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SHORT REPORT

# Significant neutralizing activities against H2N2 influenza A viruses in human intravenous immunoglobulin lots manufactured from 1993 to 2010

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**Abstract:** Influenza A H2N2 virus, also known as the Asian flu, spread worldwide from 1957 to 1967, although there have been no cases reported in humans in the past 40 years. A vaccination program was introduced in Japan in the 1960s. Older Japanese donors could have been naturally infected with the H2N2 virus or vaccinated in the early 1960s. Human intravenous immunoglobulin (IVIG) reflects the epidemiological status of the donating population in a given time period. Here, the possible viral neutralizing (VN) activities of IVIG against the H2N2 virus were examined. Hemagglutination inhibition (HI) and VN activities of IVIG lots manufactured from 1993 to 2010 in Japan and the United States were evaluated against H2N2 viruses. High HI and VN activities against H2N2 viruses were found in all the IVIG lots investigated. HI titers were 32–64 against the isolate in 1957 and 64–128 against the isolates in 1965. VN titers were 80–320 against the isolate in 1957 and 1280–5120 against the isolates in 1965. Both the HI and VN titers were higher against the isolate in 1965 than in 1957. Thus, antibody titers of IVIG against influenza viruses are well correlated with the history of infection and the vaccine program in Japan. Therefore, evaluation of antibody titers provides valuable information about IVIGs, which could be used for immune stimulation when a new influenza virus emerges in the human population.

**Keywords:** IVIG, influenza, H2N2, neutralization

## Introduction

The highly pathogenic avian influenza A H5N1 virus has spread among wild birds worldwide. As of April 2012, there have been 602 cases of human infections, with an extremely high mortality rate of about 59%.[1] Therefore, there are public health concerns regarding the possible global emergence of an H5N1 pandemic virus. However, a swine-origin pandemic influenza A H1N1 virus suddenly emerged in 2009. This novel virus spread among human populations within a short period of time because of the low level of immune responses against the virus, especially among young people. Thus, a pandemic influenza virus may be transmitted to humans because of limited immune responses against the virus in humans. The H2N2 virus could be considered one such virus, because it was prevalent in humans between 1957 and 1967, and a vaccination program was introduced in Japan in the 1960s.[2] However, although H2N2 continues to circulate among birds and pigs, this virus has not infected humans for the last several decades.[3] Based on the above background, it might be worthwhile examining the neutralizing activity of human intravenous immunoglobulin (IVIG) against the H2N2 virus.

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Generally, IVIGs are manufactured in individual lots with serum donations from over 10,000 healthy donors. Therefore, IVIG contains various antibodies against numerous human pathogens including seasonal influenza viruses. In fact, not only current (manufactured in 2008) but also previous (manufactured in 1999) IVIGs contain antibodies with significant neutralizing titers against seasonal and pandemic 2009 influenza viruses.[4] The antibodies comprising IVIG therefore reflect the epidemiological status of the donating population, in a particular time period and geographical area.

In this study, IVIG lots manufactured from 1993 to 2010 were evaluated for hemagglutination inhibition (HI) and virus neutralizing (VN) activities against the H2N2 virus.

## Material and methods

Clinical isolates of H2N2 (A/Okuda/1957, A/Izumi/5/1965, A/Kaizuka/2/1965), IVIGs manufactured from 1993 to 2010 from healthy donors in Japan (currently Kenketsu Venoglobulin®-IH; Benesis Corporation, Osaka, Japan), and IVIGs manufactured in 1993 and 1999 from healthy donors in the United States were used in this study (Table 1).

The viruses were propagated in Madin–Darby canine kidney (MDCK) cells or in the allantoic cavity of 11-day-old embryonated chicken eggs. The culture media and allantoic fluids were stored at −80°C prior to use. Viral infectivity (FFU/mL) was titrated in MDCK cells using the peroxidase-antiperoxidase (PAP) staining technique.[5] The HI test using 0.7% guinea pig erythrocytes was carried out as described previously.[5] HI titers are expressed as the reciprocal of the highest dilution of the IVIG preparation showing inhibition. The VN test was also carried out as described previously.[5] Briefly, each IVIG was serially diluted two-fold with serum-free minimal essential medium. The IVIG dilutions (30 μL of each) were mixed with 100 FFU (30 μL) of virus. After incubation for 30 minutes at 37°C, the mixture (30 μL) was applied to MDCK cells in a 96-well microplate. After incubation for 16 hours, the cells were fixed with ethanol and stained using PAP technique. The results are expressed as the reciprocal of the dilution resulting in 50% neutralization ($VN_{50}$).

**Table 1** Cross-reactivity of intravenous immunoglobulins to the influenza H2N2 virus

| IVIG | H2N2 virus | | | | | |
|---|---|---|---|---|---|---|
| | Okuda/1957 | | Izumi/5/1965 | | Kaizuka/2/1965 | |
| | HI | $VN_{50}$ | HI | $VN_{50}$ | HI | $VN_{50}$ |
| 1993JP, Lot A | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 1993US, Lot B | 64 | 320 | 128 | 5120 | 128 | 2560 |
| 1999JP-A, Lot C | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 1999JP-B, Lot D | 32 | 80 | 64 | 1280 | 64 | 1280 |
| 1999JP-C, Lot E | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 1999JP-D, Lot F | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 1999US, Lot G | 64 | 320 | 128 | 5120 | 128 | 2560 |
| 2000JP, Lot H | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 2003JP, Lot I | 32 | 80 | 64 | 1280 | 64 | 1280 |
| 2006JP, Lot J | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 2007JP, Lot K | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 2008JP, Lot L | 32 | 160 | 64 | 1280 | 64 | 1280 |
| 2009JP, Lot M | 32 | 80 | 64 | 1280 | 64 | 1280 |
| 2010JP, Lot N | 32 | 80 | 64 | 1280 | 64 | 1280 |

**Abbreviations:** HI, hemagglutination inhibition titer; IVIG, intravenous immunoglobulin; JP, Japan; US, United States; $VN_{50}$, virus neutralization titer giving 50% inhibition of infectivity.

## Results

As summarized in Table 1, the titers were significant against all three isolates of H2N2: HI titers of 32–64 and $VN_{50}$ titers of 80–320 against Okuda/1957, HI titers of 64–128 and $VN_{50}$ titers of 1280–5120 against Izumi/5/1965, and HI titers of 64–128 and $VN_{50}$ titers of 1280–2560 against Kaizuka/2/1965. Both the HI and $VN_{50}$ titers were higher against the isolate in 1965 than in 1957 (Table 1). There were no apparent differences in the HI and $VN_{50}$ titers against H2N2 virus isolates among the IVIG products prepared in the United States and Japan in different years.

## Discussion

Currently, most of the donor population has not been exposed to the H2N2 virus. IVIG is manufactured from pooled plasma. In a previous study, HI and VN activities against seasonal influenza H1N1 and H1N1-pdm2009 were examined in IVIG lots manufactured from 1999 to 2008. The IVIGs indicated high and stable HI and VN activity against H1N1. It was taken into account that the donor population may have been immunized through native infections and/or vaccine programs. Interestingly, the IVIGs also showed low but significant HI and VN activities against H1N1-pdm2009 despite most of the donor population never having been exposed to swine H1N1 or the Spanish flu.[4]

In this study, HI and VN activities against the H2N2 virus, which has not been included in vaccine programs for the past 45 years, were measured in IVIGs manufactured from 1993 to 2010 in Japan. Both the HI and $VN_{50}$ titers were higher against H2N2 isolates in 1965 than in 1957. It has been reported that people under the age of 50 years have little or no immunity to H2N2 and that people older than 50 years who have been exposed to the virus show a higher rate of resistance.[6] Therefore, older Japanese donors could have been naturally infected with the H2N2 virus or vaccinated in the

early 1960s. Consequently, antibody titers against the H2N2 virus in the general population will decrease in the future, which could lead to an H2N2 pandemic. It is not clear why such differences of HI and VN activities of IVIG preparations were observed between the United States and Japan. One possible explanation may be derived from the difference in environmental factors and population for blood donation between the two countries.

## Conclusion

The results suggest that the antibody titers against influenza viruses in IVIGs correlate with the history of infection and with vaccination programs. Therefore, the evaluation of antibody titers provides valuable information about IVIGs, which could be used for immune stimulation when a new influenza virus emerges in the human population.

## Acknowledgments

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## Disclosures

Yoshinobu Okuno and Ritsuko Kubota-Koketsu are employed by The Research Foundation for Microbial Diseases of Osaka University. Mikihiro Yunoki is employed by the Benesis Corporation. All other authors report no conflicts of interest in this work.

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# Jurkat cell proliferation is suppressed by *Chlamydia* (*Chlamydophila*) *pneumoniae* infection accompanied with attenuation of phosphorylation at Thr389 of host cellular p70S6K

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ABSTRACT

*Chlamydia* (*Chlamydophila*) *pneumoniae* infects T lymphocytes and multiplies within them. Our previous studies have indicated that *C. pneumoniae* infection suppresses proliferation of peripheral blood mononuclear cells stimulated with *Staphylococcus*-enterotoxin B; however, the mechanism of suppression was unclear. In this study, we explored the molecular mechanism involved in *C. pneumoniae* infection by using human acute T cell leukemia cell line, Jurkat E6-1. Proliferation of Jurkat cells was suppressed in an m.o.i.-dependent manner by *C. pneumoniae* infection. The suppression by the infection was particularly evident during the initial 24 h of the infection, and down modulation of cyclin D3 protein levels were observed at the same time period by immunoblot analysis. The suppression of the Jurkat cell proliferation and the down modulation of cyclin D3 protein level were only induced by viable *C. pneumoniae* infection, not by exposure to UV-killed or heat-killed *C. pneumoniae*. Phosphorylations at Thr308 and Ser473 of AKT were induced by *C. pneumoniae* infection; however, phosphorylation at Thr389 of the downstream kinase, p70S6K was inhibited by unidentified mechanism associated with *C. pneumoniae* infection. Taking into account that G1 arrest of the *C. pneumoniae* infected Jurkat cells were not observed and that p70S6K is one of the most important regulators of protein synthesis, it was suggested that the suppression of Jurkat cell proliferation by *C. pneumoniae* was at least in part mediated by down modulation of protein synthesis through attenuation of Thr389 phosphorylation of p70S6K.



## Introduction

*Chlamydia* (*Chlamydophila*) *pneumoniae* is an obligate intracellular bacterium. Respiratory tract *C. pneumoniae* infection is one of the major causes of community-acquired pneumonia, bronchitis, and sinusitis. Seroepidemiological and histopathological studies have detected *C. pneumoniae* antigens or the bacteria itself within atherosclerotic lesions, suggesting that *C. pneumoniae* infection may be related to the pathogenesis of atherosclerosis and cardiovascular diseases (Grayston 2000; Mussa et al. 2006; Watson and Alp 2008). Interaction between host cells and *C. pneumoniae* is thought to play a prominent role in the pathogenesis of these inflammatory diseases. However, the pathogenic mechanisms involved in the diseases associated with *C. pneumonia* infection are still unclear.

