A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin

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A promising approach to reduce the impact of influenza is the use of an attenuated, live virus as a vaccine. Using reverse genetics, we generated a mutant of strain A/WSN/33 with a modified cleavage site within its hemagglutinin, which depends on proteolytic activation by elastase. Unlike the wild-type, which requires trypsin, this mutant is strictly dependent on elastase. Both viruses grow equally well in cell culture. In contrast to the lethal wild-type virus, the mutant is entirely attenuated in mice. At a dose of 10^5 plaque-forming units, it induced complete protection against lethal challenge. This approach allows the conversion of any epidemic strain into a genetically homologous attenuated virus.

Because of annually recurring epidemics and the enduring threat of a pandemic, influenza remains a serious public health problem despite the availability of inactivated vaccines. A live vaccine, FluMist, manufactured by MedImmune Vaccines Inc., is now commercially available in some countries. This vaccine consists of two influenza A and one influenza B reassortants of a cold-adapted master strain. These reassortants have temperature-sensitive mutations in the polymerase subunits PB2 and PB1 and the nucleoprotein genes¹ and are decorated with the hemagglutinin and the neuraminidase of the circulating epidemic strain. Such a vaccine virus has the potential to provide the internal genes without temperature-sensitive mutation for reassortment with an epidemic virus and could thus give rise to a new strain with unpredictable traits^{2,3}.

An important step in the replication cycle of the influenza virus is cleavage of the hemagglutinin by a host protease in order to gain infectivity^{4,5} by activating the fusion potential^{6–8}. The cleavage site contains a conserved arginine or a stretch of basic amino acids like the highly pathogenic avian strains^{9–11}.

Using reverse genetics, we generated in this study a mutant of strain A/WSN/33 (H1N1) with a modified hemagglutinin cleavage site that requires elastase instead of trypsin for activation. This virus is attenuated *in vivo*, but grows *in vitro* as well as the wild-type virus does, and induces a strong protection against lethal infection with the

wild-type. Such an approach allows the conversion of any epidemic strain as a whole into an attenuated live vaccine virus. It is genetically homologous to the wild-type, and thus avoids the risk of generating pathogenic reassortants.

RESULTS

Generation of an elastase-dependent influenza A virus

We sought to make a virus that is not susceptible to *in vivo* activation at the basic hemagglutinin cleavage site but that can be activated *in vitro* by a protease not available under natural conditions. Wild-type hemagglutinin is cleaved by trypsin-like serine proteases, and cleavage generates two fragments, HA1 and HA2. We had to consider two requirements for the choice of the cleavage motif of such a protease. First, the glycine had to be retained at position P1 because this amino acid is essential at the amino terminus of the HA2 fragment for induction of fusion and cannot be replaced without compromising or possibly abolishing fusion^{10,12–14}. Second, to reduce the probability of reversion, we had to select an amino acid whose codon differs by two nucleotides from any arginine or lysine codon^{15,16}. We therefore exchanged the A at position 1,059 and the G at position 1,060 of the A/WSN/33 hemagglutinin with G and T, respectively, replacing Arg343 with valine and generating a cleavage site for porcine pancreatic elastase¹⁷ (Fig. 1). Using a previously established reverse genetics system¹⁸, we generated recombinant viruses containing either authentic (WSNwt) or mutated (which can be cleaved by elastase; WSN-E) hemagglutinin. We then sequenced the gene encoding the HA1 peptides of WSNwt and WSN-E and found no nucleotide differences (data not shown).

WSN-E is activated by elastase exclusively

Multicycle replication and thus plaque formation require proteolytic activation⁴. Therefore, we performed plaque assays on Madin-Darby canine kidney (MDCK) cells in the presence of either trypsin or elastase in the plaque overlay or in the absence of an exogenous protease for control. With WSNwt, clear plaques were only visible in the presence of trypsin. In contrast, WSN-E was exclusively activated by elastase

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Figure 1 Cleavage sites of WSNwt and WSN-E. The monobasic cleavage site of the WSNwt hemagglutinin contains an arginine, which has been replaced by a valine in the case of WSN-E.

