Characterization of cross-clade monoclonal antibodies against H5N1 highly pathogenic avian influenza virus and their application to the antigenic analysis of diverse H5 subtype viruses

Dulyatad Gronsang[1,4] · Anh N. Bui[2] · Dai Q. Trinh[2] · Vuong N. Bui[2] · Khong V. Nguyen[2] · Minh X. Can[2,3] · Tsutomu Omatsu[5] · Tetsuya Mizutani[5] · Makoto Nagai[5, 6] · Yukie Katayama[5], Rapeewan Thampaisarn[1,4] · Haruko Ogawa[1] · Kunitoshi Imai[1*]

[1] Diagnostic Center for Animal Health and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan

[2] National Institute of Veterinary Research, Dong Da, Hanoi, 10000, Vietnam

[3] Sub-Department of Animal Health, Ha Dong, Hanoi, 10000, Vietnam

[4] Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, 73170, Thailand

[5] Research and Education Center for Prevention of Global Infectious Diseases of Animals, Tokyo University of Agriculture and Technology, Tokyo, 183-8509, Japan

[6] Bioresources and Environmental Sciences, Ishikawa Prefectural University, Nonoichi, Ishikawa 921-8836, Japan

*Correspondence: Kunitoshi Imai, Phone: (81) 155-49-5892. Fax: (81) 155-49-5892. E-mail: imaiku@obihiro.ac.jp

Abstract

H5N1 highly pathogenic avian influenza viruses (HPAIVs) are a threat to both animal and public health and require specific and rapid detection for prompt disease control. We produced three neutralizing anti-hemagglutinin (HA) monoclonal antibodies (mAbs) using two clades (2.2 and 2.5) of the H5N1 HPAIV isolated in Japan. Blocking immunofluorescence tests showed that each mAb recognized different epitopes; 3B5.1 and 3B5.2 mAbs against the clade 2.5 virus showed cross-clade reactivity to all 26 strains from clades 1, 2.2, 2.3.2.1, 2.3.2.1a, b, c and 2.3.4, suggesting that the epitope(s) recognized are conserved. Conversely, the 1G5 mAb against the clade 2.2 virus showed reactivity to only clades 1, 2.3.4 and 2.5 strains. An analysis of escape mutants and some clades of the H5N1 viruses recognized by 3B5.1 and 3B5.2 mAbs suggested that the mAbs bind to an epitope, including amino acid residues at position 162 in the HA1 protein (R162 and K162). Unexpectedly, however, when five Eurasian-origin strains (EA-nonGsGD clade) and two American-origin strains (Am-nonGsGD clade) with R162 of H5 low-pathogenic AIV (LPAIV) were examined, they recognized only EA-nonGsGD clade strains. The R162 and K162 in the HA1 protein 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. The amino acid residues (158PTIKRSYNNTNQE170) in the HA1 protein were probably an epitope responsible for the cross-clade reactivity of the mAbs, considering the epitopes reported elsewhere. The 3B5.1 and 3B5.2 mAbs may be useful for the specific detection of H5N1 HPAIVs circulating in the field.

INTRODUCTION

The first outbreak of H5N1 highly pathogenic avian influenza viruses (HPAIVs) occurred in Hong Kong in 1997 in chicken populations, and the 18 human cases with 33.3% mortality were also observed at the same time [1]. Since then, these viruses have continued to cause deadly poultry outbreaks in Asia, Africa, and Europe [2]. They have also caused serious diseases highly associated with mortality in humans, leading to an increased awareness of a pandemic outbreak [3]. Recently, the H5N1 viruses appeared in North America [4].

Currently, HPAIVs have been classified as clades 0–9 based on the phylogenetic analysis of hemagglutinin (HA) genes [1, 5]. However, the continued evolution of the H5N1 virus has led to the emergence of new phylogenetic groups, except for clades 0, 3, 4, 5, 6, 8, and 9 that have not been recently detected [5-8]. Clades 1, 2 and 7 have been phylogenetically divided into additional clades. Especially, clade 2 viruses have evolved rapidly and extensively, and the continued evolution of the virus has generated second-order (clades 2.1–2.5), third-order (2.1.1–2.1.3, 2.2.1, 2.2.2, 2.3.1–2.3.4), fourth-order (2.1.3.1–2.1.3.3, 2.2.1.1, 2.2.1.2, 2.2.2.1, 2.3.2.1 and 2.3.4.1–2.3.4.4), and fifth-order groups (2.1.3.2a, b, 2.2.1.1a, and 2.3.2.1a, b, c). Some new fifth-order clade group strains (2.3.2.1a, c) have caused outbreaks in Asia, and recently, these clade strains have been detected in Africa and Europe [9-11]. It suggests that the newly emerging H5N1 HPAIVs are circulating in many regions. However, not all strains of H5 subtype are highly pathogenic: only a small number of H5 low pathogenic AIV (LPAIV) has mutated to the HPAIV [12]. LPAIVs can be classified into Eurasian and American clades (EA-nonGsGD and Am-nonGsGD) that were originated in Asia and America, respectively [13]. Therefore, an accurate, rapid, and specific diagnostic system for HPAIV and LPAIV infection is essential for the establishment of prompt disease control.

The currently available diagnostic techniques for identifying H5N1 viruses, e.g., virus isolation followed by serological tests to determine HA and NA subtypes or reverse transcription polymerase chain reaction, have some disadvantages in terms of high cost, rapidness, necessary expertise, and the need for a biosafety level- (BSL) 3 laboratory [14]. Rapid diagnostic tests using monoclonal antibodies (mAbs) against H5N1 HA proteins (anti-H5 mAbs) to immunologically detect H5N1 viruses have been reported [14-16]. However, there have been no reports as to whether the mAbs used in these tests could detect currently circulating H5N1 clade viruses, although the test principle was based on the use of broad cross-reactive H5-specific mAbs. Therefore, it is important to continuously make efforts to search for anti-H5 mAbs with broad reactivity with the epitopes on 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.

Recently, anti-H5 mouse and human mAbs recognized broad cross-reactive epitopes of several clade viruses: 1, 2.1.3.2, 2.2, and 2.3.4 [17], 0, 1, 2.2, 2.3.2.1, and 2.3.4 [18], and 0–9, except for clade 7.2 [19]. Unfortunately, the newly circulating H5N1 clades 2.3.2.1a and 2.3.2.1c were not evaluated [17-19]. Recently, Xiong et al. [20] reported that the broad cross-reactivity anti-H5 human mAbs reacted to clade 0, 1 2.1.3, 2.2, 2.2.1, 2.3.2, 2.3.2.1a, 2.3.4, and 2.5 viruses; however, some strains of clade 2.3.2.1a were not recognized by the mAbs.

Here, we tried to produce anti-H5 mAbs specific for H5N1 HPAIVs and evaluate cross-clade reactivity using H5N1 HPAIV strains isolated in Japan and Vietnam in addition to Eurasian-H5 (clade EA-nonGsGD) and American-H5 (clade Am-nonGsGD) LPAIV strains. We also analyzed possible antigenic sites on HA proteins recognized by the anti-H5 mAbs established in this study.

