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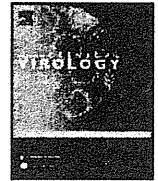
Virology Question and Answer Scheme (VIROQAS)

A 15-month-old boy with reduced consciousness and convulsion

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1. Case description

A previously healthy 15-month-old boy was admitted to our hospital with fever, reduced consciousness, and convulsion. He was the first child of unrelated healthy parents. None of his family had had a febrile illness for at least 1 month before his admission. He also had a generalized convulsion lasting for 3 min on the day of admission. He was transferred to our hospital because a local pediatrician noticed mildly decreased responsiveness.

On admission, the patient had a temperature of 40.6 °C, heart rate of 148 bpm, respiration rate of 52/min, and oxygen saturation of 98% in room air. Physical examination was unremarkable except for nuchal rigidity. Neurological examination revealed mild reduction of consciousness with a Glasgow Coma Scale score of 13. Deep tendon reflexes were normal. Laboratory examination showed hyponatremia (Na; 125 mequiv./l), whereas other hematological measures and blood chemistry were unremarkable. Serum amylase level was not measured. Cerebrospinal fluid (CSF) examination demonstrated marked pleocytosis of 868 cells/mm^3 (854 lymphocytes), with normal glucose and protein levels. Bacterial cultures from blood and CSF yielded no growth of pathogens. Head MRI on the second day of admission showed reduced diffusion in the splenium of the corpus callosum (Fig. 1). The patient was treated with steroid pulse therapy and intravenous gamma globulin. His consciousness did not recover until 4 days after admission. A reduction of diffusion in the splenium of the corpus callosum disappeared on MRI on the fifth day of admission (Fig. 1). He had no neurologic sequelae and had achieved normal psychomotor development at the last follow-up at 29 months of age.

FLAIR   DWI
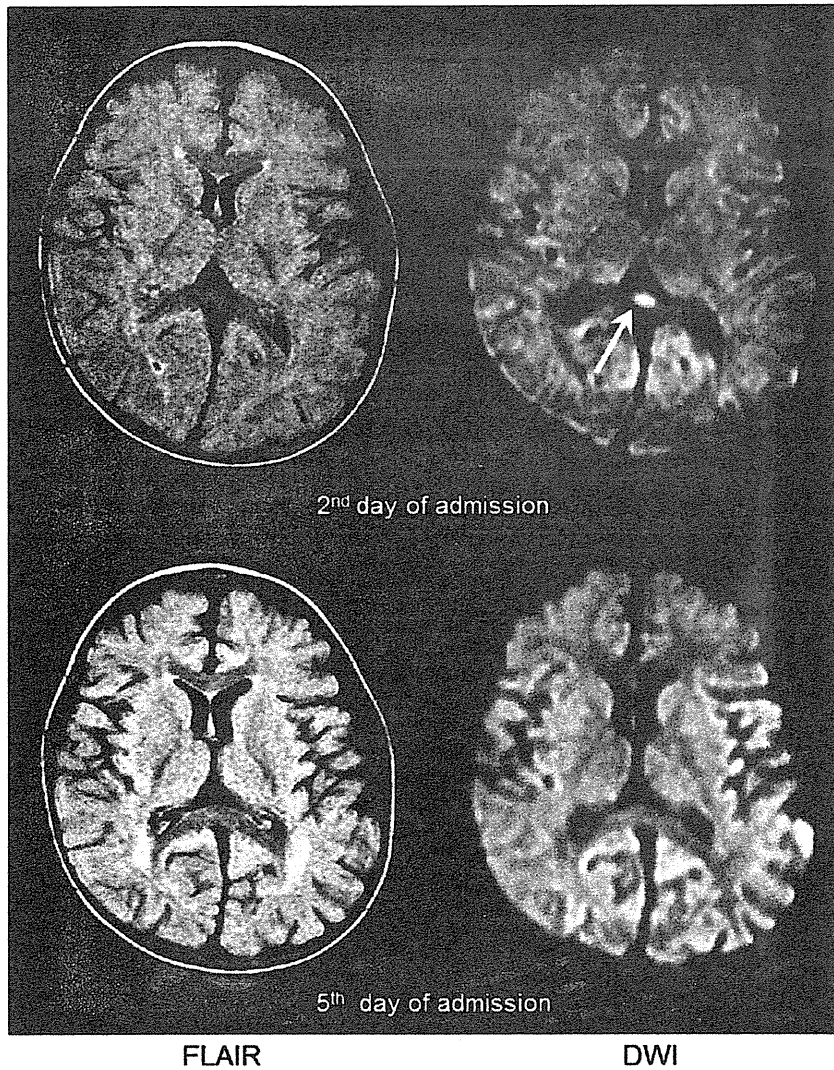
Fig. 1. MRI findings. Top: MRI on the second day of admission DWI revealed reduced diffusion in the splenium of the corpus callosum. This lesion is unclear on FLAIR. Bottom: MRI on the fifth day of admission. No abnormal findings were observed on FLAIR or DWI. FLAIR, fluid-attenuated inversion-recovery images; DWI, diffusion-weighted images.

What are the possible diagnoses?

How do you determine the cause of the disease?

See evidence-based opinion overleaf

Virology Question and Answer Scheme (VIROQAS)

Evidence-based opinion

What are the possible diagnoses?

A neuroimaging feature of our patient was transiently reduced diffusion in the splenium of the corpus callosum. Transient splenial lesions can be observed in various neurologic disorders including acute encephalitis/encephalopathy,[1] sudden withdrawal of antiepileptic drugs,[2] clustering seizures in infants,[3,4] and delirious behavior during febrile illness.[5,6] In our patient, mildly reduced consciousness, convulsion, and pleocytosis in association with fever were present. These symptoms strongly indicated acute encephalitis. Other conditions with transient splenial lesions were not compatible with our patient's symptoms. Thus, our patient was diagnosed as having mild encephalitis with reversible splenial lesion (MERS).

