Comparative safety, immunogenicity, and efficacy of several anti-H5N1 influenza experimental vaccines in a mouse and chicken models (Testing of killed and live H5 vaccine)

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Objective Parallel testing of inactivated (split and whole virion) and live vaccine was conducted to compare the immunogenicity and protective efficacy against homologous and heterosubtypic challenge by H5N1 highly pathogenic avian influenza virus.

Method Four experimental live vaccines based on two H5N1 influenza virus strains were tested; two of them had hemagglutinin (HA) of A/Vietnam/1203/04 strain lacking the polybasic HA cleavage site, and two others had hemagglutinins from attenuated H5N1 virus A/Chicken/Kurgan/3/05, with amino acid substitutions of Asp54/Asn and Lys222/Thr in HA1 and Val48/Ile and Lys131/Thr in HA2 while maintaining the polybasic HA cleavage site. The neuraminidase and non-glycoprotein genes of the experimental live vaccines were from H2N2 cold-adapted master strain A/Leningrad/134/17/57 (VN-Len and Ku-Len) or from the apathogenic H6N2 virus A/Gull/Moscow/3100/2006 (VN-Gull and Ku-Gull). Inactivated H5N1 and H1N1 and live H1N1 vaccine were used for comparison. All vaccines were applied in a single dose. Safety, immunogenicity, and protectivity against the challenge with HPAI H5N1 virus A/Chicken/Kurgan/3/05 were estimated.

Results All experimental live H5 vaccines tested were apathogenic as determined by weight loss and conferred more than 90% protection against lethal challenge with A/Chicken/Kurgan/3/05 infection. Inactivated H1N1 vaccine in mice offered no protection against challenge with H5N1 virus, while live cold-adapted H1N1 vaccine reduced the mortality near to zero level.

Conclusions The high yield, safety, and protectivity of VN-Len and Ku-Len made them promising strains for the production of inactivated and live vaccines against H5N1 viruses.

Keywords H5N1, live attenuated influenza vaccine.

Introduction

Annual influenza vaccination is the most effective method for preventing influenza virus infection and its complications. Inactivated influenza vaccine (IIV) and live attenuated influenza vaccine (LAIV) can be used for vaccination. Subvirion antigen preparations of IIV are referred to as 'split' vaccines. Whole-virus vaccines are more immunogenic particularly after the first dose of vaccine, but two doses of split vaccines are entirely effective.[1] Live attenuated vaccines are constructed by reverse genetics or by reassortment using the hemagglutinin (HA) and neuraminidase (NA) genes from epidemic strains and six remaining genome segments from attenuated, cold-adapted master donor strains (ca MDS). Cold-adapted A/Ann Arbor/6/60 (H2N2) virus is used in the USA,[2] and the cold-adapted A/Leningrad/134/17/57 (H2N2) is used in Russia.[3]

Continuous circulation of highly pathogenic avian influenza H5N1 viruses and the possibility of an influenza pandemic are incentives for the development of anti-H5 vaccine both for human and for poultry. Immunization of poultry carries a further important benefit in that an effective vaccine will limit opportunities for the transmission of H5N1 viruses to humans or other mammalian hosts, thereby mitigating

the human pandemic threat at its source. The development of a vaccine against avian influenza virus is therefore clearly desirable.[4] Recombinant live attenuated vaccine with a modified HA and intact NA genes from A/Vietnam/1203/04 (H5N1) and six remaining genome segments from A/Ann Arbor/6/60 virus were shown to be attenuated in chickens, mice, and ferrets.[2,5] The intranasal live vaccine 'Ultragrivac' was developed based on a reassortment of two viruses: A/Duck/Potsdam/1402-86 (H5N2) and the ca MDS A/Leningrad/134/17/57 (H2N2) [Virology Department, St Petersburg Institute of Experimental Medicine (Russia)].[6]

Novel approaches to the development of effective live influenza A virus vaccines have been developed recently. New vaccines were designed by exploiting the understanding of influenza virus pathogenicity at the molecular level using reverse genetics technology.[7,8] Some factors are believed to increase the pathogenicity of poultry influenza viruses; those are the introduction of multiple basic residues at the cleavage site, the presence of 627Lys and 701Asp in PB2 polymerase protein,[9,10] and the NS1 gene product that counteracts the host type I interferon response, a key component of innate immunity.[11]

One approach for the generation of the vaccine strain is the NS1 truncation strategy. Poor replication and lack of disease following delNS1 virus infection were furthermore correlated with increased levels of type I interferon. These approaches have produced effective vaccine viruses as demonstrated in mice,[12] chicken,[13,14] and swine.[15,16]

It was shown that replacement of the polybasic HA cleavage site with an elastase motif creates the attenuated virus and induced homologous and cross-protection.[17]

