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A new RNA vaccine platform based on MS2 virus-like particles produced in *Saccharomyces cerevisiae*

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ABSTRACT

mRNA vaccines are potentially attractive alternatives to DNA vaccines more often discussed, as they are generally considered safer than their DNA counterparts. The major limitations on the potency of RNA vaccines are their instability and inability to spread in vivo. Virus-like particles (VLPs) based on the bacteriophage MS2 have demonstrated remarkably high stability and may provide an improved platform for RNA-based genetic vaccination. However, no in vivo study of an MS2 VLP-mediated RNA vaccine has been reported. Therefore, we developed a model vaccine wherein MS2 VLPs packaging HIV-1 *gag* mRNAs (1544 bases) were produced in *Saccharomyces cerevisiae*, and then, used to immunize BALB/c mice. Serological analyses showed that antigen-specific antibody responses were elicited by immunization. These findings suggest that MS2 VLPs can be used in the design and construction of novel and safe phage-based mRNA delivery vectors.

1. Introduction

Since the early 1990s, DNA-based genetic vaccines have emerged as attractive approach to immunization that elicit immune responses similar to those induced by live attenuated vaccines [1–3]. However, because of the inherent features of plasmid DNA, a safety concern regarding this class of vaccines is the potential risk of integration into the host genome, leading to malignant transformation [4–6]. An alternative is to use mRNA for gene delivery and vaccination, as RNA-mediated gene expression retains the advantages of DNA vaccines without the risk of integration into the host genome [7,8] or severe side effects such as the generation of autoimmune disease or anti-DNA antibodies [9].

Several studies have used RNA immunization for immunotherapy of cancer, infectious disease, or allergy [7,9–13]. However, the potency of naked RNA vaccines is limited by their instability and inability to spread in vivo [14]. Bacteriophage vectors have potential as vaccine delivery and gene transfer vectors, because of their genetic tractability, high degree of stability, high production capacity, inexpensive production, and inherent biological safety in mammalian cells [15,16]. A previous study demonstrated that MS2 virus-like particles (VLPs) packaging functional heterologous mRNAs can be produced in *Saccharomyces cerevisiae*, which makes MS2 VLPs amenable to development into gene delivery vectors [17]. However, no in vivo study of MS2 VLP-mediated RNA vaccination has been carried out. For this reason, we undertook the current study to investigate the potential of MS2 VLPs packaging heterologous antigen-encoding RNA produced in *S. cerevisiae* as a delivery vehicle for RNA vaccination in mice. As the HIV-1 Gag region is one of the most conserved viral proteins and is widely considered a relevant antigen for the development of an anti-HIV vaccine [18], we chose the HIV-1 *gag* mRNA as a model RNA to study the MS2 VLP delivery system. Our results showed that the MS2 VLP-mediated mRNA delivery could effectively induce antigen-specific humoral immune responses in BALB/c mice.

2. Materials and methods

2.1. Plasmids

The cDNA encoding the MS2 capsid protein was amplified by PCR from the plasmid pMS27 using the primers CF and CR and inserted in-frame into the plasmid pESC-Ura (Stratagene) to generate the capsid mRNA packaging vector pMS. The cDNA encoding HIV-1 Gag was amplified by PCR from the plasmid pSG3Δenv (kindly provided by the National Center for AIDS/STD Control and Prevention, China CDC) with primers GVF and GVR, which were designed to introduce C-5 variant *pac sites* [19,20] before and after the HIV-1 Gag coding sequence, and the product was inserted in-frame into the plasmid pMS to generate the recombinant plasmid pMS-GAG. The cDNA encoding HIV-1 Gag was amplified by PCR from the plasmid pSG3Δenv with the primers GF and GR and inserted in-frame into the plasmid pCDNA-3.0 (Invitrogen) to generate the

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Table 1
Nucleic acid sequences of primers designed for plasmid construction. Restriction enzyme sites are indicated by underscoring; C-variant *pac sites* are indicated in boldface type.

