD titer by pgG+pCpG. Serum anti-HSV-2-gD specific total IgG was assessed by ELISA. As shown in Fig. 5, IgG levels were significantly increased in the pgD+pCpG group, compared with the pgD and pgD+pcDNA3Kan groups (P<0.05), at week 8 post-inoculation. The levels in the control group were significantly reduced compared with the remaining groups (P<0.001).

Difference in CD4$^+$ and CD8$^+$ T cells in each group. To determine the importance of these T cell subsets in the protection of pgD-immune mice against infection with HSV-2, CD4$^+$ and CD8$^+$ T cells were analyzed from all groups prior to virus challenge. As shown in Fig. 6, the percentage of CD8$^+$ T cells from the pgD+pCpG group was only marginally higher than in other groups. However, the percentage of CD4$^+$ T cells in the pgD+pCpG group was significantly higher than in the other groups (P<0.05), particularly compared with the control-treated pcDNA3Kan group (P<0.001).

Protection against HSV-2 challenge. To evaluate the level of protection conferred by immunization, mice were challenged with a lethal dose of 1x10^5 PFU of HSV-2, 3 weeks following the last immunization. Mice in the pgD, pgD+pCpG and pgD+pcDNA3Kan groups showed significantly higher survival rates compared with the control group (Fig. 7).

Discussion

Mice were intramuscularly immunized with eukaryotic expression plasmids encoding gD to induce protective immune responses (17-19). Certain immune adjuvants such as interleukin-12, chemokines, cytokines and the *E. coli* heat labile enterotoxin can enhance the immunogenicity of the HSV DNA vaccine (20-23). At present, there is no way to provide complete immunity against HSV in mice, and there is no appropriate HSV vaccine for humans.

The present study designed and constructed a recombinant plasmid pCpG containing 8 CpG motifs. These were investigated as immune adjuvants for a DNA vaccine. According to the results, the recombinant plasmid pCpG in combination with the DNA vaccine could protect mice infected with lethal doses of HSV-2 virus. The HSV-2 antigen specific antibodies were detected by ELISA, and the IgG levels were moderately increased in the pgD+pCpG group, compared with other groups, at week 8 post-inoculation. Peripheral T-lymphocyte subsets were examined by flow cytometric analysis, and the percentage of CD4$^+$ T cells from the pgD+pCpG group was significantly increased compared with the other groups (P<0.05). Test results proved that these mice could induce more notable cellular immunity compared with pgD+pcDNA3Kan and pgD alone in mice.

The experimental results demonstrated that cloning CpG motifs into plasmid DNA is an effective way to apply CpG motifs as adjuvants for DNA vaccines. Sato *et al* (24) demonstrated that the characteristics of CpG existed in plasmid DNA. Human monocytes transfected with plasmid DNA containing CpG motifs, transcribed large amounts of interferon-α, interferon-β, and interleukin-12. This type of immune response is highly important.

In the present study, a new recombinant plasmid pCpG based on CpG motifs was constructed. pCpG could significantly improve cell-mediated immunity induced by the HSV-2 DNA vaccine. Thus, pCpG has shown great potential as an adjuvant for the HSV-2 DNA vaccine, and may also be used for other DNA vaccines.

Acknowledgements

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