baculovirus infection phosphorylates ganciclovir. This would explain why a higher concentration of ganciclovir was needed to inhibit baculovirus proliferation in BmN cells than to inhibit HSV-1 proliferation in Vero cells. Godeau *et al.* (1992) reported that baculovirus expressing the thymidine kinase of HSV-1 was highly sensitive to ganciclovir, which supports this hypothesis.

Historically, screening of antiviral substances has involved inhibition of virus proliferation in cultured cells induced by candidate compounds. A problem with this method is that most of the candidate compounds that have antiviral activity *in vitro* are not effective for virus proliferation in host animals, due to their pharmacodynamic characteristics in the host animals. The pharmacodynamics of compounds in animal bodies are governed by adsorption, distribution, metabolism and exclusion. To determine the pharmacodynamics of each compound, experiments with model animals are essential. We propose the use of the silkworm infection model prior to the use of mammalian infection models for general screening of therapeutic compounds. We demonstrated previously that (i) antibiotics that are effective in human patients are also effective in silkworms infected with bacteria or fungi pathogenic to humans, and (ii) ED_{50} values, which provide a quantitative basis for assessing the therapeutic effect of antibiotics, are consistent between silkworms and mammals (Hamamoto *et al.*, 2004, 2005; Hamamoto & Sekimizu, 2005). These findings suggest that we can predict the pharmacodynamic characteristics of antibiotics in mammals by using the silkworm infection model. We propose that the pharmacodynamic characteristics of antiviral agents in the infection models of silkworms and mammals will be consistent. The ratio between the IC_{50} (the effective concentration of the compound needed for inhibiting virus proliferation in cultured cells by 50 %) and the ED_{50} (the evaluation of the amount of the compound needed to produce a therapeutic effect) will be useful for determining the pharmacodynamics of the compound. A small value would indicate better pharmacodynamics. We used several antiviral agents in this study that are clinically effective in humans infected with pathogenic viruses. The ED_{50} : IC_{50} value for all of the tested agents typically used for clinical purposes was <5. We propose that candidate compounds that have an ED_{50} : IC_{50} value of <5 should be considered as promising candidate antiviral agents for clinical purposes.

Antiviral effect of cinnzeylanine

Mao-to, Kakkon-to and Shosaiko-to have a long history as treatments for patients with influenza in Kampo medicine, but none of the compounds that are effective against viral infection have been identified. In this study, Mao-to had a therapeutic effect in the baculovirus-infected silkworm, and we purified an antiviral substance from a hot-water extract of cinnamon bark, one of the four components of Mao-to. Our assay measured the therapeutic effects in silkworms infected with baculovirus. The purified substance was cinnzeylanine, whose structure was identified previously. The antiviral activity of cinnzeylanine had not been determined previously. A remarkable feature of cinnzeylanine is that this compound shows therapeutic effects following administration into the midgut. Our previous study demonstrated that mammalian intestines and silkworm midgut have common permeability characteristics for chemical compounds (Hamamoto *et al.*, 2005). Therefore, we can expect that oral administration of cinnzeylanine is effective in humans. We also showed that cinnzeylanine inhibited the proliferation of HSV-1 in Vero cells. Taking these findings together, we propose that cinnzeylanine is a good candidate antiviral agent against HSV infection. To use cinnzeylanine in humans, chemical modifications of this compound are needed to increase the antiviral effect. The silkworm infection model will also be useful for optimizing chemically modified candidates.

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Timely production of A/Fujian-like influenza vaccine matching the 2003–2004 epidemic strain may have been possible using Madin–Darby canine kidney cells

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ABSTRACT

Timely production and antigenic match with those of the epidemic strains are required for influenza vaccines. A/Fujian/411/2002-like (H3N2) virus was the main epidemic influenza virus during the 2003/2004 season in the northern hemisphere. But A/Fujian-like reassortant viruses were not available until more than one year later. We evaluated the A/Kumamoto/102/2002 strain, an A/Fujian/411/2002-like strain isolated in 2002, as a potential vaccine. We compared A/Kumamoto/102/2002 viruses isolated from the same clinical sample in Madin–Darby canine kidney (MDCK) cells and eggs. Kumamoto/102/2002 isolated from eggs grew poorly and showed amino acid mutations of haemagglutinin. In contrast, A/Kumamoto/102/2002 isolated from MDCK cells grew well in MDCK suspension culture. The amino acid sequence of MDCK-derived A/Kumamoto virus was identical to that of A/Fujian/411/2002. These results suggest that culture in MDCK cells could have produced an influenza vaccine with a better antigenetic match to the predicted epidemic strain for the 2003/2004 season than the vaccine actually produced.

