

Construction of an Immunostimulatory Plasmid, pUCpGs10, and Research on its Immune Adjuvant Effect

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Abstract In order to overcome the instability of CpG ODN *in vivo*, sequence diversity, and individual differences, eleven CpG ODN fragments were meticulously selected and linked to form a Multi-CpG, which were repeatedly inserted into the cloning vector pUC19 for constructing the recombinant plasmid pUCpGs10 containing ten of Multi-CpG. Using the multi-genotype HCV E1 and multi-epitope complex HCV-T as immunogens, and

plasmid pUCpGs10 as the immune adjuvant, Balb/c mice were immunized through nasal and subcutaneous immunization. Strong-specific humoral and cellular immune response were induced, which can obviously inhibit the growth of homograft expressing HCV antigen. The immune adjuvant effect of pUCpGs10 closely matched that of Freund's complete adjuvant. The plasmid pUCpGs10 can significantly improve IgA content in serum and different mucosal extract and systematical T-cell response via intranasal immunization. In conclusions, the newly constructed immunostimulatory plasmid pUCpGs10 is able to effectively activate the humoral and cellular immune activity, and possesses activation on mucosal immune response.

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Introduction

The advent of vaccines was an epical event in human history. As an example of the importance of vaccines, the application of a vaccine against smallpox led the World Health Organization (WHO) to announce that smallpox had been eradicated in 1980. However, there are some difficulties in developing vaccines for some chronic viral infectious diseases, such as Human immunodeficiency virus (HIV) and Hepatitis C virus (HCV). Although the reasons for these difficulties are numerous, one important reason is that an effective immune response can hardly be generated in the body against the vaccine immunogen due to the viral invasion of the immune system [1, 2], as well as immune inhibition of virus-encoded protein [3–5]. To overcome these problems, the selection of an effective

immunologic adjuvant seems to be important. Currently, the aluminum adjuvant commonly used in human vaccines mainly functions by stimulating the immune response to Th2, which is insufficient for a viral vaccine. As such, new breakthroughs in the adjuvant research are needed.

In recent years, much attention has focused on oligodeoxynucleotide motif-containing unmethylated-CG-2-deoxy-nucleotide (CpG ODN), which can accelerate the secretion of a variety of pro-inflammatory cytokines and chemokines in immunocytes and improve the expression of major histocompatibility antigen (MHC) and immune costimulatory ligands (CD80 and CD86) in antigen presenting cells [6, 7]. The former offers a direct killing effect on pathogenic microorganisms and the latter advances the Th1-specific immune response in the body against invasive pathogenic microorganisms and stimulates a transformation from the Th2-specific immune response to the Th1-specific response. As a result, CpG ODN is considered a new potential immune adjuvant [8–10]. Notably, recent studies have shown that CpG ODN is also a good adjuvant of mucosal immunity [11–13], which is the first line of the body's defense system. Recent literature about CpG ODN as an adjuvant reports that synthetic CpG ODN fragments are always employed, and thio-modified bases are used to enhance stability *in vivo*, which can increase the biological activity but also the complexity of the biological effect due to the introduction of unnatural ingredients [14]. In addition, not only CpG ODN itself has multiple forms (including A, B, C categories), but even the effect of the one type of CpG ODN can show heterogeneity between species and individuals [15]. Accordingly, a CpG ODN synthesized via small fragments often does not produce satisfactory effects in each individual. In order to overcome the instability of CpG ODN *in vivo*, sequence diversity, and individual differences, we designed a recombinant plasmid, pUCpGs10, containing a number of different CpG ODNs and used as an immune adjuvant. We evaluated pUCpGs10's stimulation of cellular immunity, humoral immunity and mucosal immunity activity, laying a foundation for further research and applications of plasmid pUCpGs10.

Materials and Methods

Ethics Statement

All animal experimental protocols of the study are in accordance with the national guidelines for the use of animals in scientific research. It is also approved by Animal Care and Use Committee of Beijing Institute of Basic Medical Sciences, with the approval number BMS-090723.

Screening of CpG ODN

We compared experimental research data related to CpG ODN [16–23], selected several CpG ODN fragments with strong immune activation, and selected the fragments mainly used in human experiment but also taking into account the fragments that have shown good results in animal experiments. We mostly chose natural sequences existing in microbial genomes and adopted some synthetic fragment sequences with strong immune activation as well.

Construction and Purification of Plasmid pUCpGs10

The selected CpG ODNs were connected head to tail to form a DNA sequence containing a number of -CG-dinucleotides, which was named “Multi-CpG” and considered an immune activation unit. Multi-CpG primers were segmentally designed and chemically synthesized via the overlapping PCR method. We selected the high-copy plasmid vector pUC19 (Takara Biotechnology) as the cloning vector, which linked with the Multi-CpG fragment to obtain plasmid pUCpGs01. Subsequently, we designed primers to amplify Multi-CpG⁺ that contained the Multi-CpG sequence and some vector sequence from plasmid pUCpGs01. By utilizing the advantage of complementary restriction sites (*Xba* I and *Spe* I), the amplified Multi-CpG⁺ could be inserted into pUCpGs01 repeatedly, and a series of pUCpGs containing different Multi-CpG segments were obtained, including the final constructed plasmid pUCpGs10 containing ten segments of Multi-CpG. The purification of plasmid was performed using EndoFree Plasmid Giga Kit (Qiagen GmbH Company) according to the instruction provided by the manufacturer.