(Fig. 2a), even if only one wash with PBS was done before inoculation. These results demonstrate that neither plasmin shown to activate WSNwt^{5,19} nor another protease provided by MDCK cells or fetal calf serum is able to cleave WSN-E hemagglutinin. We analyzed the dependence of the hemagglutinin cleavage in WSNwt on trypsin and in WSN-E on elastase by western blotting. The results show that the hemagglutinin of WSN-E is cleaved by elastase, but is resistant to trypsin (Fig. 2b). The weak HA1 band, which appeared after incubation of WSNwt with elastase, can be attributed to incomplete cleavage between amino acids glycine and leucine¹⁷. This cleavage does not cause proteolytic activation, as shown by the plaque assay (Fig. 2a). In the presence of the appropriate protease, both viruses grew to similar titers, although kinetics were somewhat slower with WSN-E (Fig. 2c).

WSN-E is attenuated in mice, whereas WSNwt is lethal

To analyze WSN-E *in vivo*, we infected mice with 10^6 plaque-forming units (p.f.u.) of either WSNwt or WSN-E and observed them for 14 d. When infected with wild-type virus, mice ($n = 3$) showed signs of disease and died on day 7. In the case of WSN-E, all mice ($n = 4$) survived (Fig. 3a) without weight loss (Fig. 3b) or any visible symptoms of disease.

WSN-E undergoes restricted replication in the lung

To compare the pathogenic traits of both viruses *in vivo*, we inoculated mice with either PBS, 10^6 p.f.u. WSNwt or 10^6 p.f.u. WSN-E. After 12 h, 1 d and 3 d, we removed lung, brain and heart. Beginning with undiluted organ homogenate, we performed plaque titrations with WSNwt in the presence of trypsin and with WSN-E in the presence of elastase. We found WSNwt in the lungs up to day 3, showing a rise in titer. Wild-type virus was also detectable in brain and heart. Mice inoculated with WSN-E showed different characteristics. We did not find WSN-E in brain or heart at any time point analyzed. In the lung, viral titers stagnated from 12 h to 1 d after inoculation. On day 3, we could not detect any WSN-E virus (Fig. 3c–e). The lung titer of WSN-E at 12 h was approximately 1.4×10^6 p.f.u. per mouse lung, which is close to the inoculum dose. For WSNwt, the result was considerably different. The lung titer at 12 h was approximately 2.2×10^8 p.f.u. per mouse lung, showing that WSNwt readily multiplies in the lung. In contrast, replication of WSN-E is abortive because it is restricted *in vivo* to one replication cycle because of the absence of the appropriate protease.

Figure 2 *In vitro* properties of WSNwt and WSN-E. Plaque assay (a) and western blot (b) of WSNwt and WSN-E in the absence of an exogenous protease, in the presence of trypsin or elastase. Growth curves (c) of WSNwt (circles) in the presence of trypsin and WSN-E (squares) in the presence of elastase.

WSN-E is found only after first passage in mouse lung

To check for the emergence of revertants, we carried out sequential lung passages in mice. At the first passage, we inoculated the animals with 10^6 p.f.u. WSN-E. For the subsequent infections, we used 50 μ l lung homogenate from the previous passage. During five lung passages of either 1 d or 3 d duration, the mice remained unaffected. We determined the virus titers by plaque assay in the presence of elastase. Because WSNwt is activated by plasmin^{5,19}, this virus can be detected in absence of trypsin if only one wash is performed before inoculation. We did not detect any virus, even in undiluted lung homogenate obtained from the second to the fifth of the 1-d passages and from the first to the fifth of the 3-d passages (data not shown). This shows the absence of WSN-E and of revertants.

Reversion of WSN-E *in vitro*

After the first passage in mouse lung, the entire amount of WSN-E was in the range of 10^5 – 10^6 p.f.u.. Such virus populations are too small for the generation of double-point revertants, having an equilibrium frequency of approximately 10^{-5} to 10^{-8} (ref. 20). Therefore, we passaged the elastase-dependent WSN-E on MDCK cells in the presence

Figure 3 Pathogenicity and organ tropism of WSNwt and WSN-E in mice. Survival rates (**a**) and average weight loss (**b**) after infection with WSNwt (circles) or WSN-E (squares). Plaque titers from entire lungs (**c**), brains (**d**) and hearts (**e**) removed at 12 h, 1 d or 3 d.

of trypsin, beginning with inocula of 10^8 , 10^7 or 10^6 p.f.u. each in ten parallel cell cultures. From all 10^8 p.f.u. inocula and from six out of ten 10^7 p.f.u. inocula, we found trypsin-dependent virus with lysine at its cleavage site. We could not obtain any revertants from inocula of 10^6 p.f.u.. Therefore, the reversion frequency within the WSN-E stock is approximately 10^{-7} . The low reversion rate and the small viral loads of WSN-E in the mouse lung explain the absence of revertants during mouse passages. Another reason for the genetic stability of WSN-E *in vivo* is the restriction to one replication cycle because of the absence of the appropriate protease.