	Primer sequence (5′–3′)
CF	5′-CG*ACTAGT*GCCACCATGGCTTCTAACTTTACTCAG-3′
CR	5′-CG*TTAATTAA*ATGGCCGGCGTCTATTAGTAG-3′
GVF	5′-CG*GGATCC*GCCACCATGGA**ACATGAGGATCACCCATGT**CATGGGTG CGAGAGCGTCAGT-3′
GVR	5′-CCG*CTCGAG***ACATGGGTGATCCTCATGT**TCATTATTGTGACGAG GGGTCGTT-3′
GF	5′-CG*GGATCC*GCCACCATGGGTGCGAGAGCGTCAGT-3′
GR	5′-CCG*CTCGAG*TCATTATTGTGACGAGGGGTCGTT-3′

recombinant plasmid pCDNA3-GAG. The capsid mRNA packaging vector pMS2C has been described elsewhere [17]. The sequences and details of primers and probes are listed in Table 1. Correct construction of all plasmids was confirmed by sequencing.

2.2. Production and purification of the MS2 VLPs

The plasmids pMS2C and pMS-GAG were separately transferred into the YPH499 yeast strain (Stratagene) by the LiAC/SSDNA/PEG method [21]. The MS2 VLPs were expressed, produced, and further purified as described previously [17] and stored at 4 °C. The purified products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on 18% gels to identify pMS-GAG VLPs. The particles were also observed by transmission electron microscopy at 100 kV and 59,000× screen magnification.

2.3. Identification of the RNA packaged by MS2 VLPs by RT-PCR

RNA purification and reverse transcription (RT)-PCR reaction were performed as described elsewhere [22], except for the primers and extension times used for RT-PCR. For identification of the RNA packaged by the pMS2C VLPs, the primer CR was used in the RT reaction, and PCR was performed with the primers CF and CR at 94 °C for 5 min, followed by 35 cycles of 45 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C, and followed by 10 min at 72 °C. For identifying the RNA packaged by pMS-GAG VLPs, the primer GR was used in the RT reaction, and PCR was performed with the primers GF and GR at 94 °C for 5 min, followed by 35 cycles of 45 s at 95 °C, 30 s at 56 °C, and 90 s at 72 °C, and followed by 10 min at 72 °C. PCR products were then purified and ligated into the pEGM-T Easy plasmid (Promega) for verification by sequencing.

2.4. Durability of pMS-GAG VLPs

The freshly prepared pMS-GAG VLPs were digested by DNase I and RNase A at 37 °C for 60 min. The recombinant plasmid pCDNA3-GAG and the RNA extracted from pMS-GAG VLPs were used as controls. After digestion, the samples were analyzed by agarose gel electrophoresis (1%) with ethidium bromide staining. For the freeze–thaw test, the quantified pMS-GAG VLPs were diluted with normal human EDTA-preserved plasma to a concentration of 100,000 copies/ml. A single batch was separated into aliquots of 0.1 ml for individual time point samples, the volume required for Kehua HIV RNA real-time RT-PCR assay. Samples were frozen at −20 °C and thawed to room temperature 5 times before quantification in duplicate by Kehua HIV RNA real-time RT-PCR assay (Shanghai Kehua Bio-Engineering Co., Ltd., Shanghai, China).

2.5. Expression of VLP mRNA products in mammalian cell lines

HEK-293, HeLa, and CHO cell lines were all maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and cultured at 37 °C and 5% CO_2. Six-well plates were used for transfection. The purified RNA of pMS-GAG VLPs was transfected into cells by using the transfection reagent DOTAP (Roche), according to the manufacturer's instructions. After 24 h, the cells were harvested, and cell lysates were subjected to ELISA using the Genescreen Plus HIV Ag–Ab assay (Bio-Rad), according to the manufacturer's instructions.

2.6. Immunization of mice

Six to eight week-old female BALB/c mice (provided by the Animal Center of the Academy of Military Medical Sciences, Beijing, China) were intramuscularly vaccinated with either 15 μg of total protein of purified pMS2C VLPs (as a control) or 15 μg of total protein of purified pMS-GAG VLPs (5×10^{12} RNA copies) resuspended in sterile phosphate-buffered saline (PBS) and emulsified 1:1 in Freund's adjuvant (Sigma). The mice received 2 boosts with the same amount of MS2 VLPs in Freund's incomplete adjuvant (Sigma) at 3 and 5 weeks after initial vaccination. Mice injected with Freund's adjuvant alone were used as controls. Eight mice were used per group.

