원 저

Generation of NS1-mutant Live attenuated Human Influenza vaccine candidate

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NS1 변이형 약독화 인플루엔자 생백신 후보의 개발

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배 경 : 분자유전학의 발전으로 cDNA만을 이용하여 세포로부터 바이러스를 만들어내는 일이 가능해졌다. 인플루엔자 바이러스는 8개의 유전자 RNA 분절로 구성된 전형적인 분절형 바이러스로서 이들 바이러스 RNA을 만들어낼 수 있는 플라즈미드를 이용하여 유전자의 조작이 가능한, 여러 형태의 바이러스 합성이 가능하여졌다. 이러한 기술의 발전을 바탕으로 약독화 생백신 주로서 사용이 가능한 바이러스를 제조해 내고자 하는 일련의 시도로서 바이러스의 세포내 감염 후 나타나는 인터페론 반응을 적절히 억제하여 세포내에서 증식은 가능토록 유지하면서 독성은 약화된 형태의 사람 인플루엔자 바이러스를 만들어 내고자 하였다.
방 법 : 인터페론 조절 단백인 NS1의 N-말단 부위를 보유한 NS1-126, NS1-99 변이 유전자를 중합효소연쇄반응법을 이용하여 야생형 바이러스로부터 만들었고 나머지 유전자(PA, PB1, PB2, NP, NA, HA, M) 역시 동일한 방법으로 얻었다. 인플루엔자 바이러스 합성에 흔히 이용하는 293T/MDCK 세포주에서 바이러스 합성을 시도하였으며 7-9 일란에서 증폭하고 역전사 중합효소 연쇄반응 및 염기서열분석을 통하여 NS1 변이형 바이러스(A/Texas)를 최종 확인하였다. FDA에서 허가한 세포주(Vero 세포) 및 허가 가능 세포주(293, CEF 세포)에서 동일한 실험을 시행하였다.
결 과 : 1) 293T/MDCK 세포주로부터 유전자 재조합된 야생형 A/Texas 인플루엔자 바이러스 및 NS1 변이형 (NS1 1-126Δ NS 1-99Δ) A/Texas 바이러스를 합성하였다. 계란에서 증폭한 NS1 변이형 바이러스의 역가(10^7/mL)는 야생형 바이러스(10^9/mL/)에 비하여 10-100 배가량 낮았으며 인터페론 반응이 미숙한 조생란(7일란)에서 배양한 경우 역가가 높게 측정되었다. 2) 293T/MDCK 세포주에서 얻은 야생형 및 NS1 변이형 A/Texas 인플루엔자 바이러스의 성상을 FDA에서 허가한 Vero 세포주와 293, CEF 세포에서 확인한 결과 Vero 세포에서는 바이러스의 효율적인 성장이 관찰되지 못하였다. CEF 세포는 플라즈미드 DNA의 transfection이 불가능하였으나 바이러스의 성장은 비교적 효율적이었다. 293세포는 transfection, 바이러스 성장면에서 타 세포에 비하여 비교적 우수하였다. 3) Vero, CEF, 293 세포주에서 NS1 변이형 A/Texas 인플루엔자 바이러스 합성을 수 차례 시도하였으나 실패하였다.
결 론 : NS1 약독화 인플루엔자 바이러스는 이미 개발된 저온 적응형(cold adapted) 바이러스에 비하여 면역학적으로 우수한 반응을 유도할 것으로 기대되는 생백신의 후보이다. 비록 현재까지 FDA에서 공인하고 있는 세포주에서의 인간 NS1 약독화 바이러스의 합성에는 실패하였으나 293/CEF의 혼합배양, 돼지 호흡기 상피세포 등 사람에 있어 보다 안전한 세포주에서의 바이러스 합성시도가 계속될 전망이어서 향후 안정적인 시스템에서 바이러스를 만들어낼 수 있을 것으로 기대된다.