MERS is a recently established subtype of acute encephalitis/encephalopathy characterized by a reversible lesion with transiently reduced diffusion in the corpus callosum.[1] The neurologic symptoms in children with MERS are usually mild, including mildly reduced consciousness, delirious behavior, and brief convulsions. Hyponatremia is frequently observed in these children.[7] The outcome is usually excellent in patients with MERS. These clinical features are consistent with those of our patient. The

occurrence of MERS may be underestimated in countries outside Japan. The clinical manifestations of MERS are usually mild and transient, and disappear spontaneously.[8] The diagnosis will be difficult if diffusion-weighted images are not performed during an appropriate period.

How do you determine the cause of the disease?

In order to determine the cause of disease, interview on events within a few weeks before onset sometimes gives us an important information. The patient's mother stated that he had received mumps vaccine at a local clinic 20 days before admission. Pyrexia and vomiting appeared 17 days after vaccination. Thus, mumps virus was considered to be the most probable pathogen. However, involvement of the parotid or other salivary glands was not observed. Viruses other than mumps including influenza virus, enterovirus, or adenovirus could be the cause of MERS. Comprehensive virological examinations were necessary to determine the cause of MERS. For this purpose, virus isolation and polymerase chain reaction (PCR) are useful.

We first performed reverse transcription (RT)-PCR for mumps virus in the CSF on admission because we considered mumps virus the most likely possible pathogen. RNA extracted from the CSF of the patient and an extract from phosphate buffered saline as a negative control were reverse-transcribed and amplified with a mumps virus specific primer pair, which is designed
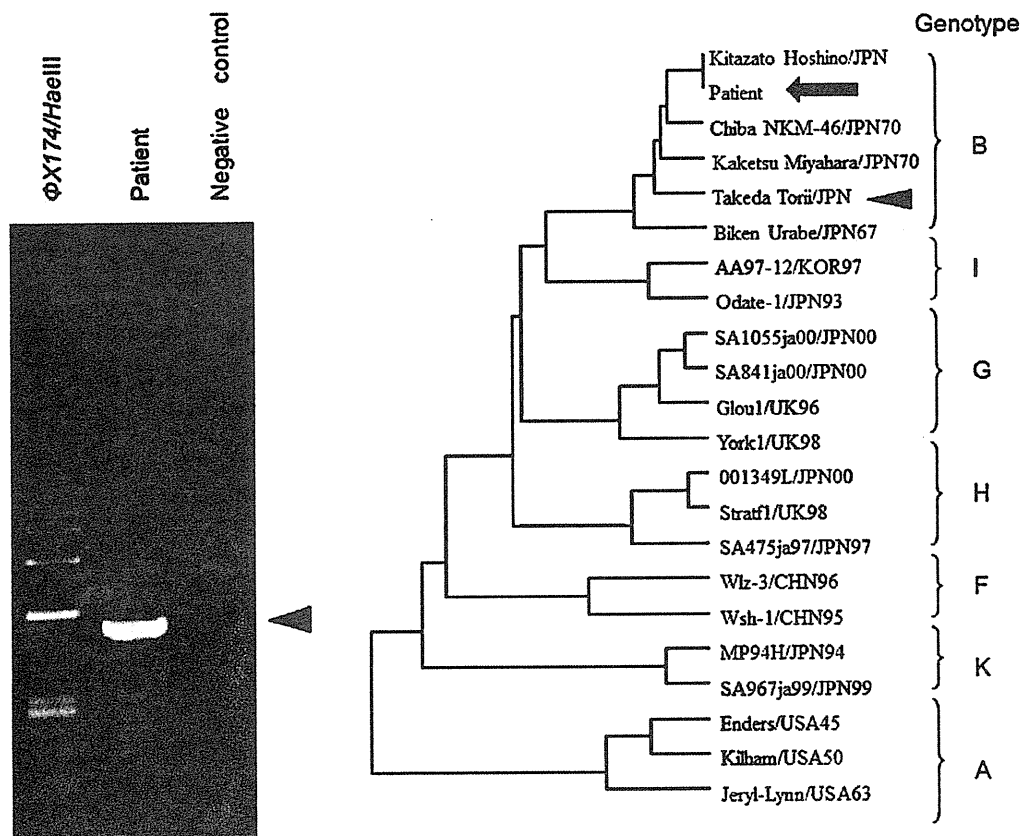
Fig. 2. Virological examinations. Left: Agarose gel electrophoresis. The specific DNA band with a length of 549 bp was amplified in the CSF of our patient (triangle), but no DNA was shown in the negative control. ΦX175 phage DNA digested with HaeIII was used as a DNA size marker. Right: Phylogenetic tree of the mumps virus strain calibrated by the bootstrap method. The SH sequence of the strain from the CSF of our patient was identical to that of the Hoshino vaccine strain (arrow). A triangle indicates the strain from the patient reported by Hara et al.[7] These two strains are categorized into genotype B.

to amplify the SH gene. The sequence of the primer pair was as follows, F/SH-F (5′-TCAAGTAGTGTCGATGATCTC-3′) and SH/HN-R (5′-AGGTGGCATTGTCTGACATTG-3′).[9] RT-PCR was performed using the Primescript one step RT-PCR kit (Takara Bio Inc., Shiga, Japan), according to the manufacturer's recommendations. The specific DNA band with a length of 549 bp was shown in the CSF sample, but none of the band was shown in the negative control (Fig. 2) by 1.5% (w/v) agarose gel electrophoresis. This indicated the presence of replicated mumps virus in the central nervous system.

It was important to determine whether mumps viral DNA from the patient originated from virulent field mumps virus or from vaccinated live attenuated mumps virus. Therefore, amplified DNA was sequenced, and the obtained sequence was compared with those downloaded from the DNA database. The mumps virus SH gene varies extensively from strain to strain and is therefore used in classification of mumps virus strains. Thirteen such groups (A to M) are referred as genotypes.[10,11] The SH sequence was identical to that of live attenuated vaccine, Hoshino strain, classified into genotype B (Fig. 2), but not to that of the pathogenic strains prevailing in the field, mainly classified into G or H. This patient is the second case of acute encephalopathy associated with mumps vaccination; the first case was related to mumps vaccine Torii strain.

