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Original Article

Preparation of genetically engineered A/H5N1 and A/H7N1 pandemic vaccine viruses by reverse genetics in a mixture of Vero and chicken embryo cells

Isabelle Legastelois,[a] Adolfo Garcia-Sastre,[b] Peter Palese,[b] Terrence M. Tumpey,[c,1] Taronna R. Maines,[d] Jacqueline M. Katz,[d] Frederick R. Vogel,[a] Catherine Moste[a]

[a]Research and Development, sanofi pasteur, Marcy L'Etoile, France. [b]Department of Microbiology, Mount Sinai School of Medicine (MSSM), New York, NY, USA. [c]US Department of Agriculture (USDA), Southeast Poultry Research Laboratory (SEPRL), Athens, GA, USA. [d]Influenza Branch, CDC, Atlanta, GA, USA

Correspondence: Isabelle Legastelois, sanofi pasteur, Building X North, 1541 Avenue Marcel Mérieux, 69280 Marcy L'Etoile, France.
Email: isabelle.legastelois@sanofipasteur.com

[1]*Present address*: Influenza Branch, Division of Viral and Rickettsial diseases, Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA.

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Background In case of influenza pandemic, a robust, easy and clean technique to prepare reassortants would be necessary.

Objectives Using reverse genetics, we prepared two vaccine reassortants (A/H5N1 × PR8 and A/H7N1 × PR8) exhibiting the envelope glycoproteins from non-pathogenic avian viruses, A/Turkey/Wisconsin/68 (A/H5N9) and A/Rhea/New Caledonia/39482/93 (A/H7N1) and the internal proteins of the attenuated human virus A/Puerto Rico/8/34 (H1N1).

Methods The transfection was accomplished using a mixture of Vero and chicken embryo cells both of which are currently being used for vaccine manufacturing.

Results This process was reproducible, resulting in consistent recovery of influenza viruses in 6 days. Because it is mainly the A/H5N1 strain that has recently crossed the human barrier, it is the A/PR8 × A/H5N1 reassortant (RG5) that was further amplified, either in embryonated hen eggs or Vero cells, to produce vaccine pre-master seed stocks that met quality control specifications. Safety testing in chickens and ferrets was performed to assess the non-virulence of the reassortant, and finally analysis using chicken and ferret sera immunized with the RG5 virus showed that the vaccine candidate elicited an antibody response cross-reactive with the Hong Kong 1997 and 2003 H5N1 strains but not the Vietnam/2004 viruses.

Conclusions The seeds obtained could be used as part of a pandemic vaccine strain 'library' available in case of propagation in humans of a new highly pathogenic avian strain.

Keywords Influenza, pandemic, reverse genetics, vaccine.

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Introduction

Influenza A virus causes widespread respiratory disease in humans and animals. It is an enveloped virus with eight segments of negative-sense RNA which can encode for 11 proteins. The hemagglutinin (HA) surface glycoprotein is a major antigenic determinant responsible for the induction of neutralizing antibodies. Antibodies generated to the neuraminidase (NA), the second surface glycoprotein, may also have a role in protection by reducing viral replication. At unpredictable intervals, influenza pandemics occur, which are generally associated with increased influenza morbidity and mortality when compared with annual inter-pandemic or epidemic influenza strains (see as a review Ref. 1) Classically, pandemics result from the emergence of viruses that express HA and usually NA toward which most people are fully naïve (antigenic shift). The source of these new influenza virus gene segments is mainly wild aquatic birds, which have the potential to carry all of the 16 existing HA subtypes of influenza A viruses.[2] Only three of these HA influenza virus subtypes (H1, H2 and H3) have been transmitted successfully to humans in the past and have been circulating in the human population during the past century.[3] Avian influenza viruses are low/non-pathogenic in their natural host (wild aquatic birds) and may become highly pathogenic upon transmission to poultry.[4] Since 1997, H5, H7 and H9 avian influenza viruses have been responsible for cases of human infection and death.