Key Words : 인플루엔자, NS1 단백, 생백신

INTRODUCTION

Influenza virus infection remains a major public health concern, resulting in severe illness and thousands of deaths each year around the world (1). There is a great need for improved influenza vaccines that

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could confer effective antibody and cell mediated immunity, and that could be administered conveniently and safely to all age groups. The vaccines available at present consist of injected killed influenza virus preparations that are designed to elicit humoral immunity against current influenza A and B virus strains. A new vaccination approach, using cold adapted live influenza viruses delivered intranasally, appears to have significant advantages over the injected killed vaccine (2, 3). Being infectious but unable to cause disease, cold-adapted virus reportedly induces not only antibody responses against the influenza virus types in the vaccine, but also cross-reactive cell mediated immunity against related strains. In addition to the ease of intranasal administration, the cold adapted virus also readily induces mucosal immune responses needed to protect against influenza infection. Although cold-adapted virus is attenuated to physical conditions, it retains undesirable properties that may interfere with immune responses. Breakthroughs in genetic manipulation and understandings in immune evasion mechanisms of influenza viruses suggest that additional approaches can be used to rationally design and develop new vaccines that could incorporate nasal delivery and attenuation, but with considerable advantages of safety and immunogenicity beyond current vaccine candidates (4, 5).

In order to develop a live virus vaccines by altering of the influenza virus gene that functions as an interferon antagonist, we tried to rescue a NS1-mutant A/Texas from various cell system by reverse genetics.

MATERIALS AND METHODS

1. Preparation of 8 viral cDNAs and 4 expression plasmids

A/Texas/36/91 viral stock from a lot prepared for the Division of Microbiology and Infectious Diseases, NIAID, NIH, was the source of the influenza virus gene segments. Viral RNA, extracted using a commercial kit, served as the template for viral cDNAs produced by reverse transcriptase using primers (data are not shown). The cDNAs were then expanded by PCR and the gene segment products extracted from agarose gels. The extracted cDNA was then ligated into pGEM-T plasmid and used to transform DH-5α bacteria. Miniprep DNAs with inserts of the correct size and sequence were digested, and the insert was into pPOLI vectors, which were then gel-purified. This procedure yielded 8 plasmids, each one contaning influenza HA, NA, NP, M, NS, PB1, PB2, and PAm respectively. Production of the PB1, PB2, PA, and NP expression plasmids was by cloning the gene segments from A/WSN/33 (H1N1) as above, but using the expression vector pCAGGS instead of pPOLI.

2. Preparation of the altered NS1 genes

Each of the altered NS1 viruses was produced form the Texas NS pPOLI plasmid by inserting stop codons into each reading frame at specific points such that only defined truncated NS1 proteins would be produced. The altered NS1 genes were shown, by sequencing, to have only the desired changes. The Texas NS1-126Δ has stop codons such that only amino acids 1-126 of NS1 would be expressed. It also has a deletion from nucleotide 402 to 482. The Texas NS1-99Δ has stop codons permitting only the residue 1-99 of NS1 to be expressed, and has deletion of nucleotides 324-382 (Figure 1).

3. Transfection of MDCK and 293T cells and rescue of recombinant virus

To produce infectious virus particles by reverse genetics, each of the 4 expression plasmids for PB1, PB2, PA, and NP, as well as each of the 8 viral RNA plasmids were transfected into a mixture of MDCK cells and 293T cells, the best cellular system to construct attenuated stains. For the altered NS1 viruses, each of the two gene segments altered as described above were used instead of the wild-type NS1 plasmid. Detailed procedures are as follows; 0.3 ug of each plasmids, 4 expression plasmids and the 8 vRNA plasmids, were diluted in 100 uL Opti-MEM and transfected into a mixture of 5X Opti-MEM abd 2 mL DMEM supplemented with 0.3% BA and 10 mM Hepes (Figure 2). To recover the NS1 mutant A/ Texas virus, the plasmid NS1 PR8 pCAGGS was transfected along with the 4 expression plasmids and the 8 vRNA plasmids. The transfected cells were incubated at 37℃. After incubation for 72 hours for wild-type virus or 96 hours for the altered NS1 viruses, the supernatants