1. Introduction

Influenza vaccines currently used around the world are manufactured from viral strains that are chosen to be antigenically similar to anticipated epidemic strains. The World Health Organization (WHO) makes a recommendation about the influenza virus strains that should be used for vaccine production for the northern hemisphere in February and for the southern hemisphere in September, based on analysis of the strains that are circulating predominantly at the time. Most influenza vaccines are manufactured by using embryonated chicken eggs as a substrate for viral growth. Therefore, only viral strains that show good replication in eggs can actually be used for vaccine production. For this reason and to avoid contamination with other human respiratory viruses, vaccine seed viruses should be isolates derived in eggs. However, some clinical isolates fail to grow or show poor replication in eggs. In such cases, to improve the growth of the desired viral strain, a reassortant virus is created by using the haemagglutinin (HA) and neuraminidase (NA) genes derived from the epidemic strain and other genes derived from the high yielding PR-8 strain [1]. However, it takes time to develop PR-8-based reassortant strains and it is sometimes impossible to establish a reassortant virus that is antigenically equivalent to the recommended strain. In recent years, the reverse genetics (RG) technique has made it easier to create reassortant strains suitable for vaccine production. However, viruses created by this technique are regarded as genetically modified organisms (GMO) in some countries. Therefore, the need for biological confinement of RG viruses during vaccine production means that this technique is not used to produce influenza vaccines at present.

In the spring of 2003, the WHO held its annual meeting to recommend influenza vaccine strains for the northern hemisphere 2003/2004 flu season. At that time, it was considered that an A/Fujian/411/2002-like (H3N2) strain might emerge as a result of antigenic drift from the A/Moscow-like strain that had been used in influenza vaccines during the previous year, but the WHO finally decided to recommend the A/Moscow-like strain vaccine again. One reason for choosing the A/Moscow-like strain over the A/Fujian-like strain was the lack of a viral strain that was antigenically equivalent to A/Fujian/411/2002 that showed good growth in eggs [2]. As a result, the vaccine manufacturers used the A/Panama/2007/99 strain (an A/Moscow/10/99-like virus) as the H3N2 component of vaccines for the 2003/2004 season. Subsequently, H3N2 strains were predominant during the flu season from

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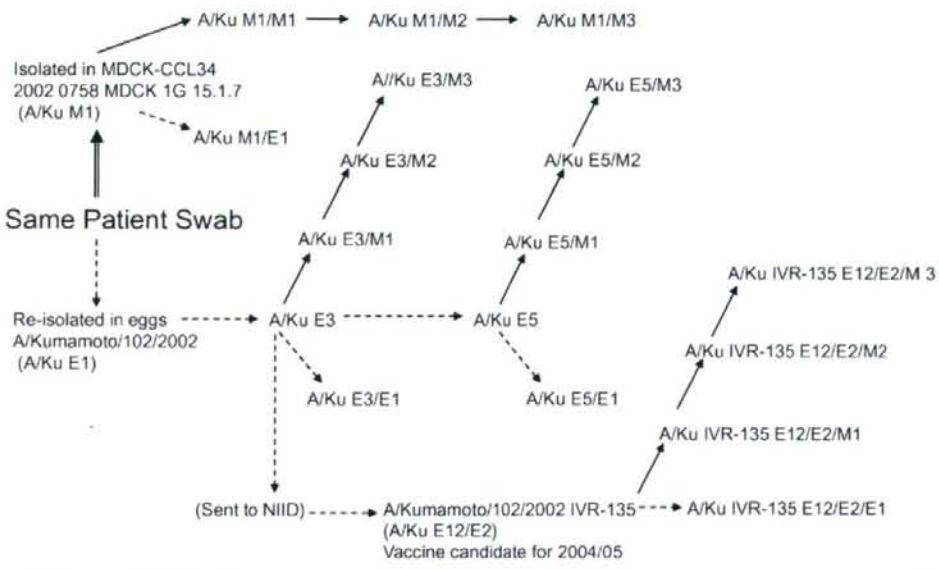

Fig. 1. History of the A/Kumamoto/102/2002 viruses. A solid line arrow shows passage in MDCK-33016. A solid double line arrow shows cultivation in MDCK CCL34. A dotted line arrow shows passage in eggs. Viral growth was measured for 3 passages each of A/Ku M1, A/Ku E3, A/Ku E5, and A/Ku IVR-135 E12/E2. HA1 gene sequences were compared for A/Ku M1, A/Ku M1/M2, A/Ku E3, A/Ku E3/M2, A/Ku E5, A/Ku E5/M2, A/Ku IVR-135. E12/E2, and A/Ku IVR-135 E12/E2/M2. Viruses purified from A/Ku M1/M3, A/Ku E3/M3, A/Ku E5/M3, and A/Ku IVR-135 E12/E2/M3 were used for immunogenicity assays. To compare the HA content, A/Ku M1/M3, A/Ku E3/M3, A/Ku E5/M3, and A/Ku IVR-135 E12/E2/M3 were re-cultured from previously passaged viruses (/M2).

the winter of 2003 to the spring of 2004, and the A/Fujian-like strain was the most prominent of the H3N2 strains [3]. This resulted in a mismatch between the influenza vaccine and the predominant epidemic influenza virus [4].

