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Different counteracting host immune responses to clade 2.2.1.1 and 2.2.1.2 Egyptian H5N1 highly pathogenic avian influenza viruses in naïve and vaccinated chickens

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ABSTRACT

In Egypt, two distinct lineages of H5N1 highly pathogenic avian influenza (HPAI) viruses, “classic 2.2.1.2” and “variant 2.2.1.1” strains, have evolved. The underlying host immune responses counteracting these viruses in chickens remain not well understood. In the present study, the cytokine responses to a classic strain (C121) and those to a variant strain (V1063) were compared in naïve and vaccinated chickens. In naïve chickens, the C121 replicated more efficiently than the V1063. Both the C121 and the V1063 increased interferon (IFN)-γ and interleukin (IL)-10 gene expression at 48 h post inoculation (hpi) in the lung and spleen but the levels of these cytokines were lower in chickens infected with the C121 than those infected with the V1063. In contrast, in chickens vaccinated with inactivated C121-based vaccine, the C121 replicated less than the V1063. Both challenge with the C121 and that with the V1063 did not increase IFN-γ gene expression at 48 hpi; rather, the C121 increased IL-4 gene expression in the lung accompanied with lower viral titer and higher HI titers. These results suggested that the pathogenicity of HPAI viruses correlated with IFN-γ-producing helper and/or cytotoxic T cell responses in naïve chickens, whereas vaccine efficacy to HPAI viruses correlated with IL-4 producing helper T cell responses in the lung in vaccinated chickens. It implies that IL-4 in the lung, in addition to the traditional serum HI titers, could be used to screen novel vaccine strategies, such as strains, adjuvant, prime/boost protocols, against HPAI in chickens.

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1. Introduction

Highly pathogenic avian influenza (HPAI) is a devastating disease of poultry and poses a potential pandemic threat in humans (Cattoli et al., 2011). The disease has become endemic in poultry in China, Viet Nam, Indonesia, India, and Egypt, causing a destructive effect on poultry production and posing serious threats to the economy in those countries (Swayne et al., 2011). Since the introduction of the H5N1HPAI 2.2.1 virus into poultry in Egypt in 2006, over 30 million birds have been killed by the virus or culled to control its spread (Abdelwhab and Hafez, 2011). After the last winter (2014–2015), Egypt now considered the country with the highest human cases worldwide with 114 deaths out of 342 infected human cases reported as of 30 April 2015, and most of the cases had a history of poultry exposure (WHO, 2015).

Since the emergence of the epizootic, Egypt has used mass vaccination of poultry as part of a national strategy to control HPAI. Late 2007, there are evidences that the use of unsuitable H5 vaccines as part of this strategy in commercial poultry resulted in the emergence of a genetically and antigenically distinctive “variant” strains that clustered in clade 2.2.1.1 (Grund et al., 2011). These variant strains harbor major changes in immunogenic epitopes of the hemagglutinin (HA) protein that enabled the virus to evade the humoral immune responses evoked by H5N2-based

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vaccine commonly used in Egypt (Hassan et al., 2012). In addition, viruses isolated from non-vaccinated backyard birds and small-scale commercial poultry and human clustered in a distinguishable clade designated as 2.2.1.2 (Arafa et al., 2015). The differences in pathogenesis between the co-evolving classic and variant strains in chickens are not well understood.

Cytokines have positive and/or negative effects on the pathogenesis of HPAI viruses, but these effects vary among strains, hosts (Kuchipudi et al., 2014) and depend on the stage of the infection (Nagai et al., 2003). Previous studies using mouse models suggested that overactive inflammatory response with cytokine dysregulation is likely to be the cause of the high fatality rate of influenza infection (Tisoncik et al., 2012). In particular, macrophage infection caused high activation and rapid proliferation of T-cell and natural killer cells, that in turn caused a cytokine storm triggering excessive induction of apoptosis. In contrast, another study reported that H5HPAI virus infection was lethal to mice lacking tumor necrosis factor (TNF) and interleukin (IL)-6 (Salomon et al., 2007).

HPAI virus infection in chickens is characterized by high mortality up to 100% within 24–48 h post infection (hpi) with no-overt clinical signs of infection until just 2 h before death (Suzuki et al., 2009). The rapid onset of the disease implies that virus–host interactions, such as immune responses, may contribute to the pathogenesis of HPAI viruses in chickens. That was probed by (Karpala et al., 2011; Kuribayashi et al., 2013) who described strong inflammatory and T-helper 1 (Th1) cytokine responses following H5N1HPAI virus infection. Also, the rapid and extensive proliferation of H5N1HPAI viruses was well correlated with excessive cytokine responses that cause fatal multiple organ failure in chickens (Kuribayashi et al., 2013). However, in peracute cases of H5N1HPAI virus infection, cytokine gene expression was not significantly increased in the lung until death (Suzuki et al., 2009).