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The A/H5N1 virus infected 18 people in Hong Kong, six of whom died.[5,6] In 2003, viruses related to the A/Hong Kong or A/Guangdong/03 (H5N1) caused three infections with one fatality. The same year, A/Netherlands/03 (H7N7) infected 89 people with one fatality. Since mid-December 2003, A/H5N1 avian influenza outbreaks in Asian countries in domestic poultry have resulted in 274 human cases with 167 reported deaths.[7] Furthermore, two human cases of conjunctivitis in Canada were due to an H7N3 avian virus in 2004.[8]

The occurrence of a pandemic depends on the ability of an animal influenza virus to cross the species barrier or re-assort with a human influenza virus and to adapt to its new host so as to efficiently spread from human to human. The threat of such an event is presently considered high as the H5N1 virus continues to spread in avian populations across Asia and, more recently, in European and African countries, despite the measures taken to stop its transmission.[9–11] In case of a pandemic, millions of doses of a pandemic influenza vaccine would be needed in a very short period of time. Therefore, the production of 'pandemic-like' vaccines is part of the pandemic preparedness planning that has been undertaken in various countries.[12] Because of the potential high pathogenicity of the avian strains for humans, it is very important to produce the vaccine in safe conditions to minimize the risk of transmission to birds or to humans during vaccine manufacturing. Conventional influenza vaccine production is performed using embryonated hen eggs; more recently, cell culture systems have been developed.[13,14]

Current epidemic trivalent influenza vaccines contain a wild-type B strain and A/H1N1 and A/H3N2 reassortants that have been obtained by the co-infection in eggs of two parental strains: (i) classically A/Puerto Rico/8/34 (H1N1) (PR8), which provides the internal protein genes conferring both attenuation[15–17] and the ability to replicate with a high yield in embryonated eggs, and (ii) the WHO recommended annual epidemic A type strain, whose HA and NA proteins will confer the expected immunity and protection. This procedure, that requires the selection of one particular reassortant from a mixture is unpredictable and time consuming.[18]

Plasmid-based reassortment or 'reverse genetics' is the method of choice for generating pandemic vaccine strains, as it can be used both to attenuate highly pathogenic avian viruses through directed genetic modifications of structures known to be potentially involved in the pathogenicity[19] and to design the desired vaccine strain without a subsequent selection step. The '12 plasmid-based system' allows the generation of infectious influenza viruses based on the use of four protein expression plasmids encoding for the three polymerase proteins (PB1, PB2 and PA) and the nucleoprotein (NP), plus eight human Polymerase I promoter (pPol-I) transcription plasmids that encode the eight viral gene segments. The 12 plasmids are transfected into cells appropriate for transfection and influenza replication, and the viral progeny is usually rescued after 3–6 days.[20–22]

In the present work, we used the '12 plasmid-based system' to generate 6:2 reassortant vaccine strains expressing the surface glycoproteins of non-pathogenic H5 and H7 avian strains. The recombinant vaccine influenza strain was constructed using eight viral RNA expression plasmids. Six were derived from the internal protein genes of the attenuated human virus PR8, one from the HA gene of either A/Turkey/Wisconsin/68 (H5N9) or A/Rhea/New Caledonia/39482/93 (H7N1) viruses and the NA gene from the A/Rhea/New Caledonia/39482/93. The avian influenza parental strains are from low-pathogenic avian isolates and express HAs that do not naturally contain the multiple basic amino acid cleavage site associated with increased virulence in avian species.[23–25] Therefore, these parental strains are classified as biosafety level (BSL) class 2 pathogens and no HA genetic modification is required for generating attenuated vaccine reassortants. Research grade constructs of such reassortants having been confirmed to be avirulent in chickens, the transfection of the 12 plasmids was performed in a mixture of Vero cells that are commonly used for polio or rabies vaccine production, and chicken embryo cells (CEC), classically used to produce measles vaccine. We focused then on the A/H5N1 reassortant virus (RG5) produced by reverse genetics because current human outbreaks due to avian strains have been caused by A/H5N1 viruses and not A/H7N1. The reassortant virus was easily amplified using either embryonated hen eggs or Vero cells, to produce corresponding pre-master virus seed stocks. The pre-master seed stocks were then biologically characterized for infectivity in MDCK and in eggs, antigenicity, genetic sequence, and absence of virulence in ferrets.

Materials and methods

Facilities

All the work was performed in BSL3 facilities until the ferret and chicken safety testing results were available, the work then continued in BSL2.

Raw materials

Certified raw materials compliant for vaccine manufacturing were used during the preparation of the vaccine pre-master seeds.

Cells, eggs and media

Vero cells from sanofi pasteur (Marcy L'Etoile, France) were grown in Iscove medium supplemented with 4% irra-

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diated donor calf serum. Chicken embryo primary (CEP) cells were isolated from 10-day-old pathogen-free embryonated vaccine grade hen eggs (Charles River, SPAFAS, North Franklin, CT, USA) and grown in DMEM HF12 with 5% irradiated fetal calf serum, according to standard procedures. After one passage, selection of Chicken Embryo Fibroblasts (CEF) occurs and CEF were used between passages 1 and 12. SPAFAS eggs were also used to perform the amplification of the RG5 virus and the viral infectivity titrations (50% egg infectious dose, EID_{50}).

