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A replication-incompetent virus possessing an uncleavable hemagglutinin as an influenza vaccine

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Abstract

Vaccination is one of the most effective measures to protect against influenza virus infection. Inactivated and live-attenuated influenza vaccines are available; however, their efficacy is suboptimal. To develop a safe and more immunogenic vaccine, we produced a novel replication-incompetent influenza virus that possesses uncleavable hemagglutinin (HA) and tested its vaccine potential. The uncleavable HA was engineered by substituting the arginine at the C-terminus of HA1 with threonine, which prevents cleavage of HA into its HA1 and HA2 subunits, preventing fusion between the host and viral membranes. Although this fusion-deficient HA influenza virus that possesses uncleavable HA (uncleavable HA virus) could undergo multiple cycles of replication in only wild-type HA-expressing cells, it could infect normal cells and express viral proteins in infected cells, but could not generate infectious virus from infected cells due to the uncleavable HA. When C57BL/6 mice were intranasally immunized with the uncleavable HA virus, influenza-specific IgG and IgA antibodies were detected in nasal wash and bronchoalveolar lavage samples and in serum. In addition, influenza-specific CD8⁺ T cells accumulated in the lungs of these mice. Moreover, mice immunized with the uncleavable HA virus were protected against a challenge of lethal doses of influenza virus, unlike mice immunized with a formalin-inactivated virus. These findings demonstrate that this fusion-deficient virus, which possesses uncleavable HA, is a suitable influenza vaccine candidate.

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Keywords

influenza virus; vaccine; fusion-deficient virus; mucosal immunity; antibody

1. Introduction

Influenza viruses cause acute respiratory disease and are responsible for epidemics and occasional pandemics as exemplified in 2009 [1,2]. Although medical treatment options are well developed, influenza continues to be a great burden on public health and economies worldwide.

Currently, inactivated and live-attenuated vaccines are available. While inactivated vaccines are safe and induce virus-specific immunoglobulin G (IgG) in sera, live-attenuated vaccines, which bear introduced mutations that are responsible for temperature sensitivity and viral attenuation [3–7], are more effective because they induce not only virus-specific IgG but also secretory Immunoglobulin A (IgA) at the site of infection following intranasal administration [8]. In addition to inducing antibodies, live-attenuated vaccines elicit the virus-specific cytotoxic T lymphocytes (CTLs). CTLs specifically lyse infected cells and prevent viral spread. CTLs are also known to have cross-reactivity between different subtypes of influenza A virus. Thus, the induction of CTLs has attracted attention in vaccine development [9]. Nevertheless adverse effects, such as runny nose, due to replication of the live-attenuated vaccine virus are an issue [10], and importantly, this live vaccine is not recommended for children under the age of 2, adults aged 50 or over, pregnant women, or immunocompromised patients [11]. To overcome current limitations, an ideal influenza vaccine continues to be sought and various strategies have been employed such as virus-like particles (VLPs) [12].

To develop a safe and more effective vaccine that has the merits of inactivated and live-attenuated vaccines, we previously proposed a replication-incompetent virus as a novel influenza vaccine. We reported that NS2-knockout VLPs could efficiently protect mice against lethal doses of influenza virus [16]. However, because of the lack of a cell line that stably expresses NS2 protein, we have to generate VLPs by transfecting plasmids each time.

To overcome this drawback, here we developed a novel replication-incompetent virus that possesses an uncleavable HA (uncleavable HA virus) and assessed its immunogenicity and vaccine efficacy in mice.

2. Materials and methods

2.1 Plasmids

To generate the construct encoding the uncleavable HA gene, we performed inverse PCR to introduce amino acid substitutions at the HA cleavage site (Supplement Fig. 1A) by using a plasmid containing the HA gene of A/California/04/09 (CA04) as a template for PCR and primers containing nucleotide replacements at positions 1031 and 1032 of the HA gene. Primer sequences are available upon request. This mutated HA gene was cloned into a plasmid under the control of the human polymerase I promoter and the mouse RNA

polymerase I terminator (referred as PolI plasmid) as described previously [14]. In addition, a plasmid for the expression of wild-type CA04 HA was also constructed by cloning the open reading frame of the CA04 HA gene into an expression plasmid pCAGGS [15].

