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Abstract of Thesis/Dissertation

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Title: Characterization of monoclonal antibodies against H5N1 subtype highly pathogenic avian influenza virus and their use for analysis of viral antigenicity and diagnosis (H5N1 亜型高病原性鳥インフルエンザウイルスに対する単一抗体の特徴づけとこれらの抗体の抗原分析と診断への利用)

Abstract

Avian influenza virus (AIV) is the member of the Genus *influenza A virus*, belonging to the Family *Orthomyxoviridae*, and antigenically classified into subtypes based on the surface proteins, hemagglutinin (HA) and neuraminidase (NA). To date, sixteen HA (H1–H16), and nine NA (N1–N9) subtypes of the AIV have been identified. AIV can be pathotyped as low pathogenic AIV (LPAIV) and highly pathogenic AIV (HPAIV), based on the pathogenicity in chickens. LPAIVs usually cause mild or asymptomatic infections in chickens, whereas HPAIVs do severe diseases with high morbidity and mortality rates. Wild aquatic birds are natural reservoirs of the LPAIVs. Occasionally, LPAIVs from the reservoirs may spread to poultry. Conversely, HPAIVs have not been maintained in the natural reservoirs, but it is believed that some strains of H5 and H7 subtype LPAIVs that were circulating in poultry populations may mutate into HPAIVs during the repeated infection and transmission among birds, and these mutated viruses may acquire ability to cause severe disease with high mortality rate in poultry.

Since the occurrence of the H5N1 outbreak in Hong Kong in 1997 and the main land of China in 2003, H5N1 HPAIVs quickly spread and have caused deadly outbreaks of poultry in many areas

including Asia, Africa and Europe. The H5N1 viruses have continued evolution during the repeated transmission. Consequently, 10 phylogenetically distinct clades (0–9) based on their H5 HA genes have been found until now. However, clades 3–6 and 8–9 H5N1 HPAIVs have not recently spread. Especially, in contrast, clade 1, 2 and 7 viruses are still continuing evolution, and these clades are now further classified into new second-order (clades 1.1, 2.2 and 7.1), third-order (clades1.1.1 and 2.2.1), fourth-order (clades 2.2.2.1, 2.3.2.1 and 2.3.4.1) and fifth-order clade groups (clades 2.3.2.1a, 2.3.2.1b, 2.3.2.1c). In Japan, there were 6 HPAI outbreaks in birds during 2004 to 2016. The first to fourth outbreaks were caused by the H5N1 strains of clade 2.5, 2.2, 2.3.2.1 or 2.3.2.1c. However, the fifth outbreak observed in 2014 was caused by the H5N8 HPAIV (clade 2.3.4.4). In 2016, the H5N6 HPAIV, which is related to clade 2.3.4.4, caused the outbreaks. Therefore, accurate, specific and rapid diagnosis of H5N1 infection is essential to contain HPAI outbreaks. Although there have been several diagnostic techniques for identifying H5N1 viruses, including viral isolation followed by serological tests to determine HA and NA subtypes or reverse transcription polymerase chain reaction, those techniques have some disadvantages in terms of expensiveness, rapidness, necessary expertise, or the need for a biosafety level-3 laboratory. In contrast, direct antigen detection with monoclonal antibodies (mAbs) from clinical samples is suitable for rapid diagnosis of the H5N1 infection. Rapid diagnostic tests utilizing mAbs against NP of influenza A virus are currently available in the field investigation of H5N1 HPAIVs. However, although these mAbs can recognize influenza A virus, they cannot identify HA or NA subtypes. Therefore, since the mAbs against the HA proteins of H5 subtype AIV (anti-H5 mAbs) can identify subtypes, they may be valuable rather than the anti-NP mAbs for direct, specific and rapid diagnosis of H5N1 infection.

Anti-H5 mAbs against the clade 2.5 H5N1 viruses isolated in Japan were previously reported. However, the mAbs could not recognize all tested strains belonging to clade 2.3.2.1. Moreover, there are no reports as to whether these mAbs could detect currently circulating H5N1 clade viruses. Therefore, it is important to continuously make efforts to search for anti-H5 mAbs with broad cross-reactivity with the epitopes on the HA proteins in the development of diagnostic systems that are highly sensitive and can rapidly detect H5N1 HPAIVs, especially the H5N1 viruses that are currently circulating and the newly emerging H5N1 clades.

The present thesis is composed of three chapters.