Many studies have reported the preferred host cell types for *C. pneumoniae*. *C. pneumoniae* can infect and multiply in endothelial cells, aortic smooth muscle cells, monocytes/macrophages (Gaydos et al. 1996; Godzik et al. 1995; Kalayoglu et al. 2001; Quinn and Gaydos 1999; Airenne et al. 1999), and lymphocytes (Haranaga et al. 2001a,b; Yamaguchi et al. 2002a,b). It has been hypothesized that circulating monocytes or lymphocytes infected with *C. pneumoniae* may act as carriers of the bacteria from the respiratory tract to the peripheral endothelial or smooth muscle cells, or as a reservoir of infected *C. pneumoniae* near and at the lesion in the development and progression of atherosclerosis. In

*Abbreviations:* PBMC, peripheral blood mononuclear cell; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; HBSS, Hank's balanced salt solution; PI3K, mammalian target of rapamycin; mTOR, phosphoinositide 3-kinase; mTORC1, multi-component mTOR complex 1; AMPK, 5′ AMP-activated protein kinase; gadd34, growth arrest and DNA damage protein 34.

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the pathogenesis of these inflammatory diseases, lymphocytes are regarded as key contributors to acute and chronic inflammation. Therefore, investigation into how *C. pneumoniae* infection modulates the functions of lymphocytes is important for understanding the development of the inflammatory diseases associated with *C. pneumoniae* infection. We have previously reported the effects of *C. pneumoniae* infection on human peripheral blood mononuclear cells (PBMCs) (Haranaga et al. 2001a,b; Yamaguchi et al. 2008, 2004; Hirai et al. 2010) and established human T lymphocytes (Yamaguchi et al. 2002a,b; Takano et al. 2005). Recently, we reported that *C. pneumoniae* infection suppresses proliferation of *Staphylococcus*-enterotoxin B stimulated PBMCs (Hirai et al. 2010). However, the mechanism by which this occurs has not been well explained. In this study, we aimed to elucidate the effect of *C. pneumoniae* on the proliferation of T lymphocytes using Jurkat, clone E6-1 cells as an infection model. The results obtained in this study suggest that *C. pneumoniae* might exert a suppressive effect on T lymphocytes via suppression of Thr389 phosphorylation of p70S6K, which is indispensable to protein synthesis and cell proliferation.

## Materials and methods

### *Cell lines*

The human acute T cell leukemia cell line, Jurkat, clone E6-1 (Jurkat; ATCC TIB-152), and the human epithelial cell line HEp-2 (ATCC CCL-23) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics (10 μg/ml gentamicin, 10 μg/ml vancomycin, and 1 μg/ml amphotericin B). HEp-2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FCS and the same antibiotics.

### *Bacteria*

*C. pneumoniae* strain TW183 was obtained from the ATCC and propagated in HEp-2 cells according to previously described methods (Yamaguchi et al. 2002a,b). Heat-killed and UV-killed *C. pneumoniae* were prepared as described in previous reports (Hirai et al. 2010; Geng et al. 2000). The number of infectious *C. pneumoniae* cells was determined as inclusion forming units, by counting the inclusion bodies formed in *C. pneumoniae* infected HEp-2 cells after staining with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-*Chlamydia* antibody specific to *Chlamydia* lipopolysaccharide (LPS) (Denka seiken, Tokyo, Japan) (Hirai et al. 2010).

### *C. pneumoniae infection*

Jurkat cells were seeded at 0.5 or $1.0 \times 10^7$ cells/well in a 24-well plate on the day of *C. pneumoniae* infection. *C. pneumoniae* cells were added to Jurkat cells at an m.o.i. of 0, 1, 10, 30, or 100. The plate was then centrifuged at $700 \times g$ for 60 min at room temperature. After centrifugation, the cells were washed twice with Hank's balanced salt solution (HBSS), and cultured in RPMI 1640 medium supplemented with 10% FCS and the previously itemized antibiotics. The Jurkat cells were collected at 0, 1, 2, 3, 4, 5, 8, 24, 48, and 72 h after infection and subjected to experiments. In some experiments, Jurkat cells were treated with 10 μM of cytochalasin D (Wako Pure Chemical Industries, Ltd., Osaka, Japan) since 30 min before and during the centrifugation. In some experiments, heat-killed and UV-killed *C. pneumoniae* were used. The treated Jurkat cells were collected after 0, 24, 48, and 72 h of exposure, for further analysis.

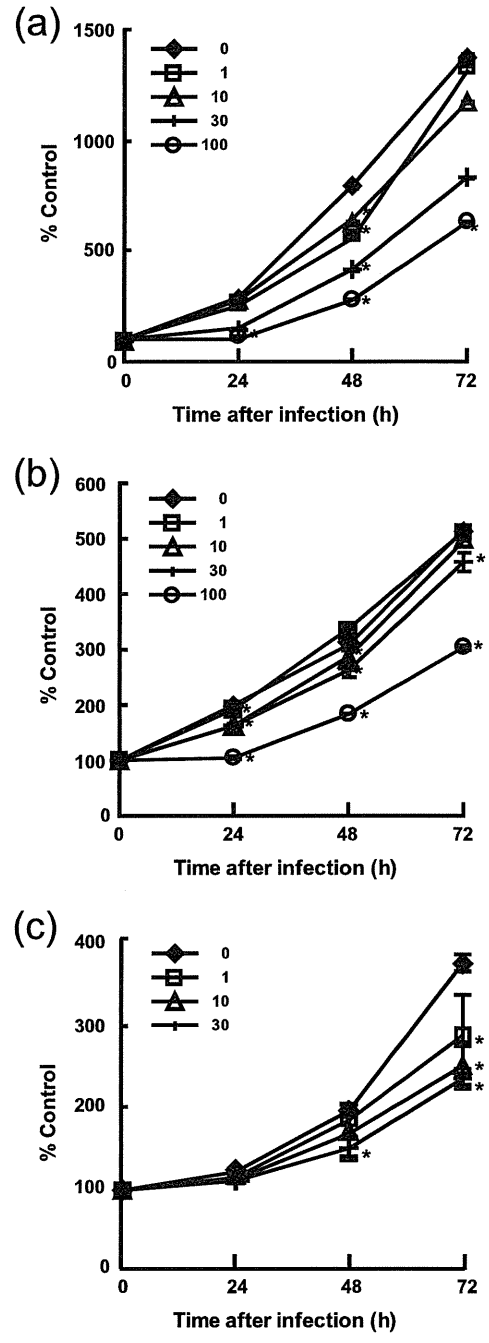


**Fig. 1.** Suppression of Jurkat cell proliferation by *C. pneumoniae* infection. Jurkat cells and HEp-2 cells were infected with *C. pneumoniae* at an m.o.i. of 0, 1, 10, 30 or 100 and of 1, 1, 3, 10 or 30, respectively. At 0, 24, 48, and 72 h after infection, cells were collected. The relative viable cell ratios of the infected Jurkat cells (a), and the relative viabilities of the infected Jurkat cells (b) and the infected HEp-2 cells (c) were measured. Representative results from 3 independent experiments are shown. The numbers indicate the value relative to the control group at time point 0, and are represented as means ± standard deviations ($n = 3$). *$P < 0.05$, significantly different from the control group at the same time point.

### *Cell proliferation assay*

After *C. pneumoniae* infection, the cells were seeded at $5.0 \times 10^4$ cells/well in a 96-well plate. The proliferation of infected cells was examined at 0, 24, 48, and 72 h after infection, using the Cell Counting Kit-8 (WST-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Cell numbers and viabilities

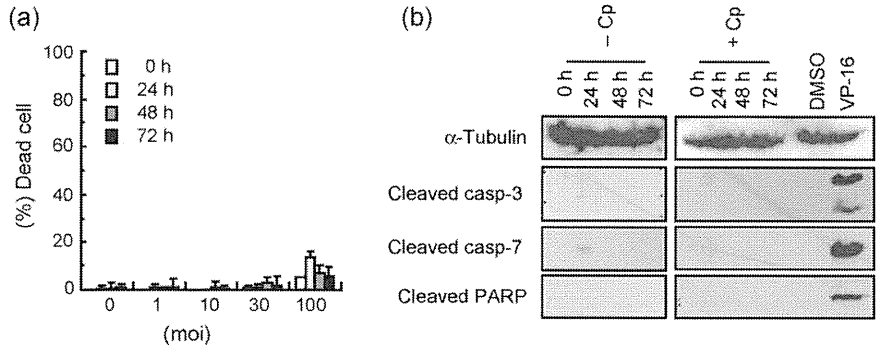


**Fig. 2.** Less activation of apoptosis machinery of the Jurkat cells infected by *C. pneumoniae*. (a) Jurkat cells were infected with *C. pneumoniae* at an m.o.i. of 0, 1, 10, 30, or 100. At 0, 24, 48, and 72 h after infection, cells were collected, and the dead cell ratios of the infected cells were measured. The ratios are represented as means ± standard deviations ($n = 3$). (b) Jurkat cells infected with *C. pneumoniae* at an m.o.i. of 0 or 30 were collected at 0, 24, 48 and 72 h after infection, protein extracts from the infected cells were subjected to immunoblotting with anti-α-tubulin, anti-cleaved caspase-3, anti-cleaved caspase-7 and anti-cleaved PARP antibodies. Representative results from 3 independent experiments are shown.

of the infected cells at these time-points were determined by the trypan-blue dye exclusion method.

*Immunoblot analysis*

Cell lysates were prepared in lysis buffer (50 mM Tris, HCl, pH 8.0, 150 mM NaCl, 10% glycerol, and 1% Triton X-100) containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Sigma) (Hirai and Wang 2002). Equal protein amounts of the lysates were analyzed by SDS-PAGE and blotting using peroxidase conjugated secondary antibody and the SuperSignal West Dura Extended Duration Substrate (Thermo, Rockford, IL). Anti-α-tubulin monoclonal antibody was obtained from Sigma. Anti-cyclin D3 monoclonal antibody was purchased from BD. Anti-cleaved PARP polyclonal antibody, anti-cleaved caspase-3 polyclonal antibody, anti-cleaved caspase-7 polyclonal antibody, anti-p70S6K monoclonal antibody, anti-phospho-p70S6K monoclonal antibody, anti-AKT monoclonal antibody and anti-phospho-AKT monoclonal antibody were purchased from CST Japan (Tokyo, Japan).

*Statistical analysis*

Statistical analyses were performed using the unpaired Student's *t* test.

## Results

*The effect of C. pneumoniae infection on the proliferation of Jurkat cell proliferation*

The number of Jurkat cells and their cell viability were examined at 0, 24, 48, and 72 h after infection (Fig. 1), to evaluate the effect of *C. pneumoniae* infection on the proliferation of Jurkat cells. The results showed that the proliferation of Jurkat cells was significantly suppressed by *C. pneumoniae* infection in an m.o.i.-dependent manner, and this suppression was most evident at the earlier time-points, within 24 h of infection. Similar results were obtained from the trypan blue dye exclusion assay (Fig. 1a) and the WST-8 cell proliferation assay (Fig. 1b).

In order to confirm whether the suppression of Jurkat cell proliferation by *C. pneumoniae* was cell type specific event, we performed similar WST-8 cell proliferation assay using human epithelial cell line HEp-2 cells (Fig. 1c). The results indicated that the infected HEp-2 cell proliferation was also suppressed by *C. pneumoniae* infection as same manner as the infected Jurkat cells. This result suggested that suppression of cell proliferation by *C. pneumoniae* was not cell type specific event.

The trypan blue dye exclusion assay showed that cell death caused by *C. pneumoniae* infection was minimal in Jurkat cells (Fig. 2a). To confirm this, cleaved caspase-3, cleaved caspase-7 and cleaved PARP were detected in the Jurkat cells infected with *C. pneumoniae* at an m.o.i. of 0 or 30 by immunoblotting (Fig. 2b). Any obvious signals corresponding to cleaved caspase-3, cleaved-caspase-7 and cleaved PARP were not observed from the Jurkat cells infected with *C. pneumoniae* at an m.o.i. of 30. Taken together, these results suggested that apoptosis was not induced in the Jurkat cells infected with *C. pneumoniae* at least up to an m.o.i. of 30.