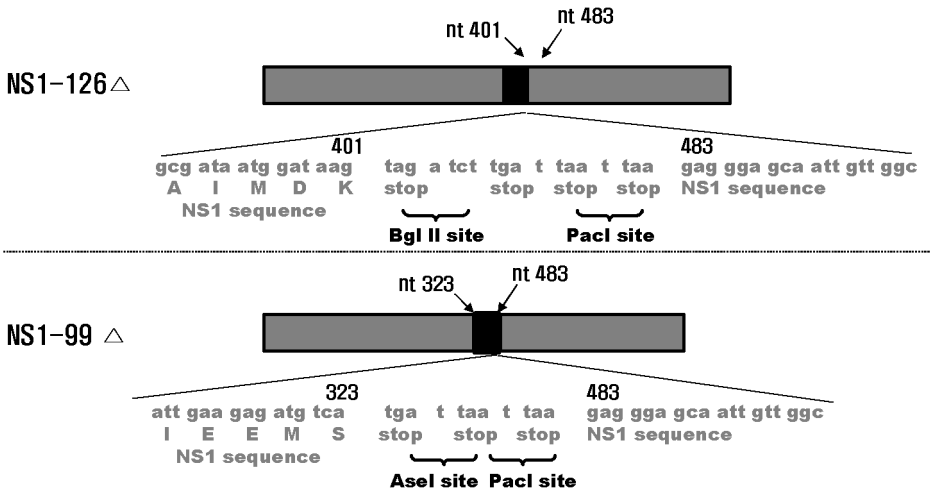

Figure 1. The Texas NS segment encodes the NS1 gene and the splice variant NEP gene. The Texas NS 1-126Δ gene has a deletion of nucleotide 402-482, four stop codons in three reading frames, a *Bgl* II site and a *Pac* I site. The Texas NS 1-99Δ gene has a deletion of nucleotide 324-482, four stop codons in three reading frames, an *Ase* I site and *Pac* I site.

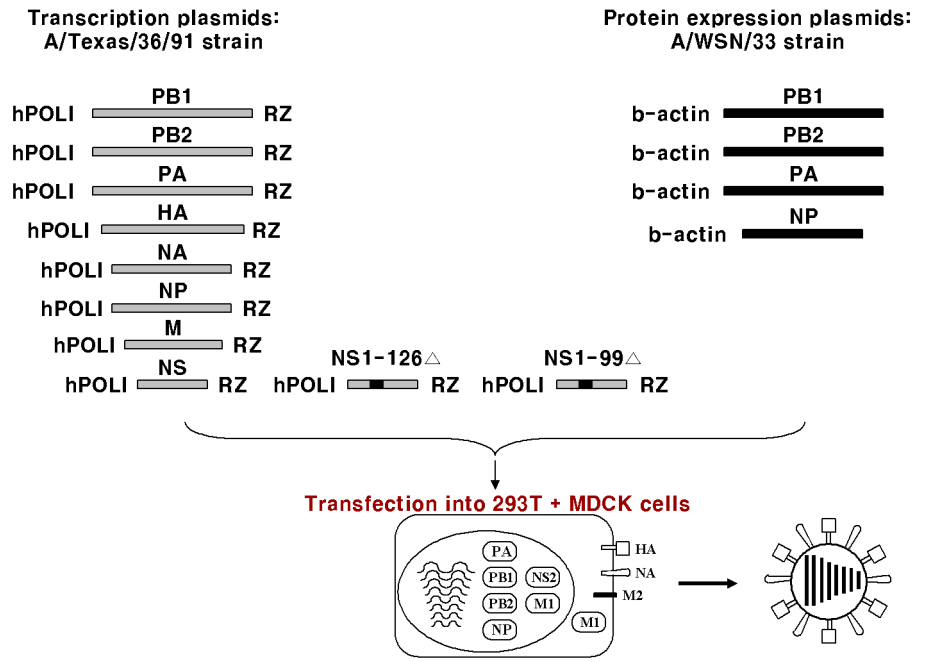

Figure 2. Plasmid-based rescue system for generating NS1 mutant influenza virus.

were harvested and an 100-200 μl of each supernatant were injected into the allantoic cavity of embryonated chicken eggs for virus expansion. Hemagglutination assays were performed to detect the presence of virus in allantoic fluid. We performed RT-PCR with specific primer for the samples with positive result from hemagglutinin assay. Analyses of restriction enzyme sites followed by sequencing confirmed the identity of each gene segment of the rescued viruses. Plaque assay of NS1 mutant virus was performed on MDCK cells in the presence of 2 ug/mL trypsin (Difco) at 37℃.

4. Growth kinetics of NS1 mutant virus in Embryonated eggs and various cell lines

In order to know the growth property of NS1 mutant virus, we measured EID_{50} in 8-day old eggs. For every 10 fold dilution, 4 eggs were used. Infected eggs were incubated at 37℃ for 48 hours. Allantoic fluid was harvested and titer was measured by HA assay. Depending on the viral titer obtained from the above method, we inoculated 100 pfu of viruses into the allantoic cavity of embryonated eggs ranging in age from 6 to 8 days. Infected eggs were incubated at 37℃ for 48 hours. Allantoic fluid was harvested and titer was measured by plaque assay.