MATERIALS AND METHODS

Viruses and cells

A total of 38 strains of influenza viruses, including different clades of the H5N1 HPAIVs isolated in Japan and Vietnam, escape mutant H5N1 viruses, low pathogenic avian influenza viruses (LPAIVs), and human influenza A virus was used in this study (Table 1). All viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs. All experiments with the viruses were conducted in a BSL-3 laboratory approved by the relevant committee at the institution.

Madin-Darby canine kidney (MDCK) cells and mouse myeloma cells (Sp2/0) were cultured as reported previously [21, 22].

Production of mAbs

Ck/Yamaguchi/7/04 (clade 2.5) and Ck/Miyazaki/K11/07 (clade 2.2) were purified as described previously [21] and inactivated with 0.1% formalin. Two female BALB/c mice (8-weeks-old) were immunized with 0.2 mL of the inactivated purified H5N1 viruses, emulsified in a squalene-based adjuvant (AddaVAX™, InvivoGen, San Diego, CA) of the inoculum at the HA titer of 1,280 by intraperitoneal injection three times at intervals of 2–3 weeks. Final immunization was performed with the same viruses in 0.1 mL of phosphate buffered saline (PBS, pH 7.4) intravenously without the adjuvant three days before cell fusion. Fusion of the mouse spleen cells with the SP2/0 cells was performed, and the fused cells were cultivated as previously described [22], except that an OPI media supplement (Sigma-Aldrich Japan) and an endothelial cell growth supplement (Sigma-Aldrich Japan) were applied. Antibody-producing hybridomas were first screened using an enzyme-linked immunosorbent assay (ELISA) and then anti-HA antibody-producing hybridomas were selected using a hemagglutination inhibition test (HIT), as described below. The anti-HA antibody-producing hybridomas were cloned by a single cell pick up method, and these hybridomas were injected into the peritoneal cavity of mice to produce ascitic fluids containing mAbs, as described previously [23]. The IgG fraction of the ascitic fluids were precipitated with a 50% saturated ammonium sulphate solution, followed by dialyzing against the PBS.

All mouse studies were conducted in compliance with the institutional rules for the care and use of laboratory animals using protocols approved by the relevant committee at the institution.

ELISA

ELISA was performed as previously described [24] with some modifications. Briefly, the inactivated purified viruses were incubated with a lysis buffer (4% Triton-X100/2M KCl) at 4°C for 30 min, and coated to wells of the ELISA microplates (Thermo Fisher Scientific, K.K., Yokohama, Japan) at 4°C overnight. The plates were blocked with 10% newborn calf serum in PBS at 37°C for 2 h. Culture supernatants of the hybridomas were added to each well and incubated at 37°C for 1 h. Peroxidase-conjugated goat anti-mouse polyvalent Igs (IgA, IgG, and IgM) (Sigma-Aldrich, Japan) were then added and incubated at 37°C for 1 h. A TMB peroxidase substrate reagent (BD Bioscience Pharmingen, San Diego, CA) was added to each well and the optical density (OD) of the samples were measured at 450 nm.

Hemagglutination inhibition test (HIT)

HITs were performed according to the Manual on Animal Influenza Diagnosis and Surveillance [25] using 0.5% chicken red blood cells. Briefly, serial 2-fold dilutions of the receptor destroying enzyme-treated-ascitic fluids containing mAbs were mixed with 4 HA units/25 μL of the viruses, and then incubated at room temperature for 30 min. The chicken red blood cells were then added to the mixtures and incubated for 30 min. The HI titer was examined as a reciprocal of the highest dilution of mAb that completely inhibited hemagglutination.

Indirect fluorescence antibody test (IFAT) and blocking IFAT

IFATs were used to determine the mAbs isotypes. Briefly, MDCK cells were inoculated with the Ck/Yamaguchi/7/04 or the Ck/Miyazaki/K11/07 and incubated for 18 h. The infected cells were fixed with acetone for 10 min and incubated with mAbs (1:100) for 1h. The infected cells were then incubated with fluorescein isothiocyanate- or Rhodamine-conjugated anti-mouse IgG subclasses (IgG1, IgG2a, or IgG2b), (Rockland Immunochemicals, Gilbertsvile, PA).

In blocking IFATs, the infected cells were reacted with the 3 mAbs (3B5.1, 3B5.2, or 1G5) produced in this study for 30 min at 37°C. Then, the 3B5.1 mAb labeled with Alexa Fluor® 488 using a Fluorescein Labeling kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan) or the 3B5.2 and 1G5 mAbs labeled with Alexa Fluor® 594 using a Zenon® Mouse IgG Labeling kit (Thermos Fisher Scientific, K.K.) were reacted for 30 min. The fluorescent signal was observed under a fluorescence microscope (Biorvo BZ-9000, Keyence, Japan).

Western blotting (WB) analysis

The inactivated purified viruses were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with or without a reducing condition, and transferred to polyvinylidene difluoride membranes using a semi-dry apparatus. The membranes blocked with 3% bovine serum albumin in PBS were incubated with ascitic fluids containing mAbs. Peroxidase-conjugated goat anti-mouse polyvalent Igs (IgA, IgG, and IgM) (Sigma-Aldrich Japan) were used as secondary antibodies. The membrane was developed with enhanced chemiluminescence WB detection reagents (GE Healthcare UK Ltd., Buckinghamshire, UK) and photographed using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

Virus neutralization test (VNT)

VNTs with a constant mAb and diluted virus condition were performed. Briefly, serial 10-fold stepwise dilutions of the virus were mixed with mAb (1:10) or virus growth medium (VGM) [21] and incubated for 2 h at 37°C. Then, the mixtures were added to the MDCK cells grown in 96-well microplates and incubated for 2 h. After four days, the 50% tissue culture infectious dose ($TCID_{50}$) of the mixture was determined [26]. The neutralizing index was calculated by subtracting the log $TCID_{50}$ of the mAb-virus mixture from that of the virus control. The mAb with a neutralizing index higher than 0.7 was regarded to have neutralizing activity.

Selection and sequencing of escape mutants

Serial dilutions of the virus were incubated with an excess of mAb for 1 h, and the mixtures were inoculated into MDCK cells and incubated for 3 d at 37°C. Escape mutants were cloned by limiting-dilution in embryonated chicken eggs. Viral RNAs were extracted from the mutant-infected allantoic fluids using ISOGEN II (NIPPON GENE, Tokyo, Japan). The construction and purification of cDNA libraries which were used in next-generation sequencing were conducted according to a previous report [27]. The sequencing was carried out on a MiSeq bench-top sequencer (Illumina, San Diego, CA) to generate 51-bp single-end reads The FASTQ-formatted sequence data was generated using MiSeq Reporter program (Illumina). The contiguous sequences were assembled from the short sequence reads using CLC Genomics Workbench version 6.5.1 (CLC bio, Aarhus, Denmark). The consensus sequence was determined using BLAST.

Analysis of epitopes recognized by mAbs

The HA1 amino acids sequences (positions 1-322, corresponding to H5 numbering) of the parental strains (Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07) and their escape mutants (3B5.1, 3B5.2, and 1G5mts) were aligned and the mutation sites were further analyzed in the HA1 protein.