Protection against lethal challenge

To investigate the potential of WSN-E to serve as a live vaccine, we immunized five groups of mice with WSN-E: four groups received live virus at dosages of 10^3 ($n = 6$), 10^4 ($n = 6$), 10^5 ($n = 6$) or 10^6 p.f.u. ($n = 5$); one group received formalin-inactivated virus ($n = 4$). An additional group of nonimmunized mice ($n = 6$) served as a positive control during challenge. The animals tolerated the immunization without any signs of illness, as in the previously described survival experiment. Four weeks later, we challenged the mice with 10^6 p.f.u. WSNwt and monitored survival and weight loss (**Fig. 4a,b**). The challenge was lethal for both groups vaccinated with formalin-inactivated virus or 10^3 p.f.u. WSN-E, as well as for the nonimmunized control animals. From the mice immunized with 10^4 p.f.u. WSN-E, four of six mice survived and partially recovered from disease. Although some animals from the group vaccinated with 10^5 p.f.u. WSN-E developed temporary weight loss and milder disease symptoms, they eventually recovered. All animals vaccinated with the highest dose of 10^6 WSN-E survived the challenge and did not develop any weight loss or other visible symptoms of illness. We removed the lungs of two mice per group for plaque assay 3 d after challenge (**Fig. 4c**). Notably, after vaccination with 10^6 p.f.u. WSN-E, no plaques were seen, even in undiluted lung homogenates. This contrasts considerably with the plaque titers of the other groups. The challenge experiment indicates that the degree of protection against disease increases with the immunization dose. Additionally, the failure of the formalin-inactivated virus to prevent death shows that WSN-E must replicate to induce protection. Taken together, these results show that WSN-E is an attenuated virus that is able to prevent lethal influenza virus infection.

Strong serum and mucosal antibody response

We immunized mice with 10^3 ($n = 6$, $n = 5$), 10^4 ($n = 5$, $n = 4$), 10^5 ($n = 5$, $n = 6$) or 10^6 p.f.u. ($n = 4$, $n = 6$) WSN-E, 10^6 p.f.u. formalin-

inactivated WSN-E ($n = 5$, $n = 4$), WSNwt at a nonlethal dosage of 10^3 p.f.u. ($n = 5$, $n = 7$), or with PBS ($n = 6$, $n = 6$). Mice were killed 4 weeks later. We challenged a subgroup of each treatment cohort with 10^6 p.f.u. WSNwt 3 d before analysis. To investigate the virus-specific antibody response, we determined the IgG titers of sera and IgA titers of bronchoalveolar (BAL) fluid and nasal wash samples by ELISA. Additionally, we performed serum hemagglutination inhibition tests. Mice that received 10^3 p.f.u. WSN-E, formalin-inactivated WSN-E or PBS did not show any antibody response before or after challenge. By contrast, the groups immunized with 10^4 , 10^5 or 10^6 p.f.u. WSN-E showed substantial levels of virus-specific IgG and hemagglutination titers in serum as well as IgA titers in BAL and nasal wash, which increased with the immunization dose (**Fig. 5a-d**). We achieved the highest antibody titers with 10^3 p.f.u. WSNwt. With 10^6 p.f.u. WSN-E, the hemagglutinin inhibition titer before challenge was 1:40 (**Fig. 5b**). Compared with nonchallenged animals, challenged mice of the WSN-E 10^6 p.f.u. group showed elevated antibody titers (**Fig. 5a-d**), especially in the nasal wash (**Fig. 5d**). This may be attributed both to antigenic boost and induction of a fast and effective memory immune response.