In January 2002, the A/Kumamoto/102/2002 virus was isolated from a clinical specimen (swab) in southern Japan using Madin–Darby canine kidney (MDCK) cells (ATCC CCL-34) and it was found to have antigenic properties similar to that of A/Fujian/411/2002. This virus was re-isolated from the same clinical specimen using embryonated hens' eggs. Subsequently, a reassortant strain was generated from this egg-derived virus and was designated as A/Kumamoto/102/2002 IVR-135. This reassortant virus became one of the influenza vaccine candidates for the 2004/2005 season. Thus, it took 2 years to establish a reassortant vaccine seed candidate after isolation of the wild strain. Finally, A/Wyoming/3/2003 was recommended as the A/Fujian/411/2002-like strain for the 2004/2005 season.

We have been developing influenza vaccines by using suspension culture of the virus in MDCK cells. It has been reported that MDCK cells show lower selectivity for influenza virus than eggs, so that the generation of reassortant viruses may be unnecessary for the production of vaccines with these cells [5]. However, few authors have attempted to confirm this with viruses isolated from both MDCK cells and eggs that originated from the same clinical specimen. We recently obtained a paired set of A/Kumamoto/102/2002-related viruses, comprising the original isolates from MDCK cells and eggs, respectively, viruses passaged in these two substrates, and a high growth reassortant developed for egg culture. Using these viruses and suspension culture in MDCK cells, we investigated whether it was possible to obtain an influenza vaccine that showed a better antigenetic match with the predicted epidemic strain than the actual vaccine produced from embryonated eggs for the 2003/2004 influenza season.

2. Materials and methods

2.1. Viruses

We studied four A/Kumamoto/102/2002-related viruses (2002 0758 MDCK 1G 15.1.7, A/Kumamoto/102/2002 E3, A/Kumamoto/102/2002 E5, and A/Kumamoto/102/2002 IVR-135), as well as A/Wyoming/3/2003 IVR-134 and A/Wyoming/3/2003 X-147.

The history of the A/Kumamoto/102/2002 viruses is summarized in Fig. 1.

In January 2002, the A/Kumamoto/102/2002 virus was isolated from a clinical specimen at the Kumamoto Prefectural Institute of Public Health and Environmental Science (Kumamoto, Japan) using MDCK (ATCC CCL-34) cells. This virus was originally named '2002 0759 MDCK 1G 15.1.7' (A/Ku M1) and was sent to the National Institute of Infectious Diseases (NIID). In March 2003, The Chemo-Sero-Therapeutic Research Institute started to re-isolate this virus from the same specimen (swab) using embryonated hens' eggs. The isolated virus was passaged 3 times in eggs and then was sent to NIID as "A/Kumamoto/102/2002 E3 (A/Ku E3)" on March 17, 2003. Next, A/Ku E3 was passaged twice more in embryonated eggs at Kaketsuken and was harvested as "A/Kumamoto/102/2002 E5 (A/Ku E5)". Growth of both A/Ku E3 and A/Ku E5 in embryonated eggs was not adequate for vaccine production. Subsequently, A/Ku E3 was sent to CSL (Melbourne, Australia) and a high growth reassortant with PR-8 was generated. This reassortant strain was designated as A/Kumamoto/102/2002 IVR-135. We obtained this reassortant through the NIID at E12/E2 (A/Ku IVR-135 E12/E2), and A/Ku IVR-135 E12/E2 became one of the influenza vaccine candidates for 2004/2005 season.

A/Wyoming/3/2003 IVR-134 E13/E2 (A/Wy IVR-134 E13/E2) and X-147 E5/E2 (A/Wy X-147 E5E2) were actually used as A/Fujian-like vaccine strains in Japan and Europe for the 2004/2005 season. These

strains were also tested for growth in MDCK cells and embryonated eggs to allow comparison of viral growth and HA gene sequences in the following experiments.

2.2. Seed virus preparation

MDCK cells (MDCK-33016) developed by Novartis Vaccine and Diagnostic (Marburg, Germany) [6,7] were used throughout the study. The cells were cultured in 100-mL spinner flasks using serum-free medium. Each viral strain was passaged twice consecutively in the MDCK-33016 cells to establish seed viruses for pilot cell culture. Inoculated cells were grown in serum-free chemically defined medium containing 1 μg/mL trypsin at 34 °C for 3 days. Then the cells were removed by centrifugation, the supernatant was collected, and its infectivity titer was measured [8].