The 3D-structural model of the HA1 protein of the Ck/Yamaguchi/7/04 strain was constructed by using the Phyre2 web server [28], based on homology modeling. The HA1 crystal structure of the A/Vietnam/1194/04 strain was downloaded from the protein data bank (PDB) (PDB ID: 2IBXA) and used as the template for this construction.

The sixteen HA1 amino acid sequences of the different clades of H5N1 HPAIVs, escape mutants, and H5 subtype LPAIVs used in this study were aligned to predict a cross-clade epitope recognized by the anti-H5 mAbs. Subsequently, the predicted cross-clade epitope was analyzed and mapped to the HA1 structural model of the Ck/Yamaguchi/7/04 strain using PyMOL [29]. A predicted cross-clade epitope was further analyzed to find the conserved amino acid residues in the HA1 protein among the 5,366 HA amino acid sequences of clades 0–9 of the H5N1 viruses, obtained from the GenBank and Influenza Research Database.

RESULTS

Production and characterization of mAbs

Three hybridomas (anti-H5 mAbs; 3B5.1, 3B5.2, and 1G5) with both HI and neutralizing activities were established (Fig. 1A). The 3B5.1 and 3B5.2 mAbs to Ck/Yamaguchi/7/04 (clade 2.5) showed higher HI titers and neutralizing indexes than the 1G5 mAb to Ck/Miyazaki/K11/07 (clade 2.2).

WB analysis showed that the 3B5.2 and 1G5 mAbs reacted only to a 75 kDa protein band of the HA0 under non-reducing conditions, indicating that these mAbs recognized the discontinuous (conformational) epitope of the HA proteins. However, the 3B5.1 mAb recognized both the 75 and 50 kDa proteins of the HA (HA0 and HA1) under non-reducing and reducing conditions, respectively (Fig. 1B). This suggested that the 3B5.1 mAb specifically recognized linear and conformational epitopes of the HA proteins.

Reactivity of the mAbs with heterologous H5 subtype influenza viruses

The 3B5.1 and 3B5.2 mAbs against Ck/Yamaguchi/7/04 (clade 2.5) had reactivity to all the 26 heterologous strains examined 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 of the H5N1 HPAIV, isolated in Japan and Vietnam. The HI titers of the 3B5.1 mAb to these heterologous H5N1 viruses ranged from 160–640, and those of the 3B5.2 mAb ranged from 10–640 (Table 2). In contrast, the 1G5 mAb against Ck/Miyazaki/K11/07 (clade 2.2) recognized only clade 1, 2.3.4 and 2.5 viruses with the HI titers ranging from 20–320. However, only one of the five strains examined in clade 2.3.4 reacted with this mAb (titer of 1:20).

The 3B5.1, 3B5.2, and 1G5 mAbs also recognized the H5N1, H5N2, and H5N3 LPAIV strains belonging to the EA-nonGsGD clade with the HI titers ranging from 80 to 1,280, except the Ck/Ibaraki/1/05 (H5N2) and Whis. sw./Shimane/499/83 (H5N3) strains belonging to the Am-nonGsGD clade (Table 3).

Blocking IFAT

Blocking IFAT was performed to verify whether the three anti-H5 mAbs could recognize different antigenic sites in the HA1 protein or not. Binding of all the fluorescein-conjugated 3B5.1, 2B5.2, and 1G5 mAbs were not blocked by any competitor mAbs, except the homologous mAbs (Fig. 2), indicating that each of the mAbs recognized different antigenic sites.

Analysis of the escape mutants and epitopes recognized by the mAbs

Three escape mutants to the anti-H5 mAbs (3B5.1 mt, 3B5.2 mt, and 1G5 mt) were generated and sequenced to identify the mutated amino acid positions in H5 HA1 associated with the mAbs binding sites (Fig. 3A). The 3B5.1 mt carried point mutations at amino acid positions 56 (S56N), and 162 (R162I) (according to mature H5 numbering), whereas the 3B5.2 mt had two mutation sites at the positions 162 (R162G) and 184 (A184G) in the HA1 protein. The 1G5mt had only a single point mutation at position 139 (G139R).

Then, these mutated amino acid positions were mapped to the HA1 crystal structure (2IBXA). The result showed that the three mutated amino acid residues (positions 139, 162 and 184) were located on the HA1 protein (Fig. 3B and C). The mutated amino acid at position 139 was located in the antigenic site A in H3 [37] and Ca in H1 HA [38], whereas the mutated amino acid at position 184 was located in the antigenic site B in H3 [37] and Sb in H1 HA [38]. The mutated amino acid at position 162 was located in the antigenic site Sa in H1 HA [38]. However, the mutated amino acid at position 56 was not located on any antigenic sites of H1 and H3 HA (Fig. 3B). The HA amino acid residue at position 184 (E184) of the clades 2.3.2.1, 2.3.2.1a, and 2.3.2.1c used in this study (data not shown) was different from that of the parental strain (G184), but these clade strains were recognized by the 3B5.1 and 3B5.2 mAbs (Table 2). Therefore, these results suggested that the HA amino acid residues at position 162 is a major epitope recognized by the 3B5.1 and 3B.2 mAb. The 1G5 mAb recognizes the amino acid residues at position 139 in the HA protein.

In this study, the 3D-structural model of the Ck/Yamaguchi/7/04 strain was analyzed to identify the binding site of 3B5.1 and 3B5.2 in HA1 protein. Two different amino acid residue portions were found to be located adjacent to or surrounding the HA1 amino acid residue at position 162. These portions consisted of the amino acid residues at positions 115–128 (115QIIPKSSWSDHEAS128) that are located in the antigenic site A in H3 and at positions 158–170 (158PTIKRSYNNTNQE170) that are located in the antigenic site Sa in H1 HA [39] (Fig. 3D). These two portions may affect the binding of the 3B5.1 and 3B5.2 mAbs.

However, because no evidence was found in this study to support that 3B5.1 and 3B5.2 mAbs recognized the HA1 amino acids at positions 115–128, the amino acid residue positions 158–170 containing the mutated sites at position 162 seemed to be an epitope recognized by these mAbs. Thus, the HA1 amino acids at positions 158–170 were analyzed to identify the antigenic sites of the 3B5.1 and 3B5.2 mAbs. The presence of 158PTIKRSYNNTNQE170 residues in the HA1 protein of the

Ck/Yamaguchi/7/04 strain was examined to verify whether the residues were conserved among the H5 subtype strains, including six H5N1 HPAIV strains and seven LPAIV (EA-nonGsGD and Am-nonGsGD clades) strains used in this study (Table 4) The amino acid sequence of six HPAIV strains was the same as that of the Ck/Yamaguchi/7/04 strain, except at position 162 of clades 2.3.2.1, 2.3.2.1a, and 2.3.2.1c (K162), and at position 163 of clades 2.3.2.1a and 2.3.2.1c (G163). However, the lysine residues at position 162 (K162) did not affect the binding ability of the 3B5.1 and 3B5.2 mAbs, although the mAbs could not react to mutants with R162I or R162G (Fig. 3A).