The influence of the multibasic cleavage site (MBS) and of the H5 HA on the attenuation, immunogenicity, and efficacy of a live attenuated influenza H5N1 cold-adapted vaccine virus were studied, and it was shown that restoring the MBS in the H5 HA of the vaccine virus improved its immunogenicity and efficacy, probably as a consequence of increased virus replication, indicating that removal of the MBS had a deleterious effect on the immunogenicity and efficacy of the DeltaH5N1 vaccine in mice.[18]

An alternative approach for the generation of live attenuated vaccines was based on using reassortants between low pathogenic avian and human influenza A viruses.[19,20]

Although numerous vaccines have been developed to protect against highly pathogenic avian influenza of subtype H5N1, there are insufficient direct comparisons of their performance.

In this study, we describe parallel testing of inactivated (whole virion and split) and several live H5N1 experimental vaccines. The H5 HAs of the strains tested have intact or removed MBS with attenuated, cold-adapted or apathogenic avian viruses used as the source of internal genes. Homologous and heterosubtypic protection in mice and homologous protection in chicken were studied.

Materials and methods

Viruses

The list of viruses used in this study is shown in Table 1. Highly pathogenic H5N1 influenza viruses A/Chicken/Kurgan/3/05 was kindly provided by Dr S. S. Yamnikova, D. I. Ivanovsky Institute of Virology, Moscow, Russia.

Table 1. Influenza A viruses used in this study

Virus	Subtype	Design	Notes
Chicken/Kurgan/3/05	H5N1 clade 2	Ch/Ku	HPAI
Vietnam/1203/04-PR8/CDC-RG	H5N1 clade 1	VN-PR8	Vaccine strain
Gull/Moscow/3100/2006	H6N2	Gull/M	No pathogenic avian virus
Leningrad/134/17/57; MDS	H2N2	Len	Cold adapted
Puerto Rico/8/34	H1N1	PR8	Vaccine strain
New Caledonia-Leningrad/134/17/57	H1N1	NC-Len	Live cold-adapted vaccine strain
Strains obtained in this study			
Attenuated Chicken/Kurgan/3/05	H5N1 clade 2	Ku-at	With substitutions of Asp54/Asn, Lys222/Thr in HA1 and Val48/Ile, Lys131/Thr in HA2
Vietnam/1203/04-Leningrad/134/17/57	H5N2	VN-Len	Cold-adapted reassortant 1/7*
Vietnam/1203/04-Gull/Moscow/3100/2006	H5N2	VN-Gull	Reassortant 1/7
Attenuated Chicken/Kurgan/3/05-Leningrad/134/17/57	H5N2	Ku-Len	Cold-adapted reassortant 1/7
Attenuated Chicken/Kurgan/3/05-Gull/Moscow/3100/2006	H5N2	Ku-Gull	Reassortant 1/7

*H5 hemagglutinin gene and seven remaining genome segments from donor strain.

Vietnam/1203/04-PR8/CDC-RG (H5N1) virus was kindly provided by Dr R. Donis, CDC, USA. Viruses Gull/M, PR8, Len, and NC-Len (Table 1) were from the repositories of Institute of Poliomyelitis and Viral Encephalities and Research Institute of Experimental Medicine (Russia). Other viruses were produced in this study.

Whole virion and split vaccines were from NPO 'Microgen', Russian Ministry of Health, Russia. All influenza viruses were propagated in 10-day-old embryonated eggs at 36 or 32°C. Cold-adapted reassortants were obtained by classical reassortment on the backbone of MDS A/Leningrad/134/17/57 as previously described.[6] For generation of reassortants with apathogenic virus H6N2 A/Gull/Moscow/3100/2006,[21] a two-step protocol was used. On the first step, cold-adapted reassortants were obtained, and on the second step, selection with antibody to H6N2 at 39°C was conducted.

Attenuation of A/Chicken/Kurgan/3/05 is described in Ref. 22. Briefly, undiluted virus in allantoic fluid was treated with 1 mg/ml trypsin at 37°C overnight before inoculating into embryonated eggs followed by propagation at 36°C. After five such passages, allantoic fluid was incubated for 1 hour at 37°C at pH 4·7–5 before inoculation of eggs. After five such passages, the eggs were incubated for 72 hours at 36°C, and eggs with live embryos were harvested. Virus was subsequently passaged three times at limiting dilution, and all the genes were sequenced.

Growth kinetics of virus in 10-day-old embryonated chicken eggs

Ten-day-old embryonated chicken eggs were inoculated with 10^2 embryonal infecting dose (EID) of viruses and incubated at 26, 32, 36, and 39°C. Allantoic fluids were harvested and subsequently assayed for viral growth at 48, 72, and 96 hours post-infection. The titer of virus present in the allantoic fluid was tested by hemagglutination assay.