2.3. Pilot viral culture in MDCK cells and eggs

The cells cultured in 100-mL spinner flasks were inoculated with seed viruses at a multiplicity of infection (MOI) of 10^{-3}, 10^{-4}, or 10^{-5} (based on the titer of the seed virus). Then the inoculated cells were cultured in serum-free medium containing 1 μg/mL trypsin at 34 °C for 3 days, after which the cells were removed by centrifugation, the supernatant was collected, and its HA titer was measured. Virus was purified directly from the medium by ultracentrifugation on a 30% glucose cushion (112,700 × *g* for 150 min) to estimate the viral yield. After centrifugation, the viral pellet was collected and suspended in phosphate-buffered saline (PBS). Then the suspension was adjusted to the original volume of the culture supernatant and its HA content was measured by a single radial immunodiffusion (SRD) assay.

As a control, the four viruses (A/Ku M1, A/Ku E1, A/Ku E5 and A/Ku IVR-135 E12/E2) and A/Wy IVR-134 E13/E2 were also cultured in eggs. The viruses were inoculated into the chorioallantoic cavity of embryonated eggs at concentrations of 10^{-2} to 10^{-4} EID_{50}/0.2 mL. The inoculated eggs were incubated at 34 °C for 3 days and then were left overnight at 4 °C. Subsequently, the allantoic fluid was centrifuged at 320 × *g* for 10 min, 10 mL of supernatant was centrifuged at 102,300 × *g* for 60 min to pellet the virus, and the pellet was re-suspended in 100 μL of PBS. Then the HA content was measured by the SRD assay. We used the reagents for A/Wyoming in all SRD assays because we could not obtain any reagents for the A/Kumamoto virus.

2.4. HA1 gene sequencing

The HA1 gene sequences of the original viruses that we had obtained and the seed viruses that we established after passaging in MDCK cells and eggs were analyzed to investigate the occurrence of selection during culture in the cells or eggs.

The method employed was reported previously [9].

In brief, viral RNA was extracted with a Catrimox-14TM RNA Isolation Kit Ver. 2.11 (TaKaRa). RT-PCR was performed using the extracted viral RNA and primers to amplify HA genes with the TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa). The primers were designed by using gene analysis software Genetix. The PCR products were separated by electrophoresis on 0.7% agarose gel and the part of the gel containing the HA gene region was cut out. Then DNA was harvested from the gel using Quantum Prep Freeze 'N Squeeze DNA (Bio-Rad), and was purified by phenol/chloroform treatment and ethanol precipitation. Finally, the gene sequence was analyzed using primers designed for sequencing and a Beckman CEQ2000XL capillary sequencer [10].

Table 1
Growth of A/Fujian-like viruses in MDCK-33016 cells.

Viruse	Passage[a]		
	1st (M1)	2nd (M2)	3rd (M3)
A/Ku M1	9.3[b]	8.9	8.6
A/Ku E3	7.9	8.9	8.9
A/Ku E5	8.1	8.6	8.1
A/Ku IVR135 E12/E2	7.3	8.6	9.0
A/Wy IVR-134 E13/E2	7.5	8.5	8.9
A/Wy X-147 E5/E2	6.8	7.9	9.3

[a] Viruses were passaged 3 times.

[b] Viral titers of culter supernatant were determined by the TCID assay. Titers are shown as log($TCID_{50}$/ml).

2.5. Immunogenicity

The animal study was approved by our institutional Animal Experimentation Ethics Committee. The immunogenicity of the four A/Kumamoto viruses (A/Ku M1/M1 and A/Ku IVR-135 E12/E2/M3) was compared in Balb/c mice.

First, these viruses were purified and inactivated according to the following procedure. Culture supernatants were subjected to ultracentrifugation to precipitate viral particles, which were then purified by sucrose density gradient centrifugation and dialyzed to remove the sucrose from the viral fraction. After dialysis, the purified viral particles were inactivated by exposure to 0.02% formalin.

Second, eight-week-old female Balb/c mice were injected intraperitoneally with the inactivated purified viral particles at a dose of 3 μg of total protein. The HA content of the purified virus was confirmed to be equivalent by SDS-PAGE (similar mobility and concentrations of the HA protein bands). Eight mice were immunized with each purified viral antigen. Immunization was performed twice at a 3-week interval, and blood samples were obtained 2 weeks after the second dose. Serum samples from the immunized mice were examined by a cross haemagglutination-inhibition (HI) antibody assay with 2 purified viral antigens that were used for immunization as described above [11].