*The suppression of Jurkat cell proliferation is dependent on viable C. pneumoniae infection*

To prove that *C. pneumoniae* infection was essential to the suppression of Jurkat cell proliferation, the cell proliferation assay using WST-8 after treatment of actin polymerization inhibitor, cytochalasin D were performed. Repeatedly, the proliferation of the Jurkat cells was suppressed by *C. pneumoniae* infection at an m.o.i. of 30. Cytochalasin D treatment itself did not effect on the Jurkat cell proliferation. Moreover, cytochalasin D restored the Jurkat cell proliferation even under presence of *C. pneumoniae* probably by blocking *C. pneumoniae* invasion into the host Jurkat cells (Fig. 3).

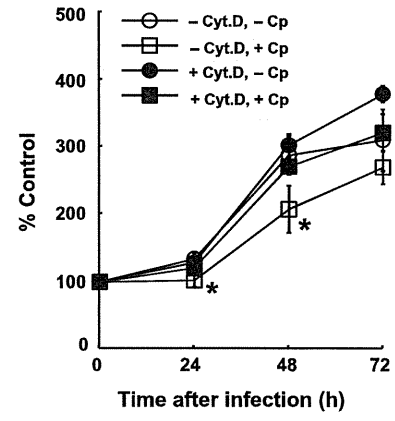


**Fig. 3.** *C. pneumoniae* infection-dependent suppression of the infected Jurkat cell proliferation. Jurkat cells were treated with or without 10 μM of cytochalasin D for 30 min and infected with or without *C. pneumoniae* at an m.o.i. of 30. At 0, 24, 48, and 72 h after infection, the relative viabilities of the infected Jurkat cells were measured. Representative results from 3 independent experiments are shown. The numbers indicate the value relative to the control group at time point 0, and are represented as means ± standard deviations ($n = 3$). *$P < 0.05$, significantly different from the control group at the same time point.

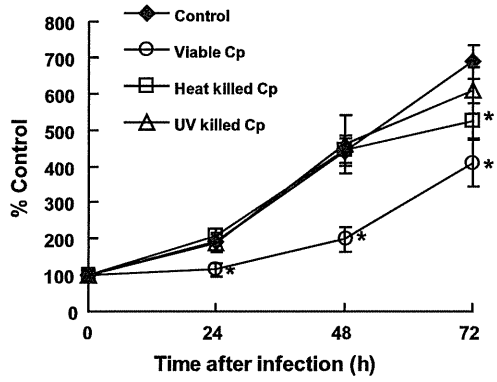


**Fig. 4.** Viable *C. pneumoniae*-dependent suppression of the infected Jurkat cell proliferation. Infection was performed with viable, heat-killed, or UV-killed *C. pneumoniae* at an m.o.i. of 10. At 0, 24, 48, and 72 h after exposure to the bacteria, aliquots of the Jurkat cells were harvested and subjected to the trypan-blue dye exclusion assay. Representative results from 3 independent experiments are shown. The values indicate the numbers relative to the control group at time point 0, and represent means ± standard deviations ($n = 3$). *$P < 0.05$, significantly different from the control group at the same time point.

This result suggested that the suppression of Jurkat cell proliferation required the establishment of *C. pneumoniae* infection.

To evaluate whether the suppressive effect of *C. pneumoniae* on proliferation required viable *C. pneumoniae*, heat-killed and UV-killed *C. pneumoniae* were substituted for live bacteria in the proliferation assay (Fig. 4). Viable *C. pneumoniae* infection at an m.o.i. of 10 consistently suppressed the proliferation of infected Jurkat cells. In contrast, neither heat-killed nor UV-killed *C. pneumoniae* showed any suppressive effects on the proliferation of the host Jurkat cells. These data suggest that viable *C. pneumoniae* infection is essential to the suppression of proliferation observed in infected Jurkat cells.

### *The suppression of Jurkat cell proliferation by C. pneumoniae infection is accompanied by a reduction in phosphorylation level at Thr389 of p70S6K*

Cyclin D3 is reported as key regulator in Jurkat cell proliferation (Boonen et al. 1999). Therefore, cyclin D3 protein level was evaluated by immunoblot analysis in order to determine whether suppression of Jurkat cell proliferation by *C. pneumoniae* infection involved cyclin D3 down modulation. As shown in Fig. 5a, cyclin D3 protein levels were diminished by *C. pneumoniae* infection. The reduction in cyclin D3 was an m.o.i. dependent (Fig. 5b) and required infection with viable *C. pneumoniae* (Fig. 5c). In spite of decreased cyclin D3 protein level, we could not observe an accumulation of infected Jurkat cells at Go/G1 phase (data not shown). This suggested that the suppression of Jurkat cell proliferation associated with *C. pneumoniae* infection did not involve the induction of cell cycle arrest at the G1 phase.

We performed preliminary experiments with chemical compounds including MEK inhibitor U0126, protein kinase A (PKA) activator CPT-cAMP, PKA inhibitor H-89, mammalian target of rapamycin (mTOR) inhibitor rapamycin. U0126, CPT-cAMP and rapamycin suppressed Jurkat cell proliferations; however, we could not obtain any evidence that MEK and PKA pathways were related to the suppression of cyclin D3 expression level. Therefore, we further analyzed phosphoinositide 3-kinase (PI3K)/AKT/mTOR/p70S6K pathway. This pathway is prominent signal transduction pathway in cell proliferation and growth (Asnaghi et al. 2004; Manning 2004; Peter et al. 2010), and *Chlamydia* infection stimulates phosphorylation of AKT by PI3K dependent manner (Coombes and Mahony 2002; Verbeke et al. 2006). Therefore, alteration of the phosphorylation status of AKT and p70S6K was investigated in the context of *C. pneumoniae* infection (Fig. 6). Even in the absence of *C. pneumoniae* infection, AKT was somewhat phosphorylated by stimulation with additional FCS after the procedure of *C. pneumoniae* infection. Consistent with previous report, phosphorylation on Thr308 and Ser473 of AKT was induced by *C. pneumoniae* infection. In case of p70S6K, Thr389 phosphorylation was induced by additional FCS as same manner as AKT; however, *C. pneumoniae* infection dramatically suppressed Thr389 phosphorylation of p70S6K in contrast to activation of AKT. Concurrently, cyclin D3 expression level was also suppressed as phosphorylation at Thr389 of p70S6K (Fig. 6). These data suggested that down modulation of the p70S6K phosphorylation by unidentified mechanism associated with *C. pneumoniae* infection could be the main mechanism for suppression of Jurkat cell proliferation.

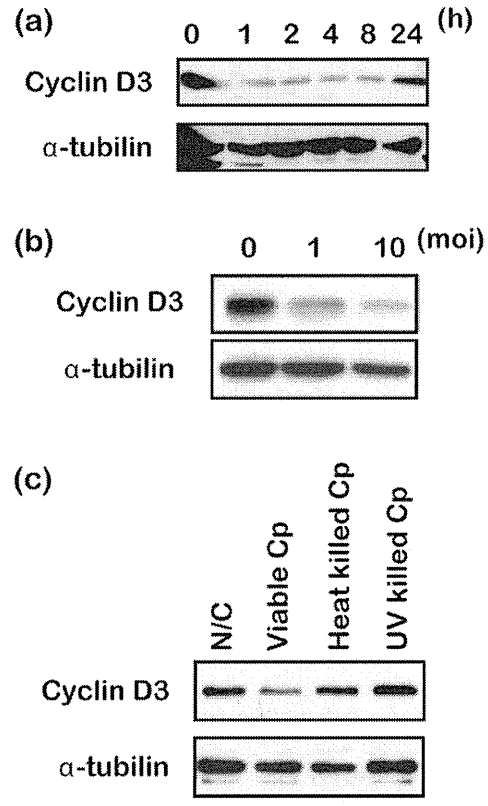


**Fig. 5.** Viable *C. pneumoniae* infection decreases cyclin D3 expression levels. (a) At 0, 1, 2, 4, 8, and 24 h after *C. pneumoniae* infection at an m.o.i. of 10, aliquots of infected Jurkat cells were harvested and lysed in an appropriate volume of lysis buffer. Equal protein amounts of the cell lysates were analyzed via immunoblotting, using anti-cyclin D3 antibody and anti-α-tubulin antibody. (b) Infected Jurkat cells were collected 2 h after *C. pneumoniae* infection at an m.o.i. of 0, 1, or 10. Equal protein amounts of the cell lysates were analyzed via immunoblotting using anti-cyclin D3 antibody and anti-α-tubulin antibody. (c) Jurkat cells were harvested 2 h after infection with viable *C. pneumoniae* at an m.o.i. of 10, or exposure to the same amount of heat-killed or UV-killed *C. pneumoniae*. Equal protein amounts of the cell lysates were subjected to immunoblotting analysis using anti-cyclin D3 and anti-α-tubulin antibodies.

## Discussion

Previously, we have reported that *C. pneumoniae* derived antigens and DNA can be detected in PBMCs (Haranaga et al. 2001a,b), and that *C. pneumoniae* infects and multiplies in T lymphocytes (Haranaga et al. 2001a,b; Yamaguchi et al. 2002a,b, 2008). It has also been reported that *C. pneumoniae* infection affects host-cell function by reducing CD3 and CD25 expression levels (Yamaguchi et al. 2008; Hirai et al. 2010). However, how *C. pneumoniae* infection

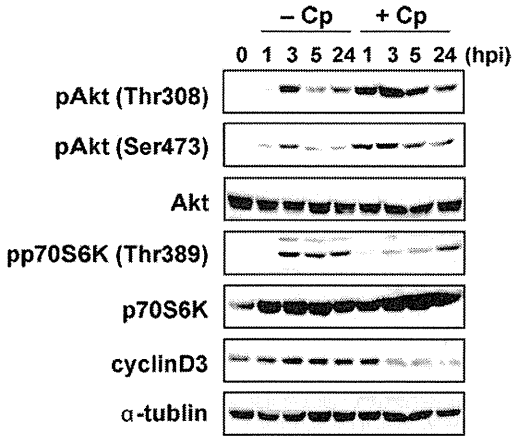


**Fig. 6.** Suppression of Thr398 phosphorylation of p70S6K by *C. pneumoniae* infection. Jurkat cells were harvested at the indicated time-points after infection with *C. pneumoniae* at an m.o.i. of 0 or 10. After preparation of cell lysates, equal protein amounts from each were subjected to immunoblotting analysis using anti-AKT, anti-phospho-AKT (Thr308), anti-phospho-AKT (Ser473), anti-p70S6K, anti-phospho-p70S6K (Thr389), anti-cyclin D3, and anti-α-tubulin antibodies.

modulates host-cell functions, and the effect these modulations have on the onset and progression of disease, is not well understood. In this study, we investigated the mechanism by which *C. pneumoniae* infection suppresses T lymphocyte proliferation using the human acute T cell lymphoma cell line, Jurkat E6-1 as a model.