Hara et al. reported an 8-year-old boy with MERS after mumps vaccination.[12] Mumps Torii vaccine strain was isolated from his CSF. The patient had transient hallucinations, nuchal rigidity, and hyponatremia. Head MRI showed symmetrical high intensities in the splenium of the corpus callosum and periventricular white matter on diffusion-weighted images. The clinical course and MRI findings were similar to those of our patient. This indicates that mumps vaccines used in Japan are possible causative agents of acute encephalitis. Although the outcome of the patients was favorable, we must be aware that acute encephalitis can be a rare complication of mumps vaccination and not just natural mumps virus infection.

Our patient did not show parotitis or involvement of the salivary glands, which are typical clinical symptoms of mumps virus infection. We must be aware that acute encephalitis/encephalopathy can occur after vaccination for mumps as an adverse effect, even without parotitis. Vigorous virological examinations should be considered when we encounter a patient with acute encephalitis/encephalopathy with onset a few weeks after mumps vaccination.

Competing interests

None of the authors has an association that may represent a current conflict of interest nor has identified any potential conflict of interest.

Ethical approval

The institutional review board of Juntendo University Faculty of Medicine had approved the study.

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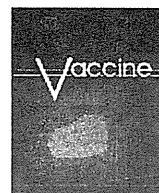
Vaccine

Differences in the priming effect of various clades/subclades of inactivated H5N1 vaccine for booster injection with heterologous clades of vaccine strains

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ABSTRACT

The prime–boost response induced by different combinations of four H5N1 vaccines (NIBRG-14 (clade 1), Indo05/2005(H5N1)/PR8-IBCDC-RG2 (clade 2.1), A/Bar-Headed Goose/Qinhai Lake/1A/05 SJ163222 (clade 2.2), and Anhui01/2005(H5N1)-PR8-IBCDC-RG5 (clade 2.3.4)) was evaluated in mice. Clade 1-primed BALB/c mice showed a booster response to all of the other three H5N1 vaccines. Clade 2.2 vaccine was also a good priming vaccine. However, mice primed with clade 2.1 or clade 2.3.4 vaccine did not respond to booster injection with clade 1 vaccine, suggesting that priming might actually inhibit the booster response with some combinations of vaccines belonging to different clades. Analysis of the mechanism involved showed that lymphocytes from primed mice secreted comparable amounts of cytokines with any combination of priming and booster vaccines. Therefore, impairment of B cell immunity specific to certain booster strains may have been involved.

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1. Introduction

H5N1 influenza viruses have caused epidemics in bird populations throughout the world since 1997, and human infections transmitted from infected birds have continuously been reported in several Southeast Asian countries and Egypt. Although the number of human infections due to H5N1 influenza virus has been limited and no human-to-human transmission has been confirmed so far, the mortality rate is very high [1]. If the virus acquires the ability to be readily transmitted from human to human, it could lead to a pandemic. Therefore, H5N1 is considered to be a candidate virus for causing a possible pandemic. Although we have recently experienced a pandemic caused by H1N1 2009 virus, the fear of H5N1 virus has not diminished.

H5N1 influenza viruses are classified into 10 clades and several subclades, and 11 human vaccine strains have been selected by the WHO as part of the preparations for an influenza pandemic [2]. Although multiple vaccine strains have been prepared as a safeguard, it remains difficult to predict which influenza virus will actually cause a pandemic. In general, manufacturing an influenza vaccine takes longer than 4 months, so it is too late to start production after a pandemic strain has been identified. Under these circumstances, pre-pandemic vaccination is one of the possible solutions [3,4].

Pre-pandemic vaccination has two advantages. First, if the antigenic difference between the pre-pandemic vaccine strain and the pandemic viral strain is small, cross-protection can be expected against infection with the pandemic virus. Such cross-protection has been demonstrated in several animal studies [5–8]. Second, even if antigenic differences between the pre-pandemic vaccine strain and the pandemic viral strain are more important, a prime–boost effect could be expected upon administration of the pandemic vaccine that would lead to a rapid and enhanced antibody response against the pandemic virus. We have already reported the prime–boost effect of alum-adjuvanted whole H5N1 vaccines in mice [9], where priming with NIBRG-14 (clade 1) significantly enhanced the booster response induced by the antigenically heterologous clade of Indo05/2005(H5N1)/PR8-IBCDC-RG2 (clade 2.1). The clinical studies have also demonstrated that the same combination of the priming vaccine and the booster vaccine elicited an effective antibody response in humans [10–12].

A peculiar immunological phenomenon has been noted in humans infected with a seasonal influenza virus in which re-infection of immunologically primed individuals with a new variant induces antibodies that are predominantly specific for the earlier variant rather than the new one. This phenomenon is known

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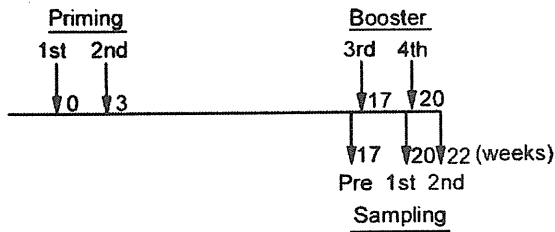E-mail address: ikeno-da@kaketsuken.or.jp (D. Ikeno).

Fig. 1. Vaccination and blood sampling schedule of Table 1.

as Original Antigenic Sin (OAS) [13–15]. It is unclear whether or not an OAS-like phenomenon occurs in a prime–boost regimen other than the combination of clade 1 for priming and clade 2 as the booster. Therefore, we investigated several different prime–boost regimens in a mouse model, particularly combinations other than clade 1 for priming and clade 2 as the booster.