Immunization and infection in mice

Six-week-old BALB/c mice were used. Groups of 20 mice were anesthetized and then inoculated intranasally with live vaccines (allantoic fluid with tested viruses) or intramuscularly with inactivated vaccines or placebo (PBS). The single immunization was performed in all experiments. On days 7, 15, and 30 post-immunization, serum samples were taken for antibody titration. The levels of antibody present in sera were assessed by ELISA with anti-mouse IgG1 and IgG2a horse radish peroxidase–labeled antibody (AbD Serotec, Düsseldorf, Germany) as described previously.[23]

Challenge with HPAIV A/Chicken/Kurgan/3/2005

Viral infectivity and aerosol infection of animals were determined as described in the Ref. 24. Briefly, all mice or chicken were marked and infected by using in-house-constructed apparatus for whole-body aerosol exposure. Virus dosage per mouse was about 10^4 tissue culture infecting dose $(TCID)_{50}$/mouse that is equivalent to more than 100 lethal dose $(LD)_{50}$. On day 4 post-challenge, lungs of three mice from each group were taken, and titers of the challenge virus were quantitated by standard 50% tissue culture infectious dose assay in MDCK culture. Survival and body weight following vaccination and challenge were monitored daily.

Immunization and infection in chickens

Ten-day-old Iso-Brown chickens were infected intranasally with non-diluted allantoic fluid with tested viruses. On day 15 post-immunization and post-challenge, serum samples were taken for antibody titration. On day 24 days, after vaccination, chickens were challenged as described earlier with A/Chicken/Kurgan/3/05 ($\sim 10^2$ EID per chicken). All birds were assessed daily for body weight, clinical signs of disease, and mortality. On day 3 post-challenge, lungs of one chicken from each group were taken, and titers of the challenge virus were determined. Feces were sampled on days 3–10 post-challenge. The levels of antibody present in sera were assessed by ELISA with anti-chicken horse radish peroxidase–labeled antibody (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test.

Results

Attenuated A/Chicken/Kurgan/3/05 (Ku-at) was obtained by the selection of highly pathogenic H5N1 influenza viruses A/Chicken/Kurgan/3/05 in conditions similar to the life cycle of wild duck influenza virus, specifically by means of treatment of stock virus with trypsin followed by incubation at acid pH. Final strain (Ku-at) was attenuated for mice and chicken embryo and had reduced pathogenicity for chicken.

Phenotypic differences Ku-at from parent virus

The fusion pH of Ku-at is 0·5 units lower than of A/Chicken/Kurgan/3/05. The pattern of receptor-binding specificity of Ku-at changed toward increasing affinity for fucosilated receptors.[22] We have previously the same type of receptor specificity change for H9 virus after the replacement of bulky 222 amino acid with one having a smaller side chain.[25]

Replacements in the proteins of attenuated A/Chicken/Kurgan/3/05

The whole-genome sequence of Ku-at reveals 18 nucleotide replacements, which produce amino acid substitutions as listed in Table 2. (GenBank accession numbers: DQ323672-DQ323679; HQ724520-HQ724527). The reassortants of

Table 2. Amino acid substitutions in Ku-at proteins

Gene	PB1	PB2	HA1*	HA1	HA2	HA2	NP	NA	NA	NS1
Amino acid number	218	256	54	222	48	131	386	46	189	37
A/ch/Kurgan/3/2005	Leu	Asp	Asp	Lys	Val	Lys	Trp	Ala	Asn	Arg
Ku-at	Met	Asn	Asn	Thr	Ile	Glu	Cys	Thr	Ser	His

*H3 numbering for HA.

Ku-at with cold-adapted master strain A/Leningrad/134/17/57 and with apathogenic H6N2 virus A/Gull/Moscow/3100/2006 had no additional amino acid replacements in HA.

Ku-at has a total of 10 amino acid substitutions compared with the parent virus (Table 2). Ku-at preserves such molecular markers of pathogenicity of HPAI H5N1 viruses as MBS, 627Lys and 701Asp in PB2 and 92Glu in NS1.[9,10] Nevertheless, it has low pathogenicity for mice. Replacements in PB1, NP, and NS of Ku-at are all likely to decrease virus pathogenicity. Trp386 in NP is part of the protein RNA-binding motif.[26] Arg37 in NS1 is located in the nuclear localization signal, which contains a sequence of basic amino acids Asp-Arg-Leu-Arg-Arg (codons 34–38).[27]

Amino acid substitutions in HA are situated in the fusion peptide pocket (48 of HA2), in the HA2 coiled-coil domain (48 and 131), and in regions where distant parts of the linear HA1 chain come into contact in the folded structure (54 of HA1) or are at the interface of subunits in the HA trimer (222 of HA1 and 131 of HA2). These replacements affect the flexibility of the molecule and could change the parameters of pH-dependent conformational changes and fusion,[28] and we indeed see that the fusion pH of Ku-at decreased 0·5 units. Such a decrease could be a significant attenuating factor during virus replication as was shown in.[29–31]