Comparison of the HI antibody titers determined for each individual mouse was done with the unpaired Student's *t*-test(two-tailed) [12].

3. Results

3.1. Comparison of viral growth and HA titers between egg and cell culture

Table 1 shows the generation of seed viruses by serial culture twice in MDCK cells.

All of the viruses showed efficient replication in these cells during both primary culture and subsequent passages, irrespective of whether they were originally isolated from MDCK cells or eggs. It is noteworthy that the A/Ku M1 virus isolated from MDCK cells showed good growth in MDCK-33016 cells during primary culture as well as in subsequent passages. Even A/Ku E3 and A/Ku E5, which were isolated and passaged in eggs, showed good growth during subsequent passaging in MDCK cells. For pilot culture in eggs, the original viruses were used as the seed.

After pilot culture in MDCK cells and eggs, the HA titers of the culture supernatants and the allantoic fluid and the HA content of partially purified virus were measured. The results are shown in Fig. 2.

When A/Ku M1 virus was cultured in embryonated eggs (A/Ku M1/E1), the virus showed no replication, with no HA titer or HA content of allantoic fluid being detected. Although A/Ku E3 and A/Ku E5 grew in eggs, the yield was lower compared with that for reas-

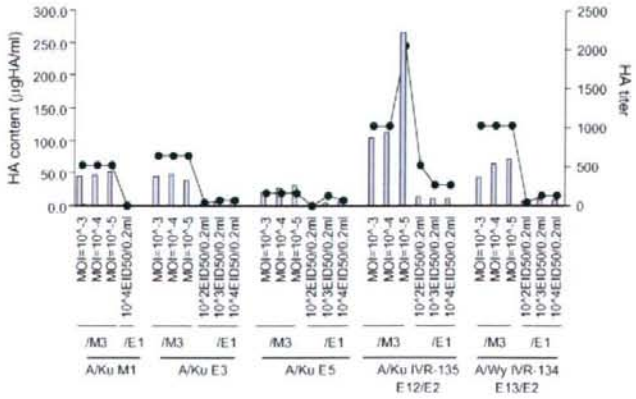

Fig. 2. HA content (bars) and HA titer (symbols) in MDCK culture medium or allantoic fluid. All cultures were performed at 3 inoculum concentrations, except for A/Ku M1/E1 because of the small volume of A/Ku M1. Viruses from MDCK cells were purified and re-suspended in PBS at the same volume as that of the culture medium. Viruses from eggs were purified and re-suspended in PBS at 1/100 of the allantoic fluid volume. The HA content was measured by SRD (reagents consisted of egg-derived A/Wyoming antigen and anti-A/Wyoming sheep serum obtained by immunization with egg-derived A/Wyoming HA antigen).

sortant viruses (A/Ku IVR-135 E12/E2/E1, A/Wy IVR-134 E13/E2/E1). When these viruses were cultured in MDCK cells, they all produced high HA titers and HA yields at any inoculated MOI. The HA content of virus partially purified from pilot culture of A/Ku M1/M3 in MDCK cells was 24–39 μg/mL, which was 2–3 times higher than the HA content of the reassortant viruses A/Ku IVR-135 and A/Wy IVR-134 cultured in eggs. The HA content of the A/Kumamoto reassortant cultured in MDCK cells (A/Ku IVR-135 E12/E2/M3) ranged from 105 to 267 μg/mL, which was 6–10 times higher than that after culture in eggs. On the other hand, the A/Wy IVR-134 E13/E2/M3 reassortant showed a similar HA yield to that of A/Ku M1/M3 in MDCK cells.

3.2. HA1 gene sequences analysis

The results of HA1 gene sequencing are summarized in Table 2. The positions of all genes and amino acids not described in the table were identical with the sequence of A/Fujian/411/2002. The gene sequences of A/Fujian/411/2002 and A/Wyoming/3/2003 were cited from The Influenza Sequence Database (http://www.flu.lanl.gov/). A/Ku M1 had nucleic acid point mutations at 3 sites (A6G, U75A, and A763C) compared with the sequence of A/Fujian/411/2002, but the sequence of translated amino acids was identical for these two viruses. A/Ku M1/M2 had a HA1 sequence identical to that of A/Ku M1. A/Ku E3 had point mutations at 3 sites

Table 2
Comparison of the HA1 sequences of A/Fujian, A/Kuamoto and A/Wyoming cultured in eggs or MDCK cells.