In the present study, we compared the cytokine responses to an Egyptian H5N1HPAI virus classic strain (C121) with those to a variant strain (V1063) in naïve and vaccinated chickens to investigate how cytokine responses counteract infection of and vaccine efficacy to the two distinct co-evolving genotypes in chickens.

2. Material and methods

2.1. Viruses

A variant virus (A/chicken/Egypt/1063/2010; V1063, GenBank accession number HQ198269) from clade 2.2.1.1 and a classic virus (A/chicken/Egypt/121/2012; C121, GenBank accession number JQ858483) from clade 2.2.1.2 were obtained from the influenza virus repository at the Reference Laboratory for Quality Control on Poultry Production (RLQP), Giza, Egypt. The viruses were propagated in specific pathogen free (SPF) and intravenous pathogenicity index (IVPI) were calculated following OIE established protocols (OIE, 2014). IVPI of C121 and V1063 were 2.8 and 2.4, respectively. All experiments were performed in biosafety level-3 (BSL3) facilities at the National Institute of Animal Health (NIAH), Tsukuba, Japan using approved protocols.

2.2. Sequencing of the HA gene

Viral RNA was extracted from the allantoic fluid using the QIAamp viral RNA mini kit (QIAGEN, Germany). The HA genes of the viruses were amplified using OneStep RT-PCR kit (QIAGEN) with the primers published by Hoffmann et al. (2001). The PCR products were subjected to electrophoresis in 1.5% agarose gel, and the specific amplicons of DNA were excised, purified from the gel using a QIAquick Gel Extraction kit (QIAGEN). Sequences were obtained using BigDye Terminator Kit 3.1 cycle sequencing kit (Applied Biosystems, USA) on a 3130 Genetic Analyzer (Applied Biosystems). Nucleotide and amino acid sequences were analyzed using BioEdit software v7.0 (Hall, 1999). A maximum likelihood phylogenetic tree was constructed using IQ-tree software version 1.1.3 (Nguyen et al., 2015). The phylogenetic tree was viewed and edited using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.48. The deduced amino acid sequences of the HA were compared with those of an ancestral H5N1 viruses of clade 2.2 (A/Bar-headed-Goose/Qinghai-65/05; GenBank accession no. DQ095622) and an H5N1 virus first isolated in Egypt (A/chicken/Egypt/2253-1-2006; GenBank accession no. CY020645). Homology models of the HA protein of H5N1 strain C121 and V1063 were created using SWISS-Model server (Biasini et al., 2014) with A/chicken/Egypt/2253-1/2006 (H5N1) as a template. Amino acid substitution mutations were viewed using RasTop version 2.2 (http://www.geneinfinity.org/rastop/).

2.3. Vaccine preparation

Propagated viruses (C121 and V1063) were inactivated by the addition of beta-propiolactone to the harvested allantoic fluid in a final concentration of 1/1000 for 4 h at room temperature and then incubated at 4 °C overnight for hydrolysis of beta-propiolactone. Moreover, complete virus inactivation was confirmed by two passages of the inactivated virus in five SPF eggs. Failure to recover the virus was confirmed by hemagglutination test. The inactivated virus was then purified from the allantoic fluid using standard sucrose density-gradient centrifugation (Kida and Yanagawa, 1979).

2.4. Experimental design

Experimental design is summarized in Table 1. Four-week-old SPF White Leghorn chickens (*Gallus gallus domesticus*) were purchased from Nisseiken Co. (Japan) and housed in BSL3 animal facilities with continuous access to food and water. All chickens were serologically tested prior to vaccination using hemagglutination inhibition (HI) test for antibodies against H5N1 viruses. All experiments were performed in BSL3 facilities

Table 1
Experimental design and sample collection time points.

Group (no. of chickens)	Vaccination	Challenge[a]	Organs and sampling time
1 (10)	Vaccinated	C121[b]	Lung, spleen, tracheal and cloacal swabs and serum at 2,6 and 14 dpc
2 (10)	Vaccinated	V1063[c]	
3 (10)	No	No	
4 (10)	No	C121[b]	Lung, spleen, tracheal and cloacal swabs at 2 dpi
5 (10)	No	V1063[c]	

Dpc = days post-challenge.
[a] Challenge at 30 days post-vaccination using 10^6 EID_{50}/bird.
[b] Bird of the classic strain.
[c] Bird of the variant strain Vaccination was done using inactivated C121 based vaccine.

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