Available online at www.sciencedirect.com
ScienceDirect
Vaccine 25 (2007) 4291–4300
Vaccine
www.elsevier.com/locate/vaccine

Mutant influenza A virus nucleoprotein is preferentially localized in the cytoplasm and its immunization in mice shows higher immunogenicity and cross-reactivity

Kenji Ohba[a,b,c,*], Shinsuke Yoshida[b], Md. Zahidunnabi Dewan[a,b], Hiromi Shimura[a], Nozomi Sakamaki[a], Fumihiko Takeshita[c], Naoki Yamamoto[a,b,*], Kenji Okuda[c]

[a] *Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[b] *AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjyuku-ku, Tokyo 162-8640, Japan*
[c] *Department of Molecular Biodefense Research, Yokohama-city University School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan*

Received 31 August 2006; received in revised form 14 February 2007; accepted 21 February 2007
Available online 12 March 2007

Abstract

Many influenza vaccines targeted to hemagglutinin (HA) show efficient immunogenicity for protecting subjects against influenza virus infection. Major antigenic changes to HA molecules can help influenza virus to develop resistance against HA-targeted vaccines. DNA vaccines encoding conserved antigens protect animals against diverse subtypes, but their potency requires further improvement. We generated a DNA-based nucleoprotein (NP)-targeted vaccine using an N-terminal mutant of NP (NPm) that efficiently localized in the cytoplasm, and examined the immune responses in mice immunized with NPm or wild-type (WT) NP DNA vaccine. Importantly, the NPm vaccine showed 1.5–2-fold higher immunogenicity than the WT NP vaccine in mice. Furthermore, NPm vaccination efficiently protected the mice against lethal challenge with influenza viruses and showed cross-reactivity toward heterologous viruses. Therefore, DNA-based vaccination with NPm may contribute to the development of protective immunity against diverse influenza virus through its ability to stimulate cellular immunity.

Keywords: NPm; Cytoplasmic localization; Immunogenicity

1. Introduction

Influenza A virus infections and complications, a major cause of human morbidity and mortality, have been prevalent worldwide every year for a long time [1–10]. Influenza virus particles have two virus-encoded membrane glycoproteins, namely hemagglutinin (HA) and neuraminidase (NA). Given that there are 16 subtypes of HA and 9 subtypes of NA [1–4,9,10], we suppose that influenza virus can exist with various combinations of membrane proteins. There are three main subtypes of influenza virus type A, designated H1N1, H2N2 and H3N2, and influenza virus type B that can infect humans [1,2]. Influenza virus infection is not particularly lethal in humans, with the exception of infants, elderly people and acquired immunodeficiency syndrome patients. However, it was recently reported that the highly pathogenic H5N1 avian influenza virus can infect humans and increase the usual mortality rates [2–9].

Influenza virus nucleoprotein (NP), a core antigen of influenza virus, is a more conserved protein than the mem-

* Corresponding authors at: Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Tel.: +81 3 5803 5178; fax: +81 3 5803 0124.
E-mail addresses: oken818.molv@tmd.ac.jp (K. Ohba), nyama@nih.go.jp (N. Yamamoto).

0264-410X/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2007.02.074

brane glycoproteins [11]. NP is bound to eight ssRNA genomes of influenza virus and protects the ssRNA against degradation by various enzymes [2,3]. Influenza virus NP characteristically has a nuclear localization signal (NLS) and an RNA-binding domain. NP translocates into the nucleus via the NLS after translation and has no ability to export back from the nucleus to the cytoplasm [12–20]. In the proliferative phase of influenza virus, NP forms a ribonucleoprotein complex (RNP-complex) with ssRNA, three polymerase proteins (PA, PB1 and PB2) and matrix protein (M1), and the RNP-complex is exported from the nucleus to the cytoplasm. The NP gene is rarely mutated and is conserved by approximately 90% in many strains of influenza type A virus. However, only a small amount of information is currently available for developing more effective vaccines that target NP [11].

The NLS consists of amino acids (a.a.) 327–345 (gene size: 975 bp) on NP. Deletion of the NLS has been reported to decrease the nuclear localization of NP. However, the nuclear localization is not completely abolished by deletion or mutation of the NLS [17–21]. One of these studies further showed that mutation of Tyr-Lys-Arg (a.a. 6–8 of NP) to Ala-Ala-Ala efficiently decreased the nuclear localization of NP, irrespective of the NLS [20].