Limulus Amebocyte Lysate (LAL) Assay

The endotoxin of purified plasmid was determined by LAL assay (Associates of Cape Cod, Inc.). A series of dilutions of the plasmid and buffer samples were prepared using the LRW as the diluent. One hundred microliters of appropriately diluted samples were mixed with 100 μ l of PyrotellTM solution in depyrogenated flint glass reaction tubes and incubated in a water bath for 1 h at 37 °C. A negative control containing only LRW and positive controls containing endotoxin standards were run concurrently. At the end of incubation, the tube was removed from the water bath and immediately inverted by 180°. The test is positive if a gel has formed and remains intact in the bottom of the tube after inversion. The test is considered negative if no gel has formed or if the gel that formed breaks or collapses upon inversion.

Cytokines Production of Stimulated Immune Cells

Five seven-week-old female Balb/c mice were terminated without immunization, and their spleen lymphocytes were separated using EZ-SepTM Mouse lymphocyte separation medium (Dakewe Biotech Company) under sterile conditions. The spleen lymphocytes were randomly divided into three groups and stimulated by PBS, PCR-amplified repeated double-stranded CpG-DNA (20 µg/ml) and the plasmid pUCpGs10 (20 µg/ml), respectively. The cytokines production of stimulated immune cells was examined by ELISA assay (Dakewe Biotech Company) according to the manufacturer's instructions.

Mice and Immunization

To validate and compare the effect of immunostimulatory adjuvant of pUCpGs10 and pUC19 parental plasmid primarily, using multi-type HCV-E1 complex epitope antigen [24] (50 µg/each) as immunogen, seven-week-old female Balb/c mice were randomly divided into six groups and were injected subcutaneously with (I) PBS + Freund's adjuvant (Sigma Company), (II) HCV-E1 + Freund's adjuvant, (III) HCV-E1 + Freund's adjuvant + pUC19, (IV) HCV-E1 + Freund's adjuvant + pUCpGs01, (V) HCV-E1 + Freund's adjuvant + pUCpGs10, and (VI) HCV-E1 + pUCpGs10. The dose of plasmid was 20 µg each mouse. Except where indicated, an interval of 1 week was invariably for detecting IgG antibody titers by indirect ELISA.

To further validate the effect of immunostimulatory adjuvant of pUCpGs10, taking both multi-type HCV-E1 complex epitope (50 µg/each) and multi-epitope complex HCV-T cell epitope (50 µg/each) as immunogens, seven-week-old female Balb/c mice were randomly divided into two groups: nasal immunization and subcutaneous immunization groups. Ten Balb/c mice were immunized and five of them for homograft tests. Group A, containing a PBS control group, an immunogen group and an immunogen plus pUCpGs10 (20 µg/each) group, were given intranasal immunization at 1, 5, 8, 9, and 10 weeks, respectively. Group B, containing a PBS control group, an immunogen group, an immunogen plus Freund's adjuvant group, and an immunogen plus pUCpGs10 group, were given subcutaneous immunization by injection at 1, 5, and 9 weeks, respectively.

Indirect ELISA Detection on Special Antibodies IgG and IgA

Three weeks after the first, second, and last immunizations, mice were bled and sera were collected in the first and second group for assaying IgG antibody titers by indirect ELISA. To avoid the influence of the IL-1 fragment in the

recombinant immunogen, another recombinant antigen expressed with pBVIL6/HCV-E1-PADRE (data not shown) was used for coating, in a final concentration of 4 µg/ml, overnight at 4 °C. The reactant was washed by PBST once and blocked by 2 % casein (4 h at room temperature). After discarding the sealing fluid and washing, the diluted immune serum of mice (100 µl/well) was added and placed at 37 °C for 60 min. After washing with PBST, the reactant was incubated with HRP conjugated goat anti-mouse IgG (Zhongshanjinqiao Company) at 37 °C for 30 min. The reactant was again washed by PBST, stained with TMB for 10 min, and the reaction was terminated by 2 M of sulfuric acid. The absorbency of the reactant at 450 nm was determined. Vaginal washing fluid, feces, lung lavage fluid, and lung serum were collected 1 week after every immunization from the mice in the first group. These collections were extracted and IgA antibody titers were examined as IgG except that the HRP conjugated goat anti-mouse IgA (Bethyl Corporation) were used.