2. Methods

2.1. Vaccine strains

NIBRG-14, Indo/05/2005(H5N1)/PR8-IBCDC-RG2, A/Bar-headed goose/Qinghai Lake/1A/05 SJ163222, and Anhui01/2005(H5N1)-PR8-IBCDC-RG5 were the vaccine strains used. These 4 clades/subclades of H5N1 have been stockpiled in Japan. NIBRG-14 is one of the vaccine reference strains, which was attenuated by reverse genetic engineering from the A/Vietnam/1194/2004(H5N1) virus (clade 1) by the UK National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). Indo/05/2005(H5N1)/PR8-IBCDC-RG2 (Indo05) and Anhui01/2005(H5N1)-PR8-IBCDC-RG5 (Anhui01) are attenuated vaccine strains derived from the A/Indonesia/5/2005(H5N1) virus (clade 2.1) or the A/Anhui/01/2005 (H5N1) virus (clade 2.3.4), respectively, by the Centers for Disease Control and Prevention (CDC, Atlanta, USA). A/bar-headed goose/Qinghai Lake/1A/05 SJ163222 (Qinghai1A) is an attenuated vaccine strain derived from A/bar-headed goose Qinghai Lake/1A/05 by St. Jude Children's Research Hospital (Tennessee, USA) [2].

The vaccine seed viruses were cultured in embryonated hen eggs, purified from allantoic fluid by zonal centrifugation, and then inactivated with formalin to prepare the inactivated whole virion. The vaccine was formulated by adding aluminum hydroxide (final concentration: 0.3 mg/ml) as the adjuvant. The formulation of the vaccine is the same as the Japanese H5N1 vaccine [16,17]. The HA concentration was determined by SDS-PAGE/densitometry analysis, which method is validated [16].

2.2. Priming effect of the alum adjuvanted whole vaccine to the other clades of vaccine

Specific-pathogen-free female BALB/c mice aged 6–8 weeks (Japan SLC, Inc.) were used in all experiments. The protocol for these animal experiments was approved by the animal experimentation ethical committee of Kaketsuken (Kumamoto, Japan).

An outline of the experiment schedule is shown in Fig. 1. Mice were primed by two intramuscular injections at a 3-week interval, each containing 0.2 μg HA/dose of H5N1 whole-virion antigen (NIBRG-14, Indo05, Anhui01, or Qinghai1A) with 0.03 mg of alum adjuvant (injection volume: 0.1 ml). Control mice were injected with alum-containing PBS. Each group of mice comprised 7 or 8 animals. Four months after priming, the mice

were boosted by injecting 0.2 μg HA/dose of H5N1 whole-virion antigen with 0.03 mg of alum adjuvant (injection volume: 0.1 ml). The strain of the booster vaccine was different from that of the priming vaccine to achieve heterologous combinations. Serum samples were obtained before the booster injection (pre), before the 2nd booster injection (1st), and 14 days after the 2nd booster injection (2nd). The antibody responses to both primed and booster strains were measured by hemagglutination-inhibition (HI) assay and micro-neutralization test as described previously [9,16].

2.3. ELISPOT assay

ELISPOT (Enzyme-Linked Immunosorbent SPOT) assays were performed using mouse IFN-γ (InterFeroN-γ) and IL-4 (InterLeukine-4) ELISPOT kits (Mabtech AB, Nacka, Sweden). BALB/c mice were immunized twice intramuscularly at a 3-week interval with 0.2 μg HA/dose of H5N1 whole-virion antigen (NIBRG-14, Indo05, or Anhui01) plus 0.03 mg of alum adjuvant (injection volume: 0.1 ml). Control mice were injected with alum-containing PBS. Each group consisted of eight mice. Single-cell suspensions of spleen cells from all mice in each group were prepared at 14 weeks after the second injection, pooled, and dispensed at 3×10^5 cells/well into polyvinylidene difluoride-coated 96-well plates for incubation with anti-IFN-γ or anti-IL4. The cells were exposed to 0.1 μg HA of each H5N1 whole antigen as the plates were incubated overnight at 37 °C, and then the cells were discarded. After washing with PBS, INF-γ and IL-4 were detected by incubation with biotinylated antibodies for these cytokines, followed by addition of streptavidin-alkaline phosphatase and development with BCIP/NBT substrate solution. The number of spots for INF-γ or IL-4 was counted by an automated ELISPOT reader (AID, Strassberg, Germany).

2.4. Transfer of antiserum to naïve mice

To prepare antisera, BALB/c mice were injected twice at a 3-week interval intramuscularly with 0.2 μg HA/dose of H5N1 whole-virion antigen (NIBRG-14 or Indo05) and 0.03 mg of alum adjuvant. The anti-NIBRG-14 and anti-Indo05 antisera were harvested at 2 weeks after the second immunization and pooled.

For treatment with the antiserum, BALB/c mice were divided into 3 groups. Group 1 received intraperitoneal injection of 0.2 ml of antiserum at one day before the 1st vaccination. (The volume of antiserum was determined by preliminary experiments. We examined 2 different serum volumes (1 ml, 0.2 ml). The results of the 1 ml and 0.2 ml volumes were approximately the same, so we chose 0.2 ml/dose because it decreased the numbers of the mice.) Subsequently, the mice were immunized twice at a 3-week interval by intramuscular injection of 0.2 μg HA/dose of heterologous antigen with 0.03 mg of alum adjuvant (mice given anti-NIBRG-14 serum were immunized with the Indo05 vaccine and vice versa, injection volume: 0.1 ml.). Group 2 mice were actively immunized as in group 1, but the antiserum was injected at one day before the 2nd vaccination. Group 3 mice were treated as in group 1, but were injected with normal BALB/c serum (control). All groups were sacrificed on Day 35 and serum samples were evaluated by the HI assay (against the vaccine strain) described previously [9].