2.2 Cells and viruses

Madin-Darby canine kidney (MDCK) and human embryonic kidney 293T (HEK293T) cells were maintained in minimum essential medium (MEM) containing 5% of newborn calf serum (NCS) and in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, respectively. MDCK cells expressing HA from CA04 [HA-MDCK (CA04)] and from A/WSN/33 (WSN) [HA-MDCK (WSN)] were established as follows: for HA-MDCK (CA04) cells, MDCK cells were co-transfected with plasmids for the expression of CA04-HA and puromycin *N*-acetyltransferase, and were cultured in MEM containing 5% NCS and 5 µg/ml puromycin dihydrochloride (Nacalai Tesque). Expression of HA in selected cell clones was confirmed by means of immunostaining with an anti-CA04 HA antibody. For HA-MDCK (WSN), MDCK cells were transfected with plasmids expressing WSN-HA and WSN-neuraminidase (NA) along with pcDNA3.1(+) (Invitrogen, Eugene, OR), and were cultured in MEM containing 5% NCS and 0.8 mg/ml Geneticin. Surviving cell colonies were picked and were tested for HA and NA expression by means of immunostaining with anti-WSN HA and NA antibodies. Antigen-positive cells were then cloned by using a limited dilution assay. Expression of HA but not NA protein was confirmed in the resulting cell clones. All cells were maintained at 37°C in 5% CO₂. Mouse-adapted CA04 virus [16] was propagated in MDCK cells as previously described.

2.3 Generation of uncleavable HA virus

The uncleavable HA virus was artificially generated by using plasmid-based reverse genetics as described previously [14]. This virus was a reassortant between CA04 and A/Puerto Rico/8/34 (PR8) viruses. Briefly, eight PolI plasmids (mutant HA and NA [16] from CA04 and all others from PR8 [17]) were transfected with five protein-expressing plasmids for the expression of the three polymerases, nucleoprotein (NP) [17] and with wild-type CA04 HA into HEK293T cells. Forty-eight hours after transfection, the supernatants containing the uncleavable HA virus were harvested and propagated once in HA-MDCK (CA04) at a multiplicity of infection (MOI) of 0.001 at 35°C for 48 h in MEM containing L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (0.8µg/ml) and 0.3% bovine serum albumin (BSA) (Sigma Aldrich). Cell debris was removed by centrifugation at 3,500 rpm for 20 min at 4°C and the supernatants were stored at -80°C until use.

2.4 Preparation of formalin-inactivated virus

For the formalin-inactivated virus, a reassortant virus between CA04 (wild-type HA and NA genes) and PR8 (the rest of the genes) (CA04 2:6 virus) was generated by using plasmid-based reverse genetics as described above. The virus was propagated in MDCK cells and the culture supernatants containing the virus were treated with 0.1% formalin (final concentration) (Sigma Aldrich) at 4°C for a week to inactivate infectivity. Inactivation of the virus was confirmed by the absence of detectable infectious virus following inoculation of formalin-treated virus into MDCK cells. After confirmation of inactivation of infectivity,

formalin-treated virus was purified by sucrose-gradient ultracentrifugation at $100,000 \times g$ for 2 h at 4°C and stored at -80°C until use.

2.5 Immunostaining assay

Twenty-four hours after infection with viruses, cells were washed twice with phosphate-buffer saline (PBS) and fixed with 100% methanol for 30 min at room temperature. To detect CA04 HA-expressing cells, these cells were reacted with anti-CA04-HA antibody. To determine the viral titer of the uncleavable HA virus, we conducted the same procedure after performing a plaque assay with HA-MDCK (WSN) cells.