Sera

Anti-A/Tk/WS/68 and anti-A/HK/213/03 post-infection ferret anti-sera, were generously provided by N. J. Cox (CDC, Atlanta, GA, USA).

Plasmids

We used the '12 plasmid-based system' for which the plasmids are described in Ref. 22. The six vRNA segments from the influenza strain PR8 and the avian HA and NA genes (including the non-coding sequences) were cloned into hPol-I transcription plasmid (Sap I site) that contains a truncated human Pol-I transcription promoter at the 5′-end and a hepatitis delta virus ribozyme positioned downstream of the vRNA cloning site. PCAGGS plasmids were used to express the proteins PB1, PB2, PA and NP of the virus A/WSN/33 (H1N1) under the control of chicken β-actin promoter.[22]

Transfection

Reassortant viruses were generated by DNA transfection, according to a protocol adapted from Ref. 22. Twelve microlitres of Lipofectamine 2000 (Invitrogen, Cergy Pontoise, France) and 238 μl of Opti-MEM 1 (Invitrogen) were incubated for 5 min at room temperature. At the same time, the 12 plasmids necessary to rescue the reassortant A/H5N1 (TW/68 HA, RNC/93 NA × PR8), RG5 or A/H7N1 (RNC/93 HA and NA × PR8) RG7 were pooled in Opti-MEM 1 and incubated for 15 min at room temperature. Both mixtures (Lipofectamine-Opti-MEM and DNA-Opti-MEM) were then mixed and incubated for 20 min at room temperature. During that time, Vero cells were trypsinized with irradiated porcine trypsin (Sigma, St. Quentin Fallavier, France) and 1–1.5 million of cells were added (1.5 ml) to each well of a six-well plate. The mixture of Lipofectamine-DNA and OptiMEM was then added to the Vero cells and incubated for 6 h at 37°C under 5% CO_2. One million of CEP or CEF cells (0.5 ml) were added to the transfected Vero cells to amplify the viral infection. The ratio of the CEC to Vero cells was optimized by varying the number of Vero and CEC added to the cultures (0.5–3 million of each cell type). One hour later, irradiated porcine trypsin (1, 5 or 8 μg/ml, about 3.5, 17.5 or 28 USP/ml, Sigma), necessary for the cleavage of the influenza HA, was added. Every 3 days aliquots of supernatants were harvested and fresh medium with trypsin was added to the culture.

Amplification of the RG5 virus in SPAFAS eggs and Vero cells

Three passages were performed in Vero cells and in SPAFAS eggs using different viral inoculum concentrations, to amplify the RG5 virus obtained by transfection and to adapt the virus to grow in Vero cells or in embryonated hen eggs. Briefly, 10-day-old SPAFAS eggs were inoculated into the allantoic cavity with 0.2 ml of virus sample. They were then incubated for 2 days at 34–35°C and 80% humidity. Allantoic fluids were then clarified by centrifugation and samples were taken for titrations. For the cell culture, 95% confluent Vero cells were washed twice with medium without serum before the addition of the viral inoculum that was adsorbed for 1 h. Iscove medium without serum but containing trypsin, was then added to the viral cultures that were incubated for 3 days at 37°C and 5% CO_2.

Sterility

Sterility control assays included tests for bacteria, fungi, cultivable and uncultivable mycoplasma detection and were performed on the pre-master seed stocks produced in eggs and in Vero cells (third passage).

Infectivity and hemagglutination titrations

Viruses produced during the transfection were first titrated for hemagglutination activity (HAU/50 μl) using chicken red blood cells, and subsequently their infectivity was assessed in SPAFAS eggs (EID_{50}/ml) and in MDCK cells (tissue culture infectious dose 50%, $TCID_{50}$/ml), using standard methods. RG5 viruses that had been amplified in Vero cells, were titrated in MDCK cells ($TCID_{50}$/ml), and those amplified in eggs were titrated using eggs (EID_{50}/ml).

Hemagglutination inhibition titration

The antigenicity of the RG5 virus was characterized by HI after amplification in eggs or in cells. The two chicken sera, anti-A/Turkey/WS/68 (H5N9) and anti-A/Rhea/NC/93 (H7N1) (provided by T. Tumpey, USDA/SEPRL, Athens, GA, USA) were treated with receptor-destroying enzyme from Vibrio cholerae (Sigma) to eliminate non-specific hemagglutinin inhibitors. Serial dilutions of each serum were then performed in PBS (Invitrogen) in 96-well plates and incubated with four HAU of RG5 virus per well for 1 h at room temperature; 50 μl of 0.5% chicken red blood cells were then added to each well and incubated for 1 h. The titre in HI antibody was given as the reciprocal of the last dilution giving no hemagglutination.