2.5. Statistics

HI titers were transformed into $\log_{10}$ values for calculation of the geometric mean titer (GMT) and 95%CL at every time of assessment. All data manipulations and statistical computations were done with Microsoft Excel software (version 2002).

3. Results

3.1. Priming effect of alum-adjuvanted whole-virion vaccines on the booster response to other clades/subclades with different antigenicity

We investigated the priming effect of alum-adjuvanted whole-virion vaccines on the recall response evoked by boosting with vaccines from different clades/subclades. Four months after priming, most of the mice had high levels of HI antibody for the homologous strain, but had lower levels of cross-reactive antibodies for the heterologous clades/subclade strains (Table 1A).

When mice were primed with the NIBRG-14 strain and boosted with the Indo05 or Anhui01 strains, HI antibodies to the booster strain as well as the priming strain were elevated after one or two booster vaccinations, compared with the HI titer of unprimed animals (shown as PBS). When Indo05 and Anhui01 were used for the priming and booster injections, respectively, as well as with the reverse combination, the HI antibody response to each strain also increased after booster immunization, although it was more specific for Indo05 and Anhui01. Unexpectedly, the combination of priming with Indo05 or Anhui01 and boosting with NIBRG-14 resulted in low titers of HI antibody to NIBRG-14 even after two booster injections. Compared with the response of unprimed mice, priming with Indo05 or Anhui01 actually suppressed the booster response to NIBRG-14. Similar results were obtained with the neutralizing antibody test (data not shown).

The prime–boost effect of the Qinghai1A vaccine was also investigated (Table 1B). Four months after priming and before booster immunization, mice retained a high titer of HI antibodies for the homologous Qinghai1A but had lower levels of antibodies cross-reacting with the heterologous booster strain, NIBRG-14 and Anhui01. Cross-reactivity between the Indo05 and Qinghai1A strains was relatively higher than with the other combinations. After one or two booster doses, HI antibody response to both the priming and booster strains increased.

3.2. Cellular immune responses of mice primed with different H5N1 strains

To investigate the mechanisms that enhanced (or suppressed) the booster response, the helper T cell activity of mice immunized with different clade/subclade strains was compared by investigating cytokine secretion from lymphocytes (Fig. 2). Lymphocytes from mice primed with any of the vaccine strains produced a similar amount of IFN-γ and IL-4 after stimulation with a combination of homologous and heterologous antigens. On the other hand, unprimed mice did not produce these cytokines. Cytokine secretion was not impaired by priming with Indo05 or Anhui01 followed by the booster with NIBRG-14 which led to failure of the booster response.

3.3. Effect of pre-existing antibody on active immunization with antigenically different strains

As a possible mechanism of OAS, Lambert et al. proposed antibody-mediated inhibition of the naïve B cell response to specific epitopes of the new strain [18]. Therefore, we investigated whether pre-existing antibodies could affect the immune response by injection of anti-H5N1 serum before active immunization with different clades/subclades (Table 2).

The HI antibody response of mice injected with the antiserum before the 1st vaccination (Group 1) was suppressed compared with the control mice injected with normal mouse serum (Group 3). There was no difference in the suppressive effect of anti-NIBRG antiserum and anti-Indo05 antiserum. On the other hand, the HI
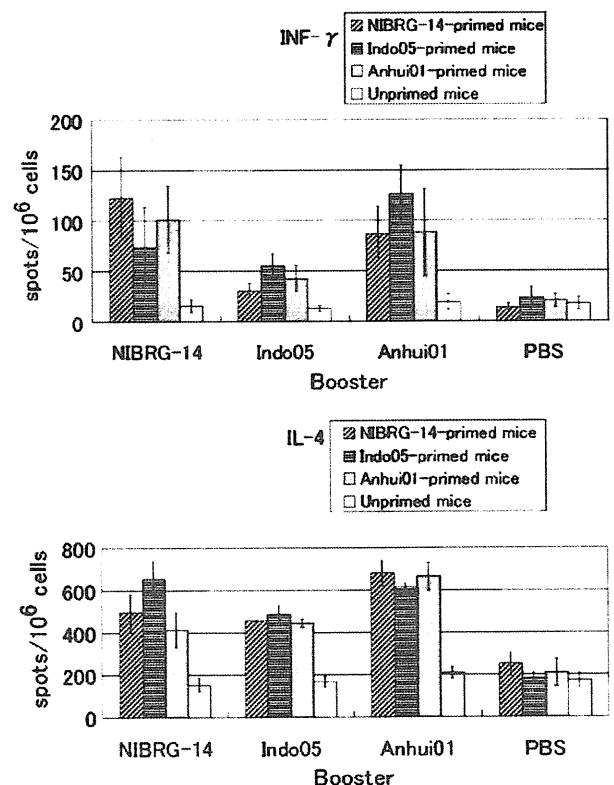

Fig. 2. BALB/c mice were injected twice intramuscularly with 0.2 μg HA/dose of an H5N1 strain plus 0.03 mg of alum adjuvant at a 3-week interval. As a control, mice were injected with alum-containing PBS alone. Spleen cells were harvested at 14 weeks after the second injection, and dispensed into polyvinylidene difluoride-coated 96-well plates with anti-IFN-γ or anti-IL4 at 3 × 10⁵ cells/well. Then cells were stimulated with 0.1 μg HA of each H5N1 whole-virion vaccine. The plates were incubated overnight at 37 °C and the number of spots was counted (spots/10⁶ cells, 95% CL).

response of mice injected with the antiserum before the 2nd vaccination (Group 2) was not impaired and was comparable to that of the control mice (Group 3).

4. Discussion

We evaluated the priming effect of four H5N1 vaccines belonging to different clades/subclades on booster vaccination with heterologous combinations. NIBRG-14 (clade 1)-primed BALB/c mice developed an enhanced cross-clade booster response after injection of any of the vaccines tested. Qinghai1A (clade 2.2)-vaccination also induced a similar priming effect. In contrast, mice primed with Indo05 (clade 2.1) or Anhui01 (clade 2.3.4) did not respond to booster vaccination with NIBRG-14 and no enhancement of secondary antibody response to both the homologous and heterologous vaccines was observed. The successful prime–boost regimen employing NIBRG-14 (priming) and Indo05 (booster) confirmed the results of our previous study [9]. In addition, prime–boost responses were confirmed with some combinations of different clade 2 vaccines, but we did not expect to find failure of the response with other combinations.