Several studies have reported that cytotoxic T lymphocyte (CTL) responses are very important in clearing influenza A virus infections in mice and humans [22–27]. To induce more effective CTL responses, it is necessary to consider the antigen-presenting system, in which antigen-presenting cells (APCs), mainly dendritic cells (DCs), process proteins, load peptides onto major histocompatibility complex class I (MHC I) and present the peptide-MHC I complexes on the cell surface [28–35]. APCs present the antigen peptides on the cell surface through processing by the proteasome and endoplasmic reticulum (ER) in the cell cytoplasm [28,29,32–34]. This antigen-processing machinery is optimized to induce rapid, strong and sustained CTL responses, and to avoid misguided attacks. It is supposed that the strong induction process of the CTL responses is dependent on the quantity of protein expression and presentation by the antigen-presenting pathway of APCs. We assumed that it is necessary to increase the quantity of protein expression in order to induce intense CTL responses. The organelles and molecules responsible for antigen processing are present in the cytoplasm. It should also be mentioned that proteins localized in the nucleus, such as influenza virus NP, show lower efficiencies for antigen presentation through the cytosolic antigen-presentation pathway.

In the present study, we show that an N-terminal mutation induced efficient localization of NP in the cytoplasm without changing the level of expression, which is important for the induction of higher immunogenicity. An NP mutant (NPm) DNA vaccine successfully induced interferon (IFN)-γ-producing cells and CTL responses to protect the immunized animals against challenges with the same or heterologous viral stains. This DNA vaccination could represent a new strategy for developing effective vaccines against influenza virus.

2. Materials and methods

2.1. Construction of pCAG/NP and pCAG/NPm

To construct the DNA vaccine, we used a pCMV/NP plasmid containing the NP gene of influenza virus type A/Puerto Rico 8/H1N1 strain. To amplify the NP gene, we used primer pairs with 5′ *Xho* I and 3′ *Sal* I linkers (5′-ACCGCTCGAGATGGCGTCTCAAGGCACCAAAC and 3′-GACGCGTCGACTTAATTATCGTATTCCTCTGCATTG). To amplify NPm mutated from Tyr-Lys-Arg (a.a. 6-8 of NP) to Ala-Ala-Ala, we used other primer pairs with 5′ *Xho* I and 3′ *Sal* I linkers (5′-ACCGCTCGAGATGGCGTCTCAAGGCGCCGCAGCATCTTACGAAC and 3′-GACGCGTCGACTTAATTATCGTATTCCTCTGCATTG) and the same template (pCMV/NP). The NP and NPm genes were amplified by polymerase chain reaction (PCR) using these primers. After treatment of the PCR-amplified NP and NPm genes with *Xho* I and *Sal* I, we inserted the digested NP and NPm genes into the *Xho* I site of the pCAG plasmid [36]. The digested end of *Xho* I and *Sal* I were able to be directly ligated. pCI/NP-Flag and pCI/NPm-Flag were generated using specific primer pairs with 5′ *Eco* RI and 3′ *Sal* I linkers (5′-ACCGGAATTCATGGCGTCTCAAGGCGCCGCAGCATCTTACGAAC and 3′-GACGCGTCGACTCACTTGTCGTCATCGTCTTTGTAGTCATTATCGTATTCCTCTGCATTG) in the pCI vector (Promega, Madison, WI). The plasmids were cultivated in *Escherichia coli* DH5α, and then purified and concentrated using Qiagen columns according to the manufacturer's instructions.

2.2. DNA sequencing

To confirm the mutation in pCAG/NPm, DNA sequencing was performed with upstream and downstream primers (5′-CCATGTTCATGCCTTC and 3′-GTGGTATTTGTGAGCC) for the NPm gene inserted in the pCAG cloning site. The DNA sequencing reactions and measurements were performed according to the manufacturer's instructions. Briefly, 500 ng of the pCAG/NPm plasmid was denatured for 5 min at 95 °C and then immediately placed on ice. Primers and Big-Dye Sequencing Enzyme (ABI, Foster City, CA) were mixed with the plasmid and the sequencing reaction was carried out using a thermal cycler. After the sequencing reaction, the samples were purified using columns, dried and dissolved in TSR. Finally, the samples were analyzed by a sequence analyzer and its analysis software (ABI).