Replacements such as Asp54/Asn in HA1, Val48/Ile and Lys131/Glu in HA2, and Ala46/Thr in NA are more typical for HA and NA proteins from wild duck viruses because most of ancient duck H5 viruses had Asn54 in HA1 and Ile48 in HA2; H3 and H4 duck viruses have Glu131 in HA2. Many of duck viruses with N1 have Thr46 in it. Thus, the selection in conditions that were similar to the life cycle of the wild duck influenza viruses resulted in the reversion of amino acids and partial loss of characteristic feature of HPAI viruses.

Generation of the reassortants H5 viruses

The selection of reassortants of H5N1 viruses with cold-adapted master strain A/Leningrad/134/17/57 was conducted at 26°C with antibodies to H2N2 virus. The derived strains VN-Len and Ku-Len were then used for obtaining the reassortants with apathogenic H6N2 virus A/Gull/Moscow/3100/2006. The selection at 39°C was conducted with antibody to H6N2 virus. It is interesting to note that in all cases, the NA gene accompanied inner genes of parent virus in spite of unfavorable antibody pressure.

Growth characteristics of viruses in chicken eggs at different temperatures

Optimal growth temperature for parent viruses Ch/Ku, Gull/M, VN-PR8, as well as Ku-at and reassortants with Gull/M was 36°C, while for MDS A/Leningrad/134/17/57 and reassortants VN-Len and Ku-Len, optimal temperature was 32°C. Yield of viruses at optimal temperature was very different. It was relatively low for A/Chicken/Kurgan/3/05, medium for A/Gull/Moscow/3100/2006, A/Vietnam/1203/04-PR8/CDC-RG, Ku-at and for VN-Gull and Ku-Gull. Yield of MDS Len and reassortants VN-Len and Ku-Len was much higher (titer 1024–2048 in HA test). Only Len, VN-Len, and Ku-Len were capable of growth at 26°C and did not grow at 39°C, i.e., they have both cold-adapted and temperature-sensitive phenotype (data not shown).

Safety and protective efficacy of the vaccine in mice

To assess the safety of the reassortant viruses in study and estimate their vaccine efficacy, we performed vaccine challenge studies in mice. Groups of 20 mice were inoculated intranasally with live vaccines and intramuscularly with inactivated vaccines and placebo. Inactivated vaccines as well as cold-adapted reassortants VN-Len, Ku-Len, and NC-Len were well tolerated. Weight dynamics and survival in these groups were nearly equal to placebo group (Table 3). The most pathogenic was parent virus Vietnam/1203/04-PR8/CDC-RG, which caused significant weight loss and 80% mortality. Ku-at caused 15% weight loss and 20% mortality, while VN-Gull and Ku-Gull caused ~10% weight loss and 10% mortality.

On day 30, mice were challenged by H5N1 A/Chicken/Kurgan/3/05 virus (~100 LD$_{50}$). On day 4 after challenge, the H5N1 virus in the mouse lungs was determined. The content of virus in the lungs was maximal in the placebo group, groups vaccinated with non-specific H1N1 vaccines and with low dose of H5N1 split vaccine.

Table 3. Post-vaccination and post-challenge survival, weight dynamics, and levels of challenge virus in the lung of mice

Vaccine		Dose	Post-vaccination		Post-challenge		
			Survival*	Weight**	Virus in lung***	Survival	Weight
Inactivated		μg HA	%	%	Log $TCID_{50}$	%	%
Whole	H5N1	2·5	97	102 ± 3	1 ± 1	95	95 ± 8
Whole	H5N1	0·5	100	105 ± 4	2 ± 1	80	83 ± 11
Split	H5N1	2·5	99	105 ± 5	4 ± 0·5	70	79 ± 14
Split	H5N1	0·5	99	105 ± 4	5·5 ± 1	17	74 ± 16
Whole	H1N1	2·5	100	101 ± 5	5·5 ± 1	0	72 ± 8
Live		$TCID_{50}$					
VN-PR8	H5N1	10^6	20	78 ± 6	1 ± 1	98	94 ± 6
Ku-at	H5N1	10^6	79	84 ± 5	1 ± 0·8	98	92 ± 6
VN-Len	H5N2	10^6	97	93 ± 4	2 ± 1	94	88 ± 7
VN-Gull	H5N2	10^6	92	88 ± 7	1·5 ± 1	97	93 ± 5
Ku-Len	H5N2	10^6	97	96 ± 5	2 ± 1·3	95	90 ± 9
Ku-Gull	H5N2	10^6	90	89 ± 10	1·5 ± 1·3	97	91 ± 6
NC-Len	H1N1	10^6	97	95 ± 6	4·7 ± 0·8	90	77 ± 5
Placebo		–	99	105 ± 3	6 ± 0·7	0	70 ± 4