The mechanism leading to such failure is uncertain, but two possibilities can be suggested: (1) failure of Th cell responses or (2) failure of B cell responses. ELISPOT assay showed that spleen cells from primed BALB/c mice produced similar amounts of cytokines irrespective of the combination of priming and boosting antigens, including combinations that led to inhibition of the booster response (Fig. 2). These data showed that helper T cell function

Table 1
Priming effect of alum-adjuvanted whole-virion vaccines for heterologous booster vaccines.

Priming	Booster	HI titer against NIBRG-14			HI titer against Indo05			HI titer against Anhui01		
		Pre	1st	2nd	Pre	1st	2nd	Pre	1st	2nd
(A) Prime–boost responses among the NIBRG-14, Indo05, and Anhui01 strains										
Indo05	NIBRG-14	29.7 (16.5–53.6)	23.8 (14.4–39.2)	32.8 (18.5–58.3)	144.9 (76.0–276.4)	226.3 (91.4–560.0)	226.3 (108.0–474.2)	33.6 (13.8–82.2)	80.0 (36.0–177.9)	59.4 (22.4–158.0)
Anhui01		28.3 (16.8–47.7)	13.0 (6.2–27.1)	25.9 (10.5–64.0)	25.9 (11.1–60.7)	28.3 (9.5–84.1)	36.7 (13.2–101.9)	95.1 (46.1–196.3)	174.5 (86.3–352.9)	160.0 (68.1–376.0)
PBS		5.0 (5.0–5.0)	10.9 (7.6–15.7)	95.1 (32.4–279.8)	5.0 (5.0–5.0)	5.0 (5.0–5.0)	10.9 (5.1–23.5)	5.0 (5.0–5.0)	5.9 (4.0–8.9)	21.8 (9.1–52.4)
NIBRG-14	Indo05	207.5 (113.9–377.9)	1660.0 (584.6–4713.6)	1810.2 (635.7–5154.3)	25.9 (13.3–50.7)	246.8 (68.7–886.9)	349.0 (138.0–882.7)	33.6 (13.1–86.4)	349.0 (110.7–1099.6)	415.0 (160.1–1075.4)
Anhui01		30.8 (18.4–51.8)	11.9 (6.6–21.3)	8.2 (4.2–16.1)	16.8 (11.3–25.1)	123.4 (55.7–273.4)	160.0 (72.0–355.8)	95.1 (63.8–141.9)	380.5 (212.0–683.0)	452.5 (247.3–828.0)
PBS		5.0 (5.0–5.0)	6.5 (4.3–9.9)	11.2 (5.2–24.1)	5.0 (5.0–5.0)	28.3 (16.8–47.7)	269.1 (180.4–401.3)	5.0 (5.0–5.0)	10.9 (6.2–19.1)	103.7 (46.8–229.9)
NIBRG-14	Anhui01	190.3 (127.6–283.7)	1076.3 (419.1–2764.3)	1280.0 (575.6–2846.4)	28.3 (15.5–51.8)	146.7 (48.5–444.0)	246.8 (105.4–577.9)	21.8 (10.8–44.1)	207.5 (70.0–615.0)	415.0 (198.8–866.4)
Indo05		23.8 (13.3–42.7)	18.3 (10.5–32.1)	10.9 (6.2–19.1)	146.7 (61.0–352.8)	452.5 (203.5–1006.4)	640.0 (305.4–1341.3)	43.6 (21.6–88.2)	246.8 (111.4–546.8)	293.4 (145.1–593.5)
PBS		5.0 (5.0–5.0)	7.1 (4.2–12.8)	10.0 (2.5–39.9)	5.0 (5.0–5.0)	5.0 (5.0–5.0)	8.4 (5.1–13.9)	5.0 (5.0–5.0)	13.0 (7.7–21.8)	80.0 (47.4–135.0)

Priming	Booster	HI titer against booster strain			HI titer against priming strain		
		Pre	1st	2nd	Pre	1st	2nd
(B) Prime-boost responses between Qinghai1A and the other three strains as boosters							
Qinhai1A	NIBRG-14	21.8 (7.9–60.6)	174.5 (92.3–329.7)	160.0 (79.6–321.4)	640.0 (379.3–1079.9)	2347.5 (1634.3–3372.1)	1413.2 (820.6–2433.8)
PBS		5.0 (5.0–5.0)	21.8 (13.6–35.0)	226.3 (123.7–414.0)	-ᵃ	-ᵃ	-ᵃ
Qinhai1A	Indo05	113.1 (57.6–222.3)	293.4 (204.3–421.5)	246.8 (147.0–414.1)	586.9 (408.6–843.0)	1280.0 (903.1–1814.2)	1076.3 (721.8–1605.1)
PBS		5.0 (5.0–5.0)	36.7 (16.1–83.6)	246.8 (111.4–546.8)	-ᵃ	-ᵃ	-ᵃ
Qinhai1A	Anhui01	30.8 (13.2–72.2)	452.5 (295.2–693.7)	349.0 (217.8–559.2)	538.2 (326.1–888.1)	1660.0 (1090.2–2527.6)	1660.0 (911.5–3022.8)
PBS		5.0 (5.0–5.0)	26.9 (13.6–53.4)	80.0 (48.8–131.0)	-ᵃ	-ᵃ	-ᵃ
NIBRG-14	Qinhai1A	67.3 (29.0–156.0)	452.5 (268.2–763.6)	452.5 (295.2–693.7)	415.0 (227.9–755.7)	905.1 (536.4–1527.3)	905.1 (536.4–1527.3)
Indo05		146.7 (68.2–315.7)	697.9 (369.4–1318.7)	538.2 (260.8–1110.4)	452.5 (268.2–763.6)	697.9 (369.4–1318.7)	452.5 (203.5–1006.4)
Anhui01		14.1 (5.4–36.8)	103.7 (33.6–320.4)	269.1 (99.9–724.5)	207.5 (106.0–406.0)	987.0 (588.1–1656.4)	761.1 (328.3–1764.6)
PBS		5.0 (5.0–5.0)	73.4 (41.9–128.4)	190.3 (106.0–341.5)	-ᵃ	-ᵃ	-ᵃ