2.6 Immunization and protection test

Four-, six-, or eight-week-old female C57BL/6 mice (Japan SLC, Japan) were intranasally immunized with 50 µl of 1.7×10^5 PFU (equivalent to 16 hemagglutination units (HAU)) of the uncleavable HA virus three times, twice, or once, respectively, at 2-week intervals. As control groups, female C57BL/6 mice (4-week-old) were intranasally immunized with 50 µl of 16 HAU of the formalin-inactivated virus (FI) or with medium three times at 2-week intervals. Three weeks after the final vaccination, six mice per group were euthanized to obtain sera, bronchoalveolar lavage (BAL), and nasal washes. In addition, three mice per group were euthanized to obtain lungs and spleen for detection of viral-specific CD8⁺ T-lymphocytes. Also three weeks after the final vaccination, mice were challenged with 10 or 100 times the 50% mouse lethal dose (MLD₅₀) of mouse-adapted CA04 virus. Eight mice per group were monitored for survival and body weight changes for 14 days after challenge. Lungs and nasal turbinates from three mice per each group were collected on days 3 and 6 after challenge to determine virus titers. Virus titers were determined on MDCK cells.

2.7 Detection of virus-specific antibodies

Virus-specific antibodies in nasal wash, BAL, and serum were detected by using an enzyme-linked immunosorbent assay (ELISA) [18]. We used undiluted samples (nasal washes and BAL) and 1:10 diluted samples (serum). In this assay, 96-well ELISA plate wells were coated with approximately 200 HAU (in 50 µl) of purified CA04 virus treated with disruption buffer (0.5M Tris-HCl [pH 8.0], 0.6M KCl, and 0.5% Triton X-100). After incubation of the samples on virus-coated plates, goat anti-mouse IgA or IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratory Inc., Gaithersburg, Md) was added to detect bound antibody.

2.8 Detection of virus-specific CD8⁺ T lymphocytes

A tetramer assay was used to detect virus-specific CD8⁺ T lymphocytes. Single cell suspensions of lung and spleen were prepared from inoculated mice three weeks after their final vaccination. After being incubated with anti-CD16/32 (BD Bioscience), the cells were mixed with a Phycoerythrin (PE)-conjugated H-2D^b tetramer specific to the NP epitope (amino acid positions 366–374, ASNENMETM) (MBL) at room temperature for 20 min. Cells were then incubated with Allophycocyanin-Cyanine-7 (cy7)-conjugated anti-CD3ε antibody (BD-Bioscience), PE-cy7-conjugated anti-CD8α antibody (BD Bioscience), and via-probe (BD Bioscience) for 30 min at 4°C and then washed with PBS containing 0.5%

BSA and 2 mM EDTA (pH 7.2). Cells were analyzed with FACS Aria II (Becton, Dickinson and Company) and Flowjo software (Tree Star, Inc.).

2.9 Ethics

All animal experiments were performed in accordance with the University of Tokyo's Regulations for Animal Care and Use, which were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo (approval number PA 10-15). The committee acknowledged and accepted both the legal and ethical responsibility for the animals, as specified in the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology.

3. Results

3.1. Replicative ability and viral protein expression of the uncleavable HA virus in vitro

To generate the uncleavable HA, the arginine (R) at the cleavage site of HA1, which is important for HA cleavage by trypsin-like protease, was changed to threonine (T) (Supplementary Fig. 1A). Since the cleavage of HA protein is essential for membrane fusion [19], a virus possessing this uncleavable HA should be unable to complete its replication cycle (Supplementary Fig. 1B). To assess this, we examined if the uncleavable HA virus replicated in MDCK and HA-MDCK (CA04) cells: the latter cells constitutively express CA04 virus HA. As expected, CA04 2:6 reassortant virus, serving as a control, replicated to a similar extent in both MDCK and HA-MDCK (CA04) cells (Fig. 1). By contrast, although no infectious particles of the uncleavable HA virus were detected in the supernatant of MDCK cells, the uncleavable HA virus could replicate efficiently in HA-MDCK (CA04) cells, to a level comparable to that of CA04 2:6 reassortant virus (Fig. 1). We also tested whether the virus could revert to the wild-type by passaging it in MDCK cells. After three passages, we did not observe any cytopathic effect (data not shown), suggesting that the mutation was stable.