In addition, five EA-nonGsGD clade strains recognized by the 3B5.1 and 3B5.2 mAbs had the same amino acid sequence (158PTIKRSYNNTNQE170) as the Ck/Yamaguchi/7/04 strain, except at position 169 of the Dk/Hong Kong/820/83 (Q169K) and at position 170 of the Md/Hokkaido/24/09 (E170D) (Table 4). However, these mutated amino acid residues (Q169K and E170D) did not affect the binding ability of 3B5.1 and 3B5.2 mAbs. Unexpectedly, in two Am-nonGsGD strains, the Ck/Ibaraki/1/05 strain which possessed P158K, I160L, S163N, N165T, and Q169V substitutions and the Whis. sw/Shimane/499/83 strain which possessed K161E, S163T, and Q169V substitutions, the R162 residues in their HA1 protein were not recognized by 3B5.1 and 3B5.2 mAbs.

Conservation of the HA1 epitope (158–170) among divergent H5N1 HPAIVs recognized by the mAbs

The 158PTIKRSYNNTNQE170 epitope was aligned and compared among 5,366 strains of clades 0–9 of the H5N1 viruses to analyze the conservation of this epitope (Table 5). The result showed that the HA1 amino acid at positions 158, 160, 164, and 166 (P158, I160, Y164, and N166) were highly conserved (>90%) among all clades 0–9 of the H5N1 viruses. The R162 and K162 in the HA1 amino acid residues were also highly conserved among all clades of the H5N1 viruses, except clades 2.2.1.1a (E162), 2.2.2 (I162), 2.2.2.1 (I162), 2.3.1 (I162), 2.3.4.4 (I162), 7.1 (V162), and 7.2 (V162). The amino acid positions 159, 163, 165, and 167–170 (T159, S163, N165, T167, N168, Q169, and E170) were completely mutated and highly conserved in clades 2.3.2.1b (I159 and K169), 7.1 (P159, N163, and T165), 7.2 (P159, N163, T165, and A167), 2.1.3.2a (T163), 2.1.3.2b (T163), 2.3.2.1c (G163), 2.2.1.1 (H165), 2.2.1.1a (H165), 2.1.3 (E168), and 2.3.4.4 (R169) and mutated and moderately conserved (50–90%) in clades 1.1.1 (D168), 2.3.4.1 (S168), 2.3.2.1c (R169), and 7.2 (K170).

DISCUSSION

In this study, three neutralizing anti-H5 mAbs against clades 2.2 (1G5 mAb) and 2.5 (3B5.1 and 3B5.2 mAbs) viruses were successfully established (Fig. 1A). Interestingly, the 3B5.1 and 3B5.2 mAbs reacted to all HPAIV 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 isolated in Japan and Vietnam during 2003–2012, indicating that the mAbs recognized the cross-clade epitope; whereas the 1G5 mAb did not react to all clades examined (Table 2).

Although four amino acid mutations (S56N, R162I, R162G, and A184G, H5 numbering) were found in the escape mutants (3B5.1 and 3B5.2 mts) (Fig. 3A), the mutations at positions 56 and 184 did not seem to affect the binding ability of the 3B5.1 and 3B5.2 mAbs since the amino acid at position 56 was buried into the HA1 protein, and clades 2.3.2.1, 2.3.2.1a, and 2.3.2.1c containing the E184 (data not shown) were recognized by those mAbs (Table 2). In contrast, the positively charged amino acid residue (R162) seemed to highly associate with the binding ability of the 3B5.1 and 3B5.2 mAbs, since the change from positively charged amino acid residue (R162) to the neutral charged residue (I162 and G162) might lead to the inhibition of the binding ability of the 3B5.1 and 3B5.2 mAbs (Fig. 3A). However, although the H5N1 clade 2.3.2.1, 2.3.2.1a, and 2.3.2.1c strains have the lysine residue (K162) and not Arginine, they were also recognized by these mAbs. Unfortunately, since the HA amino acid sequences of the clade 2.3.2.1b strains used in this study were not available, we examined the 35 HA sequences of clade 2.3.2.1b available in the database. We found that all strains contained the amino acid residue K162 as shown in Table 5. Therefore, the 3B5.1 and 3B5.2 mAbs may mainly interact with the positively charged amino acid residues at position 162 in the HA1 protein.

The mutation of amino acids in position 162 of the HA1 protein caused a loss of binding of some anti-H5 mouse mAbs [40-42]. The VN04-2 mAb to the A/Vietnam/1203/04 (clade 1) strain reacted to clade 2.1.1 (R162), but not to clade 2.1.3.1, 2.2, and 2.3.4 strains with the same residue R162 [40]. Four anti-H5 mouse mAbs to Duck/Novosibirsk/56/05 (clade 2.2) (3G9, 5G9, 5F12, and 6E2) seemed to recognize R162, because they failed to react with the escape mutants with the amino acid residue change R162G/K/W; however, these mAbs could not recognize the clade 1 (R162) and clade 2.3.2.1 (K162) and 2.3.2.1c (K162) strains [41, 42]. On the other hand, our mAbs (3B5.1 and 3B5.2) could recognize clade 1 (R162) and clades 2.1.3.1 (R162), 2.2 (R162), 2.3.2.1(K162), 2.3.2.1c (K162), and 2.3.4 (R162). Thus, our mAbs also recognized the H5N1 strains with K162 in the HA1 protein (Table 4), a result that is contradictory to that observed in previous studies [40-42]. Although the reason for this contradiction is

unclear, the antigenic structures of these regions, including the amino acid residue at position 162 that our mAbs (3B5.1 and 3B5.2) recognized, may be different from those that other mAbs recognized.

Although several broad cross-clade reactivities of anti-H5 HA human and mouse mAbs have been reported [17-20], there is not much information on the reactivity to the newly emerging H5 HPAIVs (clades 2.3.2.1a or 2.3.2.1c). Although a human mAb (FLD194) recognized A/Hubei/01/10 strain (clade 2.3.2.1a; up to date at this time) bearing Q119 in the HA1 protein (H5 numbering) in addition to clade 0, 1, 2.1.3, 2.2, 2.2.1, 2.3.1, 2.3.2, 2.3.4, and 2.5 strains, it could not react to the other two strains of the same clade 2.3.2.1a (A/chicken/Bangladesh/11RS1984-33/11 and A/chicken/Bangladesh/14VIR2665-23/14) bearing R119 and K119, respectively [20].

Thus, we may need to examine as many strains as possible from different origins within clades to define the broad cross-reactivity of established mAbs. Conversely, our mAbs (3B5.1 and 3B5.2) could react to all six strains of clade 2.3.2.1a strains bearing K119 (Table 2) and EA-nonGsGD LPAIV strains bearing R119 (Table 3). Although the clade 2.3.2.1a strains used in the previous study [20] were not available in this study, the two strains with the R162 or K162 residues in the HA1 protein seemed to be recognized by the 3B5.1 and 3B5.2 mAbs.