Priming vaccines were injected into BALB/c mice twice at a three-week interval. After 4 months, booster vaccines were injected twice at a three-week interval (A: Prime–boost responses among the NIBRG-14, Indo05, and Anhui01 strains, B: Priming with Qinghai1A and use of the other 3 strains as boosters). HI antibody titers for booster and priming strains (GMT, 95% CL) were measured before the first booster vaccination (Pre), two weeks after the first booster vaccination (1st), and two weeks after the second booster vaccination (2nd). All vaccines were given at are 0.2 μg HA/dose. The grey indicates the HI titer against booster strain, which is the most important results.

ᵃ Not tested because animals were primed with PBS (control).

Table 2
Effect of pre-existing antibodies on active immunization for antigenically different strains.

Vaccine	Antiserum	Group 1 (antiserum: before the 1st vaccine)	Group 2 (antiserum: before the 2nd vaccine)	Group 3 (normal serum: before the 1st vaccine)
NIBRG-14	Indo05	13.2 (6.5–26.9)	121.3 (48.9–300.8)	69.6 (24.6–196.8)
Indo05	NIBRG-14	8.7 (2.1–36.2)	242.5 (57.0–1031.3)	320.0 (120.6–849.2)

Antiserum was obtained from mice after 2 intramuscular injections of 0.2 mg HA/dose of antigen with 0.03 mg of alum adjuvant at a 3-week interval. All group mice were immunized twice with at a 3-week interval by intramuscular injection of 0.2 mg HA/dose of heterologous antigen with 0.03 mg of alum adjuvant. Group 1 mice were injected intraperitoneally with 0.2 ml of antiserum (anti-NIBRG-14 or anti-Indo05) at one day before the 1st vaccination. Group 2 mice were injected with antiserum before the 2nd vaccination. Group 3 mice were treated as for group 1, but received normal serum (control). All groups were sacrificed on Day 35 and serum was harvested, then HI antibody titers for vaccine strains (GMT, 95%CL) were measured.

was normal even when there was failure of the prime–boost response. Therefore, impairment of B cell immunity was suggested.

As a possible B cell-related mechanism of OAS, Kim et al. proposed that OAS may occur due to competition for common epitopes between Ag-specific memory and naïve B cells [19]. Alternatively, Lambert et al. proposed that pre-existing cross-reactive antibodies may inhibit the activation of naïve B cells. Our experiments are in accordance with the proposal of Lambert et al. because anti-H5N1 antiserum inhibited the antibody response when injected before primary vaccination suggesting that antibody-mediated inhibition of naïve B cells is one of the mechanisms leading to failure of the prime–boost effect (Table 2). However, the reason why the prime–boost regimen was successful in other combinations is unclear. As shown in Table 1, antibodies for Indo05, Anhui01, and Qinghai1A had little or modest cross-reactivity with NIBRG-14. Therefore, the antibodies present after booster injection of Indo05, Anhui01, or Qinghai1A into NIBRG-14-primed mice should have been specific to NIBRG-14. However, in fact antibodies specific to strains other than NIBRG-14 were also present, so there might be a mechanism that overcomes antibody-mediated inhibition of naïve B cell activation, e.g., naïve B cells could be activated by direct binding of influenza virus to cell surface sialic acid irrespective of their B cell receptor's specificity [20], or very small populations of cross-reactive memory B cells might be generated by primary vaccination.

Although we found an OAS-like phenomenon after H5N1 vaccination in mice, other groups have reported that cell culture (Vero)-derived whole virus (H5N1) vaccines based on wild-type strains could elicit heterologous prime–boost reactions in CD1 mice (priming: clade 2.1, booster: clade 1) [21]. Therefore, the prime–boost response may be dependent on the vaccine formulation or the genetic background of vaccinated hosts. In a human study, it was shown that an AS03-adjuvanted split vaccine (clade 1) induced an increased HI antibody response to a clade 2 booster vaccine while split vaccines without AS03 did not [11]. There has also been a similar report about MF59-adjuvanted vaccines [22]. One of the reasons for successful priming would be that adjuvanted H5N1 vaccines could stimulate a wide range of antibody repertoire and expand cross-reactivity to different clades/subclades in primary immunization [23,24]. Our results show that the antibody response was strongly suppressed only in the naïve mice and not in the primed mice by transferring cross-reactive antiserum. Therefore, the priming with such adjuvants to induce a broader cross-reactivity might overcome the OAS-like suppression. For the similar reasons, universal vaccines might contribute towards overcoming such OAS-like suppression [25].

Although we have recently experienced an influenza pandemic with H1N1 2009 virus, there are still many remaining issues regarding the vaccination program. As the next candidate for causing a pandemic, H5N1 virus still has great potential. The optimum prime–boost regimen, as well as other remaining issues, should be decided before any pandemic due to H5N1 occurs. In particular, prime–boost regimens with clades

2.1 or 2.3.4 for priming and clade 1 as the booster have not yet been investigated in humans. Therefore, it is urgent to confirm whether an OAS-like phenomenon occurs with human vaccination.