2.7. Detection of antigen-specific antibody responses by ELISA

Seven days after the final immunization, serum was recovered from the mice by tail bleeding. Plates coated with HIV-1 p24 antigen (kind gifts from Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China.) were used to capture anti-Gag antibodies by an end-point dilution ELISA assay (in duplicate) on samples from individual animals. Serial 2-fold dilutions beginning at 1:100 of sera in blocking buffer were prepared, and antigen-coated plates were incubated with 100 μL of each dilution at 37 °C for 1 h. Subsequently, the plates were washed three times with PBS containing 0.05% (w/v) Tween-20 (PBST), and then incubated with HRP-conjugated goat anti-mouse IgG antibody (1:20,000, Sigma–Aldrich). After incubation at 37 °C for 1 h, 100 μL of tetramethyl benzidine (TMB) solution was added to each well. After incubation at 37 °C for 30 min, color development was terminated by addition of 50 μL of 2 M H_2SO_4, and the optical density (OD) was measured at 450 nm (with 620 nm as a reference) by using an ELISA plate reader (Labsystems, Finland).

3. Results

3.1. Analysis of MS2 VLP production in S. cerevisiae

The purified pMS2C VLPs and pMS-GAG VLPs were subjected to 1% agarose gel electrophoresis and a single band between 750 bp and 1000 bp was observed for each sample (Fig. 1A). In order to confirm that MS2 VLPs were produced in the *S. cerevisiae* expression system, purified MS2 VLPs were analyzed on denaturing SDS–polyacrylamide gels and observed by transmission electron microscopy. The molecular weight of the proteins in each sample was approximately 14 kD (Fig. 1B), and the diameter of the MS2 VLPs was approximately 30 nm (Fig. 1C). These results indicated that the MS2 VLPs were successfully expressed in the *S. cerevisiae* system.

3.2. Analysis of the packaged RNA

As expected, the sizes of the full-length RT-PCR amplification products of the RNA extracted from pMS2C VLPs and pMS-GAG VLPs were 429 bp (Fig. 2A) and 1529 bp (Fig. 2B), respectively. These results were further verified by sequencing (data not shown).