To further ensure that the uncleavable HA virus replicated in HA-MDCK cells, but not in MDCK cells, we visualized the infected cells by immunostaining (Fig. 2). Whereas CA04 2:6 virus efficiently spread in both cell types (Fig. 2, left panels), the uncleavable HA virus only infected and expressed viral protein (HA in this case) in individual MDCK cells (Fig. 2, upper middle panel) although it efficiently spread in HA-MDCK (WSN) cells (Fig. 2, lower middle panel). These results indicate that the uncleavable HA virus is replication-incompetent, capable of only a single cycle of infection and unable to produce infectious progeny in normal MDCK cells.

3.2. Induction of virus-specific immunity by the uncleavable HA virus in mice

To assess the ability of the uncleavable HA virus to induce virus-specific immunity against influenza virus, we immunized mice with the uncleavable HA virus or FI. To examine virus-specific antibody production, IgG and IgA against CA04 in the nasal wash, BAL, and serum of immunized mice were measured by using an ELISA (Fig. 3). Although only low levels of

IgG and IgA were detected in mice immunized three times with FI or inoculated with medium, antibody levels increased as the number of vaccinations increased in mice immunized with the uncleavable HA virus. Notably, both the virus-specific IgG and IgA titers in BAL, and the IgG in serum from mice immunized only once with the uncleavable HA virus were higher than the respective titers from mice immunized with FI three times.

We also examined the virus-specific (NP in this case) CD8⁺ T cell response by using a tetramer assay. NP-specific CD8⁺ T cells were identified in the lungs and spleens only of mice immunized with the uncleavable HA virus (Fig 4). Of note, NP-specific CD8⁺ T cells predominantly accumulated in the lungs rather than the spleens of the immunized mice (Fig. 4), and the number of NP-specific CD8⁺ T cells increased as the number of vaccinations increased (Fig. 4B).

These results demonstrate that the uncleavable HA virus could elicit both humoral and cellular immunity more efficiently than FI against influenza virus at the local sites where influenza virus replicates.

3.3. Protection against influenza virus in mice immunized with the uncleavable HA virus

Finally, we evaluated the protective ability of the uncleavable HA virus. To this end, we monitored body weight changes and survival of mice immunized with the uncleavable HA virus after challenge. The body weights of mice inoculated with medium decreased rapidly to approximately 70%–80% (Fig. 5A) and most mice (7 out of 8) succumbed to their infection (Fig. 5B). The body weights of mice immunized with FI also clearly decreased to 80% (Fig. 5A) and several mice (3 and 5 out of 8 with 10 and 100 MLD₅₀, respectively) died (Fig 5B). By contrast, with 10 MLD₅₀, mice immunized once with the uncleavable HA virus showed slightly reduced body weights, and mice immunized twice and three times with this virus showed no body weight changes (Fig. 5A left panel). Moreover, all mice immunized with this virus survived (Fig. 5B left panel). With 100 MLD₅₀, although mice immunized once with this virus showed similar body weight reductions as mice immunized with medium and FI, the mice immunized twice and three times with the uncleavable HA virus showed only slight or no body weight loss, respectively (Fig. 5A, right panel). All mice immunized with the uncleavable HA virus survived, except for 3 out of 8 mice immunized only once with the uncleavable HA virus (Fig. 5B, right panel).

We also determined virus titers in the lungs and nasal turbinates of mice immunized with the uncleavable HA virus after challenge (Table 1). Virus titers in both organs of mice immunized with the uncleavable HA virus were appreciably lower as the number of vaccinations increased, especially on day 6 post-infection, than those in organs of mice immunized with medium or with FI. Taken together, these results indicate that the uncleavable HA virus has potential as an influenza vaccine.

4. Discussion

Currently, embryonated hen's eggs are typically used to produce both inactivated and live-attenuated vaccines, although cell-based vaccines are approved in Europe. However, vaccine production in eggs has several issues. One problem is the alteration of antigenicity from that

of the circulating virus during cultivation of the vaccine strain in embryonated hen's eggs [20–22]. Another is allergic reactions to components derived from eggs present in vaccines grown in eggs [23]. In contrast, the antigenicity of viruses grown in cells closely matches that of viruses isolated from influenza patients [22, 24]. Since the uncleavable HA virus in this study only grew in HA-expressing cells and to a level comparable to a virus possessing wild-type HA (Fig. 1), production of this vaccine should not only be feasible, but this type of vaccine should maintain its antigenicity; however, the cell line needs to be validated to ensure that it is free of anything harmful, such as tumorigenicity.