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Sequencing

Viral RNA from the RG5 virus amplified in Vero cells or in eggs was extracted with the Qiagen RNeasy MiniKit (Qiagen, Courtaboeuf, France), and treated with the Ambion DNA-free Kit (Applied Biosystems, Courtaboeuf, France). The viral RNA was then submitted to RT and PCR using RT Superscript II Kit (Invitrogen), the PCR Expand High Fi Kit (Roche, Meylan, France), and primers designed to amplify the entire HA or the entire NA gene. The sequencing was performed by Genome Express (Meylan, France) on the two strands. The sequencing reactions were performed by PCR according to Applied Biosystems (Big Dye Terminator) or Amersham Pharmacia Biotech (ET terminator) protocols. Unincorporated nucleotides were removed by exclusion columns. All samples were processed on either 'slab gel' sequencing instruments or capillary sequencing instruments.

HI and neutralization reactivity of serum samples from RG5-vaccinated animals

This study was performed at the USDA. Briefly, RG5 egg-amplified whole virus was sucrose purified, formalin inactivated and inoculated subcutaneously into two chickens or two ferrets (10 μg with adjuvant). For chicken vaccination, one volume aqueous vaccine virus antigen was mechanically emulsified in four volumes of oil phase (Drakeol 6 VR containing 10% Arlacel 80 and 1% Tween 80) as previously described.[26] Animals received a secondary subcutaneous immunization 3 weeks after the initial inoculation. Sera were collected 21 days after the last immunization and treated with receptor-destroying enzyme from Vibrio cholerae (Denka-Seiden, Tokyo, Japan) before testing for the presence of HI and neutralization antibodies by standard methods.[27]

Chicken pathogenicity test

Chicken pathogenicity test was performed at USDA/SEPRL by TM Tumpey with RG5 and RG7 research grade reassortant viruses obtained from the laboratory of P Palese (MSSM, NY) where transfections were performed with the same plasmids as described above, but on a mixture made of MDCK and 293T cells[22] and reassortants were amplified by one passage in eggs. Viruses were inoculated intravenously into each of ten 4-week-old White Rock Chickens with standard 0.2 ml of a 10^{-1} dilution (in sterile isotonic saline) of fresh infective allantoic fluid. Eight birds per group were used for pathotyping (examined at 24-h intervals for 14 days: at each observation, each bird was scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead) and oropharyngeal and cloacal swabs were taken from all birds on day 3 post-infection to determine infectious virus titres. Swabs were placed in 1 ml of sterile brain heart infusion medium and clarified homogenates were titrated for virus infectivity in eggs from initial dilutions of 1:2. The limit of virus detection for this assay was $10^{0.8}$ EID_{50} per ml. After 14 days, sera were harvested for evidence of sero-conversion by HI and birds were killed. Two birds per group were killed on 3 days post-infection or day of death for histochemistry and immunohistochemistry.

Ferret pathogenicity test

The ferret pathogenicity test was performed at the CDC in an animal BSL3 laboratory with enhancements required by the USDA and the Select Agent Program, following WHO recommendations.[28] Six male ferrets (7–12 months old) that were serologically negative for currently circulating influenza A viruses were used for each pathotyping experiment. Each ferret was inoculated intranasally with 10^7 EID_{50} in 1.0 ml of either RG5 amplified in eggs or 10^7 $TCID_{50}$ of the same virus amplified in Vero cells. Three ferrets were killed on day 3 post-inoculation and their spleen, lungs, whole blood, nasal turbinates and brain were harvested and tested for the presence of infectious virus (in MDCK). The remaining three ferrets were monitored for clinical symptoms (weight loss, lethargy, respiratory and neurological symptoms) for 14 days post-infection and nasal washes were collected on days 1, 3, 5 and 7 post-infection.

Results

Rescue of influenza RG5 and RG7 reassortants in Vero and chicken embryo cells

Several transfection assays were performed to rescue the reassortant RG5 and RG7 viruses in different cell types. The use of the '12 plasmid-based system' resulted in poor amplification with Vero cells alone in comparison with that observed with the use of MDCK cells or eggs. Because CEC are known to efficiently replicate influenza strains,[29] we used a mixture of Vero cells (high transfection efficiency) and CEC that are used for vaccine production (e.g. measles and ALVAC canarypox virus-based vectors).[30] In these studies, various concentrations of trypsin were used (1, 5 and 8 μg/ml), as trypsin aids in the infection of cells with most influenza strains. Harvests of supernatants and additions of fresh trypsin were done every 3 days after transfection. For transfection, the optimal quantities of DNA were: (i) 1 μg of each RNA expression pPol-I plasmid to rescue the reassortant RG5 virus, 0.3 μg for rescuing the RG7; and (ii) 0.5 μg of each protein expression pCAGGS plasmid.