Hu et al. [19] reported that a human mAb (65C6) binds a conformational epitope, comprised of amino acid residues at positions118, 121, 161, 164, and 167 (H5 numbering) in the HA1 protein, that is conserved among clade 0, 1, 3–9, 2.1.3.2, 2.2, 2.2.1, 2.3.2.1, 2.3.4, 2.4, and 2.5 strains, except for clade 7.1. However, the amino acid mutation at position 162 did not significantly affect the binding ability of the 65C6 mAb, but did abolish the binding ability of the 3B5.1 and 3B5.2 mAbs used in this study (Fig. 3A). These results may suggest that our mAbs recognized different antigenic structures than those recognized by the 65C6 mAb, although each site seemed to be structurally very similar and closeby.

The cross-clade epitope in the HA1 protein at the R162 residue has been reported by Zuo et al [43]. They suggested that four major vulnerable antigenic sites (VS1–4) exist on the A/Anhui/1/05 HA1 protein based on epitopes recognized by the H5-specific human and murine mAbs, aligning with other reports. One of the mAbs (65C6) recognized the cross-clade conformational epitope in VS1 (115QIIPKSSWSDH125 and 158PTIKRSYNNTNQE170, H5 numbering) that roughly corresponded to Site A on H3 HA and Sa on H1 HA. However, the 158PTIKRSYNNTNQE170 region only includes amino acid residues at positions 158–163 (H5 numbering) of the antigenic site Sa on H1 HA, but not Site A on H3. In this study, 3B5.1 and 3B5.2 mAbs could not bind to the mutant strains with the mutation

R162I or R162G, which may suggest that the epitope recognized by these mAbs overlapped the epitope. (158PTIKRSYNNTNQE170).

Therefore, we tried to analyze whether the 158PTIKRSYNNTNQE170 epitope was conserved among the 5,366 sequences of various clade H5N1 HAs currently available in the database. Shown in Table 5, the R162 or K162 residues in the HA1 protein were highly conserved among 36 clades, including clades 2.3.2.1a and 2.3.2.1c, out of the 43 H5N1 clades reported, except for seven clades (2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1 and 7.2). Out of the 18 amino acid residues of the epitope, in four or five residues (159, 162, 163, 165, and 167) of clades 7.1 and 7.2, a very low level of residue conservation was revealed compared with other clades (Table 5). It was expected that our mAbs would fail to react to these clade strains; thus, we believe that clades of the H5N1 HPAIV containing the R162 or K162 residue in the HA1 protein could be recognized by our mAbs (3B5.1 and 3B5.2), leading to the detection of clade 2.3.2.1a and 2.3.2.1c viruses currently circulating in Asia, Africa, and Europe [11-13].

Shown in Tables 3 and 4, the 3B5.1 and 3B5.2 mAbs recognized the EA-nonGsGD clade (Eurasian-lineage) H5 LPAIV strains (H5N1, H5N2, and H5N3) with residue R162 in the HA1 protein. Unexpectedly, these mAbs could not recognize the Am-nonGsGD clade (American-lineage) H5 LPAIV strains (H5N2 and H5N3), even with the residue R162. In the American-lineage strains, the amino acid residue at position 158 in the HA1 protein of the Ck/Ibaraki/1/05 strain (K158) was the positive charge, whereas the residue at position 161 of the Whis.sw./Shimane/499/83 strain (E161) was the negative charge. Those positively and negatively charged amino acid residues were different from the Eurasian-lineage strains of H5 HPAIV and LPAIV (P158 and K161) (Table 4). The shift in the charge from positive to negative that resulted in conformational change of the HA1 surface affecting on the binding of an anti-H5 mAb has been reported [17]. Herein, the negatively charged amino acid residue (E161) of the Whis.sw./Shimane/499/83 may have affected the binding of the 3B5.1 and 3B5.2 mAbs. However, it was unclear whether the positively charged residue (K159) of the Ck/Ibaraki/1/05 strain similarly affected the binding of these mAbs. We suspected that the HA1 structure of this Am-nonGsGD clade strain was complex and different from that of the HPAIV and EA-nonGsGD clade strains.

Unfortunately, the 3B5.1 and 3B5.2 mAbs may not recognize the clade 2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1, and 7.2 strains, containing the residue E162, I162, or V162 in the HA1 protein (Table 5). Therefore, in order to detect clades 2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1, and 7.2, additional mAb should be produced. A combination of our mAbs (3B5.1 and 3B5.2) and the complementary mAbs to the clade 2.2.1.1a, 2.2.2, 2.2.2.1, 2.3.1, 2.3.4.4, 7.1, and 7.2 strains could be useful in the development of a

diagnostic test with broad cross-clade reactivity for the detection of all H5N1 clade strains. Previously, a mAb-based dot ELISA was developed for the universal detection of H5N1 viruses including clades 0, 1, 2.1, 2.2, 2.3, 4, 7, and 8 by using two complementary anti-H5 mAbs [44].

The outbreaks caused by H5N1 HPAIVs are still serious problems that have an impact on both animal and public health. Therefore, rapid diagnostic tests are essential for the detection and monitoring of circulating H5N1 viruses to prevent an outbreak. Our results indicated that the 3B5.1 and 3B5.2 mAbs have potential for this application due to their broad cross-reactivity to the H5N1 viruses. These mAbs could be available to develop diagnostic tests for recently circulating H5N1 viruses, 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 [11-13].

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Figure and Table legends

Fig. 1 Characterization of anti-H5 mAb to H5N1 HPAIV (A). General properties of anti-H5 mAbs (3B5.1, 3B5.2 and 1G5) including HIT, VNT and antibody isotypes were shown. *The HIT and VNT were performed using the homologous viral strain. Identification of anti-H5 mAbs by Western blot analysis using the inactivated purified Ck/Yamaguchi/7/04 (clade 2.5) and Ck/Miyazaki/K11/07 (clade 2.2) strains as the antigens under reducing and non-reducing conditions (B). One anti-H5 mAb (3B5.1) had reactivity to the HA1 proteins (50 KDa) under a reducing condition and to the HA proteins (75 KDa) under a non-reducing condition of Ck/Yamaguchi/7/04. Two anti-H5 mAbs (3B5.2 and 1G5) had reactivity to the HA proteins (75 KDa) Ck/Yamaguchi/7/04 and Ck/Miyazaki/K11/07. 1203/4: anti-H5 mAb to A/Vietnam/1203/4; M: molecular marker; +: reducing condition; -: non-reducing condition; Bl: Blank; Ya: inactivated purified Ck/Yamaguchi/7/04 antigen; Mi: inactivated purified Ck/Miyazaki/K11/07 antigen.

Fig. 2 Blocking immunofluorescence antibody tests. Blocking tests were conducted using anti-H5 mAbs (3B5.1, 3B5.2, and 1G5) as competitors and the mAbs directly labeled with green or red fluorescence. The 3B5.1 mAb was conjugated with Alexa Fluor®488; 3B5.2 with Alexa Fluor® 594; and 1G5 with Alexa Fluor® 594.