Data obtained in this study indicate that Jurkat cell proliferation is suppressed by *C. pneumoniae* infection, which is consistent with our previous report (Hirai et al. 2010). A recent report suggests that *C. pneumoniae* inhibits proliferation of activated human T lymphocytes by inducing apoptosis (Olivares-Zavaleta et al. 2011). In this study, the lower live-to-dead cell ratios and the less activation of apoptosis machinery, such as cleaved caspase-3, were observed in the infected Jurkat cell cultures. Considering the same results obtained in the infected PBMCs in our previous study (Hirai et al. 2010), we hypothesized that the suppression of proliferation induced by *C. pneumoniae* infection might not be solely due to cell death (infection-induced apoptosis).

It has been reported that various agents can induce cell cycle arrest in T lymphocytes at the G0/G1 phase (Fei et al. 2009; Sharif et al. 2010; Koyanagi et al. 2007; Wilson et al. 2005; Dohda et al. 2007; Gutzkow et al. 2003). In addition, cyclin D3 is reportedly the critical G1 cyclin in a leukemic T cell line (Boonen et al. 1999; Casanovas et al. 2004). Reduction of cyclin D3 protein levels in Jurkat cells results in G1 arrest of the cells (Boonen et al. 1999; van Oirschot et al. 2001). Therefore, we investigated expression levels of cyclin D3 in Jurkat cells infected with *C. pneumoniae*. In the infected Jurkat cells, cyclin D3 protein level was down regulated in an m.o.i.-dependent manner, and it was reliant on exposure to viable *C. pneumoniae* bacteria (Fig. 4). However, contrary to our expectations, we did not observe marked G1 arrest in *C. pneumoniae* infected Jurkat cells (data not shown). These observations suggested that cyclin D3 was not the main target utilized by *C. pneumoniae* to suppress host cells proliferation, and that alternative suppression mechanisms were yet to be unveiled.

Despite the protein half-life of cyclin D3 protein being relatively short (De Santa et al. 2007), protein levels of cyclin D3, unlike cyclins B1, A, and E, are relatively stable throughout the entire cell cycle (Gong et al. 1995). This suggests that the down regulation of cyclin D3 protein levels observed in this study was probably due to transcriptional and/or translational retardation. By our preliminary investigation using semi-quantitative RT-PCR, it was indicated that mRNA levels of cyclin D3 were not being altered by *C. pneumoniae* infection. Furthermore, proteasome inhibitor, MG-132 treatment maintained protein level of cyclin D3 in the Jurkat cells infected with *C. pneumoniae* (data not shown). Therefore, we focused our investigations on probing essential signal transduction pathways for the translational regulation of cyclin D3 as an index of translation level in the Jurkat cells infected with *C. pneumoniae*.

Various reports have suggested that the signal transduction pathways responsible for Jurkat cell proliferation and cyclin D expression include the mitogen-activated protein (MAP) kinase pathway (Terada et al. 1999), the PKA pathway (Gutzkow et al. 2003; van Oirschot et al. 2001), and the mTOR pathway (Hleb et al. 2004). Among these pathways, our preliminary consideration with certain chemical compounds suggested mTOR pathway might be responsible for the suppression of Jurkat cell proliferation and cyclin D3 protein level by *C. pneumoniae* infection.

Various molecules are involved in protein translation, including the cascade consisting of PI3K, AKT, mTOR, p70S6K and translational control machineries (Ma and Blenis 2009; Tee and Blenis 2005). Downstream serine/threonine kinase, p70S6K, is a direct substrate of multi-component mTOR complex 1 (mTORC1), and activation of p70S6K is initiated by phosphorylation at Thr389 which is most correlated phosphorylation site with p70S6K activation (Weng et al. 1998). Activated p70S6K modulates translation-initiation-factor functions, to facilitate protein synthesis (Ma and Blenis 2009). Once activity of p70S6K is weakened by de-phosphorylation at Thr389, protein synthesis is also attenuated (Ma and Blenis 2009). Consistent with this, data obtained in this study showed that the lower phosphorylation status of p70S6K induced by *C. pneumoniae* infection was accompanied by lower expression levels of cyclin D3 (Fig. 6). However, phosphorylations at Thr308 and Ser473 of upstream AKT were still induced by *C. pneumoniae* infection as the previous report (Coombes and Mahony 2002). This discrepancy between phosphorylation status, i.e. activation status of AKT and p70S6K suggested suppression of Jurkat cell by *C. pneumoniae* might be mediated by regulatory mechanism surrounding mTORC1.

At present, many factors involved in the modulation of mTORC1 activity by *C. pneumoniae* remain to be explained. AMPK which is one of upstream kinases of mTOR is an important molecule for sensing intracellular energy and nutrition (Hardie 2011; Canto and Auwerx 2010). AMPK is activated when the concentration of intracellular ATP is lowered, and activated AMPK phosphorylates Raptor to inhibit association between Raptor and mTOR (Gwinn et al. 2008). Consequently, inactivation of mTORC1, dephosphorylation of p70S6K, retardation of protein synthesis, and cell cycle arrest occurs. Consistently, inhibition of AMPK by a chemical inhibitor restored phosphorylation level at Thr389 of p70S6K and cyclin D3 expression level even under *C. pneumonia* infection in our preliminary experiment. During the Chlamydial life cycle, host-cell ATP is consumed by the intracellular pathogen, and therefore, activation of AMPK associated with *C. pneumoniae* infection is highly plausible. Growth arrest and DNA damage protein 34 (Gadd34) was also reported as an inhibitor of mTOR molecule under host cellular energetic stress such as energy depletion, and virus infection (Watanabe et al. 2007; Minami et al. 2007). Additionally, it is well known that *C. pneumoniae* secretes effector proteins through the type 3 secretion system (Bailey et al. 2007; Subtil et al. 2001). In this regard, forced expression of Chlamydial effector protein, CopN, arrests the cell cycle at the G2/M phase (Huang et al. 2008). Therefore, the contribution of these Chlamydial secretion proteins should also be considered.

In conclusion, *C. pneumoniae* infection weakened p70S6K phosphorylation at Thr389 in the infected Jurkat cells. Consequently, the infected Jurkat cell proliferation was suppressed via attenuation of protein synthesis in earlier period after the infection. Further

details of the mechanisms of suppression induced by *C. pneumoniae* infection remain to be elucidated.

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# Antigenic analysis of highly pathogenic avian influenza virus H5N1 sublineages co-circulating in Egypt

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Highly pathogenic avian influenza virus H5N1 has spread across Eurasia and Africa, and outbreaks are now endemic in several countries, including Indonesia, Vietnam and Egypt. Continuous circulation of H5N1 virus in Egypt, from a single infected source, has led to significant genetic diversification with phylogenetically separable sublineages, providing an opportunity to study the impact of genetic evolution on viral phenotypic variation. In this study, we analysed the phylogeny of H5 haemagglutinin (HA) genes in influenza viruses isolated in Egypt from 2006 to 2011 and investigated the effect of conserved amino acid mutations in the HA genes in each of the sublineages on their antigenicity. The analysis showed that viruses in at least four sublineages still persisted in poultry in Egypt as of 2011. Using reverse genetics to generate HA-reassortment viruses with specific HA mutations, we found antigenic drift in the HA in two influenza virus sublineages, compared with the other currently co-circulating influenza virus sublineages in Egypt. Moreover, the two sublineages with significant antigenic drift were antigenically distinguishable. Our findings suggested that phylogenetically divergent H5N1 viruses, which were not antigenically cross-reactive, were co-circulating in Egypt, indicating that there was a problem in using a single influenza virus strain as seed virus to produce influenza virus vaccine in Egypt and providing data for designing more efficacious control strategies in H5N1-endemic areas.

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## INTRODUCTION

Since the emergence of highly pathogenic avian influenza virus (HPAI) subtype H5N1 in China in 1996, the H5N1 virus has evolved to form 10 phylogenetically distinct clades (0–9) (WHO/OIE/FAO H5N1 Evolution Working Group, 2008), spread into South-East and East Asia, and

The GenBank/EMBL/DDBJ accession numbers for representative sequence data determined in this study are AB496981–AB497040, AB498019–AB498038 and AB551129–AB551136.

Three supplementary figures are available with the online version of this paper.

caused an epidemic in poultry and occasional infections in mammals (WHO, 2012c). In April 2005, HPAI H5N1 viruses caused large outbreaks in wild waterfowl at Qinghai Lake in China (Chen *et al.*, 2005) and one genotype, clade 2.2, unexpectedly spread west to central and southern Asia, Europe and Africa, including Egypt (Salzberg *et al.*, 2007; Wang *et al.*, 2008). Since the initial outbreaks in Egypt, a distinct third-order H5N1 clade, clade 2.2.1, has evolved in Egypt and diverged further into phylogenetically separate branches within clade 2.2.1 (Cattoli *et al.*, 2009). By early 2009, none of these new sublineages had become dominant and all of the sublineages continued to co-circulate in birds in Egypt (Abdel-Moneim *et al.*, 2009; Arafa *et al.*, 2010).

Clade 2.2 H5N1 virus was first isolated in poultry in Egypt in February 2006, possibly after its introduction from infected migrating ducks (Saad *et al.*, 2007). Thereafter, HPAI H5N1 spread swiftly nationwide among birds, including chickens, ducks, turkeys, geese and quail, and was declared endemic in Egypt in July 2008. Other countries with endemic HPAI H5N1 are Indonesia, China and Vietnam (OIE, 2011). To control and attempt to eradicate H5N1 viruses, the Egyptian authorities used a blanket vaccination programme and attempted to heighten biosecurity and quarantine measures in both commercial and household sectors (Peyre *et al.*, 2009). Nevertheless, HPAI H5N1 is still endemic in Egypt and continues to pose a severe threat to the poultry industry (Hafez *et al.*, 2010), causing more than a US$ 1 billion annual loss (Meleigy, 2007). In addition, among countries surveyed by the WHO, Egypt has the second-highest number of human H5N1 infections (WHO, 2012a). As of April 2012, 166 HPAI H5N1 cases, with 59 fatalities, have been reported in Egypt. In particular, the cumulative number since 2009 is notable: of 249 HPAI H5N1 cases worldwide, 123 cases (49 % of the total) were in Egypt. Most human infections were linked to close contact with and/or slaughtering of infected birds and no sustained human–human transmission has been documented to date in Egypt (WHO, 2012b, c). However, the long-term endemic status of HPAI H5N1 in Egypt could increase the opportunity for emergence of potential pandemic strains through intra- and interspecies transmission.

Influenza virus haemagglutinin (HA) is a virion-surface glycoprotein and the primary target for neutralizing antibodies (Skehel & Wiley, 2000; Smith & Helenius, 2004). The protein is initially synthesized as precursor HA0 and cleaved to yield HA1, a variable external subunit, and HA2, a conserved transmembrane subunit (Stevens *et al.*, 2006). Most of the HA1 molecule forms a globular head containing the binding pocket for cell-surface sialylglycans (or sialylgangliosides), the primary receptor for influenza viruses (Suzuki, 2005). HA affinity for sialylglycans is one of the determinants of influenza A virus host range (Horimoto & Kawaoka, 2005; Suzuki, 2005). Human and avian influenza viruses differ in their recognition of host-cell receptors: human viruses mainly bind α2,6-linked sialylglycan, while avian viruses have a high affinity for α2,3-linked sialylglycan (Couceiro *et al.*, 1993; Ito *et al.*, 1998). HA is also an antigenically variable protein in which a large number of point mutations accumulate, mainly in HA1 epitope regions (Nelson & Holmes, 2007).