We demonstrated that antibody responses (IgA and IgG) were efficiently induced by the uncleavable HA virus (Fig. 3), like NS2-knockout VLPs [13]. In addition to antibody responses, we tested the virus-specific CTL response, which we did not examine with our NS2-knockout VLPs, and found that a CTL response to an internal viral protein (i.e., NP) was also induced by this virus (Fig. 4). The reason for this induction is that viral proteins (NP in this case) are expressed in cells infected with this replication-incompetent virus, suggesting that this virus could act similarly to a live-attenuated virus in terms of induction of immunity despite not generating infectious progeny.

Our findings suggest that a combination of this type of virus and cells expressing a seasonal virus HA can serve as a platform for a bivalent vaccine as follows: If an uncleavable H5 HA gene is partnered with the rest of the genes from a seasonal influenza virus, the resulting virus can serve as a vaccine against H5 and seasonal influenza viruses. That is, antibody responses against H5 HA (expressed from the viral HA gene) and HA (derived from the cell line) would likely be induced as would CTL responses against H5 HA and the internal viral proteins (expressed from the genes of the seasonal virus). Likewise, if a gene, encoding a protein responsible for inducing immunity, from another respiratory pathogen, such as *Streptococcus pneumoniae*, which causes secondary bacterial infections following influenza virus infection, or respiratory syncytial virus, which causes severe manifestations in infants, is inserted into the HA gene, the resultant virus may be used as a vaccine against both influenza virus and the other respiratory pathogen.

In conclusion, a replication-incompetent virus that possesses a modified HA gene, when partnered with an HA-expressing cell line, has potential as a novel vaccine candidate.

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Abbreviations

MDCK Madin-Darby canine kidney

HEK293T	human embryonic kidney 293T
HA	hemagglutinin
HA-MDCK	HA-expressing MDCK
MOI	multiplicity of infection
PFU	plaque-forming unit
FI	Formalin-inactivated virus
BAL	bronchoalveolar lavage
ELISA	Enzyme-linked immunosorbent assay
WSN	A/WSN/33
PR8	A/Puerto Rico/8/34
CA04	A/California/04/09
MLD₅₀	50% mouse lethal dose

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Highlights

- Uncleavable HA virus did not replicate in normal cells.
- Uncleavable HA virus efficiently elicited mucosal and systemic immunity.
- Mice immunized with uncleavable HA virus were protected from virus challenge.

Fig. 1. Growth kinetics of CA04 2:6 and uncleavable HA viruses in MDCK and HA-MDCK cells. Both cell types were infected with viruses at an MOI of 0.001. Supernatant was collected every 12 h, and viral titers in the supernatants of both cell types were determined by means of the conventional plaque assay or a plaque assay with immunostaining with an anti-HA antibody using HA-MDCK cells. The broken line indicates the detection limit for virus titers (5 PFU/ml).

Fig. 2.

HA protein expression in virus-infected cells. MDCK and HA-MDCK (WSN) cells were infected with CA04 2:6 and uncleavable HA viruses; 24 h after infection, the cells were fixed and stained by using an anti-CA04 HA antibody. Cells colored brown are Ca04 HA-expressing cells.

Fig. 3.

Induction of virus-specific IgG and IgA in nasal wash, BAL, and serum of mice immunized with the uncleavable HA virus. Virus-specific antibodies were detected by means of an ELISA. Samples from six mice from each group were obtained 3 weeks after the final vaccination. Results are expressed as the mean absorbance ($\pm$ standard deviations) of undiluted samples (nasal wash and BAL) or of 1:10 diluted samples (serum). Statistically significant differences among the groups were assessed by using the Tukey-Kramer method. Asterisks (*) or (**) indicate a significant difference from samples from mice inoculated with FI (*, $P < 0.05$; **, $P < 0.01$). The numbers on the x-axis indicate the times of vaccination of uncleavable HA virus. M: medium. FI: Mice were immunized with formalin-inactivated vaccine three times with 2 week intervals.

