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Intranasal administration of a live non-pathogenic avian H5N1 influenza virus from a virus library confers protective immunity against H5N1 highly pathogenic avian influenza virus infection in mice: Comparison of formulations and administration routes of vaccines

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ABSTRACT

Outbreaks of highly pathogenic avian influenza viruses (HPAIVs) would cause disasters worldwide. Various strategies against HPAIVs are required to control damage. It is thought that the use of non-pathogenic avian influenza viruses as live vaccines will be effective in an emergency, even though there might be some adverse effects, because small amounts of live vaccines will confer immunity to protect against HPAIV infection. Therefore, live vaccines have the advantage of being able to be distributed worldwide soon after an outbreak. In the present study, we found that intranasal administration of a live H5N1 subtype non-pathogenic virus induced antibody and cytotoxic T lymphocyte responses and protected mice against H5N1 HPAIV infection. In addition, it was found that a small amount (100 PFU) of the live vaccine was as effective as 100 μg (approximately 10^{10-11} PFU of virus particles) of the inactivated whole particle vaccine in mice. Consequently, the use of live virus vaccines might be one strategy for preventing pandemics of HPAIVs in an emergency.

1. Introduction

Highly pathogenic avian influenza viruses (HPAIVs) have raised the concern that a pandemic will cause enormous damage worldwide after HPAIVs acquire the ability for human-to-human transmission because most humans do not possess immunity against these viruses [1,2]. It would probably take several months to prepare and distribute inactivated vaccines against HPAIVs [3,4]. However, since small amounts of non-pathogenic live vaccines against HPAIVs should be effective for protection against virus infection, it would be possible to produce live vaccines soon after an outbreak of HPAIVs and prevent a pandemic. In the present study, we examined whether nasal inoculation with a small amount of a live vaccine could prevent H5N1 HPAIV replication in mouse lungs.

Live vaccines are generally more effective than inactivated vaccines in protection against virus infection [5,6]. In the case of measles virus vaccines, it has been shown that inactivated virus did not work as a vaccine to induce protective immunity [7,8]. Both live and inactivated vaccines are available for seasonal influenza

Abbreviations: CPE, cytopathic effects; CTL, cytotoxic T lymphocyte; CFSE, carboxyfluorescein diacetate succinimidyl ester; CMTMR, (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine); HPAIV, highly pathogenic avian influenza virus; LD_{50}, 50% lethal dose; NP, nucleoprotein; OVA, ovalbumin; PFU, plaque-forming unit; $TCID_{50}$, 50% tissue infectious dose.

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virus infection [9]. However, live vaccines against influenza viruses cannot be used for children younger than 2 years of age, persons over 50 years of age, and immunocompromised patients because of side effects [10–12]. It is also possible that live vaccines will acquire pathogenicity by mutation in vaccinated hosts. In contrast, although inactivated vaccines do not have these risks, inoculation is required every year because of their weak potency for eliciting immunological memory [5].

We have established a virus library that contains 144 strains of non-pathogenic avian influenza virus with combinations between 16 hemagglutinins (HA) and 9 neuraminidases (NA) [13]. We previously reported that whole virus particles inactivated by formalin (whole particle vaccines) induced antibody and cytotoxic T lymphocyte (CTL) responses more vigorously than did ether-split vaccines and that whole particle vaccines conferred more effective protection against H3N2 and H5N1 viruses than did ether-split vaccines [14]. Since viruses in the library are non-pathogenic in chickens, we have postulated that these viruses are also non-pathogenic in mice and probably in humans without an attenuation process and gene recombination [15].

In the present study, we compared antigen-specific responses induced by a live vaccine against H5N1 HPAIV with those induced by a whole particle vaccine, since immunological rationales for differences between two types of vaccines have not been revealed [16–19]. We also examined the effects of different routes of inoculation with the live vaccine. We found that intranasal administration of the live vaccine induced antibody and CTL responses more effectively than did subcutaneous administration and conferred protection against infection, whereas subcutaneous immunization with the whole particle vaccines was more effective than intranasal immunization in protective efficacy. In addition, quantitative analysis revealed that a small amount (100 PFU) of the live vaccine was as effective as 100 μg (approximately 10^{10-11} PFU of virus particles) of the whole particle vaccine in mice. Therefore, live viruses from the non-pathogenic avian influenza virus library might be vaccine candidates for worldwide distribution soon after outbreaks of new subtypes of influenza viruses, including HPAIVs.