Interestingly, H5N1 was introduced into Egypt from a single infected source (Eladl *et al.*, 2011; Watanabe *et al.*, 2011b). In contrast, in other countries, such as China and Nigeria, co-circulation of different H5 sublineages has allowed antigenic shift due to genetic reassortment among the sublineages (Chen *et al.*, 2006; Fusaro *et al.*, 2010). Continuous replication of H5N1 virus in Egypt during the last 5 years has provided an opportunity to study the relationship between genetic evolution and selection of influenza virus phenotypes, including antigenicity, receptor-binding specificity and pathogenicity. Although previous studies focused on genetic evolution of influenza viruses, there are relatively few reports analysing the effect of genetic evolution on biological characters (Cattoli *et al.*, 2011a).

Several commercial inactivated H5 vaccines, produced using different H5 virus strains, were used during the H5N1 epidemic in Egypt (Peyre *et al.*, 2009). However, mass vaccination has failed to control the continual H5N HPAI outbreaks in Egypt (Hafez *et al.*, 2010). The vaccination campaign limited the first wave of 2006 outbreaks. However, antigenic variants were detected in several vaccinated farms in 2007 and are now the dominant strains in vaccinated and non-vaccinated flocks in Egypt (Abdelwhab & Hafez, 2011a). Several studies have suggested that immune pressure due to the vaccines resulted in major antigenic drift of the H5N1 virus, generating phylogenetically distinct clade 2.2.1 variants (denoted here as sublineage C) (Cattoli *et al.*, 2011b; Eladl *et al.*, 2011). There are conflicting data on whether commercially available vaccines provide protection against these new antigenic drift variants. Some studies have reported inadequate protection (Abdelwhab *et al.*, 2011b; Grund *et al.*, 2011; Peyre *et al.*, 2009), while others have reported sufficient protection (Kim *et al.*, 2010; Terregino *et al.*, 2010). These discrepancies were probably due to selection of strains in those studies with phylogenetically discordant or unrepresentative sequences. In addition, polyclonal antibodies in infected chicken and ferret sera, used in conventional analyses, might complicate sensitive determination of the effect of HA amino acid mutations on antigenicity due to cross-reactivity with other viral structural proteins, such as neuraminidase and nucleoprotein (Kaminski & Lee, 2011).

In this study, we performed phylogenetic analyses of HA genes in HPAI H5N1 strains isolated in Egypt from 2006 to 2011, identified the amino acid mutations that were conserved in each of the newly formed H5 sublineages, and investigated the effect of these mutations on the antigenicity and immunogenicity of the HAs with monoclonal and polyclonal antibodies. The results of this study should be useful for understanding antigenic drift among H5N1 viruses in Egypt and for planning more efficacious control strategies for endemic HPAI H5N1.

## RESULTS

### Phylogenetic analysis of H5N1 HAs and identification of conserved mutations

The phylogeny of H5N1 influenza viruses in Egypt was investigated by analysing 62 HA sequences of representative viruses isolated from birds and humans from 2006 to 2011. Phylogenetic analysis showed that all of the HA genes clustered in clade 2.2.1 with an overall monophyletic topology, indicating that these viruses diverged from a single origin (Fig. 1). In addition, most human H5N1 viruses isolated in 2009–2011 clustered in two sublineages (A and B) and most avian H5N1 viruses isolated in 2009–2011 clustered in two different sublineages (C and D), as described previously (Abdel-Moneim *et al.*, 2009; Balish *et al.*, 2010; Watanabe *et al.*, 2011b). This phylogenetic tree topology was the same as that for a phylogenetic tree reconstructed from 492 HA sequences from H5N1 viruses isolated in Egypt and posted in GenBank (data not shown). Analysis of the 492 H5 HA sequences also identified amino acid mutations that were conserved in each of the sublineages compared with ancestral Egyptian viruses; these conserved mutations are listed in Fig. 1. Most HPAI H5N1 viruses isolated in Egypt at the time of

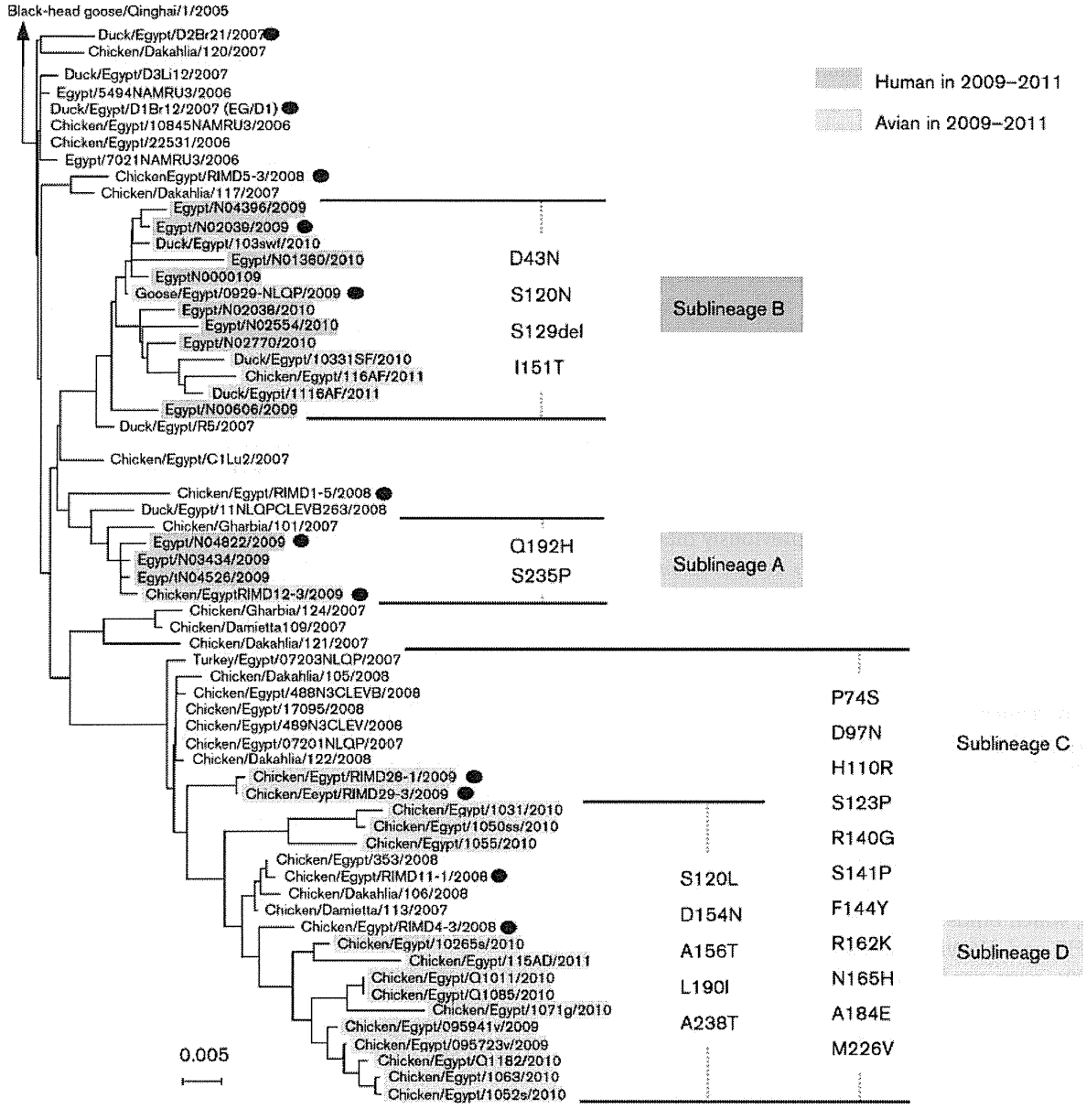


**Fig. 1.** Phylogenetic tree of HA genes of H5N1 viruses isolated in Egypt. This tree includes HA sequences of 51 H5N1 influenza A viruses isolated in Egypt, available in GenBank, and 11 HA sequences determined in our study. Strains whose HA sequences were also analysed for antigenicity are marked with ●. Amino acid mutations conserved in each of the sublineages are shown using H5 numbering. Colours are used to highlight human and avian virus strains isolated in 2009–2011. Bar, 0.005 nucleotide substitutions per site.

this study were in sublineages B and D, which were recently classified as group 2.2.1/C and subclade 2.2.1.1, respectively, in the WHO classification. In addition, viruses closely related to ancestral viruses were isolated at low frequency. Sublineages A and B were probably formed in early 2008, and contained 96 of the 97 isolates from human infection cases in Egypt since 2008. We previously found that HA mutations Q192H and S129del/I151T (H5 numbering), which were conserved in sublineages A and B, respectively, increased HA binding affinity for α2,6-linked sialylglycan, possibly accounting for the increase in human H5N1 infections in Egypt (Watanabe *et al.*, 2011b). However, sublineages C and D were probably formed in 2007, mainly from isolates from vaccinated birds in commercial farms (Balish *et al.*, 2010). These two sublineages shared several amino acid mutations in the HA globular head and retained the classical binding preference for α2,3-linked sialylglycan (Watanabe *et al.*, 2011b). Previous studies suggested a vaccine-driven emergence of sublineage C viruses (Abdel-Moneim *et al.*, 2011; Cattoli *et al.*, 2011b). Sublineage D diverged from sublineage C in 2007, with additional mutations compared with the ancestral viruses. Viruses in the most recent phylogenetic branches were in sublineage D. Therefore, no single H5N1 sublineage has become dominant, and phylogenetically distinct viruses have persisted in Egypt.

## Antigenic analysis using HA recombinant viruses

A panel of five mAbs against the HA of A/crow/Kyoto/53/2004 (H5N1), which had the antigenicity of other contemporary Asian strains, was used for antigenic analysis of H5N1 viruses circulating in Egypt. We have previously mapped the epitopes recognized by these mAbs to conserved regions in the globular head of the HAs of East Asian H5 viruses and shown that they have broad cross-neutralizing activity against Asian H5 lineage strains (Du *et al.*, 2009). The broad cross-reactivity of the mAbs with H5N1, H5N2 and H5N3 HAs was confirmed in this study (Fig. S1, available in JGV Online). The pattern of mAb reactivity with HA of A/duck/Egypt/D2Br21/2007, one of the ancestral H5 viruses in Egypt, was comparable to that of the Asian H5 lineage, indicating an antigenic similarity between the Asian H5 lineage and ancestral Egyptian H5 viruses. In these studies, mAbs C43, which binds influenza nucleoprotein (Okuno *et al.*, 1993), and C179, which binds the HA stalk with cross-reactivity to H1, H2, H5 and H6 viruses (Okuno *et al.*, 1993; Smirnov *et al.*, 1999), were used as controls. The reactivity of these mAbs with 11 HAs that contained sequences representative of sublineage A, B, C and D viruses was analysed by immunofluorescence assays. Both a human- and a bird-derived virus HA were included as representatives of sublineages A and B.

To investigate specific reactivity between the mAbs and HAs, we generated recombinant H5N1 viruses, each containing one of the sublineage HA genes and the other genes from A/duck/D1Br12/2007 (EG/D1), and infected Madin–Darby canine kidney (MDCK) cells with these viruses. Antigenic analysis of the infected cells with the panel of mAbs showed different mAb reactivity patterns against the HAs of recombinant viruses from different sublineages (Fig. 2). All mAbs reacted similarly to cells infected by viruses with sublineage A and B HAs, except for mAb 4G6, which did not react with sublineage B HAs due to the mutation at the epitope (D43N) recognized by mAb 4G6 (Du *et al.*, 2009). However, the mAbs did not react with cells infected with viruses with sublineage C and D HAs. The reactivity of the mAbs with the Egyptian HAs correlated with their neutralizing activity: parental, sublineage A and sublineage B viruses were neutralized by the mAbs with different efficacies, while sublineage C and D viruses were not neutralized by the mAbs (data not shown). These results indicated that ancestral, sublineage A and sublineage B viruses isolated in Egypt shared epitopes in the HA globular head with antigenicity similar to the Asian H5 lineages, but sublineage C and D HAs did not have this antigenicity.