DISCUSSION

Our goal was to generate influenza A virus with an atypical hemagglutinin cleavage site that is resistant to activation during natural

Figure 4 Protection against lethal challenge with WSNwt. Survival rates (a) and average weight loss (b) of challenged mice immunized with formalin-inactivated WSN-E (right-facing triangles), 10³ p.f.u. WSN-E (triangles), 10⁴ p.f.u. WSN-E (inverted triangles), 10⁵ p.f.u. WSN-E (diamonds) or 10⁶ p.f.u. WSN-E (squares) and nonimmunized (circles). Lung plaque titers (c) from the third day.

infection but can readily be activated under *in vitro* conditions. We accomplished this by replacing the original trypsin-specific cleavage site Arg-Gly with the elastase-sensitive site Val-Gly. Elastase mutants have previously been obtained after conventional cell-culture passages in the presence of this enzyme²¹. This study shows, however, that by reverse genetics generation of such mutants has become a fast and reproducible procedure suitable for routine production. WSNwt and the elastase-substituted WSN-E grew to similar titers in cell culture. In mouse lung, WSN-E was present only temporarily and did not cause any disease. But after infection with wild-type virus, we observed much higher lung titers, spread of virus to other organs and 100% lethality. Thus, the cleavage site mutant proved to be equivalent to wild-type virus regarding growth rates *in vitro*, but was completely attenuated *in vivo*. Because of these properties, WSN-E is a promising candidate for a live vaccine.

Unlike WSNwt, WSN-E titers in the mouse lung hardly exceeded the intranasal inoculum, indicating that replication was restricted to very few cycles. Accordingly, mice had to be vaccinated with relatively high doses of WSN-E (10⁶ or 10⁵ p.f.u.) for immunity against lethal challenge. But when inoculated with 10⁶ p.f.u. inactivated WSN-E, mice neither survived a challenge with WSNwt nor developed a detectable antibody response. It may be argued that the formalin inactivation was too harsh, but the protein amount used was approximately 80 ng per immunization dose. Moreover, we prepared the formalin-

inactivated WSN-E from the same nonconcentrated virus stock used for the live immunization and inoculated it intranasally only once (like the live WSN-E). Such inactivated vaccines are made from concentrated virus and usually administered three times to the mice²². The failure of immunization with formalin-inactivated virus showed that WSN-E replication was required for protection.

One intranasal inoculation of WSN-E induced a substantial, dose-dependent local and systemic immune response despite very limited presence in lung. A dosage of 10⁵ or 10⁶ p.f.u. induced substantial

Figure 5 Serum and mucosal antibody response. Serum IgG titers (a), serum hemagglutination inhibition (HI) titers (b), bronchoalveolar lavage (BAL) IgA titers (c), and nasal wash IgA titers (d) of nonchallenged (black bars) and challenged (gray bars) mice immunized with PBS, 10³ p.f.u. WSN-E, 10⁴ p.f.u. WSN-E, 10⁵ p.f.u. WSN-E, 10⁶ p.f.u. WSN-E, 10⁶ p.f.u. formalin-inactivated WSN-E and 10³ p.f.u. WSNwt.

hemagglutination inhibition titers, serum IgG and mucosal IgA titers. They were lower than those induced by 10^3 p.f.u. WSNwt because its longer replication enables antigenic stimulation. But the challenged animals showed almost comparable systemic and mucosal immunoglobulin titers when immunized with 10^6 p.f.u. WSN-E. This indicates that a notable immunological memory had already been induced in these animals.

A frequent objection against the use of live influenza virus vaccines is the possibility of reversion to pathogenicity. With WSN-E, we obtained no evidence for revertants after five passages in mice. This may be explained in part by the highly restricted replication of this virus in the lung which results in small viral loads. The absence of appropriate proteases for WSN-E *in vivo* allows only one (or very few) replication cycle(s) leading to self-limiting replication. This is the main difference from other attenuated viruses undergoing many replication cycles *in vivo*. For cold-adapted viruses, a duration of viral shedding up to 11 d in susceptible humans was reported²³. An important advantage of the short self-limiting replication is the decreased probability of any reversion, including the cleavage site motif itself and other attenuating mutations. Another factor contributing to the absence of reversion *in vivo* is the double mutation within the cleavage site. For back-mutation, two nucleotides at once have to be replaced because suppressor mutants outside of the cleavage region seem to be impossible. This explains the low reversion frequency in cell culture. In hen eggs, a factor X-like protease is present²⁴, which should cause a considerably higher proportion of revertants. Therefore, eggs might not be suitable for vaccine production. In cell culture, the substitution of trypsin by elastase for propagation of WSN-E leads both to positive selection for elastase-dependent virus and to negative selection against revertants.