To obtain additional information about the rescue system, the susceptibility of Vero, CEF, and 293 cells to influenza virus infection was evaluated. Cells were infected with rA/WT-Texas, NS1 mutant viruses isolated from coculture of 293T and MDCK cell. The growth of viruses in these cells were compared with their growth kinetics in MDCK cells.

5. Rescue trial with Vero, CEF, and 293 cells

We tried to rescue the NS1 mutant viruses from Vero cells because these cells were originally used for recovering recombinant influeza viruses from plasmid DNA (5). Novavax, Inc. supplied FDA approved cell line (p145, L# 6304). And we prepared CEF cells (Chicken Embryo Fibroblast) from 7-8 day old embryonated egg for cell line for viral rescue. The viral rescue from human embryonic kidney (293) cells was also included in our experiment because these cells are used for adenovirus vector production for gene therapy to treat patients with cancer. The indicated cell line (p53) was obtained from Vector Core and GMP Production Facility, Institute for Gene Therapy of Mount Sinai School of Medicine. Transfection procedure and growing conditions were same as above.

RESULTS

1. Viral rescue from 293T/MDCK cells

We successfully procured the rA/WT-Texas, 2 NS1 mutants (NS1-126Δ NS1-99Δ) from 293T/MDCK cells. However, HA titer from 2 NS mutant viruses were lower than that of wild-type (Table 1) and so was the rescue efficiency. We expanded the NS1-126Δ NS1-99Δ mutants in 8 day old embryonated eggs. The presence of corresonding NS segments was confirmed by RT-PCR performed on vRNA isolated from alantoic fluid. (Figure 3). The results were also confirmed by sequencing RT-PCR products. We measured these viruses by plaque assay. Viral titer were also lower in NS1 mutant viruses (Table 1). Interestingly, plaque size of the NS1 mutant viruses were markedly reduced as compared with WT A/Texas virus.

Because the usual way of expanding the viral strains for preparing current vaccine is in embryonated eggs, the growth of mutant viruses was tittered also in the eggs, The EID_{50}/mL for mutants was 10^7. Compared to the WT virus the growth of mutant viruses is attenuated in MDCK cells (Figure 5) and 8 day old embryonated eggs.

Virus titers from allantoic fluid of eggs infected with

Table 1. Viral Titer of rA/WT-Texas and NS1 Mutant Viruses

	HA titer	pfu/mL	EID_{50}
rA/WT-Texas	1024	10^7	2×10^9
NS1-126Δ	64	10^5	4.7×10^7
NS1-99Δ	64	3×10^5	3.2×10^7

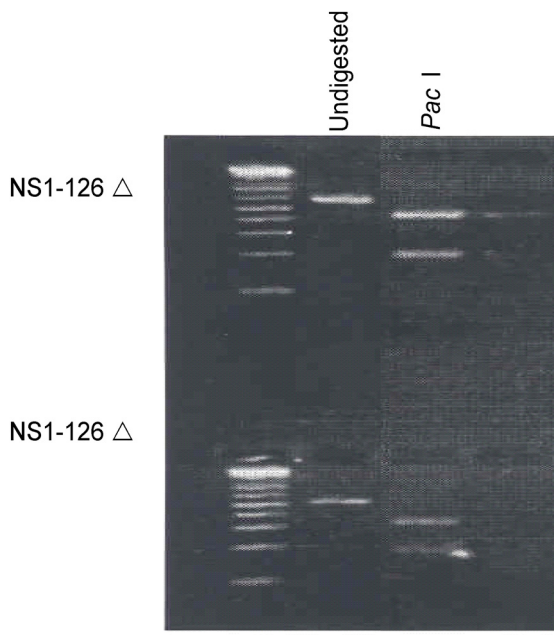

Fig. 3. RT-PCR and restriction enzyme digestion of NS1 mutant influenza virus.