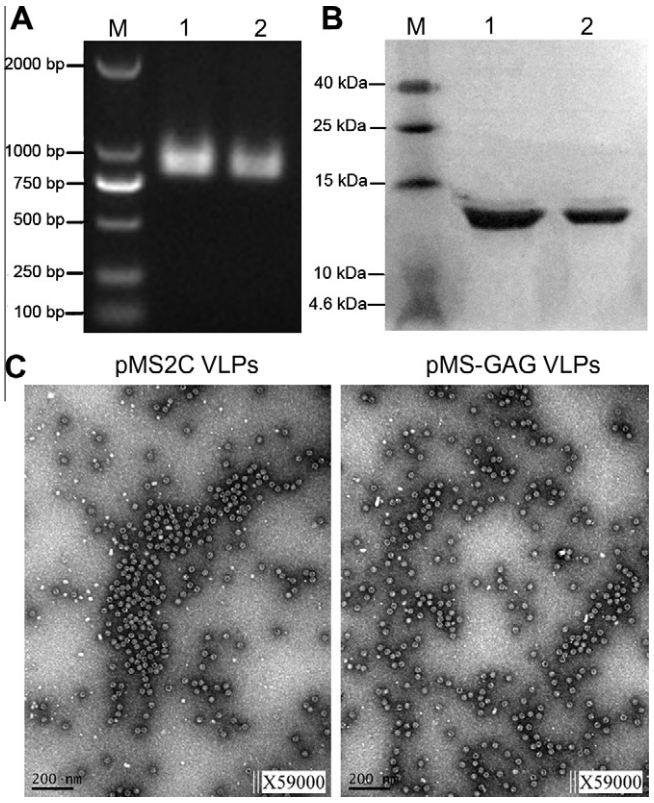

Fig. 1. Identification of pMS2C and pMS-GAG VLPs. (A) The MS2 VLPs were purified by gel exclusion chromatography and analyzed on a 1% agarose gel, producing bands between 750 bp and 1000 bp in size. Lane 1, pMS2C VLPs; Lane 2, pMS-GAG VLPs; Lane M, molecular weight marker. (B) The MS2 VLPs were purified by gel exclusion chromatography, loaded onto an SDS–polyacrylamide gel, and subjected to electrophoresis in tricine buffer. Proteins were visualized by staining the gel with Coomassie brilliant blue. Lane 1, pMS2C VLPs; Lane 2, pMS-GAG VLPs; Lane M, molecular weight marker. (C) Identification of pMS2C and pMS-GAG VLPs by transmission electron microscopy. The diameter of the MS2 particles was approximately 30 nm. Photographs were taken with a screen magnification of 59,000×.

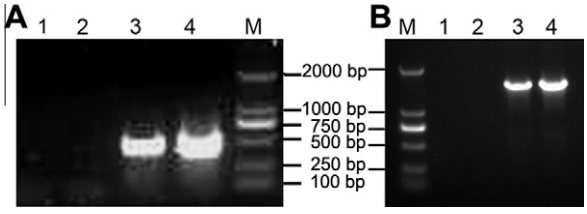

Fig. 2. Ethidium bromide-stained 1% agarose gel of RT-PCR amplification products of RNA extracted from MS2 VLPs. (A) Lane 1, negative control (no template); Lane 2, negative control (without RT); Lane 3, RT-PCR products of RNA extracted from pMS2C VLPs; Lane 4, positive control (pMS27 plasmid). (B) Lane 1, negative control (no template); Lane 2, negative control (without RT); Lane 3, RT-PCR products of RNA extracted from pMS-GAG VLPs; Lane 4, positive control (pSG3Δenv plasmid).

3.3. Durability of the pMS-GAG VLPs

The pMS-GAG VLPs were completely resistant to DNase I and RNase A treatment. By contrast, naked DNA and RNA were both rapidly degraded (Fig. 3A). Samples were frozen at −20 °C and thawed to room temperature 5 times before quantification in duplicate by Kehua HIV RNA real-time RT-PCR assay. The results showed that the pMS-GAG VLPs were largely intact after 5 freeze–thaw cycles (Fig. 3B).

3.4. Expression of VLP mRNA products in mammalian cell lines

To test the functionality of the RNA extracted from pMS-GAG VLPs, we transfected the extracted RNA into 3 different cell lines (HeLa, HEK-293, and CHO). Expression of the transfected HIV-1 *gag* RNA was quantified in duplicate by the Genescreen Plus HIV Ag–Ab assay (Bio-Rad). The RNA extracted from pMS-GAG VLPs was efficiently translated in all the 3 cell lines (Table 2).

3.5. Titration of antibody response by ELISA

To evaluate whether MS2 VLP-mediated RNA vaccination could induce antibody responses, BALB/c mice were immunized as described in Materials and Methods. Antibody responses elicited by immunization with adjuvant alone, pMS2C VLPs, and pMS-GAG VLPs were all assessed 7 days after final immunization. Results showed that MS2 VLP-mediated RNA vaccination induced strong antibody responses. The sera of the adjuvant-only and pMS2C VLP groups, all of which tested negative for anti-Gag IgG, were assigned titers of 1:100 (Fig. 4). These results indicated that antigens encoded by phage-packaged mRNA were successfully delivered and that they induced antigen-specific antibodies in mice.

4. Discussion

Naked RNA is susceptible to degradation by RNases. As demonstrated previously [22–24], armored RNA technology wherein MS2 phage assembly is engineered to encapsulate an RNA fragment of a target gene overcomes the problem of RNA instability. As expected, our results showed that HIV-1 *gag* mRNA packaged into pMS-GAG VLPs was protected by the capsid protein of MS2 from degradation by DNase I and RNase A. The pMS-GAG VLPs also survived 5 freeze–thaw cycles and were stable at 4 °C for at least 4 months (data not shown). Therefore, the MS2 VLPs produced in *S. cerevisiae* fulfill the stability requirements for a new mRNA transfection vector.