Live influenza vaccines presently approved for human application are reassortants generated by coinfection of a cold-adapted temperature-sensitive master strain from which the six segments coding for the internal virion components are derived and the circulating strain which provides the hemagglutinin and neuraminidase genes^{25,26}. The faster generation of such reassortants by reverse genetics entirely from plasmids^{1,18,27} is feasible as well. These live vaccines are well tolerated and effective^{28–30}. But such an attenuated virus may give rise to a new viral strain with unpredictable traits by exchanging the internal genes, especially those without temperature-sensitive mutations (genes encoding the polymerase subunit PA, matrix and nonstructural proteins)¹, with the circulating strain. Experimental evidence for generation of a pathogenic virus from reassortment of two apathogenic strains has indeed been obtained^{2,3}. Such a scenario is avoided when a cleavage-site mutant is used that contains all eight genes of the circulating strain. A cleavage site mutant would deliver all antigens identical to the circulating strain and, therefore, be the most authentic vaccine. The possible advantage of this feature is indicated by studies showing that the internal influenza virion components prime a helper response cooperating in the antibody response against the hemagglutinin^{31,32}. The immunogenic relevance of the internal components is also underlined by the observation that live virus, and to some extent inactivated whole-virus vaccine, can induce heterotypic protection in contrast to subunit vaccines³³. Furthermore, it has been reported³⁴ that mice could be protected successfully from lethal infection with A/HongKong/156/97 (H5N1) by prior immunization with the A/Quail/HongKong/G1/97 (H9N2) isolate, which harbors internal genes 98% homologous to the H5N1 isolate.

In vaccine production, some circulating strains may grow to inadequate titers. Therefore, the propagation of such a seed virus rescued from genes of the epidemic strain may be delayed unpredictably. A solution would be to adapt an epidemic strain to cell lines suitable

for vaccine production and to use its internal genes as a backbone each year. The internal genes evolve considerably more slowly than the surface glycoproteins³⁵. Thereby, both high-growth properties and sufficient antigenic homology of the internal viral proteins can be provided. Moreover, this backbone can carry additional attenuating mutations.

To show that our approach is generalizable to highly pathogenic influenza strains, we recently generated an elastase-dependent mutant of the strain SC35M. This virus is an H7N7 isolate, carries a polybasic cleavage site and is highly pathogenic both for chickens and mice³⁶. Like WSN-E, the SC35M mutant is strictly dependent on elastase and grows to similar titers in cell culture like the wild-type (G. Mehmetoglu & J.S., unpublished data).

Because proteolytic activation is essential for the replication of each influenza virus, the conversion of any epidemic strain or of viruses with pandemic potential, such as highly pathogenic H5N1 strains, into a live vaccine by altering the cleavage site is possible. Major assets of cleavage site mutants are antigenic identity to the parent strain, nonexistent risk of generating new pathogenic reassortants, complete attenuation *in vivo* and *in vitro* growth equivalent to wild-type. Such an attenuated virus is an ideal candidate for a live vaccine.

METHODS

Cells. We cultured 293T human embryonic kidney cells in Dulbecco minimal essential medium containing 10% FCS and MDCK cells in minimal essential medium supplemented with 10% FCS.

Recombinant viruses. For generation of the recombinant influenza viruses, we used a previously described reverse genetics system¹⁸. This system is based on transfection of eight plasmids encoding the genes of strain A/WSN/33 (H1N1) under control of the human RNAI promoter and mouse RNA polymerase I terminator and a truncated RNA polymerase II (immediate-early CMV) promoter. To rescue WSNwt, we used the eight original plasmids¹⁸ mentioned above. For WSN-E, we mutagenized the hemagglutinin-encoding plasmid pHW184-HA to replace Arg343 with valine. We changed the nucleotides corresponding to positions 1,059 and 1,060 in the A/WSN/33 hemagglutinin gene from AG to GT by using the Quikchange Kit (Stratagene). The primer sequences are: 5'-CCCATCCATTCAATACGTAGTCTATTGGAGCCA-3' and 5'-TGGCTCCAAATAGACCTACGTATTGAATGGATGGG-3'.