Fig. 3 Characterization of the escape mutants (A), * selected by the mAbs against Ck/Yamaguchi/7/04, ** selected by the mAbs against Ck/Miyazaki/K11/07. The mutated HA amino acid residues and antigenic sites of the 3B5.1 mt and 3B5.2 mt of the Ck/Yamaguchi/7/04 (B) and 1G5 mt of the Ck/Miyazaki/K11/07 strain (C) on the HA1 structural protein model. Two HA1 amino acid residue portions of the Ck/Yamaguchi/7/04 strain located adjacent to the HA1 amino acid position 162 (D). The HA1 amino acid position 162 is labeled in blue. The amino acid residue positions 115QIIPKSSWSDHEAS128 are labeled in magenta. The 158PTIKRSYNNTNQE170 residues are labeled in yellow.

Table 1 Influenza viruses used in this study

Table 2 Antigenic characterization of different clades of the HPAIV subtype H5N1 isolated in Japan and Vietnam using the three mAbs produced.

Table 3 Antigenic characterization of LPAIVs and other subtypes of influenza A viruses using the three mAbs produced.

Table 4 Comparison of the HA1 epitope at positions 158–170 among the H5 viruses used in this study.

Table 5 Conservation of the HA1 epitope at positions 158–170 among divergent clades of the H5N1 viruses.

Table 1 Influenza viruses used in this study

Influenza viruses	Strains	Clades	Virus source and references
HPAIVs	A/chicken/Yamaguchi/7/04(H5N1),[Ck/Yamaguchi/7/04]	2.5	**[30]
	A/chicken/Yamaguchi/7/04(H5N1) [3B5.1-escape mutant]		*****
	A/chicken/Yamaguchi/7/04(H5N1) [3B5.2-escape mutant]		*****
	A/chicken/Miyazaki/K11/07(H5N1),[Ck/Miyazaki/K11/07]	2.2	**[31]
	A/chicken/Miyazaki/K11/07 (H5N1),[1G5-escape mutant]		*****
	A/whooper swan/Hamanaka/11 (H5N1, [Who.sw/Hamanaka/11]	2.3.2.1c	*****[32]
	A/whooper swan/Hokkaido/1/08 (H5N1) [Who.sw/Hokkaido/1/08]	2.3.2.1	***[33]
	A/chicken/Vietnam/HN-06/03 (H5N1), [Ck/VN-HN/06/03]	1	*
	A/chicken/Vietnam/HT-33/03 (H5N1), [Ck/VN-HT/33/03]	1	*
	A/chicken/Vietnam/TB-31/04 (H5N1), [Ck/VN-TB/31/04]	1	*
	A/chicken/Vietnam/HN-40/04 (H5N1), [Ck/VN-HN/40/04]	1	*
	A/chicken/Vietnam/HT-30/10 (H5N1), [Ck/VN-HT/30/10]	2.3.2.1a	*
	A/chicken/Vietnam/VP-02/11 (H5N1), [Ck/VN-VP/02/11]	2.3.2.1a	*
	A/chicken/Vietnam/QT-06/11 (H5N1), [Ck/VN-QT/06/11]	2.3.2.1a	*
	A/chicken/Vietnam/HD-04/11 (H5N1), [Ck/VN-HD/04/11]	2.3.2.1a	*
	A/chicken/Vietnam/HD-01/12 (H5N1), [Ck/VN-HD/01/12]	2.3.2.1a	*
	A/chicken/Vietnam/TB-10/11 (H5N1), [Ck/VN-TB/10/11]	2.3.2.1a	*
	A/chicken/Vietnam/NB-05/11 (H5N1), [Ck/VN-NB/05/11]	2.3.2.1b	*
	A/molly duck/Vietnam/BN-02/12 (H5N1), [Mol.dk/VN-BN/02/12]	2.3.2.1b	*
	A/duck/Vietnam/VP-03/12 (H5N1), [Dk/VN-VP/03/12]	2.3.2.1b	*
	A/duck/Vietnam/QB-69/12 (H5N1), [Dk/VN-QB/69/12]	2.3.2.1b	*
	A/duck/Vietnam/BN-06/12 (H5N1), [Dk/VN-BN/06/12]	2.3.2.1b	*
	A/duck/Vietnam/HN-72/12 (H5N1), [Dk/VN-HN/72/12]	2.3.2.1c	*
	A/duck/Vietnam/HN-74/12 (H5N1), [Dk/VN-HN/74/12]	2.3.2.1c	*
	A/duck/Vietnam/HN-75/12 (H5N1), [Dk/VN-HN/75/12]	2.3.2.1c	*
	A/muscovy duck/Vietnam/HN-77/07 (H5N1), [Mdk/VN HN/77/07]	2.3.4	*
	A/muscovy duck/Vietnam/HN-01/09 (H5N1), [Mdk/VN-HN/01/09]	2.3.4	*
	A/muscovy duck/Vietnam/BN-16/09 (H5N1), [Mdk/VN-BN/16/09]	2.3.4	*
	A/muscovy duck/Vietnam/HN-13/09 (H5N1), [Mdk/VN-HN/13/09]	2.3.4	*
	A/muscovy duck/Vietnam/TB-05/10 (H5N1), [Mdk/VN-TB/05/10]	2.3.4	*
LPAIVs	A/mallard/Hokkaido/24/09 (H5N1), [Md/Hokkaido/24/09]	EA-nonGsGD	*** [34]
	A/duck/Japan/11UO0023/11 (H5N2), [Dk/Japan/11UO0023/11]	EA-nonGsGD	*****
	A/duck/Japan/11UO0032/11(H5N2), [Dk/Japan/11UO0032/11]	EA-nonGsGD	*****
	A/duck/Japan/11UO0083/11(H5N2), [Dk/Japan/11UO0083/11]	EA-nonGsGD	*****
	A/duck/Hong Kong/820/80 (H5N3), [Dk/Hong Kong/820/80]	EA-nonGsGD	***
	A/chicken/Ibaraki/1/05 (H5N2), [Ck/Ibaraki/1/05]	Am-nonGsGD	**[35]
	A/whistling swan/Shimane/499/83 (H5N3)[Whis.sw/Shimane/499/83]	Am-nonGsGD	****
	A/chicken/Yokohama/aq-55/01 (H9N2), [Ck/Yokohama/aq/55/01]		**[36]
Human influenza A virus	A/Puerto Rico/8/34 (H1N1), [PR/8/34]		*****

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*****Available in our laboratory, Japan.