Enzyme-Linked Immunospot Assay

Mice were terminated not executed 10 days after the last immunization, and their spleen lymphocytes were separated using EZ-SepTM Mouse lymphocyte separation medium (Dakewe Biotech Company) under sterile conditions. The determination of IFN-γ secreting cells was carried out according to the Enzyme-linked immunospot assay (ELISPOT) kit (Dakewe Biotech Company) instructions. In brief, the plate was incubated (37 °C, 5 % CO₂) for 36 h, and cells and medium in the wells were discarded. Adding ice-cold deionized water, cells were lysed in an ice-bath of 4 °C for 10 min, the plate was washed five times sequentially. After adding 100 µl/well of biotin-labeled antibody, the plate was incubated at 37 °C for 1 h and subsequently washed five times. Next, 100 µl/well of HRP-avidin was added, and the plate was again incubated at 37 °C for 1 h and washed five times. Finally, 100 µl/well of AEC staining solution was added, the plate was kept in a dark place at room temperature for 25 min. When the spots grew to appropriate sizes, wells were washed by deionized water to terminate the color reaction. After drying at room temperature, spot counting and statistical analysis were performed by the ELISPOT automatic reading instrument.

Subcutaneous Homograft Test

For homograft tests, including a blank control group, an intranasal immunization with pUCpGs10 adjuvant group, a subcutaneous immunization with Freund's adjuvant group and a pUCpGs10 adjuvant group, the mice was subcutaneously inoculated with SP2/0-N cells (expressing HCV/

NS3 protein, established by our laboratory [25]) (2×10^5 cells/each mouse) 10 days after the last immunization. In brief, SP2/0-N cells in logarithmic phase were harvested, and the cell density was adjusted to 2×10^6 cell/ml. A 100 μ l cell suspension was subcutaneously inoculated on the right side of each mouse's back. Through observing the growth of homograft, recording the initiation of tumor formation (the longitudinal diameter of the tumor nodule reached to 0.3 cm), regularly measuring the longitudinal and transverse diameter of the tumor, the homograft tumors were taken out surgically and weighed volume 30 days after the inoculation.

Statistical Analysis

Statistical analyses were performed using the software SPSS 12.0. $P < 0.05$ was considered as statistically significant.

Results

Selection of CpG ODN Sequence

Through a review of the literatures [16–23], 11 CpG ODN containing fragments with immune activity were selected (Table 1) and connected head to tail, forming a 237 bp DNA sequence including more than 40 -CG- dinucleotides, which was named Multi-CpG. The Multi-CpG contained A, B, and C-class CpG ODN.

Synthesis of Multi-CpG Fragment and Construction of Plasmid pUCpGs10

According to the Multi-CpG sequence, a number of primers were designed and chemically synthesized. Using these primers, the full-length sequence of Multi-CpG was synthesized by a center template PCR method through three steps. The size of PCR product of the ultimate synthesis of Multi-CpG was 237 bp.

Due to its high-copy property in transformed bacteria, plasmid pUC19 was chosen as a cloning vector. The Multi-CpG was inserted into vector pUC19 via double digestion with *EcoR* I and *Xba* I, which resulted in a plasmid, pUCpGs01 (identified by sequencing). Employing universal primers containing *Spe* I and *Hind* III restriction sites, respectively, a Multi-CpG + fragment with a segment of the vector sequences was amplified. Because *Spe* I and *Xba* I share the same sticky end, the post-digestion Multi-CpG + fragment can be inserted into plasmid pUCpGs01, which was digested with *Xba* I and *Hind* III, and produced pUCpGs02. The sites that were linked with *Xba* I and *Spe* I cannot be digested; therefore, the Multi-CpG + fragments can also be inserted into pUCpGs02 according to the above method, until obtaining plasmid pUCpGs10 containing 10 fragments of Multi-CpG (shown in Fig. 1). The purification of plasmid was performed using EndoFree Plasmid Giga Kit, and the endotoxin of purified plasmid was determined by LAL assay. The LAL assay showed that the endotoxin in the resultant plasmid was reduced to less than 0.1 EU/ml, which was safe and did not influence the results of the experiments.

The Immunostimulatory Activities of the PCR-Amplified Repeated Double-Stranded CpG-DNA and the Plasmid pUCpGs10

In order to validate the immunostimulatory activity of Multi-CpG, the cytokines production of mice spleen lymphocytes stimulated by the PCR-amplified repeated double-stranded CpG-DNA or the plasmid pUCpGs10 were examined. The results showed that two kinds of Multi-CpG stimulation resulted in significant production of IFN- γ , IL-6, and IL-10 in mice spleen lymphocytes, which were higher than those of control group. The representative data were shown in Fig. 2. At 24 h, the PCR-amplified repeated double-stranded CpG-DNA and the plasmid pUCpGs10 exhibited IFN- γ inducing activity, but the activity of the

Table 1 The selected fragments of CpG ODNs

Sequence name	Sequence	Reference
C274	TCGTCGAACGTTTCGAGATGAT	[19, 22]
BCG-A4a	ACCGATGACGTCGCCGGTGACGGCACCACG	[20]
ODN1681	ACCGATGTCGTTGCGGTGACG	[17]
ODN2395	TCGTCGTTTTTCGGCGCGGCCG	[23]
ODN1018P*	TGACTGTGAACGTTTCGAGA	[19]
ODN2006P*	TCGTTTTGTCGTT	[21]
AACGTC-30	ACCGATAACGTCGCCGGTGACGGCACCACG	[16]
MB-4531	AGCAGCGTTTCGTGTCGGCCT	[18]
MB-4531-F14	TGACTCGTTTCGTGTCGCATG	[18]
MB-5519	CTGGCGTAGCGCCTTCGGCCT	[18]
ODN K3	ATCGACTCTCGAGCGTTCTC	[21]