A major obstacle for RNA-based gene vaccines is cellular delivery. We hypothesized that the MS2 VLP packaging system could resolve this problem, as unmodified phage particles engineered to contain foreign antigen genes are capable of gene delivery in vivo, possibly by internalization by antigen-presenting cells (APCs) [15,25,26]. In support of this presumption, we showed that MS2 VLP-mediated mRNA delivery could effectively induce antigen-specific humoral immune responses in BALB/c mice (Fig. 4). Previous studies have demonstrated that MS2 VLPs can be transferred into target mammalian cells by chemically or covalently conjugating ligands [27–29] or cell-penetrating peptides [30] to the outer capsid surface of MS2 VLPs. It would be interesting to explore whether such targeting methods could improve the MS2 packaging system vector by increasing the efficiency of delivery of genes of interest to targeted cells or enhancing the spread of antigen.

Cloning capacity is an important factor the development of MS2 VLP-mediated mRNA delivery vectors. The maximum length reported for heterologous RNA packaged by MS2 VLPs in prokaryotic expression systems is 3034 bases, which is close to that of the full-length genome of wild bacteriophage MS2 (3569 bases) [22]. However, the lengths of heterologous RNAs packaged by MS2 VLPs produced in *S. cerevisiae* are all less than 1 kb [17], and the length of the model RNA in this study is 1544 bases. As the lengths of some antigen-encoding genes or combinations of antigen-encoding gene segments of interest are greater than 1554 bases [7,31–33], more experiments are needed to investigate the maximum cloning capacity of MS2 VLPs for further use as mRNA delivery vectors.