Table 2 Antigenic characterization of different clades of the HPAIV subtype H5N1 isolated in Japan and Vietnam using the three mAbs produced

Clades	Strains	HI titers		
		3B5.1	3B5.2	1G5
1	Ck/VN-HN/06/03	160	40	160
	Ck/VN-TB/31/04	320	10	160
	Dk/VN-HN/40/04	640	20	320
	Ck/VN-HT/33/03	320	20	40
2.2	Ck/Miyazaki/K11/07	640	640	320
2.3.2.1	Who.sw/Hokkaido/1/08	320	20	<10
2.3.2.1a	Ck/VN-HT/30/10	160	20	<10
	Dk/VN-VP/02/11	640	40	<10
	Dk/VN-QT/06/11	640	80	<10
	Ck/VN-HD/04/11	640	40	<10
	Mdk/VN-HD/01/12	320	20	<10
	Ck/VN-TB/10/12	640	40	<10
2.3.2.1b	Mol. dk/VN-BN/02/12	640	40	<10
	Dk/VN-VP/03/12	640	40	<10
	Dk/VN-QB/69/12	640	80	<10
	Dk/VN-BN/06/12	320	10	<10
	Ck/VN-NB/05/11	320	20	<10
2.3.2.1c	Dk/VN-HN/72/12	640	80	<10
	Dk/VN-HN/74/12	640	80	<10
	Dk/VN-HN/75/12	640	80	<10
	Who.sw/Hamanaka/11	320	20	<10
2.3.4	Mdk /VN-HN/01/09	160	320	<10
	Mdk /VN-BN/16/09	320	320	20
	Dk/VN-HN/13/09	320	320	<10
	Mdk /VN-TB/05/10	320	40	<10
	Mdk /VN-HN/77/07	160	80	<10
2.5	Ck/Yamaguchi/7/04	2,560	640	80

Table 3 Antigenic characterization of the LPAIVs and other subtypes of influenza A viruses using the three mAbs produced

Clades	Subtypes	Strains	HI titers		
			3B5.1	3B5.2	1G5
EA-nonGsGD	H5N2	Dk/Japan/11UO0023/11	1,280	160	80
		Dk/Japan/11OG1032/11	1,280	160	80
		Dk/Japan/11OG1083/11	1,280	160	80
Am-nonGsGD	H5N2	Ck/Ibaraki/1/05	<10	<10	<10
EA-nonGsGD	H5N3	Dk/Hong Kong/820/80	1,280	160	80
Am-nonGsGD	H5N3	Whis.sw/Shimane/499/83	<10	<10	<10
EA-nonGsGD	H5N1	Md/Hokkaido/24/09	1,280	160	80
	H9N2	Ck/Yokohama/aq/55/01	<10	<10	<10
	H1N1	PR/8/34	<10	<10	<10

Table 4 Comparison of the HA1 epitope at positions 158–170 among the H5 viruses used in this study

Strains	GenBank accession number	Clades	HIT[a]	Amino acid positions in the HA1 protein (158–170)												
				158	159	160	161	162	163	164	165	166	167	168	169	170
Ck/Yamaguchi/7/04 (H5N1)	AB166862	2.5	+	P	T	I	K	R	S	Y	N	N	T	N	Q	E
Ck/VN-HT/33/03 (H5N1)		1	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Ck/Miyazaki/K11/07 (H5N1)		2.2	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Who.sw/Hokkaido/1/08 (H5N1)	AB436550	2.3.2.1	+	.	.	.	.	K	.	.	.	.	.	.	.	.
Ck/VN-HT/30/10 (H5N1)		2.3.2.1a	+	.	.	.	.	K	G	.	.	.	.	.	.	.
Who.sw/Hamanaka/11 (H5N1)	CY110738	2.3.2.1c	+	.	.	.	.	K	G	.	.	.	.	.	.	.
Mol.dk/VN-HN/77/07 (H5N1)		2.3.4	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Dk/Hong Kong /820/80 (H5N3)	LC042047	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	K	.
Dk/Japan/11UO0023/11 (H5N2)	KR265592	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Dk/Japan/11OG1032/11 (H5N2)	KR265541	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Dk/Japan/11OG1083/11 (H5N2)	KR265560	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	.
Md/Hokkaido/24/09 (H5N1)	AB530992	EA-non GsGD	+	.	.	.	.	.	.	.	.	.	.	.	.	D
Ck/Ibaraki/1/05 (H5N2)	AB261853	Am-non GsGD	−	K	.	L	.	.	N	.	T	.	.	.	V	.
Whis.sw/Shimane/ 499/83 (H5N3)		Am-non GsGD	−	.	.	.	E	.	T	.	.	.	.	.	V	.

[a] (+) = HIT-positive to the 3B5.1 and 3B5.2 mAbs
(−) = HIT-negative to the 3B5.1 and 3B5.2 mAbs (HI titers <10)

Table 5 Conservation of the HA1 epitope at positions 158–170 among the divergent clades of the H5N1 viruses.

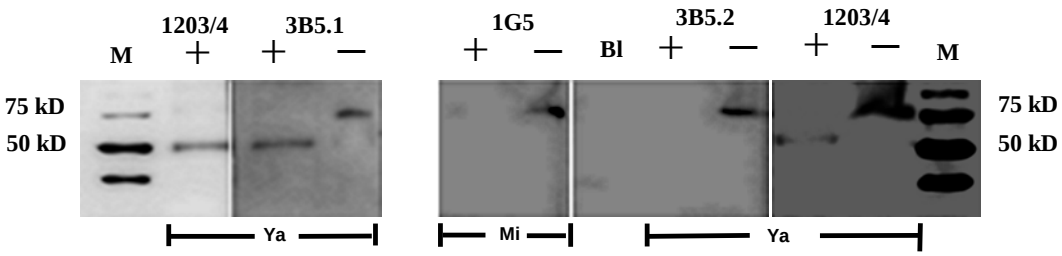
Clades	Number of strains (5,366)	Percentage homology of amino acid residues in HA1 protein***												
		P158	T159	I160	K161	R(K)162	S163	Y164	N165	N166	T167	N168	Q169	E170
0	141	100	99.3	99.3	100	100	100	100	100	100	97.9	100	99.3	100
1	622	99.7	99.7	100	100	99.8	99.8	100	99.8	100	100	100	99.5	100
1.1	68	100	100	100	100	100	100	100	100	100	100	95.6	100	100
1.1.1	25	100	100	100	92	100	100	100	100	100	100	80(D)*	100	100
1.1.2	124	100	96.8	100	100	100	100	100	100	100	100	98.4	99.2	100
2.1.1	66	100	100	100	100	100	100	100	100	100	100	100	100	100
2.1.2	39	100	97.4	100	100	100	100	100	100	100	100	100	100	100
2.1.3	38	100	100	100	100	100	100	100	100	100	100	97.4(E)*	100	100
2.1.3.1	29	100	93.1	100	100	100	69**	100	100	100	100	100	100	100
2.1.3.2	305	100	95.1	100	99.3	98.4	97.1	100	91.8	100	99.3	100	99.7	100
2.1.3.2a	55	100	96.4	100	100	96.4	100(T)*	100	94.6	100	100	100	100	100
2.1.3.2b	69	100	98.6	100	100	100	98.6(T)*	100	100	100	100	97.1	100	100
2.1.3.3	24	100	100	100	100	100	100	100	100	100	100	100	100	100
2.2	609	100	99.5	100	100	91	100	100	100	100	100	100	99.8	100
2.2.1	476	99.8	100	100	100	89.7**	99.4	100	99.8	100	100	99.8	99.6	100
2.2.1.1	95	100	88.4**	100	79**	93.7	90.5	96.8	96.8(H)*	100	97.9	100	92.6	100
2.2.1.1a	73	100	90.4	100	100	78.1(E)*	98.6	100	100 (H)*	100	97.3	100	97.3	100
2.2.1.2	343	99.1	100	100	100	99.4	100	100	99.7	99.7	99.7	100	99.7	100
2.2.2	67	100	100	100	100	100 (I)*	100	100	100	100	100	100	100	100
2.2.2.1	56	100	100	100	100	89.3(I)*	96.4	100	100	100	100	96.4	100	100
2.3.1	18	100	100	100	100	61.1(I)*	94.4	100	100	100	100	100	100	100
2.3.2	104	100	100	94.2	100	100	100	100	100	100	100	100	100	100
2.3.2.1	69	100	100	100	100	100	100	100	100	100	100	100	63.8**	100
2.3.2.1a	264	100	98.1	100	98.1	100	77.3**	100	99.6	100	99.2	100	99.6	100
2.3.2.1b	35	100	100(I)*	100	85.7**	100	97.1	100	71.4**	100	100	100	94.3(K)*	100
2.3.2.1c	521	99.8	98.8	100	99.4	100	91.2(G)*	100	100	100	98.3	100	60.7(R)*	99.8
2.3.3	30	100	100	100	100	100	100	100	100	100	100	100	100	100
2.3.4	512	100	99.8	100	99	94	95.7	99.8	99.8	100	100	98.4	92	100
2.3.4.1	28	100	100	100	100	100	96.4	100	100	100	100	53.6(S)*	100	100
2.3.4.2	45	100	100	100	100	100	100	100	100	100	100	93.3	100	97.8
2.3.4.3	84	100	100	100	100	100	100	100	100	100	100	100	98.8	98.8
2.3.4.4	32	100	100	100	100	46.9(I)*	100	100	100	100	100	100	90.6(R)*	100
2.4	22	100	100	100	95.5	95.5	100	100	100	100	100	100	100	100
2.5	20	100	95	100	100	95	100	100	100	100	100	100	100	100
3	56	100	100	100	100	100	100	100	98.2	100	100	100	100	100
4	26	100	100	100	100	96.2	96.2	100	100	100	100	100	100	100
5	28	100	100	100	100	100	100	100	100	100	100	53.6**	100	100
6	15	100	100	100	100	100	93.3	100	100	100	100	100	86.7**	93.3
7	46	100	69.6**	100	100	76.1**	67.4**	100	73.9**	100	91.3	100	100	100
7.1	18	100	100 (P)*	100	100	94.4(V)*	94.4(N)*	100	100(T)*	100	100	100	100	100
7.2	22	100	95.5(P)*	100	100	100 (V)*	100(N)*	100	100(T)*	100	100(A)*	95.5	100	55.6(K)*
8	4	100	100	100	100	100	100	100	75**	100	100	100	100	100
9	43	100	100	100	100	100	100	100	100	100	100	100	100	100