## Prevalence of mutations characteristic of sublineages C and D

To investigate the effect of vaccination on antigenic drift of sublineage C and D viruses, the prevalence of conserved amino acid mutations in these sublineages was compared between HAs from H5N1 viruses isolated from 28 vaccinated and 10 non-vaccinated geographically distant poultry flocks in northern Egypt during 2007–2009, which was the putative time when these sublineages arose (Arafa *et al.*, 2010; Balish *et al.*, 2010). In H5N1 viruses isolated from vaccinated flocks, mutations in HA characteristic of sublineage C were identified in 30–80 % of viruses isolated in 2007, 54–77 % of viruses isolated in 2008, and 80–100 % of viruses isolated in 2009, although the number of virus strains from vaccinated flocks was small (Table 1). In H5N1 viruses isolated from vaccinated flocks, mutations in HA characteristic of sublineage D were identified in 10–30 % of viruses isolated in 2007, 8–23 % of viruses isolated in 2008, and 0–80 % of viruses isolated in 2009 (Table 1). In contrast, in H5N1 viruses isolated from non-vaccinated flocks, the prevalence of HA mutations characteristic of sublineage C was essentially zero in 2007 and 2008 and 100 % in 2009, and was essentially zero for HA mutations characteristic of sublineage D in 2007, 2008 and 2009, although the number of virus strains from non-vaccinated flocks was small. For H5N1 viruses isolated in Asia, the prevalence of HA mutations characteristic of sublineages C and D was low in 2007, 2008 and 2009, with a few exceptions (at HA residues 120, 141, 154, 156 and 162). These results indicated that sublineage C and D viruses spread preferentially among vaccinated flocks in northern Egypt, implying vaccine-driven evolution of these viruses.

## Antigenic analysis of recombinant viruses with specific mutations

To compare antigenic variation among sublineage HAs, the effect of strain-specific amino acids had to be excluded. Therefore, mutations characteristic of each sublineage were introduced in the HA gene of EG/D1, and recombinant

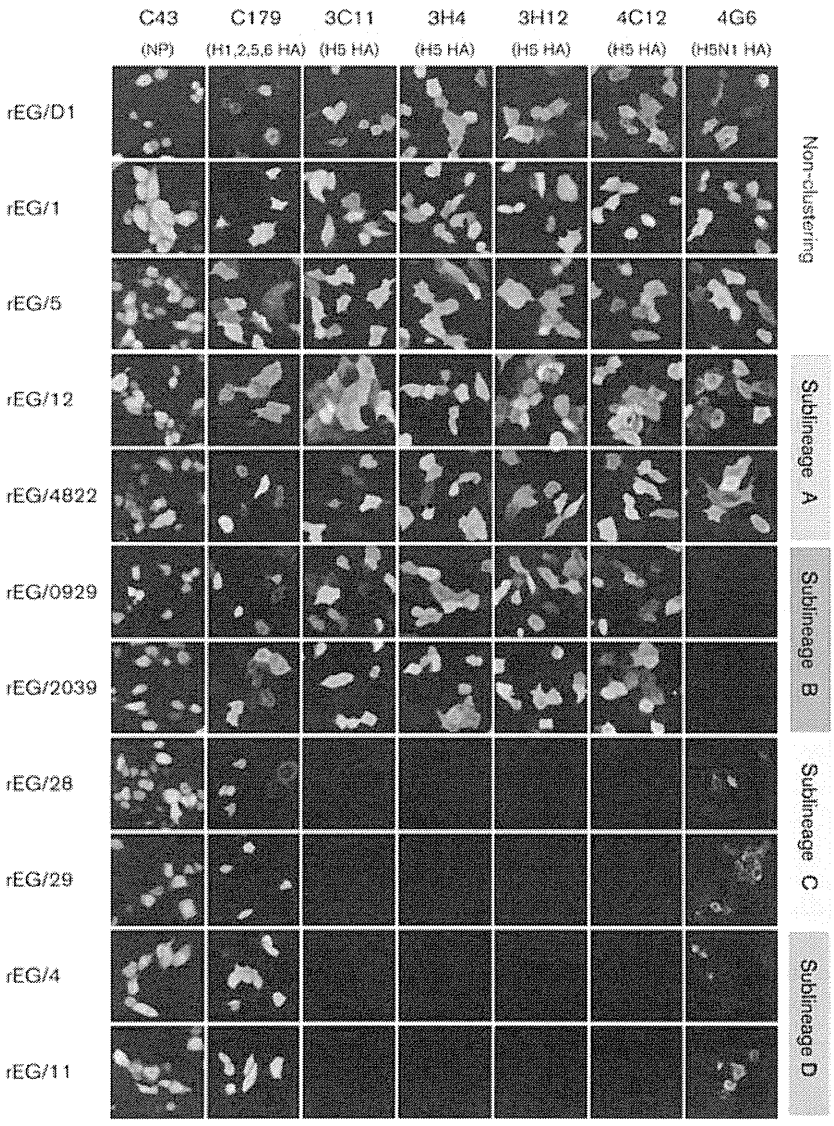


**Fig. 2.** Antigenic variation in H5N1 viruses isolated in Egypt. MDCK cells were infected with recombinant viruses with different HAs at an m.o.i. of 0.5 and the reactivity of the HAs against a panel of mAbs against influenza A viruses was determined by immunofluorescence assays. mAbs 3C11, 3H4, 3H12, 4C12 and 4G6 were cross-reactive to HA1 of the Asian H5 lineage, and mAbs C43 and C179 were controls. The recombinant virus designations are on the left and the phylogenetic sublineages are on the right. The mAb designations are at the top, with their antigens in parentheses.

viruses with the mutated HA gene and the unmodified other seven protein genes were generated. For sublineage D, the 11 mutations found in both sublineages C and D were also introduced. The antigenicity of these viruses was investigated by using them to infect MDCK cells and analysing the reactivity patterns of the infected cells with the panel of mAbs described above. With one exception, HA mutations characteristic of sublineages A and B had little effect on the reactivity patterns of the mAbs (Fig. 3). In contrast, no reactivity was seen in cells infected by viruses with HA mutations characteristic of sublineages C and D. These results confirmed that sublineage C and D HAs generally had different antigenicity from ancestral Asian and Egyptian H5 viruses.

## Glycosylation of recombinant HAs with specific mutations

The HA1 proteins of Egyptian H5 lineage viruses contain different combinations of three *N*-linked glycosylation sites (NGS) at residues 72, 154 and 165. To determine the attachment of *N*-glycans to the globular head of sublineage HAs, recombinant HAs with specific mutations were prepared as described in Methods. Each protein carried the mutations conserved in one of the sublineages. In addition, variant forms of sublineage D HA were prepared in which amino acid residues were introduced in the NGS of sublineage D HA. Electrophoretic analysis of the proteins showed different patterns of *N*-glycosylation among the sublineage HAs (Fig. 4). The mobility shift for sublineage D HA1 with mutated NGS showed that the slower mobility of HA1 with mutations characteristic of sublineage D was due to glycosylation of residue 154N as a result of amino acid changes D154N and A156T in sublineage D HA1. Amino acid changes P74S and N165H, which were conserved in both sublineage C and D HAs, resulted in the generation and loss of glycosylation sites, respectively, which explained the lack of a mobility shift for sublineage C HA1. The *N*-glycosylation patterns identified in sublineage HAs are summarized in Table 2. The effect of *N*-glycosylation changes in sublineage D HA on antigenicity was analysed by immunofluorescence assays. The results suggested that

**Table 1.** Prevalence of HA mutations characteristic of H5 sublineages C and D in viruses isolated in Egypt and Asia

| Sublineage/mutation in HA* | Strains (%) with mutations isolated in:† | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Egypt ($n$=38) | | | | | | Asia ($n$=363) | | |
| | From vaccinated flocks ($n$=28) | | | From non-vaccinated flocks ($n$=10) | | | | | |
| | 2007 ($n$=10) | 2008 ($n$=13) | 2009 ($n$=5) | 2007 ($n$=4) | 2008 ($n$=3) | 2009 ($n$=3) | 2007 ($n$=198) | 2008 ($n$=112) | 2009 ($n$=53) |
| C | | | | | | | | | |
| P74S | 50.0 | 61.5 | 100.0 | 0.0 | 0.0 | 100.0 | 0.5 | 18.8 | 1.9 |
| D97N | 50.0 | 61.5 | 100.0 | 0.0 | 0.0 | 100.0 | 1.5 | 0.9 | 1.9 |
| H110R | 40.0 | 53.8 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.9 | 0.0 |
| S123P | 40.0 | 61.5 | 100.0 | 0.0 | 0.0 | 100.0 | 15.2 | 5.4 | 15.1 |
| R140G | 30.0 | 61.5 | 80.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.9 | 0.0 |
| S141P | 80.0 | 61.5 | 100.0 | 0.0 | 0.0 | 100.0 | 40.4 | 32.1 | 20.8 |
| F144Y | 70.0 | 61.5 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.9 | 0.0 |
| R162K | 60.0 | 76.9 | 100.0 | 0.0 | 33.3 | 100.0 | 32.3 | 20.5 | 62.3 |
| N165H | 40.0 | 61.5 | 80.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.9 | 0.0 |
| A184E | 60.0 | 61.5 | 100.0 | 0.0 | 0.0 | 100.0 | 5.1 | 38.4 | 64.2 |
| M226V | 40.0 | 61.5 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.9 | 0.0 |
| D | | | | | | | | | |
| S120L | 20.0 | 7.6 | 0.0 | 0.0 | 0.0 | 100.0 | 31.7 | 39.2 | 75.4 |
| D154N | 30.0 | 23.0 | 20.0 | 25.0 | 0.0 | 0.0 | 93.0 | 79.4 | 41.5 |
| A156T | 10.0 | 23.0 | 80.0 | 0.0 | 0.0 | 0.0 | 76.4 | 47.3 | 24.5 |
| L190I | 10.0 | 23.0 | 20.0 | 0.0 | 0.0 | 0.0 | 0.5 | 17.8 | 1.8 |
| A238T | 10.0 | 7.6 | 40.0 | 0.0 | 0.0 | 0.0 | 1.0 | 8.0 | 3.7 |

*Mutations are shown according to H5 numbering.

†Percentage of H5N1 viruses that have mutation(s) characteristic of sublineages C and D for each geographical region, type of flock and year of virus isolation. Sequence information is from GenBank and from sequences analysed in this study.