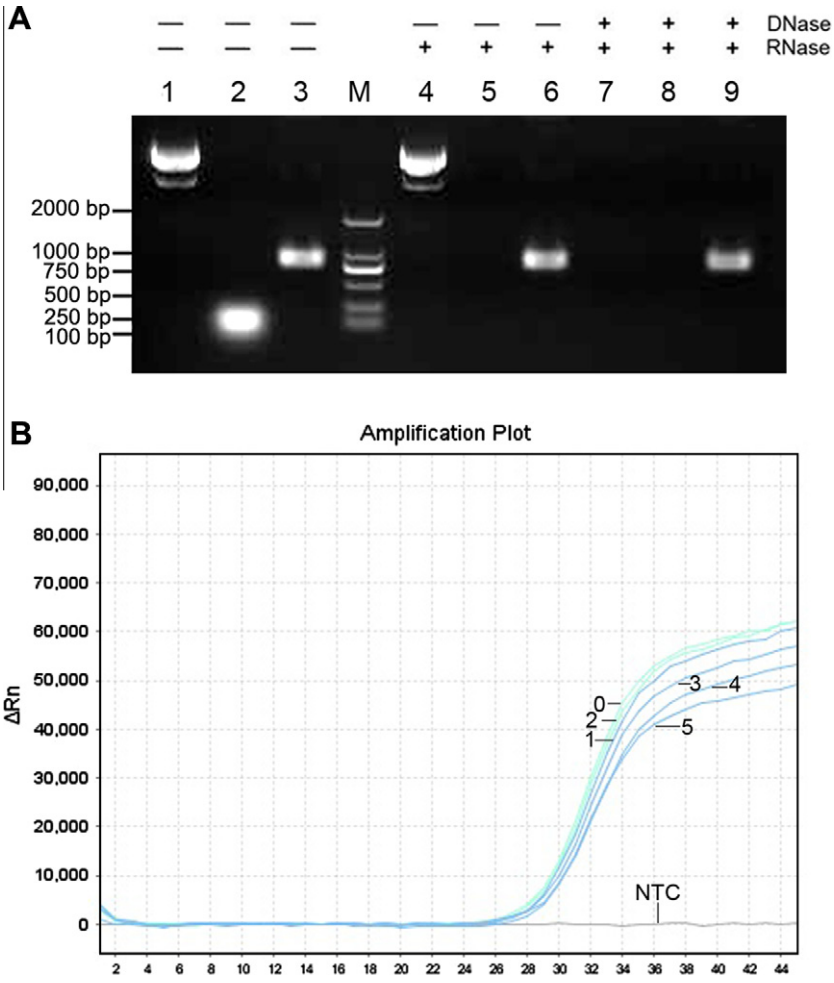

Fig. 3. Durability of pMS-GAG VLPs. (A) Nuclease resistance of purified pMS-GAG VLPs. Plasmid DNA, RNA extracted from pMS-GAG VLPs, and intact pMS-GAG VLPs were incubated with RNase A and/or DNase I at 37 °C for 1 h, separated by gel electrophoresis in a 1% non-denaturing agarose gel, and detected by ethidium bromide staining and UV fluorescence. Lane M, molecular weight marker. Lanes 1, 4, and 7: pCDNA3-GAG plasmid; lanes 2, 5, and 8: RNA extracted from pMS-GAG VLPs; lanes 3, 6, and 9: pMS-GAG VLPs. (B) Stability of pMS-GAG VLPs subjected to 5 freeze–thaw cycles. The diluted pMS-GAG VLPs were frozen at −20 °C and thawed to room temperature five times. NTC represents no template control; 0 represents original pMS-GAG VLPs; 1 represents 1 freeze–thaw cycle; 2 represents 2 freeze–thaw cycles; 3 represents 3 freeze–thaw cycles; 4 represents freeze–thaw cycles; and 5 represents 5 freeze–thaw cycles.

Table 2
Genescreen Plus HIV Ag–Ab assay of the pMS-GAG VLP RNA translation products. The RNA purified from pMS-GAG particles was translated in three different mammalian cell lines. As a negative control, the RNA purified from pMS-GAG particles was added to the cells in the absence of DOTAP. A positive control was performed by transfecting 2.5 μg of pCDNA3-GAG plasmid into cells in parallel with the pMS-GAG RNA. Cell lysates were subjected to ELISA using the Genescreen Plus HIV Ag–Ab assay (Bio-Rad). The criteria for distinguishing among positive or negative ELISA results were determined from the manufacturer's instructions. +: positive; −: negative.

	pMS-GAG RNA-DOTAP	pMS-GAG RNA + DOTAP	pCDNA3-GAG
HeLa	−	+	+
HEK-293	−	+	+
CHO	−	+	+

In conclusion, we successfully produced MS2 VLPs packaging functional HIV-1 *gag* mRNAs in *S. cerevisiae*. The bacteriophage capsid proteins effectively protected RNAs from RNase. Our results revealed that in mice, the MS2 VLP-mediated mRNA delivery system can generate strong antibodies specific for the mRNA-encoded antigens. This vaccination strategy can potentially be applied to other infectious diseases as well as to allergies or cancers.

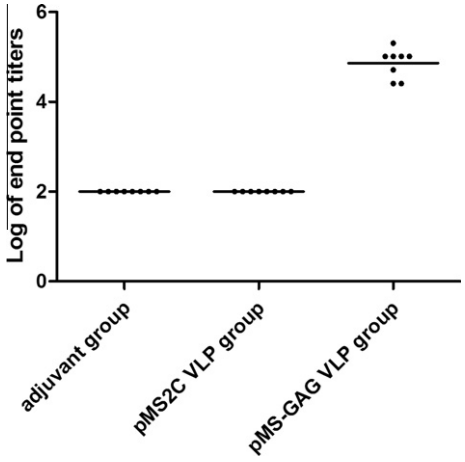

Fig. 4. Antibody titers in female BALB/c mice immunized with different antigens. Anti-Gag antibody titers were determined by end-point dilution ELISA assay using plates coated with HIV-1 p24 antigen. Dots represent antibody titers of sera derived from each mouse 7 days after the final immunization. Eight mice were used per group. The sera of the adjuvant and pMS2C VLP groups, all of which tested negative for IgG antibody, were assigned titers of 1:100. The sera of all eight mice in the pMS-GAG VLP group were strongly positive for anti-Gag antibodies.

Conflict of interest

None declared.

Acknowledgment

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