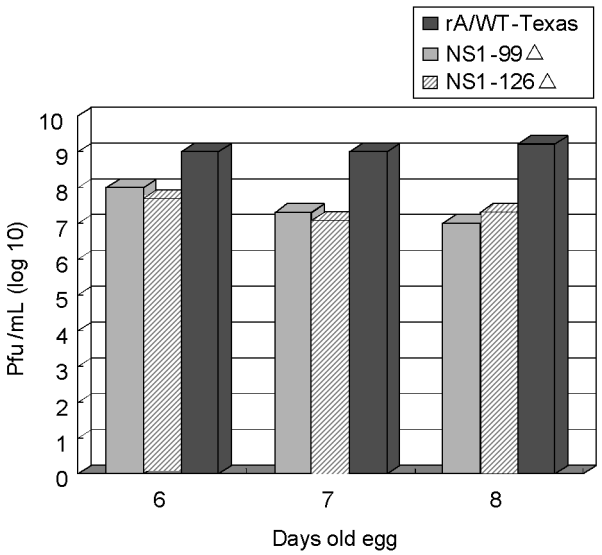

Figure 4. Embryonated eggs were infected with either rA/WT-exas and NS1 mutant virus, and allantoid fulid was titrated on MDCK cells.

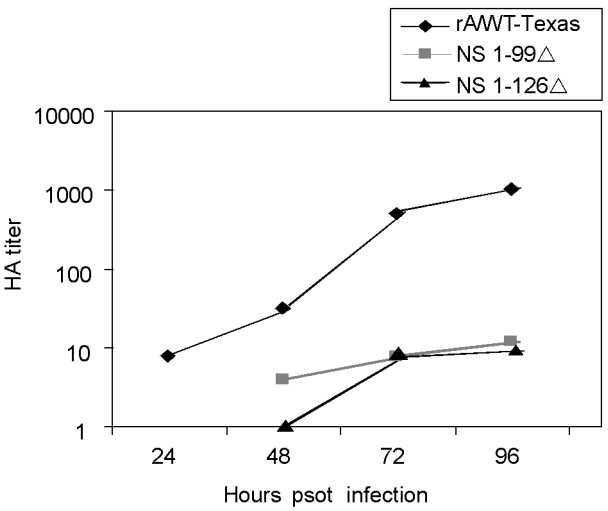

Figure 5. Viral HA titer in MDCK cells (M.O.I=0.001).

NS1 mutant and WT A/Texas virus were also measured by plaque assay (Figure 4) Yield of NS1 mutant virus decreased with the age of infected egg. The maximal titer was 1×10^8 pfu/mL for 6-day old eggs. WT A/Texas virus consistently grew to titers greater than 1×10^9 pfu/mL, regardless of the age of the egg.

2. Growth kinetics of rA/WT Texas and NS1 mutants (rescued from 293T/MDCK) in various cell lines

In general, NS1 mutant viruses were attenuated in interferone competent MDCK cells as expected (Figure 5). After infection at a M.O.I. of 0.001 only WT virus replicated to detectable titers. No virus was recovered from supernatants collected from Vero cells following infection at a M.O.I of 0.001 or 0.01 (figure not shown). Although viral titer was low, CEF cells and 293 cells were capable of growing the NS1 mutant viruses as well as rA/WT Texas virus (Figure 6).

3. Viral rescue from Vero cells, CEF and 293 cells

We couldn't get any virus from Vero cells. Following the observation that CEF cells may promote viral growth, we also tried to recover viruses from a mixture of Vero and CEF cells. However, these attempts were not successful. NS1 mutant viruses were also not prepared form 293 cells. To summarize, we concluded that Vero cells were not good cell line for creating recombinant viruses. Furthermore, 293 or CEF cells were not adequate for the NS1 mutant A/Texas rescue as a mono-cell system.