2. Materials and methods

2.1. Influenza viruses and vaccines

The influenza A virus A/Aichi/2/1968 (H3N2) (Aichi) is a low pathogenic influenza virus [20]. A genetic reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1, formally described as A/R(duck/Mongolia/54/01-duck/Mongolia/47/01)/2004 (H5N1), National Center for Biotechnology Information taxonomy database ID: 376899), was generated by mixed infection with A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) [14]. PB2, PB1, PA, HA, NP, and M genes of Vac-1 were derived from the H5N2 virus, and NA and NS genes were derived from the H7N1 virus [21]. The influenza virus A/Vietnam/1194/2004 (H5N1) (VN1194) is a highly pathogenic strain [22]. The percent sequence similarities between Vac-1 and VN1194 are 92% in HA and 90% in NA at the amino acid level. Live viruses used for inoculation were prepared from the culture supernatant of infected Madin-Darby canine kidney (MDCK) cells. For preparation of whole particle vaccines, viruses were propagated in the allantoic cavities of 10-day-old embryonated hen's eggs at 35 °C for 36–48 h. Then the viruses were purified by ultracentrifugation (112,500 × *g* for 90 min) of allantoic fluid through a 10–50% sucrose density gradient. Formalin-inactivated vaccines were prepared with 0.1% formalin at 4 °C for a week. The purified fixed viruses were then suspended in PBS. Inactivation of the viruses was confirmed by the absence of detectable hemagglutination after one passage of the treated viruses in 10-day-old embryonated hen eggs [14]. 1.4×10^{11} 50% egg infectious dose (EID_{50}) of purified Vac-1 was used to prepare 100 μg of whole particle vaccine of Vac-1. The amount of whole particle vaccines was indicated as that of entire protein including HA and the other viral proteins. The whole particle vaccine of H5N1 virus used in the present study contained 15, 170 HA units of HA antigen in 100 μg vaccine. The nucleotide sequences of HA, NA and NP of Vac-1 grown in embryonated eggs and MDCK cells were analyzed and amino acid sequences were compared in Table 1. There were three nucleotide replacements accompanying amino acid changes between Vac-1 grown in MDCK cells and that grown in embryonated eggs, whereas there was no difference in NA and NP genes between these two viruses.

Table 1
Comparison of amino acids of HA in H5N1 viruses used in this study.

Virus	Amino acid positions in HA		
	282	371	492
Vac-1 live vaccine	E	K	A
Vac-1 whole particle vaccine	K	E	E

Amino acid positions are determined based on the positions of H5 protein of Vac-1. The sequences of NA and NP genes were identical between live vaccine and whole particle vaccine of Vac-1. The position 282 in HA1 is located outside of a sialic acid binding site. The positions 371 and 492 in HA2 are located near the virion lipid membrane. VN1194 possesses identical amino acids on these three positions as Vac-1 used for whole particle vaccines.

2.2. Immunization

C57BL/6 mice (B6) (6–10 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Aichi or Vac-1 was subcutaneously (in 100 μl PBS) or intranasally (in 15 μl PBS) inoculated into mice. VN1194 (3×10^4 50% tissue infectious dose ($TCID_{50}$) = 100 × 50% lethal dose (LD_{50}) in 30 μl PBS) was intranasally inoculated into mice. All experiments were performed with the approval of the Shiga University of Medical Science.

2.3. In vivo CTL assay

Spleen cells (2×10^7 cells/ml) from naïve B6 mice were incubated with 0.5 μM ovalbumin (OVA) OVA257-264 peptide (SIINFEKL) or influenza virus nucleoprotein (NP) NP366-374 peptide (ASNENMETM for Vac-1, ASNENMEAM for VN1194) for 2 h at 37 °C. After washing twice with PBS, the recovered cells (2×10^7 cells/ml) were labeled with different concentrations of carboxyfluorescein diacetate succinimidyl ester (CFDA-SE/CFSE) (0.25 μM or 2.5 μM, Molecular Probes, Eugene, OR) or (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) (CMTMR) (5 μM, Molecular Probes) at room temperature for 10 min. Labeling was stopped with one half volume of fetal calf serum followed by two additional washes. Five million cells carrying each peptide were mixed and injected intravenously into immunized mice. At 14 h after injection, the spleens were harvested to prepare single cell suspensions. CFSE/CMTMR-positive cells were analyzed by a flow cytometer with exclusion of dead cells by ethidium monoazide bromide (Molecular Probes) staining. Cells from infected mice were treated with PBS containing 4% paraformaldehyde before analysis [14,23].

2.4. Virus titration

MDCK cells were cultured in Eagle's MEM supplemented with 10% FCS, L-glutamine, and antibiotics. Diluted suspensions of lung