Mice were exposed to single vaccination in indicated dose and challenged by H5N1 A/Chicken/Kurgan/3/05 virus (~100 LD_{50}) on day 30 post-vaccination.
*Survival: Percentage survival of three experiments;
**Weight: Average mouse weight, percentage of start value (average for three experiments) determined on day 6 post-vaccination or post-challenge.
***Challenge virus in the lung, $\log_{10}$ of $TCID_{50}$ value, average for nine mice of three experiments.

Virus titers were lower in high dose split virus group, lower still in groups vaccinated with live cold-adapted vaccine, and sometimes undetectable in groups vaccinated with whole H5N1–inactivated vaccine and live Ku-at, VN-Gull, and Ku-Gull viruses.

Protection from death after specific vaccination generally correlated with the decrease in virus in the lungs. It was maximal (100% in a part of the experiments) in groups vaccinated with high dose of whole H5N1–inactivated vaccine and with live H5 viruses. High dose of H5N1 split vaccine reduced mortality, while a low dose conferred little or no protection.

Changes in body weight after challenge (Figure 1) demonstrated the difference in clinical course for mice vaccinated with inactivated or live, specific or non-specific vaccine. The group immunized with the high dose of H5N1 whole vaccine displayed the least weight loss after challenge, while immunization with non-specific inactivated H1N1 vaccine had no effect on weight loss compared to unimmunized controls. The group vaccinated with specific live vaccine showed a moderate weight loss by day 6 post-challenge followed on days 7–8 by net weight gain. The group vaccinated with the H1N1 live attenuated vaccine demonstrated rapid weight loss during the first post-chal-

lenge days, but by days 8–10, mice started recovering and gaining weight and nearly 90% of them survived.

Immunogenicity of the vaccine in mice

To evaluate the immunogenic properties of tested experimental vaccines and estimate the balance between TH2 and TH1 cells (humoral and cell-mediated immunity), the level of anti-H5N1 IgG1 and IgG2 was measured on day 7, 14, and 30 post-vaccination and on day 14 post-challenge (Table 4).

On day 7, IgG1 was not detected. IgG2a was detected in mice vaccinated with whole-virion vaccine and was lower in mice vaccinated with live viruses, and only a few of the mice had a barely detectable level of IgG2a in the 'split' group.

On day 14, the level of IgG1 was large in groups, vaccinated with live vaccines. In groups vaccinated with whole vaccine, it was lower and in 'split' group, not detectable. The level of IgG2a on day 14 was high in live vaccine and whole vaccine groups, while in 'split' group, it was tenfold lower.

From day 14 to day 30, levels of both types of antibodies strongly increased in groups vaccinated with live vaccines with little or no change in groups receiving inactivated vac-
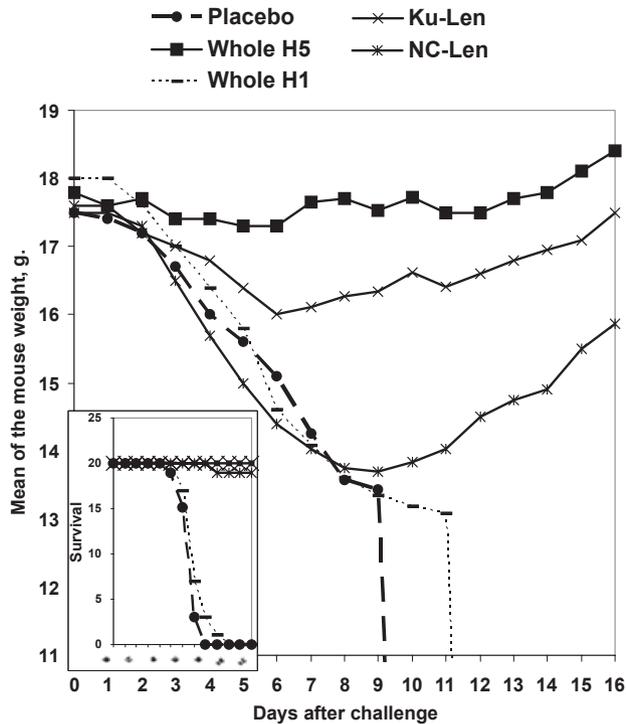
Figure 1. Weight dynamics and survival of mice after challenge with HPAI H5N1 virus A/Chicken/Kurgan/3/05. Groups of 20 mice were vaccinated with live vaccines (10^6 TCID$_{50}$) or with inactivated whole virion vaccines (2 μg HA in single dose). On day 30 mice were challenged by H5N1 A/Chicken/Kurgan/3/05 virus ($\sim$100 LD$_{50}$). Abrupt drop of weight curves denotes the death of all mice. Insertion represents the survival curves of the same experiment.

cines. The differences between whole H5, split H5, and live vaccines groups were statistically significant ($P < 0.01$ in Student's t-test). An important point is that mice vaccinated with cold-adapted live vaccines had well-balanced IgG1/IgG2a serum antibody levels, testify to sufficient humoral and cell-mediated immune response.