P* means that only some parts of the sequences were selected

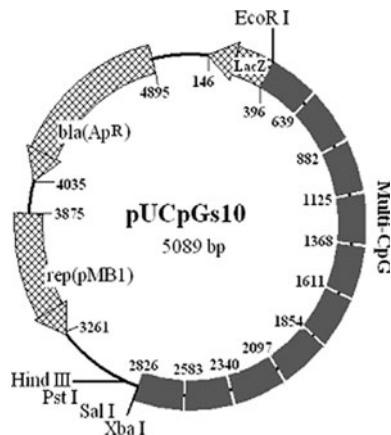


Fig. 1 The map of the newly constructed plasmid pUCpGs10. Ten Multi-CpGs were inserted into cloning vector pUC19 repeatedly producing the plasmid pUCpGs10, which containing 10 fragments of multi-CpG

plasmid pUCpGs10 was less potent than that of PCR-amplified repeated double-stranded CpG-DNA. At 48 h, their activities were no differences. Just as for IFN- γ , the IL-6 production of mice spleen lymphocytes increased from 24 h after stimulation in both PCR-amplified repeated double-stranded CpG-DNA group and the plasmid pUCpGs10 group. The IL-10 response was CpG specific as the control did not stimulate any significant production of this cytokine. From 6 h after stimulation, the plasmid pUCpGs10 exhibited IL-10 inducing activity, which is more potent than that of PCR-amplified repeated double-stranded CpG-DNA. At 48 h, both kinds of Multi-CpG exhibited IL-10 inducing activity, and their activities were no differences.

The Effect of Immunostimulatory Adjuvant of pUCpGs10

The serum IgG antibody titers in mice were summarized in Table 2. From the IgG antibody titers in group III, IV, and V 2 and 3 weeks after the subcutaneous immunization, the effect of immunostimulatory adjuvant of pUCpGs10 was

stronger than pUC19 parental plasmid and pUCpGs01 plasmid apparently. The pUC19 parental plasmid has little immunostimulatory effect, which maybe because that the pUC19 contains some CpG motifs. pUCpGs01, containing one Multi-CpG, has more stronger immunostimulatory effect than that of pUC19. From the IgG antibody titers in group II and VI, the effect of immunostimulatory adjuvant of pUCpGs10 (1:12800) was stronger than incomplete Freund's adjuvant (1:3200). Furthermore, the effect of immunostimulatory adjuvant of pUCpGs10 plasmid alone (group VI) was as strong as the Freund's incomplete adjuvant + pUCpGs10 plasmid (group V), which shows the potential immunologic adjuvant effect of the pUCpGs10 plasmid.

Detection on Antibody IgG and IgA

As shown in Table 3, after the last immunization, the serum IgG antibody titers in mice from nasal (group A3) and subcutaneous (group B4) immunization groups under pUCpGs10 adjuvant were 1:51200 and 1:3276800, respectively, which shows that the strong humoral responses were activated out only in nasal immunization mice but also in subcutaneous immunization mice. The antibody titer in the subcutaneous immunization group (group B3) under Freund's adjuvant was 1:1638400, lower than that of pUCpGs10 adjuvant (group B4). As shown in Fig. 3a and b, the IgA antibody titers in vaginal washing fluid and feces extracting fluid from the intranasal immunization group showed a gradually increasing trend with the immunization times. In addition, the IgA antibody titer in the pUCpGs10 adjuvant group was significantly higher than that in the single-antigen group ($P < 0.01$), and the IgA titer in serum and lung lavage fluid displayed the same result (Fig. 3c).

Determination on IFN- γ Secreting Cells

Under the immunogen alone, only weak T-cell immunity could be detected in both nasal and subcutaneous

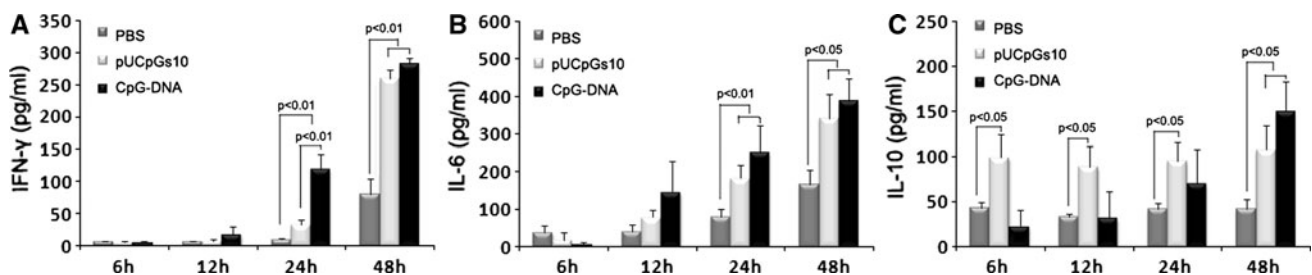


Fig. 2 Cytokines production of stimulated mice spleen lymphocytes. **a** IFN- γ , **b** IL-6, **c** IL-10. The mice spleen lymphocytes were stimulated by PBS, PCR-amplified repeated double-stranded CpG-DNA (20 μ g/ml), and the plasmid pUCpGs10 (20 μ g/ml),