Virus	Sequence at the indicated HA1 position													
	Nucleotides									Amino acids				
	6	76	382	469	557c	656	676	747	763	127	156	186	219	226
A/Fujian/411/2002[a]	A	U	A	U	G	C	G	G	A	T	H	G	S	V
A/Ku M1	G	A	–[b]	–	–	–	–	–	C	–	–	–	–	–
A/Ku M1/M2	G	A	–	–	–	–	–	–	C	–	–	–	–	–
A/Ku E3	G	A	–	–	U[e]	A[f]	A[g]	–	C	–	–	V[e]	Y[f]	I[g]
A/Ku E3/M2	G	A	–	–	U	A	A	–	C	–	–	V	Y	I
A/Ku E5	G	A	–	–	U	A	A	–	C	–	–	V	Y	I
A/Ku E5/M2	G	A	–	–	U	A	A	–	C	–	–	V	Y	I
A/Ku IVR-135 E12/E2	G	A	–	A[d]	U	A	A	–	C	–	Q[d]	V	Y	I
A/Ku IVR-135 E12/E2/M1	G	A	–	A	U	A	A	–	C	–	Q	V	Y	I
A/Wyoming/3/2003[a]	G	A	G[c]	–	U	A	A	A	C	A[c]	–	V	Y	I
A/Wy IVR-134 E13/E2	G	A	G	–	U	A	A	A	C	A	–	V	Y	I
A/Wy IVR-134 E13/E2/M2	G	A	G	–	U	A	A	A	C	A	–	V	Y	I
A/Wy X-147 E5/E2	G	A	G	A	U	A	A	A	C	A	Q	V	Y	I
A/Wy X-147 E5/E2/M2	G	A	G	A	U	A	A	A	C	A	Q	V	Y	I

Direct reverse transcriptase PCR was used for the amplification of the HA1 gene with the following sense and anti-sense primers:
sense primer 1, 5′-CAGGGGATAATTCTATTAACCA-3′; sense primer 2, 5′-ATCAGATCCTTGATGGAG-3′;
sense primer 3, 5′-GCTCAATAATGAGATCAG-3′; sense primer 4, 5′-ACTCTGAAATTGGCAACA-3′;
sense primer 5, 5′-CTAACTGACTCAGAAATG-3′;
anti-sense primer 1, 5′-TGAGGCAACTAGTGACCT-3′; anti-sense primer 2, 5′-CTATGAAACCCGCGA-3′;
anti-sense primer 3, 5′-TGCACTCAAATGCAAATGTTGCACC-3′.

[a] Reported sequences are cited from the influenza sequence database (http://www.flu.lanl.gov/).
[b] Same as A/Fujian.
[c] Nucleotide mutation inducing amino acid substitution at position 127.
[d] Nucleotide mutation inducing amino acid substitution at position 156.
[e] Nucleotide mutation inducing amino acid substitution at position 186.
[f] Nucleotide mutation inducing amino acid substitution at position 219.
[g] Nucleotide mutation inducing amino acid substitution at position 226.

Table 3
Cross haemagglutinin-inhibition test of mouse serum with A/Kumamoto viruses grown in MDCK 33016 cells.

Ha antigen	Mouse antiserum	
	A/Ku M1/M3	A/Ku IVR-135 E 12/E2/M3
A/Ku M1/M3	147[a] ($p<0.05$[b])	87
A/Ku IVR-135 E 12/E2/M3	80 ($p<0.05$[b])	147

[a] Geometric mean titer.
[b] Significant difference when individual serum was analyzed by unpaired Student's *t*-test(two-tailed).

(G557T, C656T, and G676A) compared with A/Ku M1. These point mutations were estimated to cause the following amino acid substitutions: G186V, S239V, and V246I, respectively. The HA1 gene sequences of A/Ku E3/M2 and A/Ku E5/M2 were identical with that of A/Ku E3. No HA1 gene additions or deletions were observed during passage of these viruses in MDCK cells.

In the A/Ku IVR-135 E12/E2 strain, in addition to the mutations seen in A/Ku E3, another mutation was observed (T469A). It was estimated that A/Ku IVR-135 E12/E2 had an additional amino acid substitution (H156Q) related to this mutation.

The HA1 gene sequence of the A/Ku IVR-135 E12/E2/M2 strain generated by two passages of A/Ku IVR-135 E12/E2 in MDCK cells was identical with that of A/Ku IVR-135 E12/E2.