In chapter I, production and characterization of anti-H5 mAbs against clade 2.5 and 2.2

H5N1 HPAIVs isolated in Japan were described. Two neutralizing anti-H5 mAb (3B5.1, 3B5.2) against the 2.5 clade virus (Ck/Yamaguchi/7/04) and one mAb (1G5) using the 2.2 clade virus (Ck/Miyazaki/K11/07) were successfully established. All three mAbs were IgG1 subclass and were able to recognize the HA proteins of the H5N1 viruses. 3B5.2 and 1G5 mAbs recognized the conformational epitope on the HA proteins (HA0), whereas the 3B5.1 recognized the linear and conformational epitope on the HA proteins (HA0 and HA1). The 3B5.1 and 3B5.2 mAbs reacted to each strain from 6 clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1c and 2.3.4. These results suggested that the 3B5.1 and 3B5.2 mAbs may possess broad reactivity against H5N1 clade viruses. In contrast, the 1G5 mAb showed reactivity only to clade 1 and 2.5 viruses. Therefore, the 3B5.1 and 3B5.2 mAbs may be useful for the specific detection of various H5N1 viruses circulating in the field.

In chapter II, the anti-H5 (3B5.1, 3B5.2 and 1G5) mAbs were further evaluated for the reactivity to various clade H5N1 HPAIVs, Eurasian-H5 (clade EA-nonGsGD) and American-H5 (clade Am-nonGsGD) LPAIV strains. Additionally, the possible antigenic sites (epitopes) on HA proteins recognized by these anti-H5 mAbs were analyzed. The 3B5.1 and 3B5.2 mAbs showed cross-clade reactivity to all 26 strains of clades 1, 2.2, 2.3.2.1, 2.3.2.1a, 2.3.2.1b, 2.3.2.1c and 2.3.4 including currently circulating H5N1 clades, but the 1G5 mAb showed reactivity only to clade 1, 2.3.4 and 2.5 strains. These results suggest a possibility that the epitope(s) recognized by the 3B5.1 and 3B5.2 mAbs are conserved among H5N1 HPAIVs. Blocking immunofluorescence tests showed that each mAb recognized different epitopes. An analysis of escape mutants of the 3B5.1 and 3B5.2 mAbs and some H5N1 clade viruses recognized by the mAbs suggested that the mAbs bind to an epitope, including amino acid residues at position 162 in the HA1 protein (R162 or K162). The R162 and K162 in the HA1 proteins were highly conserved among 36 clades, including clade 2.3.2.1a and 2.3.2.1c currently circulating in Asia, Africa, and Europe, of 43 H5N1 clades reported. Unexpectedly, however, when five Eurasian-origin strains (EA-nonGsGD clade) and two American-origin strains (Am-nonGsGD clade) with R162 of H5 LPAIV were examined, they recognized only EA-nonGsGD clade strains. The reasons of this contradictory are unknown.

The amino acid residues (158-PTIK**R**SYNNTNQE-170) in the HA1 protein were reported to be one of conserved epitopes responsible for the cross-clade reactivity of anti-H5 mAbs. The present study suggested that the 3B5.1 and 3B5.2 mAbs may recognize this cross-clade epitope, because the mAbs could not react to escape mutants with the mutation R162I or R162G. Due to the present data on epitope mapping, I think that the 3B5.1 and 3B5.2 mAbs may be available to develop accurate, specific and rapid diagnostic tests for recent H5N1 HPAIVs, especially for clade 2.3.2.1a and 2.3.2.1c strains that have been circulating and causing lethal infections of poultry in Asia, Africa, and Europe.

In chapter III, a simple and rapid latex agglutination test (LAT) utilizing the neutralizing 3B5.1

mAb was evaluated for the detection of H5N1 HPAIVs. Polystyrene latex beads were coupled with the 3B5.1 mAb (3B5.1 mAb-beads), and then the sensitivity and specificity of the established LAT (3B5.1 mAb-LAT) were evaluated. In the 3B5.1 mAb-LAT, mixing of 3B5.1 mAb-beads with allantoic fluids containing HPAIVs and LPAIVs, spiked chicken fecal samples containing the H5N1 HPAIV resulted in agglutination. In addition, Eurasian H5 LPAIVs (H5N1, H5N2 and H5N3) were detected in the 3B5.1 mAb-LAT, but not American H5 LPAIVs (H5N2 and H5N3). The specificity of the 3B5.1 mAb-LAT was evaluated using H9N2 and H1N1 viruses, and avian paramyxoviruses-1, 6 and 14. Nonspecific agglutination and cross-reactivity to those viruses were not observed. Unfortunately, however, the sensitivity of the 3B5.1 mAb-LAT was low, which test could detect the virus with approximately higher than $10^{8.8}$ EID_{50}/ml or 32 hemagglutination units. Therefore, the further study should be done for improving the test sensitivity.

The usefulness of the rapid and direct detection of H5 antigens from clinical samples using broad-reactive mAbs is unquestionable in diagnosis of HPAIV infection. However, H5 viruses have evolved rapidly and extensively, and the continued evolution of the virus has generated new clades. Therefore, it is critical to continuously make efforts to search for anti-H5 mAbs with broad reactivity with all clade viruses based on antigenic and genetic analysis for the development of diagnostic systems for H5 HPAI.