respectively. The PCR-amplified repeated double-stranded CpG-DNA and the plasmid pUCpGs10 also induced in significant production of IFN- γ , IL-6 and IL-10 in mice spleen lymphocytes

Table 2 Specific IgG antibodies titer in the sera from immunized mice

Group	Name	One week after immunization	Two weeks after immunization	Three weeks after immunization
I	PBS control group	1:200	1:200	1:200
II	HCV-E1 + Freund's adjuvant	1:800	1:1600	1:3200
III	HCV-E1 + Freund's adjuvant + pUC19	1:1600	1:3200	1:6400
IV	HCV-E1 + Freund's adjuvant + pUCpGs01	1:3200	1:6400	1:6400
V	HCV-E1 + Freund's adjuvant + pUCpGs10	1:6400	1:12800	1:12800
VI	HCV-E1 + pUCpGs10	1:6400	1:12800	1:12800

Table 3 Specific IgG antibodies titer in the sera from nasally and subcutaneously immunized mice

Group	Name	The first immunization	The second immunization	The last immunization
A1	PBS control group	<1:400	<1:400	<1:400
A2	Antigen	<1:400	<1:400	1:12800
A3	Antigen + pUCpGs10	1:800	1:25600	1:51200
B1	PBS control group	<1:400	<1:400	<1:400
B2	Antigen	1:3200	1:102400	1:409600
B3	Antigen + Freund's adjuvant	1:12800	1:409600	1:1638400
B4	Antigen + pUCpGs10	1:12800	1:409600	1:3276800

Note Seven-week-old female Balb/c mice were randomly divided into two groups: group A: nasal immunization, group B: subcutaneous immunization

immunization, which is shown in Fig. 4 bearing the number of spots formed by IFN- γ -secreting cells (2 ± 2 SFC/ 5×10^5 and 9 ± 7 SFC/ 5×10^5). The group with the pUCpGs10 adjuvant significantly improved the corresponding system T-cell immune response, and subcutaneous immunization (153 ± 73 SFC/ 5×10^5) was superior to intranasal immunization (24 ± 8 SFC/ 5×10^5), displaying significant differences compared to the single-antigen group ($P < 0.01$). The difference between the group with the pUCpGs10 adjuvant and group with Freund's adjuvant (163 ± 92 SFC/ 5×10^5) was not significant ($P > 0.05$).

The Anti-Tumor Effect of Experimental Animals

As shown in Fig. 5a and b, tumor formation rate in the subcutaneous immunization Freund's adjuvant group and pUCpGs10 group were only 20 and 40 %, respectively, at 30th day after the mice were inoculated with tumor cells and no mice died since the transplanted tumor growth. Furthermore, tumor appeared latest in the pUCpGs10 group (18 days after tumor transplant). Therefore, it was revealed that antigen combined with pUCpGs10 plasmid could significantly inhibit tumor growth and thus prolong survival time under subcutaneous immunization.

The longitudinal diameter and transverse diameter of tumors in mice were measured every 3 days, and their growth curve was plotted. Tumors in immunization groups were remarkably smaller than those in the control group

($P < 0.01$). As shown in Fig. 5c, tumors in the intranasal immunization group were slightly larger than those in the subcutaneous immunization group, but without a significant difference ($P > 0.05$). Thirty days after tumor transplant, tumor mass were weighted and the inhibition rates of tumors were calculated (shown in Table 4). The results showed that the inhibition rates of tumors under subcutaneous immunization in the pUCpGs10 group was the same as that of Freund's adjuvant group, which was higher than the pUCpGs10 group under intranasal immunization. It seemed that the effect of plasmid pUCpGs10 was well-matched to Freund's adjuvant and can stimulate strong, specific cellular immune responses, and its effect with subcutaneous immunization was superior to its effect with intranasal immunization.