Reassortant RG5 virus was consistently rescued 6 days after transfection when 5 μg/ml of trypsin was added. With 8 μg/ml of trypsin, HAU titres were low (1 HAU/50 μl) and the viruses were obtained only after 13 days. On days 3, 6, 9 and 13 post-transfection, samples of the culture supernatant fluids were collected and analyzed for HA activity. The HA-positive supernatant fluids were then

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pooled to prepare reassortant virus stock. Titres of 16 HAU/50 μl, $10^{4.2}$ EID_{50}/ml and $10^{4.4}$ $TCID_{50}$/ml were obtained using 5 μg/ml of trypsin. Interestingly, whereas the RG5 reassortant virus was rescued easily using either CEP or CEF cells, the RG7 virus was only consistently rescued when using CEF. For the RG7 virus, the highest HAU titre was obtained 6 days after transfection (256 HAU/50 μl) when 1 μg/ml trypsin was used. The pool of the RG7 virus supernatants obtained using 1 μg/ml of trypsin was titrated again and exhibited titres of 256 HAU/50 μl, $10^{5.2}$ EID_{50}/ml and $10^{5.4}$ $TCID_{50}$/ml.

Preliminary safety testing in chickens

Prior to initiating these studies, a preliminary chicken safety test was performed using research grade counterparts of the RG5 and RG7 reassortants obtained by transfection on a mixture of MDCK and 293T cells. The RG5 and RG7 viruses produced using the MDCK/293T reverse genetics system were shown to be identical to the Vero/CEC-produced viruses by their HA and NA sequences. Control viruses used in these studies included the wild-type parental strains A/Turkey/Wisconsin/68 (H5N9), A/Rhea/NorthCarolina/39482/93 (H7N1), the PR8 parental virus and its counterpart obtained by reverse genetics. Fourteen days after infection, all animals seroconverted, but neither morbidity nor mortality was seen with any of these viruses. Oropharyngeal swabs were positive only for the two groups infected with the wild-type parental strains A/Turkey/Wisconsin/68 (H5N9) and A/Rhea/NorthCarolina/39482/93 (H7N1) (Table 1). Cloacal swabs remained virus negative for the two groups infected with the RG5 and RG7 reassortants. These results indicated that the RG5 and RG7 reassortant vaccine candidates were not pathogenic in chickens.

Amplification in eggs and in Vero cells

As the avian strains infecting humans in recent years are mainly A/H5N1 strains, it was decided to continue the work only with the RG5 reassortant. The virus was further amplified in eggs and in Vero cells to obtain pre-master seed stocks that could be used to produce an egg- or cell-based vaccine. Indeed, egg and cell substrates will be both helpful to produce the vaccine in case of a pandemic. Three passages were performed to reach infectious titres equal to or higher than 10^{8} $TCID_{50}$/ml or EID_{50}/ml, to ensure substantial viral productivity for the production of potential clinical lots. During the second passage in Vero cells the concentration of trypsin was lowered from 5 μg/ml during transfection to 3 μg/ml to minimize detachment of the cells. As shown in Table 2, for both amplifications the infectious titres increased during the successive passages to reach 10^{8} $TCID_{50}$/ml for the Vero pre-master and $10^{8.45}$ EID_{50}/ml for the pre-master prepared in eggs. The HAU titre increased between the transfection supernatant and the first passage in eggs (from 16 to 256 HAU/50 μl) or in Vero cells (from 16 to 128 HAU/50 μl) but then stabilized through the third passage.