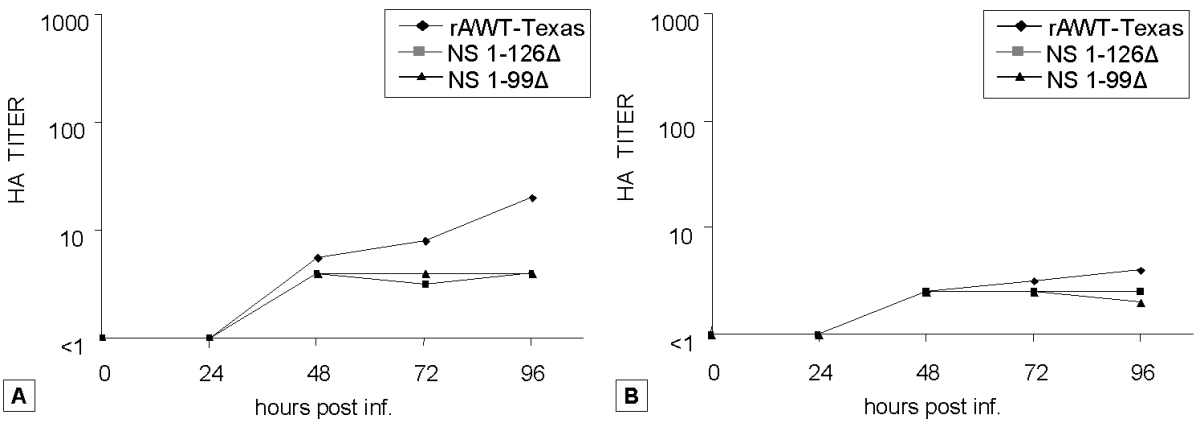

Figure 6. Viral HA titer in CEF (A) and 293 (B) cells (M.O.I=0.01).

DISCUSSION

The main option for reducing the impact of influenza is vaccination. There are two available types of influenza vaccine up to now; one is inactivated and the other is live-attenuated vaccine. Such live-virus vaccines can be administered by nasal spray-a distinct advantage over the more difficult and costly route of intramuscular injection using needles. Live viruses can also induce local neutralizing immunity and cell-mediated immune responses, which may be associated with a longer-lasting and more cross-protective immunity than that elicited by chemically inactivated virus preparations. Finally, overall protection may be better in certain age groups (children 6 months to 9 years), and there is also evidence of a drastic reduction in secondary bacterial infections causing otitis media, and thus in the need for use of antibiotics (2). The further use of live influenza vaccines will shed light on the benefits, potential risks, and economic and logistic consequences from this approach. Continuing surveillance and monitoring will be needed to safeguard against unexpected complications that might arise from the widespread use of cold-adapted vaccines.

The advent of techniques to engineer site-specific changes in the genomes of negative-strand RNA viruses (6, 7) has made it possible to consider new vaccine approaches. Specially, it is now possible to tailer-make strains with unique properties that lead to attenuation. For example, exchanging the promoter region of the NA gene of an influenza A virus with that of an influenza B virus gene attenuates the strain in mice (8). Alternatively, engineered changes in the PB2 gene led to making a live influenza A virus candidate (9, 10) with interesting biological characteristics.

A further improvement in reverse genetics techniques now allows the rescue of influenza virus vaccine candidates from cells transfected with plasmids. This plasmid-only rescue system makes it possible to engineer deletions in the genomes of influenza viruses with improved stability.

Palese group have shown that the NS1 proteins of influenza viruses have interferone (IFN)-antagonist activity (11). Following infection by a virus, the host usually mounts an antiviral IFN response. Many viruses have evolved mechanisms to subvert the host IFN response by expressing an anti-interferon protein/or proteins. It has been demonstrated previously that the influenza A virus NS1 protein exhibits IFN antagonist activity, allowing virus to replicate in IFN competent systems (11, 12). So, in the case of influenza virus, changing the NS1 protein can result in an altered virulence characteristic. Genetic alternation of influenza virus NS1 results in viral growth attenuation, suggesting that NS1 modification may be an important new approach for generating live attenuated viral vaccines. In humans, a virus lacking the whole NS1 gene may be too attenuated to be suitable for vaccines. However, we have found that viruses expressing the N-terminal 99 or 126 amino acids of the NS1 protein possess intermediate IFN-antagonist activity in mice. These viruses show significant growth attenuation in immunologically mature BALB/c mice, which is directly related to the length of the remaining amino terminal portion of NS1 (7). Furthermore, when administrated to mice, the NS1 modified viruses are much less virulent than wild type mouse adapted virus. In challenge studies in mice, animals immunized with genetically altered NS1 influenza virus gave measurable antibody titers and were protected against lethal challenges. Complete versus partial deletion of the NS1 gene also affects immunogenicity of the genetically altered viruses, and this needs to be evaluated in humans as well. The fact that the IFN response should be higher in humans infected with NS1 mutants than in those infected with wild-type virus may lead to a vigorous humoral immune response. This is based on the finding that type I IFNs can potently enhance the primary antibody response to proteins and can act as adjuvants in mice (13). If this immuno-stimulatory effect is also observed in humans inoculated with NS1-mutant viruses, lower amounts of virus may be used for vaccination. If the inoculum size could be reduced by a factors of 100 relative to the dosage needed for the cold-adapted vaccine, protection of large segments of the population in developing countries would become feasible.