On day 14 after challenge, titers of anti-H5 IgG1 and especially IgG2a were very high in all surviving mice.

Evaluation of live H5 viruses in chickens

The 10-day-old chickens were infected intranasal with 10^4 EID$_{50}$ of HPAI virus A/Chicken/Kurgan/3/05, 10^6 EID$_{50}$ of viruses Ku-at, Vn-Gull, and Ku-Gull and 10^7 EID$_{50}$ of viruses VN-Len and Ku-Len (Table 5). Chickens infected by A/Chicken/Kurgan/3/05 all died by day 3. Chickens infected by Ku-at virus lost weight and died by day 8 after infection. VN-Len, VN-Gul, Ku-Len, and Ku-Gull caused no weight loss or mortality in chickens, and anti-H5 antibodies were detected in all sera taken on day 14. On day 24, chickens were challenged by whole-body aerosol exposure with HPAI A/Chicken/Kurgan/3/05 ($\sim$$10^2$ EID$_{50}$/-chicken). Chickens of placebo group died by day 2. Most chickens vaccinated with VN-Len and Ku-Len group died

by day 3–6 after challenge, while most chickens vaccinated with VN-Gull and Ku-Gull survived challenge. Challenge virus was detected in chicken lungs of all groups, while virus in feces was not detected in Vn-Gull and Ku-Gull groups. On 14 days after challenge, the levels of anti-H5 IgG were very high in all surviving chickens.

Discussion

Our data directly comparing of LAIV and IIV show that one dose of split vaccine is insufficiently protective, while whole virion IIV in dose of 2 μg HA/mice is immunogenic and protective against homologs challenge. The single vaccination with cold-adapted H5 LAIVs is safe and effective. The increase in antibody levels after vaccination with LAIVs persists for a longer time than after IIV vaccinations demonstrated by antibody measurements and the results of challenge after vaccination.

The effect of the MBS on virus pathogenicity is revealed by comparing VN-gull with Ku-gull and VN-Len with Ku-Len. We did not detect a difference in pathogenicity within these strain pairs suggesting that mutations decreasing the flexibility of HA can affect the pathogenicity for mice to nearly the same extent as presence or absence of the MBS. The Ku-Len (cold-adapted H5 LAIV with MBS) is as apathogenic for mice as the commercial vaccine NC-Len (H1N1). This is in agreement with data of Suguitan,[18] who showed that restoring the MBS in the H5 HA of the vaccine virus improved its immunogenicity and efficacy while preserving attenuation. The high yield of VN-Len and Ku-Len in chicken embryonated eggs makes them promising strains for the production of both live and inactivated vaccines.

Our data demonstrate a fundamental difference between IIV and LAIV after heterosubtypic immunization. The IIV has no apparent efficacy in that case, while LAIV conferred almost complete cross-protection. Analogous results were obtained Kreijtz et al.,[32] who showed that primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. It was also shown that vaccination with live elastase-dependent heterosubtypic reassortants led to cross-protection [17] and that vaccination with LAIV provided a prophylactic effect against both heterologous and heterotypic infections.[33]

All experimental vaccine strains were safe for 10-day-old chickens under intranasal application. All strains give rise to anti-H5 antibodies, at variance with Suguitan et al.[5] data, which showed that the recombinant live attenuated H5N1 vaccine with six inner genome segments from A/Ann Arbor/6/60 did not infect chicken at all. Probably A/Leningrad/134/17/57 is less thermosensitive than A/Ann Arbor/6/60, and viruses VN-Len and Ku-Len may propagate in upper respiratory tract of chicken. Nevertheless,