Characterization of the reassortant RG5 virus by HI

RG5 virus pre-masters obtained either in eggs or in cell culture were characterized by HI using anti-A/Turkey/WS/68 (H5N9) and anti-A/Rhea/NC/93 (H7N1) chicken sera. Both A/H5N1 pre-masters amplified in eggs or in Vero cells had HI titre of 2560 with the anti-A/Turkey/WS/68 (H5N9), a value similar to that obtained with the avian wild-type A/Turkey/WS/68 (H5N9) virus (TM Tumpey, CDC, data not shown). The HI titre with the anti-A/Rhea/NC/93 (H7N1) was <10. We concluded that

Table 1. Results in chickens infected intravenously with 0.2 ml of a 10^{-1} dilution (in sterile isotonic saline)* of fresh infective allantoic fluid of each research grade RG5 and RG7 viruses

Group	Virus isolation: Oropharyngeal swabs	Virus isolation: Cloacal swabs	Sero-conversion HI
PR/8 wt	0/8	7/8	8/8
Transfectant PR/8	0/8	4/8	8/8
TW/68 (LPAI, H5N9) wt	4/8	7/8	8/8
Rhea/NC (LPAI, H7N1) wt	6/8	7/8	8/8
RG5	0/8	0/8	8/8
RG7	0/8	0/8	8/8

*Stock virus titers ranged from $10^{8.2}$ to $10^{9.2}$ EID_{50}/ml.

Table 2. Results of amplification in eggs and in cells of the RG5 virus obtained by plasmid transfection in Vero/CEC

	Passage	Quantity of virus	HAU/ 50 μl	EID_{50}/ ml	$TCID_{50}$/ ml
Transfection supernatant	0	/	16	$10^{4.2}$	$10^{4.4}$
Amplification in Vero cells	1	MOI 0.001	64	ND	$10^{6.2}$
	2	MOI 0.001	64	ND	$10^{7.1}$
	3	MOI 0.001	128	ND	10^{8}
Amplification in SPAFAS eggs (Charles River, USA)	1	3000 EID_{50}	256	$10^{4.95}$	ND
	2	10000 EID_{50}	256	$10^{7.95}$	ND
	3	10000 EID_{50}	128	$10^{8.45}$	ND

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the fact that the virus was obtained by reverse genetics did not impact on its recognition by the anti-A/Turkey/WS/68 (H5N9) serum.

Sequencing of the HA and NA genes of RG5

The totality of the H5 and N1 genes was sequenced (Genome Express) on RT-PCR fragments covering the entire H5 and N1 genes from both RG5 virus pre-masters produced in embryonated hen eggs and in Vero cells.

The H5 and N1 sequences from the pre-master performed in embryonated hen eggs and the N1 sequence from the pre-master performed in cells contained no mutation when compared to the sequence of the initial plasmids.

The H5 sequence from the pre-master performed in Vero cells contained one mutation when compared with the plasmid sequence. The mutation (Tyr to His at amino acid 119) probably appeared during the three passages in Vero cells as the embryonated hen egg adapted pre-master counterpart did not contain this mutation. This amino acid change is not located in receptor binding or antigenic sites of the HA[31] and it had been described as naturally occurring in HA of other low pathogenic avian influenza strains such as A/Duck/HK/205/77 (H5N3).[32]

HI and neutralization reactivity of serum samples from animals immunized with the egg-amplified reassortant RG5 virus

To assess the antigenic cross-reactivity between selected A/H5N1 wild-type viruses circulating since 1997 and the RG5 reassortant, two chickens and two ferrets were immunized twice with adjuvanted RG5 virus that had been amplified in eggs, sucrose purified and formalin inactivated (10 μg total protein for chicken and ferrets). The animal sera were tested in HI and virus neutralization assays against: (i) two human strains isolated during influenza outbreaks in Hong Kong: A/Hong Kong/483/97 (A/H5N1) and A/Hong Kong/213/03 (A/H5N1); (ii) two H5N1 isolates that circulated during influenza outbreaks in Vietnam in 2004: A/Duck/Vietnam/17/04 and A/Duck/Vietnam/23/04; and (iii) the wild-type A/Turkey/Wisconsin/68 (A/H5N9). In an HI and virus neutralization test, the ferret anti-sera showed that the HA of the RG5 reassortant present in the vaccine candidate was related antigenically to the Hong Kong 1997 and 2003 H5 viruses. However, this virus showed little or no antigenic cross-reactivity with the avian Vietnam/2004 viruses (Table 3). Similarly, chicken H5 anti-sera confirmed the antigenic cross-reactivity observed with the H5 Hong Kong human isolates but not with the H5 Vietnam/2004 strains isolated from ducks.