Virus propagation. After rescue, we plaque-purified and grew both WSNwt and WSN-E on MDCK cells in minimal essential medium containing 0.2% bovine serum albumin. For propagation of WSNwt and WSN-E, we used 0.5 µg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) and 5 µg/ml porcine pancreatic elastase (Serva Electrophoresis GmbH), respectively. For growth curves, we inoculated MDCK cells with virus at multiplicity of infection of 3×10^{-2} .

Plaque assay. We performed the plaque assays on MDCK cell monolayers essentially as described³⁷. Before inoculation, we washed the cells five times with PBS. For passage experiments, we performed only one wash. For WSN-E, we used elastase instead of TPCK-treated trypsin in the plaque overlay.

Passage experiments *in vitro*. We grew WSN-E in 10 separate flasks (165 cm²) on MDCK cells in minimal essential medium containing 0.2% bovine serum albumin in the presence of 0.5 µg/ml TPCK-treated trypsin. The next day, we transferred entire supernatants to ten flasks with fresh MDCK cells. After 2 d, we transferred half of each volume of supernatant into ten fresh flasks. We performed another two passages of 2 d unless a cytopathic effect occurred. From the supernatants of the last passage, we performed RT-PCR for sequencing of the cleavage site of the hemagglutinin.

Western blot. We washed confluent MDCK monolayers, grown in dishes 6 cm in diameter, five times with PBS and infected them with WSNwt or WSN-E at multiplicity of infection of 10. We incubated both viruses in the presence of

either TPCCK-treated trypsin, elastase or no protease for 16 h in minimal essential medium containing 0.2% bovine serum albumin. After SDS-PAGE (10%) from pelleted supernatants, we used a polyclonal rabbit antiserum to A/WSN/33 and an rabbit-specific swine monoclonal antibody, conjugated with horseradish peroxidase (Dako) as secondary antibody (each 1 h at 22 °C, 1:2,000) followed by chemiluminescence (Supersignal West Pico Chemiluminescent Substrate kit from Pierce).

Formalin inactivation. We treated WSN-E at a concentration of 5×10^7 p.f.u./ml (equivalent to 10^6 p.f.u. in 20 μ l used for intranasal inoculation) with 0.1% formaldehyde and incubated it for one week at 4 °C. By plaque assay, we confirmed the absence of live virus. The hemagglutination titer of formalin-inactivated WSN-E was 1:64, that of live WSN-E 1:128. The protein content of 10^6 p.f.u. WSN-E virus stock was approximately 80 ng.

Animal experiments. The animal experiments were performed according to the guidelines of the German animal protection law. All animal protocols were approved by the relevant German authority, the Regierungspräsidium Gießen. We intranasally inoculated 4-week-old female Balb/C mice (Charles River) with 20 μ l virus suspension under anesthesia after an intramuscular injection of ketamine hydrochloride at a dosage of 200 mg/kg. At respective time points, mice were killed by cervical dislocation and entire organs removed. After homogenization in 1 ml PBS, we determined the organ titers by plaque assay in the presence of the appropriate protease in the overlay (inoculum size 333 μ l).

Antibody assays. We detected serum IgG antibodies and IgA antibodies in bronchoalveolar lavage fluid and nasal washes by ELISA. According to previously described methods³⁸, we obtained BAL fluid and nasal wash samples. First, we coated Maxisorp 96-well plates (Nunc) with total WSNwt virus protein at 4 °C overnight. Then, we detected the bound antibodies by mouse-specific IgG or IgA labeled with horseradish peroxidase (BD Pharmingen) and BM Blue POD substrate (Roche Diagnostics). Finally, we expressed the titers as reciprocal of the dilution that yielded an optical density of 0.1. We performed the hemagglutination inhibition test³⁹ after incubation overnight with receptor-destroying enzyme (Dade-Behring) at 37 °C and heat inactivation at 56 °C for 30 min using chicken erythrocytes and 4 hemagglutinating units of WSNwt.

Accession number. The GenBank accession number for the A/WSN/33 hemagglutinin precursor is J02176 and for TPA: *Mus musculus* ribosomal DNA, complete repeating unit it is BK000964.

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COMPETING INTERESTS STATEMENT

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