Discussion

CpG ODN motifs are 6 oligodeoxynucleotides with a CG center (NNCGNN) and the flanking sequences and spacer regions among some CpG motifs could influence their activity and specificity [18]. Currently, a number of CG-containing fragments are used in research, e.g., CpG 10101 [26] and CpG7909 [27], which have been used in clinical research. Due to the diversity of CpG ODN motifs [23], there are differences in optimal sequence sensitivities of CpG ODNs among different individuals in the same

Fig. 3 The specific IgA antibody level from each group of mice after immunization. **a** Vaginal washing fluid, **b** fecal material, **c** serum and lung mucosa. The vaginal washing fluid sample was $5\times$ diluted, the collected fecal sample was suspended in PBS (200 mg/ml), and the centrifugation supernatant was used to IgA detection. The serum was $50\times$ diluted, and lung lavage fluid was $5\times$ diluted. The IgA antibody titer in the pUCpGs10 adjuvant group was significantly higher than that in the single-antigen group ($P < 0.01$)

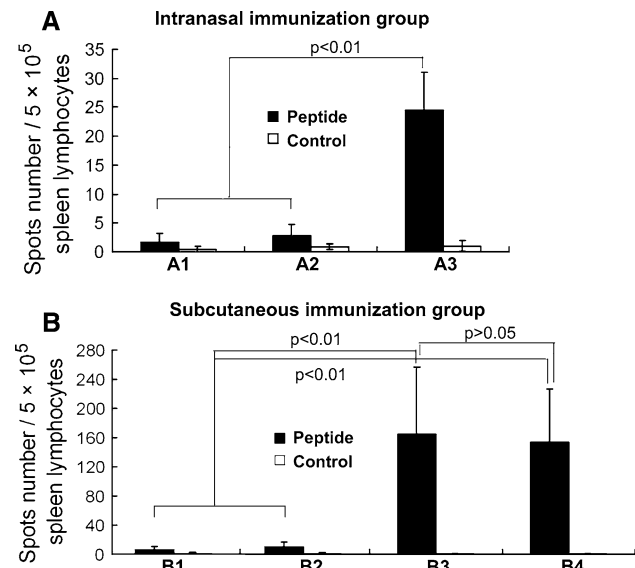
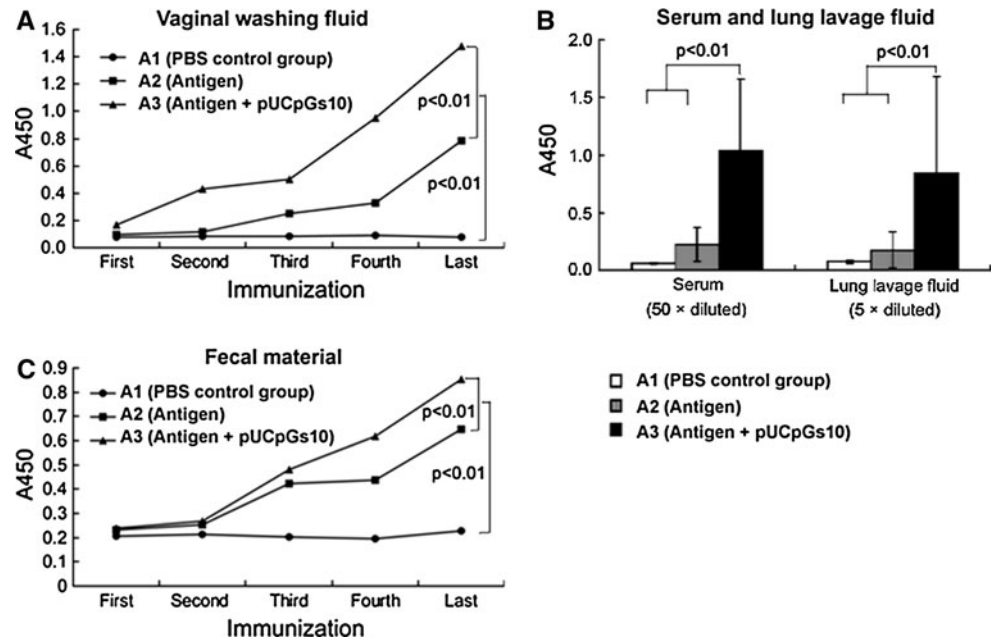


Fig. 4 ELISPOT detection results on IFN- γ secreting cells. **a** Group A, intranasal immunization group. *A1* PBS control group, *A2* Antigen, *A3* Antigen + pUCpGs10. **b** Group B, subcutaneous immunization group. *B1* PBS control group, *B2* Antigen, *B3* Antigen + Freund's adjuvant, *B4* Antigen + pUCpGs10. The group with the pUCpGs10 adjuvant significantly improved the corresponding system T-cell immune response, and subcutaneous immunization (153 ± 73 SFC / 5×10^5) was superior to intranasal immunization (24 ± 8 SFC / 5×10^5), displaying significant differences compared to the single-antigen group ($P < 0.01$)

species [15]. Therefore, the strategy of using many different CpG ODN fragments was employed in our study, as it is necessary to select a variety of sequences to meet various goals. The selection of the CpG ODN sequences depicted in Table 1 was based on the following reasons:

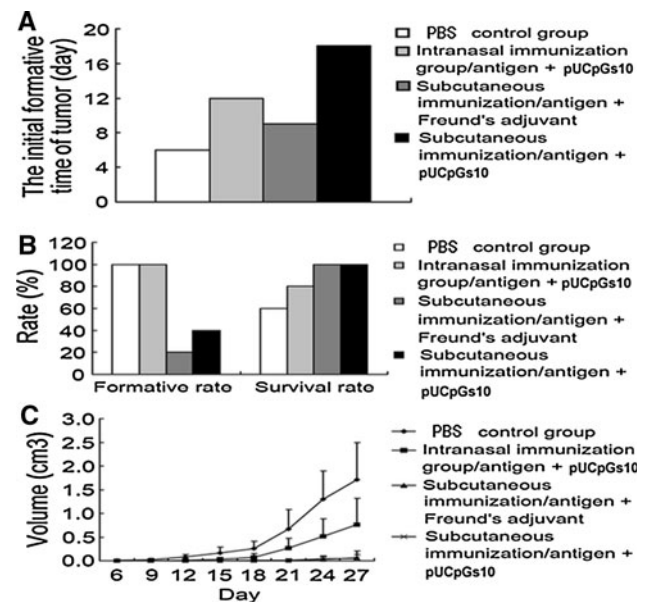


Fig. 5 The inhibition of pUCpGs10 plasmid on the subcutaneously inoculated SP2/0-N cells. **a** The initial formative time of transplanted tumor from each group of mouse. **b** The formative rate of transplanted tumor and survival rate of each group of mouse after 30 day of attack tumor. **c** The growth curves of transplanted tumor from each group of mice. It seemed that the effect of plasmid pUCpGs10 was well-matched to Freund's adjuvant and can stimulate strong, specific cellular immune responses, and its effect with subcutaneous immunization was superior to its effect with intranasal immunization

First, fragments bearing high immunization activity and comprehensive roles such as C274 [19] are able to stimulate normal human PBMC to secrete a variety of immunoreactive substances (e.g., IFN- α , IFN- γ , IP-10, MIG and MCP-2). In addition, its activity is about 10 fold higher than others.

Table 4 The weight and control rate of tumor from each group of mouse

Group	The weight of tumor (g)				Average weight of tumor (g)		The inhibition rate (%)
A1	2.43	–	–	2.47	2.39	2.43	Control
A3	1.23	0.99	2.25	–	1.23	1.43	41.15
B3	0	1.23	0	0	0	0.25	89.71
B4	0.84	0	0.39	0	0	0.25	89.71

– indicates that the mice had died before 30 days of tumor attack thus without weighting their tumor mass

Seven-week-old female Balb/c mice were immunization as the same as Table 3. *A1* PBS control group, *A3* nasal immunization/antigen + pUCpGs10, *B3* subcutaneous immunization/antigen + Freund's adjuvant, *B4* subcutaneous immunization/antigen + pUCpGs10. After immunization, the mice were subcutaneously inoculated with SP2/0-N cells. The homograft tumors were taken out surgically and weighed and after 30 days of tumor challenged

Second, natural sequences were desired if possible. For example, in Table 1, BCG-A4a [20], MB-4531 and MB-5519 are natural sequences from microbial genomes. The latter two sequences with especially high active were chosen by Lee et al. [18] from the *Mycobacterium bovis* (MB) genome through systematic analysis.

Third, we sought to choose primarily the sequences functioning in human cells, while also considering other species. For example, ODN1681 also produces a good immune stimulation in Atlantic salmon [17].

The reason for constructing the plasmid containing Multi-CpG instead of directly (chemically) synthesizing Multi-CpG was that there are already some reports of inserting CpG motifs into a plasmid and causing immune activation and even causing more activation by inserting more CpG motifs [28, 29]. In addition, convenience and economic considerations were taken into account. However, in the researches mentioned above, increasing the number of CpGs simply by inserting the same CpG motif (GTCGTT or AGCGTG) repeatedly improved the intensity of stimulation, but which was not enough to solve the CpG polymorphism and produce a broad-spectrum activity in various species, including humans. Accordingly, 11 different CpG-containing fragments (Table 1) were selected to be synthesized and connected into Multi-CpG, which was inserted into the plasmid pUCpGs01 for gaining broad-spectrum activity. Subsequently, the property of connections between same sticky ends of two enzymes was used to construct the pUCpGs10 containing 10 Multi-CpGs, which accounted for approximately half of the whole vector. This was used to try to solve the intensity of stimulation and broad-spectrum activity. After finishing construction of the vector, the vector replication process is done by microorganisms without introducing complex factors such as modified bases. Recently, the safety of plasmids and deoxyribonucleic acids has been confirmed by gene therapy or DNA vaccine research on many plasmids and vectors [30–32]. Lastly, plasmids bear high in vivo stability and are susceptible to be taken up into the

endosome [33] by immune cells (such as PDC), which is very important because the binding receptor (TLR9) of CpG ODN was just located to the lysosomal capsule membrane [34].