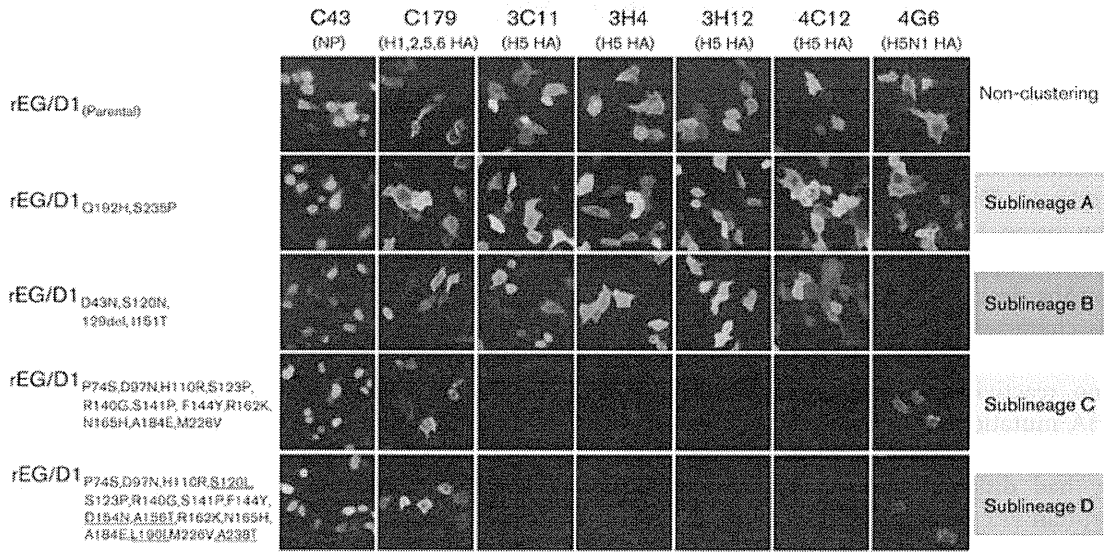


**Fig. 3.** Effect of HA conserved mutations in different sublineages on antigenicity. MDCK cells were infected with recombinant viruses with HAs containing the mutations conserved in one of the sublineages and the other genes from virus EG/D1, and the reactivity of the HAs against a panel of mAbs was determined by immunofluorescence assays as described in the legend to Fig. 2. The mutations introduced into each EG/D1 HA are on the left and the HA sublineages are on the right. Mutations specific to sublineage D are underlined. The mAb designations are at the top, with their antigens in parentheses.

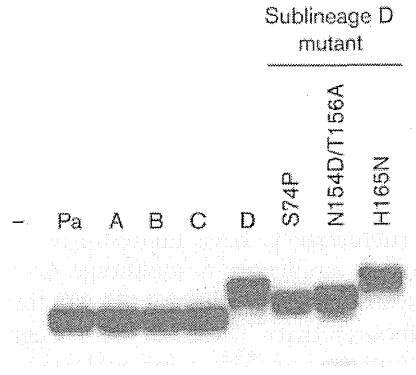


**Fig. 4.** Glycosylation patterns of HA1 proteins. Flag-tagged HA1 proteins with mutations characteristic one of the sublineages were produced in 293T cells as described in Methods. Variant forms of sublineage D HA1 with mutated NGS were also produced. Electrophoretic patterns of recombinant HA1 proteins were visualized by Western blotting with an anti-Flag antibody. Sublineage designations are at the top: Pa, parental HA1; A, sublineage A HA1; B, sublineage B HA1; C, sublineage C HA1; D, sublineage D HA1. –, Mock sample from empty-plasmid-transfected cells.

the mutations at NGS and the other sites (non-NGS) in sublineage D HA impacted antigenic variation synergistically (Fig. S2).

### Variations in immunogenicity of recombinant viruses with specific mutations

To evaluate the immunogenicity of sublineage HAs, recombinant HA proteins with specific mutations were prepared and purified as described in Methods. Electrophoretic analysis of the purified proteins showed the specificity and purity of the HA1 preparations (Fig. S3). We examined whether the amino acid changes in each sublineage affected induction of serum antibody responses to HA1. Groups of mice were vaccinated intra-peritoneally with purified HA1s, and serum HAI and neutralizing antibody titres against viruses with homologous and heterologous HAs were determined post-immunization. Sera from mice immunized with sublineage A and B HA1s had similar haemagglutination inhibition (HAI) titres against homologous virus (453 and 570) and against each other (403 and 570), and much lower HAI titres against viruses with sublineage C and D HA1s (7–36) (Table 3). Sera from mice immunized with sublineage C and D HA1s had significantly different HAI titres against homologous virus (508 and 127), lower titres against each other (18 and 50), and much lower titres against viruses with sublineage A and B HA1s (18–57) (Table 3). There was good correlation between the patterns of HAI and neutralizing antibody titres for sublineages A–D (Table 3). These results indicated that there was significant antigenic drift in sublineage C and D HAs after divergence of these phylogenetic branches from sublineage A and B HAs, and that mouse polyclonal antibodies induced by sublineage C or D HAs are not cross-reactive with sublineage A and B HAs.

**Table 2.** Glycosylation patterns on HAs investigated in this study

Glycosylation at the indicated residue in the globular head of HAs was determined by the mobility shift of HAs with mutated NGS as shown in Fig. 4. Residues are shown according to H5 numbering. +, Glycosylated; –, non-glycosylated.

| Sublineage | *N*-Glycosylation at residue: | | |
|---|---|---|---|
| | 72 | 154 | 165 |
| Parental | – | – | + |
| A | – | – | + |
| B | – | – | + |
| C | + | – | – |
| D | + | + | – |
| $D_{S74P}$ | – | + | – |
| $D_{N154D,T156A}$ | + | – | – |
| $D_{H165N}$ | + | + | + |
| $D_{S74P,N154D,T156A}$ | – | – | – |

## DISCUSSION

In this study, we elucidated the antigenic drift among the H5N1 viruses currently co-circulating in Egypt. Our analyses indicated that the HAs in sublineage C and D viruses were antigenically different from those in ancestral Asian and Egyptian H5 viruses and had undergone significant antigenic drift since their divergence from the sublineage A and B phylogenetic branches. To our knowledge, this is the first report that sublineage D HAs were generally not cross-immunogenic with other sublineage HAs, including even sublineage C HAs, although sublineages C and D form a single phylogenetic branch and have a number of amino acid mutations in common.

Viruses in the sublineage C phylogenetic branch were first isolated in 2007. Our survey of vaccination records found that sublineage C viruses preferentially circulated in vaccinated poultry flocks during this time. These data supported previous suggestions that emergence of sublineage C may have resulted from suboptimal vaccinations in Egypt (Abdel-Moneim *et al.*, 2011; Cattoli *et al.*, 2011b), although there is no direct evidence of this. Indeed, seven of the 11 mutations conserved in sublineage C HAs were in residues corresponding to the A and B antigenic sites in H3 HAs, which contain epitopes with high neutralizing efficiency (Kaverin *et al.*, 2007; Wiley *et al.*, 1981) (Fig. 5). H5N1 variants with some of the mutations characteristic of sublineage C HAs have been isolated in Egypt since April 2007, and viruses with all of the mutations characteristic of sublineage C HAs have been isolated since December 2007. The first date, April 2007, was about 13 months after the launch of mass vaccination in industrial poultry sectors in Egypt, and about 5 months before the reported emergence of sublineage C (Arafa *et al.*,

**Table 3.** Serum antibody response in mice immunized with H5N1 viruses isolated in Egypt with HA mutations characteristic of each sublineage

Values are geometric mean antibody titres (GMT) in serum from six mice, obtained 2 weeks after the second administration of virus with HA mutations characteristic of the indicated viral sublineage. Homologous titres are shown in bold.

| Sublineage | HAI GMT* (95 % confidence interval) | | | | | NT GMT† (95 % confidence interval) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Parental | A | B | C | D | Parental | A | B | C | D |
| Parental | **359** (208–621) | 254 (140–460) | 254 (174–370) | 28 (13–61) | 11 (6–19) | **7241** (876–59 843) | 7 241 (876–59 843) | 10 240 (1691–62 017) | 113 (38–341) | 113 (38–341) |
| A | 403 (277–587) | **453** (304–674) | 570 (244–1335) | 50 (19–136) | 18 (13–24) | 5120 (564–46 482) | **10 240** (452–231 811) | 20 480 (5731–73 188) | 320 (90–1144) | 226 (75–682) |
| B | 403 (277–587) | 403 (223–730) | **570** (424–767) | 57 (26–121) | 32 (15–67) | 5120 (1433–18 297) | 10 240 (801–130 773) | **20 480** (5731–73 188) | 453 (150–1364) | 320 (90–1144) |
| C | 22 (10–53) | 18 (9–36) | 36 (21–62) | **508** (240–1077) | 18 (10–31) | 226 (27–1870) | 453 (150–1364) | 640 (106–3876) | **14 482** (4806–43 634) | 453 (150–1364) |
| D | 7 (5–11) | 7 (4–13) | 11 (5–23) | 50 (13–195) | **127** (70–230) | 80 (22–286) | 113 (38–341) | 113 (38–341) | 320 (90–1144) | **2560** (423–15 504) |

*Each HAI titre against the challenge virus is the reciprocal of the highest serum dilution that inhibited haemagglutination by 8 virus haemagglutination units.
†Each neutralizing titre (NT) against the challenge virus is the reciprocal of the highest serum dilution that reduced infection of 100 virus f.f.u. by >50 %.

2010; Balish *et al.*, 2010). Similar findings were reported in China, where possible vaccine-escape variants emerged 1 year after implementation of vaccination in poultry (Smith *et al.*, 2006).

Sublineage D HAs were more phylogenetically and antigenically distant from ancestral, sublineage A and B HAs than were sublineage C HAs. Interestingly, sublineage D, which diverged rapidly from sublineage C in 2007, was antigenically distinct from sublineage C with little polyclonal antibody cross-reactivity. These data were in agreement with a previous antigenic analysis using sublineage D strain A/Egypt/3300-NAMRU3/2008 (Balish *et al.*, 2010). Sublineage D viruses also circulated preferentially in vaccinated poultry flocks, implying vaccination-driven phylogenetic emergence. The sublineage D viruses had five conserved amino acid changes, which were in residues corresponding to H3 antigenic sites A and B, in addition to 11 mutations also found in sublineage C viruses (Fig. 5). These amino acid changes included D154N and A156T mutations, which have resulted in 154N glycosylation. Post-translational *N*-glycosylation of the HA protein can influence its antigenicity and receptor-binding affinity (Skehel *et al.*, 1984; Wang *et al.*, 2010). As expected, HA1 with sublineage D characteristic mutations, including 154N and 156T, migrated more slowly electrophoretically than other sublineage HA1s with residues 154D and 156A, due to glycosylation of residue 154N in sublineage D HA1s. No other putative glycosylation sites were generated by the sublineage D conserved mutations. These data confirmed that the D154N and A156T amino acid changes resulted in acquisition of a glycosylation site and that the 154N residue in sublineage D HAs was indeed glycosylated. These amino acid changes may produce substantial differences in immunogenicity between sublineage C and D HAs, especially in HAI antibody levels in the sera of immunized mice.