Table 4. Post-vaccination and post-challenge IgG1 and IgG2a antibody to H5N1 in mice sera

| | Dose | Antibody to H5N1, log of geometrical mean of the ELISA titer | | | | | | | |
| | | 7 days post-vaccination | | 14 days post-vaccination | | 30 days post-vaccination | | 14 days post-challenge | |
Vaccine	μg HA	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
Inactivated									
Whole H5	2·5	<2	2·6	2·5	3·6	2·6	3·4	4·7	5·4
Split H5	2·5	<2	2·1	<2	2·2	2	2·4	4·5	5·3
Live	**TCID$_{50}$**								
Ku-at	10^6	<2	2·2	3·1	4·2	3·7	4·6	4·5	5·3
VN-Len	10^6	<2	2·3	2·9	3·2	3·3	4	4·1	5·1
VN-Gull	10^6	<2	2·4	3·4	3·9	3·7	4·2	4·4	5·2
Ku-Len	10^6	<2	2·3	3	3·3	3·6	3·8	4·6	5·3
Ku-Gull	10^6	<2	2·2	3·2	4·1	3·8	4·7	4·6	5·4
Placebo	–	<2	<2	<2	<2	<2	<2	–	–

Mice received a single dose of the immunizing virus. Serial 2-fold dilutions of serum were prepared, beginning with a 1:100 dilution. An undetectable antibody titer was assigned a value of 50 (Log$_{10}$ = 1·7).

immunogenicity and protectivity of VN-Len and Ku-Len were lower than immunogenicity and protectivity of VN-Gull and Ku-Gull.

One complication for the potential use of live vaccines in poultry is the difficulty in differentiating vaccinated poultry from naturally infected birds based on serology. A strategy known as 'differentiating infected from vaccinated animals' (DIVA) is used to obviate these difficulties.[34] H5N2 formulations of our experimental vaccines meet the DIVA criterion.

In summary, the use of LAIVs offers substantial benefits compared to inactivated vaccines. Intranasal administration and replication in nasopharyngeal epithelial cells mimic the natural infection route of influenza virus, which elicits a well-developed and balanced immune response to the pathogen after challenge. Cross-protective immunity against a heterosubtypic virus strain can be induced by LAIV. Utilization of last year's live vaccines can reduce severity of the disease caused by heterosubtypic pandemic strains.

Acknowledgements

Table 5. Safety, immunogenicity, and protectivity of live H5 viruses in chickens

| Virus | Dose EID$_{50}$ | Post-vaccination | | | Post-challenge survival |
		Weight*	Survival**	Ab.***	
Ch/Ku	10^4	–	0/4	–	–
Ku-at	10^6	82 ± 7	0/4	–	–
VN-Len	10^7	135 ± 13	9/9	2·4 ± 0·5	2/8
VN-Gull	10^6	133 ± 13	9/9	3·3 ± 0·7	7/8
Ku-Len	10^7	137 ± 12	9/9	2·3 ± 0·5	2/8
Ku-Gull	10^6	130 ± 19	9/9	3·2 ± 0·7	8/8
Placebo	–	140 ± 10	4/4	–	0/4

The 10-day-old chickens were once infected intranasal with indicated dose of viruses and on day 24, were challenged by H5N1 A/Chicken/Kurgan/3/05 virus (~100 LD$_{50}$).
*Weight: average chicken weight, percentage of start value, determined on day 6 post-vaccination.
**Survival: Survival: number survived/number infected.
***Ab: antibody to H5N1, log of geometrical mean of the ELISA titer.

Disclaimer

The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

References

1 Cox RJ, Hovden AO, Brokstad KA, Szyszko E, Madhun AS, Haaheim LR. The humoral immune response and protective efficacy of vaccination with inactivated split and whole influenza virus vaccines in BALB/c mice. Vaccine 2006; 24:6585–6587.

2 Subbarao K, Chen H, Swayne D et al. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 2003; 305:192–200.

3 Alexandrova GI, Polezhaev FI, Budilovsky GN et al. Recombinant cold-adapted attenuated influenza A vaccines for use in children: reactogenicity and antigenic activity of cold-adapted recombinants and analysis of isolates from the vaccines. Infect Immun 1984; 44:734–739.

4 Steel J, Lowen AC, Pena L et al. Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. J Virol 2009; 83:1742–1753.

5 Suguitan AL Jr, McAuliffe J, Mills KL et al. Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. PLoS Med 2006; 3:1541–1554.

6 Desheva JA, Lu XH, Rekstin AR et al. Characterization of an influenza A H5N2 reassortant as a candidate for live-attenuated and inactivated vaccines against highly pathogenic H5N1 viruses with pandemic potential. Vaccine 2006; 24:6859–6866.

7 Palese P, Shaw ML. Orthomyxoviridae: the viruses and their replication; in Knipe DM, Howley PM (eds): Fields Virology, 5th edn, vol. II. Philadelphia, PA: Lippincott Williams and Wilkins, 2006; 1648–1689.

8 Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A. Rescue of influenza A virus from recombinant DNA. J Virol 1999; 73:9679–9682.

9 Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 2001; 293:1840–1842.

10 Gabriel G, Dauber B, Wolff T, Planz O, Klenk H-D, Stech J. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. PNAS 2005; 102:18590–18595.

11 Garcia-Sastre A, Biron CA. Type 1 interferons and the virus-host relationship: a lesson in detente. Science 2006; 312:879–882.