Fig. 4.

Induction of virus-specific CD8⁺ T cells in mice immunized with the uncleavable HA virus. Virus-specific CD8⁺ T cells were detected in the lungs and spleen of inoculated mice by using a tetramer assay. (A) Representative dot plot for CD3ε⁺ CD8α⁺ cells from the spleen or lungs of a mouse inoculated with medium or with the uncleavable HA virus. (B) The percentage of virus-specific CD8⁺ T cells (NP-tetramer⁺ CD8α⁺ population) in the CD3ε⁺ CD8α⁺ cell populations in the lungs and spleen of inoculated mice. Results are expressed as the mean values (± standard deviations). Statistically significant differences among the groups were assessed by using the Tukey-Kramer method. Asterisks (* or **) indicate significant differences from samples from mice inoculated with FI (*, $P < 0.05$; **, $P < 0.01$).

Fig. 5. Body weight changes and survival curves for mice challenged with wild-type virus. Eight mice per group were intranasally challenged with 10 or 100 MLD₅₀ of mouse-adapted CA04 3 weeks after the final vaccination. Body weights were measured (A) and survival rate was monitored (B) for 14 days after challenge.

Table 1

Protection against challenge with lethal doses of influenza virus in mice immunized with an uncleavable HA virus^a

challenge dose	inoculum	days post-infection	organ	Virus titer (mean $\pm$ SD log ₁₀ [PFU/g])	challenge dose	inoculum	days post-infection	organ	Virus titer (mean $\pm$ SD log ₁₀ [PFU/g])
10MLD ₅₀	Medium	3 dpi	NT ^b	5.1 $\pm$ 0.6	100MLD ₅₀	Medium	3 dpi	NT	5.7 $\pm$ 0.4
			Lung	7.0 $\pm$ 0.4				Lung	7.5 $\pm$ 0.2
		6 dpi	NT	4.2 $\pm$ 0.3			6 dpi	NT	NA ^c
			Lung	6.4 $\pm$ 0.4				Lung	NA
	FI ^d	3 dpi	NT	4.6 $\pm$ 0.5		FI	3 dpi	NT	5.7 $\pm$ 0.7
			Lung	7.3 $\pm$ 0.0				Lung	7.1 $\pm$ 0.7
		6 dpi	NT	4.0 $\pm$ 0.2			6 dpi	NT	3.9 $\pm$ 0.5
			Lung	6.0 $\pm$ 0.3				Lung	5.3 $\pm$ 0.4
	Once	3 dpi	NT	5.2, 5.8		Once	3 dpi	NT	4.9 $\pm$ 0.4
			Lung	7.8 $\pm$ 0.2				Lung	7.5 $\pm$ 0.5
		6 dpi	NT	ND ^e			6 dpi	NT	3.3, 3.5
			Lung	3.4, 3.2				Lung	3.7 $\pm$ 2.3
	Twice	3 dpi	NT	4.3, 3.9		Twice	3 dpi	NT	6.0
			Lung	6.1 $\pm$ 0.8				Lung	1.6, 6.5
		6 dpi	NT	ND			6 dpi	NT	1.6
			Lung	2.5, 2.5				Lung	ND
	Three times	3 dpi	NT	ND		Three times	3 dpi	NT	ND
			Lung	2.2				Lung	5.8, 2.4
		6 dpi	NT	ND			6 dpi	NT	ND
			Lung	2.0				Lung	ND

^aTwelve mice from each group were intranasally inoculated with 10 or 100 MLD₅₀ of mouse-adapted CA04 (50 μ l per mouse) 3 weeks after the final vaccination. Three mice per group were sacrificed on days 3 and 6 post-infection, and lungs and nasal turbinate were collected to determine virus titers. Results are expressed as the mean titer (log₁₀ PFU/g) $\pm$ standard deviations. When virus was not recovered from all three mice, individual titers are given.

^bNT: Nasal turbinate

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NA: Not applicable because the mice died
FI: formalin-inactivated virus. Mice were immunized three times with two week intervals.
ND: Not detected (detection limit, 10 PFU/lung or 5 PFU/NT)