Therefore, two different modifications of the in-

fluenza virus NS1 protein have been created (Figure 1) for testing in human trials to determine whether the effects are similar to those found in the murine model. The NS1-126Δ gene segment has stop codons inserted into each reading frame such that only aminoacids 1-126 of NS1 would be expressed. The plasmid also has a deletion from nucleotide 402 to 482. Similarly NS1-99Δ expresses only the first 1-99 amino acids of NS1. It contains deletion from nucleotide 324 to 482.

We tried to rescue the rA/WT-Texas and two NS1 mutant virus from 293T/MDCK cells, which are known as highly efficient system for influenza viral rescue. Two NS1 mutant A/Texas virus were rescued as well as WT A/Texas form 293T/MCDK cells.

In case of NS1 mutant virus, the EID_{50}/mL for mutants was 10^7. Compared to the rA/WT virus, the growth of mutant viruses was attenuated in 8 day old embryonated eggs. However, viral titer was higher in 6 or 7 day old eggs compared with that in 8 day old eggs. This may be due to lower IFN response in younger aged eggs.

Although 293T/MDCK cells proved efficient for rescuing NS1 mutant A/Texas, they are not suitable for generating human vaccine candidates. Aforementioned cells are not listed among FDA approved cell lines. So, we did rescue trial with another cell lines; Vero, CEF, and 293 cells.

Depending on the growth kinetics data from these cell lines, vero cell was not suitable for viral rescue because of it's low efficiecy for viral growth. As for the CEF cells, although viral growth titer is much lower than that in MDCK cells, their titer is still measurable. However, CEF cells could not be used alone because of their negligible transfection efficacy. We used the human pPOLI plasmid for viral RNA transcription, which may not work in CEF cells. Therefore, Vero and CEF cells are proved to be unsuitable for rescuing of A/Texas virus. In view of 293 cells, even though transfection efficacy is lower than that of 293T cells, transfection and viral growth is measurable compared with other cell lines. However, though we tried repeatedly, we couldn't get the NS1 mutant virus from 293 cells. Only WT A/Texas was generated from 293 cells with low efficiency.

In summary, NS1 mutant A/Texas was easily rescuable from 293T/MDCK cells whereas, rescue trials from another available cell lines (such as CEF, vero cells) resulted in failure due to their low efficiency. However, 293 cells are still able to transfect or grow NS1 mutant A/Texas and CEF cells are capable of growing NS1 mutant A/Texas more efficiently than 293 cells. So, 293/CEF co-culture seems to be a reasonable nest step and possible candidate for NS1 mutant rescue.

ABSTRACT

Background : Influenza virus reverse genetics has reached a level of sophistication where one can confidently generate virus entirely form cloned cDNA. This system makes it possible to generate attenuated live virus vaccine candidate. We tried to generate human influenza A viruses encoding altered viral NS1 proteins in various available cell lines.

Materials and methods : Eight (HA, NA, NP, M, NS, PBI, PB2, PA) viral and four (PB1, PB2, PA, NP) expression plasmids were generated from A/T exas/36/91 influenza virus by RT-PCR and cloning with POL-I and pGEM-T vector. Two NS1 mutant cDNA (NS1-126△, NS1-99△) were also generated. We transfected these plasmids into the 293T/MDCK, 293, CEF and Vero cells and incubated with culture media for 2-3 days. And then, we inoculated cell soups into the embryonated eggs. After 3-4 days of incubation, we harvested allantoic fluid and checked viral titer by HA assay. Finally we did RT-PCR and sequencing to confirm the virus.

Results : Finally we got the NS1 mutant A/Texas influenza viruses from 293T/MDCK cells, but not from FDA approved cells. However, whereas 293 cells are capable of being transfected and of growing the NS1 mutant viruses with low titer, CEF cells are only capable of growing this mutant viruses.

Conclusion : 293 and CEF cells could not be used alone for acquiring NS1 mutant A/Texas influenza viruses. However, 293/CEF co-culture seems to be a resonable next step for NS1 mutant virus rescue for human using.

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