From our investigation, we validated that two kinds of Multi-CpG, the PCR-amplified repeated double-stranded CpG-DNA and the plasmid pUCpGs10, also induced in significant production of IFN- γ , IL-6, and IL-10 in mice spleen lymphocytes, which indicates that the present Multi-CpG not only stimulated the immune response to Th2, but also induces strong Th1-specific response. Thus, the present Multi-CpG has potential as a vaccine adjuvant. Due to the stronger immune activation of pUCpGs10 compared to the pUCpGs01 and pUC19 vector (Table 2), we only used pUCpGs10 as an immune adjuvant in the nasal immunization and subcutaneous immunization experiments. Immunogens were a multi-genotype HCV/E1 antigen and multi-epitope complex HCV-T-cell epitope antigen. The latter was a recombinant protein containing a number of cellular epitopes from HCV encoding proteins. The study of an HCV vaccine is currently a problem in the field of biomedical research [3–5]. In addition, peptide immunogens have the defect of insufficient immunogenicity compared to the whole pathogenic microorganisms, and there is a resulting higher demand on the immune adjuvant. Therefore, employing the two types of antigens as immunogen is not only an evaluation of the pUCpGs10 as an adjuvant but also a tool to provide useful lessons for HCV vaccine research.

According to the subcutaneous immunization results of our research, vector pUCpGs10 showed strong adjuvant activity in terms of assisting antigen generating specific humoral immune response (Tables 2, 3), helping the cellular immune response produce IFN- γ secretion (Fig. 4b) and animal tumor challenge experiments, when compared with without adjuvant. Even when comparing with the strongest animal immune adjuvant, namely complete Freund's adjuvant (CFA), pUCpGs10 has the same effect, which is quite satisfying. In CFA, live *Bacillus Calmette-Guerin* (BCG)

was added into a “water-in-oil” emulsion, while pUCpGs10 is used alone in this study. In addition, the adjuvant role of pUCpGs10 should be further enhanced by combine with other adjuvants such as alumina gal, as the synthetic CpG ODN adjuvant does in literature [35–38].

The potent mucosal adjuvant effect of the novel pUCpGs10 is worth mentioning. Because the vast majority of pathogenic microorganisms (e.g., Tuberculosis, Salmonella, Influenza, RSV, and HIV) invade the human body through the mucous membranes, effectively stimulating mucosal immunity to block the pathogen at its site of invasion would be preferable. Studies have shown that the immune system can be categorized as two relatively independent compartments, the systemic immune system and the mucosal immune system. Traditional vaccination stimulates a strong systemic immune (humoral and cellular immune) response but cannot stimulate mucosal immune response (IgA secretion and local CTL responses). It is only through mucosal immunization that effectively stimulates both the mucosal and systemic immune responses [39]. Mucosal immunity is implemented by an independent mucosa-associated lymphoid tissue (MALTs), and due to MALT-activated lymphocytes containing a specific markers as known homing receptors (such as the $\alpha 4\beta 1$, $\alpha 4\beta 7$, CCR9 or CCR10), can be further distributed to other mucosal tissues expressing the corresponding elements, such as MadCAM1, CCL28 or CCL25. Therefore, nasal immunization not only activates the immune response in the respiratory tract but also affects genital tract immunity and part of gastrointestinal mucosal immunity as well. In addition, the incomparable safety, convenient administration, needleless injection, and patient acceptability, has caused mucosal vaccination to be favored by most immunology workers in recent years [11–13].

Nevertheless, a strong immune response is not easily activated through mucosal immunization. Quick degradation of the general immunogen in the oral cavity or nasal mucosa makes the immunogen and adjuvant difficult to achieve MALTs. For example, Xiaowen et al. [40] recently applied an inactivated influenza virus H5N2 as an intranasal vaccine and found that only when combining immunogen with Oligo-B (synthesizing CpG ODN) or/and rIL-2 specific cellular immunity and secretion of IgG and IgA antibodies can be stimulated in MALTs. Consequently, research on mucosal immune adjuvant has become a hot topic. Cholera toxin (CT) or heat-labile enterotoxin (LT) was adopted as an adjuvant in the early years and can effectively activate mucosal immune response, but it does not suit human needs due to its toxicity [20]. Undoubtedly, CpG ODN is an ideal choice as a mucosal adjuvant. Moreover, its identified mucosal immunoadjuvant effect has been verified by different synthetic CpG ODNs [11–13, 40].

In this study, we achieved a satisfactory result by adapting pUCpGs10 as a nasal mucosal immune adjuvant. In addition to effectively stimulating the remarkable increase of secreting specific IgA antibody in the respiratory tract (lungs lotion) (Fig. 3c), an increased secretion of specific IgA in vaginal secretions and intestine was observed (Fig. 3a, b). Besides, intranasal mucosal immunization can also effectively stimulate systemic immune response, manifesting as serum IgG antibody (Table 3). In addition, the number of spleen lymphocytes secreting IFN- γ was significantly increased under the pUCpGs10 adjuvant (Fig. 4). There were also some suppressive effects on the formation of tumors in immune mice, although the immune response was not as good as that found in subcutaneous immunization.

Taken together, plasmid pUCpGs10 not only activated immunogen-specific cell-mediated immunity and humoral immune responses but also strongly activated the mucosal immune response. Its adjuvant activity is the same as CFA, which provides experimental evidence for further study and application of plasmid pUCpGs10 and sheds new light on the research and development of new adjuvants and lays the foundation for multi-epitope HCV vaccine research.

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