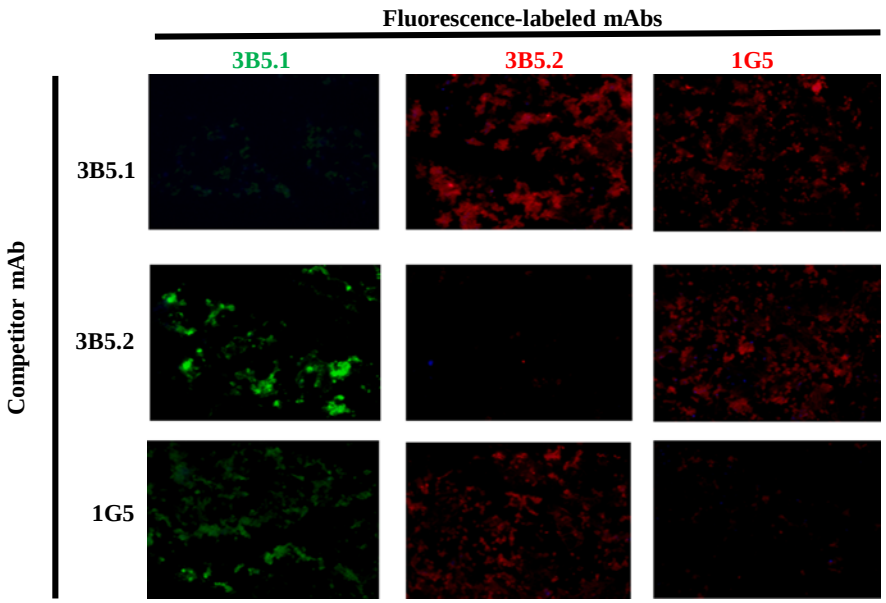
*= Amino acid residues were different and shown in parentheses.
**=Percentage of the conserved amino acid residues was less than 90.
***=The major amino acid residues were shown.

A

Immunogens (Viruses)	mAbs	Isotypes	HIT* HI titers	VNT* Neutralizing index
Ck/Yamaguchi/7/04 Clade 2.5	3B5.1	IgG1	2,560	>7
	3B5.2	IgG1	640	6
Ck/Miyazaki/K11/07 Clade 2.2	1G5	IgG1	320	3.75

B

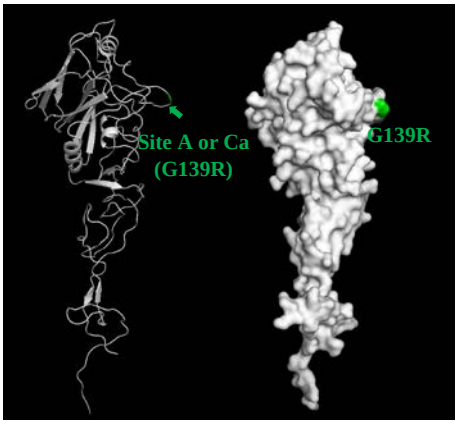



Fluorescence-labeled mAbs
3B5.1
3B5.2
1G5
Competitor mAb
3B5.1
3B5.2
1G5

A

Escape mutant	mAbs	HIT	Mutation in the HA1 protein			
			Position of nucleotide	Nucleotide change	Position of amino acid	Amino acid change
3B5.1 mt*	3B5.1	Negative	167	G to A	56	S to N
		(<1:10)	485	G to T	162	R to I
3B5.2 mt*	3B5.2	Negative	484	A to G	162	R to G
		(<1:10)	551	C to G	184	A to G
1G5 mt**	1G5	Negative	415	G to A	139	G to R
		(<1:10)				

C



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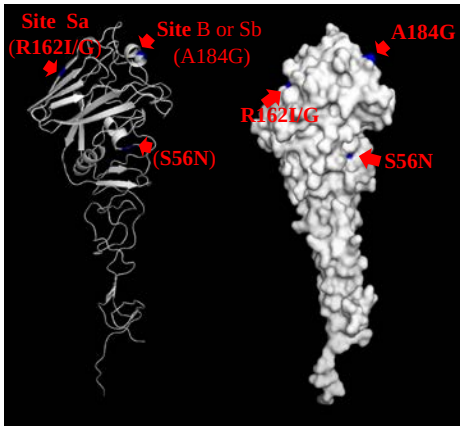

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