Safety testing in ferrets of RG5

To evaluate the pathogenicity of the RG5 virus pre-masters amplified in eggs or in Vero cells, six male ferrets were intranasally inoculated with 10^7 EID_{50} of each virus. None of the ferrets showed signs of lethargy, loss of appetite or respiratory or neurological symptoms over the 14-day post-infection period. Changes in temperature and weight loss were minimal in all animals (see Table 4). Virus titres ranging from $10^{2.0}$ EID_{50}/ml to $10^{3.3}$ EID_{50}/ml were detected in ferrets infected with either test virus on days 1 and 3 post-infection. Virus was not detected in the nasal washes of any ferret after day 3 post-infection. Virus titres in nasal turbinates determined at day 3 post-infection ranged from $10^{1.5}$ to $10^{3.25}$ EID_{50}/ml for the RG5 egg virus and $10^{2.5}$–$10^{4.5}$ EID_{50}/ml for the RG5 cell virus where $10^{1.5}$ EID_{50}/ml was the lower limit of detection. No virus was detected in the lungs, spleen or whole blood of any ferret. Reassortant RG5 cell virus was detected in the olfactory bulb region of the brain of two ferrets. However, no virus was detected in the anterior or posterior regions of these ferrets' brains. Other investigators have reported that PR8 virus was detected in the brains of two of two ferrets infected intra-nasally with $10^{7.0}$ EID_{50} virus (J. Wood, personal communication). Detection of apathogenic human H3N2 viruses in the central nervous system has also been reported.[33,34] Presence of virus at this site without the detection of neurological

Table 3. HI and neutralization reactivity of serum samples from animals immunized by the egg-amplified RG5 virus

H5N1 viruses	HI and (neutralization) titre against post-infection ferret or chicken anti-sera			
	Chicken		Ferret	
	Unvaccinated	RG5	Unvaccinated	RG5
A/HK/483/03	<10 (<10)	80 (80–160)	<10 (<10)	320 (640)
A/HK/213/03	<10 (<10)	80–160 (160)	<10 (<10)	640–1280 (1280)
A/Duck/Viet/17 or 23/04	<10 (ND)	<10 (ND)	<10 (ND)	40 (ND)
A/Tk/WS/68	<10 (<10)	160–320 (320)	<10 (<10)	1280 (1280)

ND, not done.

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Table 4. Results in ferrets infected intranasally with $10^{7.0}$ EID_{50} of RG5 vaccine candidate viruses

Virus	Number tested	Number of animals							
		Clinical symptoms to day 14 p.i.				Presence of infectious virus*			
		Weight loss	Lethargy	Respiratory	Neurological	Nasal	Lungs	Spleen	Brain
RG5 egg	3	0	0	0	0	2/3	0/3	0/3	0/3
RG5 cell	3	1**	0	0	0	3/3	0/3	0/3	2/3***

*Day 3 p.i (post-infection).
**2% weight loss.
***Virus detected in the olfactory bulb only, not in anterior or posterior sections of the brain.

symptoms (ataxia, torticollis and hind-limb paresis) is not considered to be a marker of virulence. These results indicated that the RG5 egg and cell vaccine reassortant candidates were not pathogenic in ferrets.

Discussion

The '12 plasmid-based' virus generation system that uses the human RNA Pol-I promoter requires the use of cell lines derived from humans or non-human primates because of the species specificity of the Pol-I-mediated transcription.[35] Generally, the investigators use a mixture of 293 or 293 T cells that have high-transfection efficiency to rescue reassortant viruses and MDCK cells that amplify all influenza strains to high titre.[22,36–40] However, the cloning of the chicken Pol-I promoter sequence was reported by Massin *et al.*,[41] which would be interesting to use in the present CEC system. As a prerequisite for the present work, such research grade RG5 and RG7 reassortants were prepared, exhibiting the HA and the NA of non-pathogenic H5 and H7 avian strains (TW/68 HA, RNC/93 NA × PR8 and RNC/93 HA and NA × PR8). The HA of the North American TW/68 virus was selected because it was previously shown to be strongly immunogenic and capable of eliciting cross-protection against the Asian H5N1 (A/Hong Kong/156/97) virus in chickens.[25] As expected, these 6:2 RG reassortants were confirmed to be non-pathogenic in chicken following the criteria of the US Animal Health Association 1994.[42] However, 293 and 293T cells are not approved for vaccine production and at present only limited experience exists on vaccines prepared on MDCK cells. The Vero cell line derived from African Green monkey kidney cells has been approved since the 1980s for vaccine production. This cell line has been recommended by the WHO for primary vaccine virus generation by DNA transfection.[43]

Several investigations have shown that Vero cells can be used to obtain influenza viruses by reverse genetics;[20,44,45] however, in these studies little or no virus was obtained directly in Vero supernatant fluids and the rescued viruses generally required to be re-amplified in eggs, in MDCK or in MDBK cells. We confirmed these results using our Vero cell line and the 12 plasmid-based reverse genetics system. Recently, Neumann *et al.* presented a system in which the eight RNA POL-I transcription cassettes for viral RNA synthesis are combined on one plasmid allowing the rescue of influenza virus in Vero cells.[46] It must be shown that the rescue can be performed under current quality assurance conditions required for vaccine production.