12 Hai R, Martínez-Sobrido L, Fraser KA, Ayllon J, García-Sastre A, Palese P. Influenza B virus NS1-truncated mutants: live-attenuated vaccine approach. J Virol 2008; 82:10580–10590.

13 Wang L, Suarez DL, Pantin-Jackwood M et al. Characterization of influenza virus variants with different sizes of the non-structural (NS) genes and their potential as a live influenza vaccine in poultry. Vaccine 2008; 26:3580–3586.

14 Wang L, Yassine H, Saif YM, Lee CW. Developing live attenuated avian influenza virus in ovo vaccines for poultry. Avian Dis 2010; 54(1 Suppl):297–301.

15 Richt JA, Lekcharoensuk P, Lager KM et al. Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. J Virol 2006; 80:11009–11018.

16 Vincent AL, Ma W, Lager KM et al. Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. Vaccine 2007; 25:7999–8009.

17 Gabriel G, Garn H, Wegmann M et al. The potential of a protease activation mutant of a highly pathogenic avian influenza virus for a pandemic live vaccine. Vaccine 2008; 26:956–965.

18 Suguitan AL Jr, Marino MP, Desai PD et al. The influence of the multi-basic cleavage site of the H5 hemagglutinin on the attenuation, immunogenicity and efficacy of a live attenuated influenza A H5N1 cold-adapted vaccine virus. Virology 2009; 395:280–288.

19 Murphy BR, Sly DL, Tierney EL et al. Reassortant virus derived from avian and human influenza A viruses is attenuated and immunogenic in monkeys. Science 1982; 218:1330–1332.

20 Subbarao K, Webster RG, Kawaoka Y, Murphy BR. Are there alternative avian influenza viruses for generation of stable attenuated avian-human influenza A reassortant viruses? Virus Res 1995; 39:105–118.

21 Lomakina NF, Gambaryan AC, Boravleva EY et al. Character of apathogenic influenza A viruses found in Moscow, Russia. Mol Gen Microbiol Virol 2009; 24:37–45.

22 Lomakina NF, Boravleva EY, Kropotkina EA, Yamnikova SS, Drygin VV, Gambaryan AS. Attenuation of A/chicken/Kurgan/3/2005 (H5N1) influenza virus by selection in an environment simulating a life cycle of wild duck viruses. Mol Gen Microbiol Virol 2011; 26:132–139.

23 Danesh-Bahreini MA, Shokri J, Samiei A, Kamali-Sarvestani E, Barzegar-Jalali M, Mohammadi-Samani S. Nanovaccine for leishmaniasis: preparation of chitosan nanoparticles containing Leishmania superoxide dismutase and evaluation of its immunogenicity in BALB/c mice. Int J Nanomedicine 2011; 6:835–842.

24 Gambaryan AS, Boravleva EY, Matrosovich TY et al. Polymer-bound 6′ sialyl-N-acetyllactosamine protects mice infected by influenza virus. Antiviral Res 2005; 68:116–123.

25 Gambaryan AS, Tuzikov AB, Pazynina GV et al. 6-sulfo sialyl Lewis X is the common receptor determinant recognized by H5, H6, H7 and H9 influenza viruses of terrestrial poultry. Virol J 2008; 5:85 PMID: 18652681.

26 Elton D, Medcalf L, Bishop K, Harrison D, Digard P. Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding. J Virol 1999; 73:7357–7367.

27 Greenspan D, Palese P, Krystal M. Two nuclear location signals in the influenza virus NS1 nonstructural protein. J Virol 1988; 62:3020–3026.

28 Reed ML, Yen HL, DuBois RM et al. Amino acid residues in the fusion peptide pocket regulate the pH of activation of the H5N1 influenza virus hemagglutinin protein. J Virol 2009; 83:3568–3580.

29 Brown EG, Liu H, Chang Kit L, Baird S, Nesrallah M. Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. Proc Natl Acad Sci U S A 2001; 98:6883–6888.

30 Hsu CN, Wang CH. Sequence comparison between two quasi strains of H6N1 with different pathogenicity from a single parental isolate. J Microbiol Immunol Infect 2006; 39:292–296.

31 Reed ML, Bridges OA, Seiler P et al. The pH of activation of the hemagglutinin protein regulates H5N1 influenza virus pathogenicity and transmissibility in ducks. J Virol 2010; 84:1527–1535.

32 Kreijtz JH, Bodewes R, van Amerongen G et al. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. Vaccine 2007; 25:612–620.

33 Seo SU, Byun YH, Lee EY et al. Development and characterization of a live attenuated influenza B virus vaccine candidate. Vaccine 2008; 26:874–881.

34 Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez JF. Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol 2003; 32:47–55.