Compared with A/Fujian/411/2002, A/Wyoming/3/2003 showed differences at 4 sites (A382G, G557U, G676A, and G747A), in addition to the mutations detected in A/Kumamoto/102/2002. The amino acid sequence of A/Wyoming/3/2003 also differed at 4 sites (T127A, G186V, S219V, and V226I) compared with that of A/Fujian/411/2002. The A/Wy IVR-134 E13/E2 virus used for our experiment was found to have the same sequence as that of A/Wyoming/3/2003 from the database. A/Wy IVR-134 E13/E2/M2 had the same HA1 gene sequence as that of A/Why IVR-134 E13/E2. A/Wy X-147 E5/E2 showed a point mutation (U469A) compared with the original A/Wyoming/3/2003 strain and this mutation was estimated to cause an amino acid substitution (H156Q). The HA1 gene sequence of A/Wy X-147 E5/E2/M2 was identical with that of A/Wy X-147E5/E2.

All the viruses generated from two passages in MDCK cells (/M2) were found to have completely identical HA1 gene sequences with those before passaging. That is, no additional mutations were caused by passaging in MDCK-33016 cells.

3.3. *Immunogenicity*

The results of the cross HI assay are shown in Table 3. Although there was no 4-fold significant difference between the geometric mean titers, a significant difference was detected when the unpaired Student's *t*-test was used. Serum from A/Ku M1/M3-immunized mice showed a significantly lower HI antibody titer ($p<0.05$) for A/Ku IVR-135 E12/E2/M3 antigen than for A/Ku M1/M3 antigen. Serum harvested from A/Ku IVR-135 E12/E3/M3-immunized mice showed a low reactivity for A/Ku M1/M3 antigen but a significant difference was not detected.

4. Discussion

When an influenza vaccine is produced, two important factors are timeliness and antigenic match with those of the circulating strains. Because the recent egg isolates of type A viruses have not grown very well in eggs, reassortants have been used for vaccine production. Although it was expected that A/Fujian-like strains would appear during the 2003/2004 flu season, none of the vaccine candidates, including the A/Ku E3 or E5 viruses, showed satisfactory growth in eggs. Vaccine manufactures had to wait until the next year to obtain high growth reassortant viruses for vaccine production. Therefore, there are limits to egg culture in terms of timely production. As an alternative substrate for viral growth, MDCK cells have long been suggested. If these cells were used, timely production of vaccine could be expected because of the low selectivity of this substrate. To confirm this expectation, we evaluated the growth of viral isolates (isolated in MDCK cells and eggs) from the same clinical specimen in MDCK cells and eggs. Based on the results obtained with the A/Ku M1 virus, we demonstrated that viruses showing poor growth in eggs could still replicate well in MDCK-33016 cells.

A/Ku M1 grew well in MDCK-33016 cells for 3 passages. Assuming actual vaccine production, A/Ku M1/M1, A/Ku M1/M2, and A/Ku M1/M3 would correspond to the master seed virus, working seed virus, and vaccine, respectively. A very high HA yield was observed in the culture medium of A/Ku M1/M3 cells compared with the allantoic fluid of eggs inoculated with any of the viruses. Therefore, if we had used A/Ku M1 cultured in MDCK 33016 cells for vaccine production, we could have supplied a vaccine matching the A/Fujian-like virus for the 2003/2004 flu season. Even if the A/Ku E3 or A/Ku E5 virus had been chosen, growth of the seed virus and vaccine production could have been performed in a timely manner by culture in MDCK-33016 cells.

In this study, the HA content of culture medium and allantoic fluid were measured by an SRD assay designed for the A/Wy IVR-134 strain grown in eggs. If an assay for A/Kumamoto grown in MDCK cells had been available, the HA content of culture media could have been measured more precisely [13].

When the reassortant A/Ku IVR-135 E12/E2 virus was cultured in MDCK cells, a 5- to 10-fold higher yield was achieved than that in allantoic fluid. However, the reassortant A/Wy IVR-134 E13/E2/M3 virus showed a 2- to 3-fold higher yield in MDCK cells than in allantoic fluid, which was similar to the yields of other non-reassortant viruses. It may be necessary to investigate further whether a high growth reassortant virus is needed for MDCK cells or not.

Lu reported that the His-183 HA1 gene of the A/Fujian/411/2002 strain causes impairment of growth in MDCK cells [14]. However, all of the viruses that we studied had His-183 and still grew well in these cells. They used a PR-8-based RG [15,16] recombinant virus that differed from the wild-type strain with respect to the configuration of the genome, and this may partly account for the discrepancy between their results and ours.

The A/Kumamoto/102/2002 virus accumulated point mutations with an increase of passaging in eggs and the number of resultant amino acid mutations also increased. Three mutations in the HA1 gene (G557T, C656A, and G676A) were found in the A/Ku E3 virus after 3 passages in eggs and these mutations generated 3 amino acid substitutions (G186V, S219Y, and V226I, respectively). The substitutions were identical with those of other H3N2 influenza viruses, as reported elsewhere [17–20]. These sites are thought to be related to receptor binding activity, which suggests that viruses recognizing the receptors commonly expressed in eggs may become predominant during passage in eggs [21–23].