Influenza virus receptor affinity can be altered during virus evolution by selection of immune-escape variants (Hensley *et al.*, 2009). Unexpectedly, sublineage A and B viruses, which acquired increased affinity for α2,6-sialylglycan in combination with residual affinity for α2,3-sialylglycan, retained the antigenicity of ancestral Asian and Egyptian viruses. However, mAb 4G6 did not react with sublineage B HAs, indicating a minor antigenic drift in these HAs. Viruses in sublineages A and B were first isolated in early 2008, several months after viruses in sublineages C and D were first isolated. The factors involved in the emergence of sublineages A and B remain uncertain. Previous studies reported that sublineage B virus hosts were restricted to aquatic poultry, mainly ducks (Arafa *et al.*, 2010; Cattoli *et al.*, 2011b), implying that specific mutations were selected and conserved among these avian virus strains. We also reported genome diversification in H5N1 viruses that infect wild ducks (Watanabe *et al.*, 2011a) and host-specific genetic evolution in these viruses in Egypt (Ibrahim *et al.*, 2011). It is possible that antigenic changes may gradually accumulate during influenza virus circulation in waterfowl, unrelated to whether there is vaccination

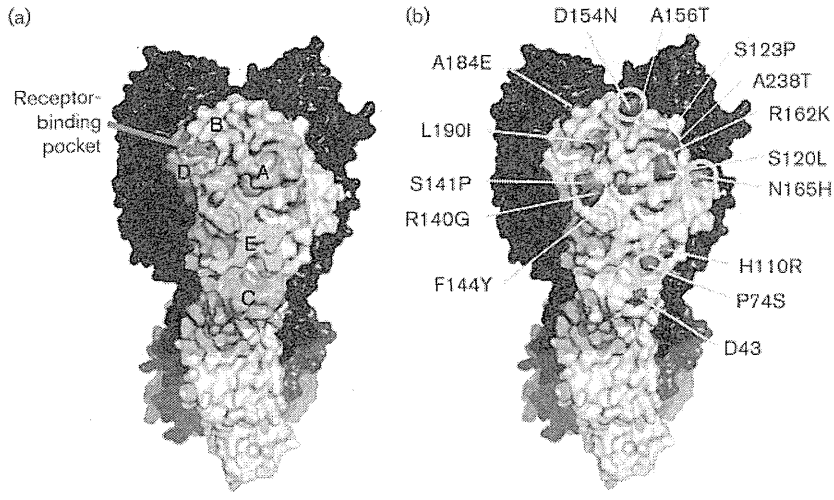


**Fig. 5.** Locations of amino acid mutations conserved in sublineage C and D HAs. (a) Three-dimensional structure of the H5 HA trimer, showing the locations of H3 HA antigenic sites A–E. (b) Locations of amino acid mutations conserved in sublineage C and D HAs. The mutated residues, which were conserved in sublineage C and D HAs, are shown in red. Orange circles indicate *N*-glycosylation sites. Residue D43, which is recognized by mAb 4G6, is shown in blue. The crystal structure of the HA of A/Vietnam/1194/2004 (H5N1) (PDB ID 2IBX) was used as a template.

in the field. Aquatic poultry probably plays a substantial role in H5N1 evolution in Egypt, along with terrestrial birds. Therefore, the ecology of sublineage A and B viruses in aquatic birds needs to be studied further.

The standard method for control of an HPAI outbreak is testing and culling of all poultry in a farm (Suarez, 2010). However, it has been suggested by the OIE that, when outbreaks spread to a broad area and become uncontrollable, ring vaccination would be an additional method to reduce nascent virus production and, thereby, suppress further virus infection (OIE, 2003). Vaccination of poultry is now considered a preventive or auxiliary control approach in several H5N1-endemic countries, including Egypt (Peiris *et al.*, 2007; Swayne & Kapczynski, 2008). The problem is that antigenically divergent groups of viruses, which are not cross-reactive, are co-circulating in Egypt. A similar situation was also found in Indonesia even though major antigenic variation had not been detected there as of 2008 (Wibawa *et al.*, 2011). Only several of the recently isolated H5N1 strains circulating in Indonesia had lost reactivity to mAb 4G6 (Mieko Kosaka, personal communication). This makes selection of one influenza virus strain as the vaccine seed virus especially problematic. Furthermore, random rearing of many bird species and their hybrid breeds with uncontrolled confinement is common in rural areas (Suarez, 2010), leaving ducks and geese free to fly away. Therefore, circulation of viruses in each sublineage in Egypt was not restricted in terms of geography or host species, complicating efforts to use a vaccine produced against antigens from a single virus strain. In the future, when vaccination is implemented as part of a comprehensive control strategy for endemic HPAI in Egypt, the most promising method may be development and use of multivalent or universal vaccines. Broad-spectrum efficacy would need to be revised periodically (Swayne, 2009), although vaccination in backyard settings was suspended provisionally in July 2009 in Egypt until a new vaccine strategy could be adopted (FAO, 2011).

It is not known whether sublineage B and D viruses, which are now the dominant strains co-circulating in Egypt, continue to evolve without the selective pressure due to vaccination. However, since the ecology of influenza viruses and that of many avian species in Egypt are closely related, evolution will continue to produce genetically divergent viruses in Egypt and, perhaps, other countries where H5N1 infection is endemic (Watanabe *et al.*, 2012). Continuous circulation among hosts may allow H5N1 virus to acquire amino acid changes enabling more bird–human transmission and eventually human–human transmission. Large-scale surveillance of avian influenza viruses in endemic areas should be expanded to better understand evolution of the virus and enable more efficacious control strategies in these regions.

## METHODS

**Generation of recombinant viruses.** Recombinant H5N1 viruses were generated using a plasmid-based reverse-genetics system as described previously (Fodor *et al.*, 1999; Watanabe *et al.*, 2011b). Each virus generated by reverse genetics (denoted here as rEG/X) carried the HA gene of the virus being studied, with the other genes coming from A/duck/D1Br12/2007 (EG/D1), one of the ancestral influenza virus strains isolated in Egypt. The 11 recombinant viruses, each containing an HA from an Egyptian virus, were EG/D1, A/chicken/Egypt/RIMD/1-5/2008 (EG/1), A/chicken/Egypt/RIMD4-3/2008 (EG/4), A/chicken/Egypt/RIMD5-3/2008 (EG/5), A/chicken/Egypt/RIMD11-1/2008 (EG/11), A/chicken/Egypt/RIMD12-3/2008 (EG/12), A/chicken/Egypt/RIMD28-1/2009 (EG/28), A/chicken/Egypt/RIMD29-3/2008 (EG/29), A/goose/Egypt/0929-NLQP/2009 (EG/0929), A/Egypt/N04822/2009 (EG/4822) and A/Egypt/N02039/2009 (EG/2039). The HA genes of EG/0929, EG/4822 and EG/2039 were synthesized using the sequences registered in GenBank (http://www.ncbi.nlm.nih.gov/nucleotide) and site-directed mutagenesis PCR. Mutant HA genes were generated by PCR-based site-directed mutagenesis of the HA genes of EG/D1, EG/11, EG/12 and EG/29. The HA genes of the virus stocks were sequenced to detect the possible emergence of revertants or unwanted mutations during amplification as described previously (Watanabe *et al.*, 2007).

**Cells.** MDCK and 293T cells were purchased from the RIKEN BioResource Center Cell Bank (http://www.brc.riken.jp/lab/cell/english/). Chicken embryo fibroblast (CEF) cells were prepared from 11-day-old embryonated eggs. These cell lines were maintained as described previously (Watanabe *et al.*, 2009).

**Virus propagation.** The recombinant viruses generated in this study were propagated by single passage in the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluids were harvested 3 days post-infection and stored at −80 °C. Virus titres were assayed as f.f.u. by focus-forming assays (Di Lonardo *et al.*, 2002) on MDCK cells. All experiments with live H5N1 viruses were performed in Biosafety Level 3+ (BSL 3+) conditions at Osaka University, as approved for work with these viruses by the Ministry of Agriculture, Forestry and Fisheries, Japan.

**Genetic analysis.** For phylogenetic analysis of HA genes, we used HA sequences of 51 representative H5N1 influenza A viruses isolated in Egypt from 2006 to 2011 and obtained from GenBank, and 11 HAs representative of the 21 HAs sequenced in our studies. Phylogenetic analysis of the 62 HA nucleotide sequences was done by the neighbour-joining method using MEGA4 software (Tamura *et al.*, 2007), with the nucleotide sequences covering most of the HA genes. Estimates of the variability of the reconstructed phylogenetic trees were calculated for 1000 bootstrap replicates. For reconstruction of a detailed phylogenetic tree, 398 HA sequences from avian viruses and 98 HA sequences from human viruses isolated in Egypt from 2006 to 2011 were obtained from a GenBank search and analysed. For comparison, 366 published HA sequences of H5N1 influenza A viruses recently isolated in Asia were obtained from GenBank. The prevalence of mutations characteristic of H5N1 sublineages C and D was calculated for 17 HA sequences in previous studies and 21 HA sequences in our studies, all isolated from different poultry sectors in northern Egypt (detailed data are available on request). These sequences were aligned by the MAFFT program (Katoh *et al.*, 2002) and the HA1 regions were compared with each other.

**Antigenic analysis.** The antigenic specificity of HAs in Egypt was assessed by immunofluorescence assays with a panel of five mouse mAbs against A/crow/Kyoto/53/2004 (H5N1), which we produced and characterized previously (Du *et al.*, 2009). MDCK cells were infected with recombinant viruses at an m.o.i. of 0.5 and analysed for reactivity with each of the mAbs by immunofluorescence using an FITC-conjugated secondary antibody.

**Purification of recombinant HA1s.** Each HA1 expression plasmid was produced by fusing an HA1 gene with the C terminus of a Flag tag and inserting it into a pcXN2 vector (Watanabe *et al.*, 2009). Insertion of the correct sequences for wild-type and mutant HA1s was confirmed by DNA sequencing and Western blot analysis of the expressed proteins. Recombinant HA1s were produced by transfecting 293T cells with plasmid DNAs using TransIT-LT1 (Mirus Bio), according to the manufacturer's instructions. At 5 days post-transfection, culture supernatants containing released HA1s were collected and centrifuged twice at 8400 *g* for 20 min. The supernatants were incubated with pre-equilibrated anti-Flag M2 agarose (Sigma-Aldrich) for 5 h at 4 °C with gentle rotation. The beads were then collected by centrifugation at 4700 *g* for 40 s and washed four times with PBS containing 0.1 % Tween 20 (PBST). The proteins immunoprecipitated by anti-Flag agarose were eluted with 3× Flag peptide (Sigma-Aldrich) in PBST and dialysed against PBS for 40 h at 4 °C. Western blotting was performed as described previously (Watanabe *et al.*, 2009).

**Immunization of mice.** Groups of six mice (Japan SLC Inc.) were immunized with two intraperitoneal injections of 100 μg purified HA1 protein with Freund's complete adjuvant, with 2 weeks between injections. Two weeks after the second administration, sera were collected and specific antibody responses were examined by HAI and microneutralization assays as described below. Control mice were immunized with PBS with Freund's adjuvant. All animal studies were conducted under the applicable laws and guidelines for the care and use of laboratory animals in the Research Institute for Microbial Diseases, Osaka University.

**Serological analysis.** Antibody levels against homologous and heterologous viruses in post-immunization mouse sera were determined by HAI and microneutralization assays as follows. Sera were treated overnight with receptor-destroying enzyme (Denka-Seiken) at 37 °C for 18 h and heat-inactivated at 56 °C for 45 min. HAI assays were performed as described previously (Okuno *et al.*, 1993), with serial twofold dilutions of mouse serum starting from a 1 : 10 dilution. The HAI antibody titre was defined as the reciprocal of the highest serum dilution that inhibited haemagglutination. For HAI calculations, it was useful to assign HAI titres <10 HAI a value of 5 HAI. For neutralization assays, serial twofold dilutions of serum starting from a 1 : 10 dilution were incubated with an equal volume of virus containing 100 f.f.u. in a 96-well U-bottom plate for 60 min at 37 °C. The virus–serum mixture was transferred to an MDCK cell monolayer and incubated at 37 °C for 8 h. The neutralization antibody titre was defined as the reciprocal of the highest serum dilution that neutralized >50 % of the viruses in a focus-forming assay.

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