In the present study, several attempts were made to obtain reassortant viruses directly in Vero cell supernatants but without success. The addition of CEC 6 h post-transfection of Vero cells is an acceptable alternative to MDCK cells to consistently amplify the viral progeny budding from the transfected Vero cells to a high infectious titre ($10^{4.4}$–$10^{5.4}$ $TCID_{50}$/ml). CEC are routinely used to produce commercialized measles and mumps vaccines as well as ALVAC vector-based vaccines under development.[30] CEC are extracted from 10-day embryonated SPAFAS eggs (Charles River). These high-quality eggs are used annually to grow the epidemic influenza master and working seeds of the egg-based trivalent vaccine. It is well established that influenza viruses grow efficiently in CEFs (secondary CEC) to high titres.[29] However, the potential for increased virulence must be monitored when secondary avian cell lines are used as indicated by M. Orlich who described an increased growth potential and virulence in chickens of an A/Turkey/Oregon/71 (H7N3) influenza virus adapted to grow in CEC. This increase in pathogenicity was due to an insertion of 54 nucleotides adjacent to the cleavage site of the HA which corresponds to a region in the 28S ribosomal RNA of egg cell origin.[47] This sequence was not found in the RG5 virus HA gene. Additionally, both RG5 virus pre-master seeds prepared in eggs and in cells were tested for safety in ferrets, which are a highly permissive model to evaluate the virulence of influenza viruses.[48] In

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these studies, both viral preparations were shown to be non-pathogenic.

In addition to approved cell lines, certified raw materials compliant for vaccine production were used to prepare the pre-master seed stocks, from the transfection to the last amplification step. No tryptone (pancreatic digest of casein) was used in the bacterial growth medium (LB 2× modified) employed for the plasmid preparation, the bovine RNase were of US origin, porcine trypsin was used instead of l-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin from bovine origin, and no bovine serum albumin (BSA) was used during transfection to limit the use of raw material coming from ruminants. TPCK trypsin and BSA are known to increase influenza virus rescue efficiency; however, using the present technique the viruses were obtained consistently by 6 days in the supernatant of Vero/CEC, without the requirement for re-amplification in a different substrate (e.g. MDCK or eggs).

To prepare pre-master seed stocks, RG5 strain was amplified in Vero or in eggs. The increase of virus titres during passage in eggs or in Vero cells is characteristic of an adaptation of the virus to its substrate or the removal of defective interfering particles in the preparation and is observed equally in both substrates.

These studies show the potential advantage of the 12 plasmid-based reverse genetics system for the production of influenza vaccines in cells instead of eggs. Reassortants can be obtained in a mixture of Vero/CEC and amplified in Vero cells without the need of any additional amplification steps in eggs, reducing the possibility of antigenic modification due to egg passages.[49,50] Moreover, plasmid transfection is an efficient purification step for eliminating potential adventitious agents present in the human influenza virus isolates as proteins are denatured and removed during the initial chemical treatment in the preparation of the plasmid.

The RG5 pandemic influenza-like pre-master seed strain obtained in the present studies by reverse genetics is capable of eliciting cross-reactive antibodies to the human Hong Kong viruses from 1997 and 2003, but not to the 2004 avian Vietnam isolates (HI results). The lack of antigenic cross-reactivity is not surprising as it was shown that sera from ferrets immunized with the Hong Kong viruses do not recognize the Vietnam strains.[51] However, HI results are not always predictive of the protection that a vaccine against an avian strain might induce.[52]

In conclusion, we generated by reverse genetics two influenza reassortants, RG5 and RG7 exhibiting the HA and NA from avirulent avian strains and the PR8 internal backbone. These viruses were produced under conditions allowing use for vaccine production, i.e. according to quality assurance requirements, such as use of cells approved for vaccine manufacturing.

Pre-master seed lots of the A/H5N1 reassortant, subsequently produced in either certified eggs or Vero cells, have been prepared under the same conditions of quality control and have been biologically characterized for their antigenicity, genetic sequence and absence of virulence in ferrets. These production-ready pre-master seed stocks could be used as part of a pandemic vaccine strain 'library' and, if antigenically appropriate, for the expedited production of an inactivated egg- or cell-based pandemic influenza vaccine.

Conflict of interest

AG-S and PP are consultants for MedImmune and Vivaldi and are authors of vaccine patents owned by Mount Sinai School of Medicine.

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