The reassortant A/Ku IVR-135 E12/E2 virus was generated through more than 10 passages in eggs and showed an additional H156Q amino acid substitution related to the T469A gene substitution. Amino acid 156 of HA1 is located at the tip of the HA molecule within an important region that determines antigenicity [24–28], so mutations at this site may influence the immunogenicity of vaccines. When immunogenicity was compared among the viruses generated by culture in MDCK cells, serum from mice immunized with A/Ku M1/M3 showed a weaker response to A/Ku IVR-135 E12/E2/M3 antigen. Serum from mice immunized with A/Ku IVR-135 E12/E2/M3 also showed a weaker response to A/Ku M1/M3

antigen. This suggests that the A/Kumamoto/102/2002 virus had developed amino acid substitution in the HA region that affects its immunogenicity during passage in eggs to form reassortants.

No new substitutions of the HA1 amino acid sequence were detected in any of the viruses (including A/Ku M1) passaged in MDCK-33016 cells, unlike the case for eggs. Viruses that were passaged in eggs and then cultured in MDCK cells did not revert to the amino acid sequence of the original virus cultured in MDCK cells. Unlike eggs, MDCK cells show weak selection pressure for the quasispecies of inoculated influenza virus and are therefore unlikely to allow a minor strain with a different amino acid sequence that grows well to become a major strain as a result of passaging. Therefore, these results indicate that MDCK cells can provide vaccines better matched to the circulating viral strain than culture in eggs.

Taken together, our findings suggest that HA1 (especially the amino acid at position 156) plays a key role in the immunogenicity of A/Fujian-like viruses. Jin et al. reported that the antigenicity of A/Wyoming/3/2003 can be converted to match that of A/Panama by simultaneous T155H and H156Q substitution of its HA1 sequence [17]. None of the viruses used in our study had both of these substitutions, although A/Ku IVR-135 and A/Wy X-147 possessed the latter substitution. Therefore, the production of influenza vaccine using A/Kumamoto/102/2002 IVR-135 and A/Wyoming/3/2003 X-147 might not have led to a vaccine with adequate antigenicity matching the predicted endemic wild strain if it had been attempted. Based on the actual events during 2003 in the northern hemisphere, it seems that an influenza vaccine matching the antigenic profile of A/Fujian could have been supplied for the 2003 winter season if vaccine manufacturing technology based on MDCK-33016 cells had been employed.

The MDCK-33016 cells used in our study were grown as a suspension culture in completely chemical medium and were therefore suitable for large-scale culture. The HA content in the culture supernatant of MDCK-33016 cells in the present study was at least twice that obtained in allantoic fluid. It is difficult to directly compare cell and egg culture with respect to vaccine production. However, the supernatant from a 1000-L bioreactor would be equivalent to allantoic fluid from at least 200,000 eggs and should produce more than 200,000 doses of vaccine, if the recovery of vaccine antigens is same as with egg-grown vaccines. Accordingly, the use of MDCK-33106 cells could make it possible to promptly supply a vaccine with superior immunogenicity to that of vaccines produced using eggs.

Under the current system of influenza vaccine production, only viruses passaged in specific pathogen-free (SPF) eggs are available as vaccine seeds. Serial passaging of viruses in eggs probably leads to the accumulation of gene and amino acid mutations, resulting in antigenic changes. When cultured in MDCK cells, influenza virus proliferates well, even during the first passage. Therefore, the first and second passages yield viral banks and the third passage allows the production of a vaccine. Thus, long-term passaging that is likely to induce amino acid mutations is not necessary in the case of MDCK cell-based vaccine production. A number of studies have shown that influenza virus cultured in cells has superior immunogenicity to that of virus cultured in eggs [28–30].

From the point of view of rapid production and antigenic matching, a vaccine based on a virus isolated from cells such as MDCK cells is preferable. According to available guidance [31], however, troublesome adventitious agent testing of seed viruses would be needed before vaccine manufacture because many agents must be detected by PCR or another method before vaccine production. To avoid such tests, a seed virus created by RG technology may be preferable for cell-based influenza vaccine production. But if the virus seed is made by RG technology, another new test which confirms the sequence of HA and NA genes may be needed [32]. Unexpectedly, our study suggested that the genes related to viral growth in cells might differ between wild-type viruses and RG viruses with the PR-8 backbone, so more data about the growth of wild-type and RG influenza viruses in MDCK cells are needed.

Finally, because vaccine strains are often changed annually, a generic production system with low cost and high yield similar to the current egg system that can be used with any virus is required. Therefore, other issues need to be addressed, such as systems for the generation of seed viruses and establishment of SRD reagents.

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