Acknowledgments

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Original Article

Newly Established Monoclonal Antibodies for Immunological Detection of H5N1 Influenza Virus

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SUMMARY: The H5N1 subtype of the highly pathogenic (HP) avian influenza virus has been recognized for its ability to cause serious pandemics among humans. In the present study, new monoclonal antibodies (mAbs) against viral proteins were established for the immunological detection of H5N1 influenza virus for research and diagnostic purposes. B-cell hybridomas were generated from mice that had been hyperimmunized with purified A/Vietnam/1194/2004 (NIBRG-14) virion that had been inactivated by UV-irradiation or formaldehyde. After screening over 4,000 hybridomas, eight H5N1-specific clones were selected. Six were specific for hemagglutinin (HA) and had in vitro neutralization activity. Of these, four were able to broadly detect all tested clades of the H5N1 strains. Five HA-specific mAbs detected denatured HA epitope(s) in Western blot analysis, and two detected HP influenza virus by immunofluorescence and immunohistochemistry. A highly sensitive antigen-capture sandwich ELISA system was established by combining mAbs with different specificities. In conclusion, these mAbs may be useful for rapid and specific diagnosis of H5N1 influenza. Therapeutically, they may have a role in antibody-based treatment of the disease.

INTRODUCTION

The highly pathogenic (HP) H5N1 avian influenza virus caused the first outbreak in humans in Hong Kong in 1997. This outbreak resulted in the infection of 18 people and resulted in six deaths (1,2). Thereafter, it was determined that H5N1 avian influenza virus was continuously circulated among geese in Southeastern China. Eventually, it spread to other Southeast Asian countries, where it severely damaged poultry farms (3,4). Subsequent H5N1 outbreaks in humans occurred in China and Vietnam in 2003 and in Indonesia in 2005. The most recent endemic has occurred in Egypt. According to a World Health Organization report, the H5N1 avian influenza virus had infected 565 people and resulted in 331 deaths by August 19, 2011 (5). Therefore, although sporadic, this fatal human infection is persistent and has the potential to cause serious future pandemics.

In humans, infection with HP H5N1 avian influenza virus causes high fever, coughing, shortness of breath, and radiological findings of pneumonia (6–8). In severe cases, rapidly progressive bilateral pneumonia develops, causing respiratory failure and may be responsible for the high mortality associated with this virus. de Jong et al. analyzed human cases of H5N1 infection and found that a high viral load and the resulting intense inflammatory response caused severe symptoms; furthermore, viral RNA was frequently detected in the rectum, blood, and nasopharynx (9). Thus, it is essential to detect HP influenza virus infection early and rapidly in order to provide early interventions that protect patients from devastating respiratory failure that arises from a high viral load. Additionally, early viral detection would facilitate rapid identification of infected patients and prevent unregulated contact with other people.

The present diagnostic standard for HP H5N1 influenza is the presence of the neutralization antibody. However, it takes more than 1 week for H5N1-specific antibodies to develop, and a well-equipped biosafety level 3 (BSL3) laboratory is required for the virus neutralization assay. A simpler method is the hemagglutination-inhibition assay using horse erythrocyte. This method has been widely performed on paired acute and convalescent sera from patients with HP H5N1 influenza virus infections. Although this method has acceptable sensitivity, its specificity has been questioned (7).

Isolating the virus from patient samples is the gold standard for diagnosing an infection; however, this is not always possible. For example, the method of sample preparation and preservation strongly influence the ability to isolate the virus. Moreover, a BSL3 laboratory is essential. At present, the most sensitive and rapid method for initial diagnosis of H5N1 virus infections is by conventional or real-time reverse-transcriptase polymerase chain reaction (RT-PCR). However, this proce-

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dure requires expertise in molecular virology and expensive equipment and reagents. Moreover, because of its high sequence specificity, this approach could fail to identify mutant influenza viruses that continually evolve due to a high mutation rate (8).

For screening suspected H5N1 influenza virus in the field, the ideal approach would be to employ an immunology-based technique that detects viral antigens. Such a method is simple and rapid. However, its sensitivity and specificity depend highly on the antibodies used. Thus, an immunological assay that uses appropriate specific antibodies against H5N1 in combination with specific antibodies against other subtypes of influenza virus or viruses that cause febrile diseases would be useful for screening in areas with endemic influenza-like illness. While there are several rapid influenza virus diagnostic systems available for seasonal influenza (10), few exist for H5N1 influenza. Therefore, we have developed a simple and rapid diagnostic system with high sensitivity and specificity for H5N1 influenza virus.

Influenza virus belongs to the family *Orthomyxoviridae*; its genome consists of a negative-sense, single-stranded RNA with eight segments, each encoding structural and non-structural proteins (11). Influenza A viruses are classified into several subtypes based on the hemagglutinin (HA) and neuraminidase (NA) serotypes. In total, there are 16 HA and 9 NA serotypes. The H5N1 viruses are divided into clades 1 and 2 based on their HA genotypes. Clade 2 has been further subdivided into five sub-clades (12). Clade 1 viruses were predominant in Vietnam, Thailand, and Cambodia in the early phase of the 2004–2005 outbreak, whereas clade 2.1 viruses were endemic in Indonesia at that time (8). These two viruses are the major prototypes for the preparation of prepandemic H5N1 vaccines. We used inactivated purified clade 1 virion [A/Vietnam/1194/2004 (NIBRG-14)] as an immunizing antigen to establish mouse monoclonal antibodies (mAbs) specific for H5N1 influenza virus. Characterization of these mAbs revealed that they could detect H5N1 viruses when used in an immunofluorescence staining assay (IFA), Western blotting analysis, immunohistochemistry, and antigen-capture sandwich ELISA. In addition, the mAbs had significant in vitro neutralization activity against H5N1 viruses, and some broadly detected both clade 1 and 2 viruses.

MATERIALS AND METHODS

Viruses and cell culture: The NIBRG-14 (H5N1) virus, which possesses modified HA and NA genes derived from the A/Vietnam/1194/2004 strain on the backbone of six internal genes of A/Puerto Rico/8/34 (PR8), was provided by the National Institute for Biological Standards and Controls (NIBSC; Potters Bar, UK). A/Indonesia/05/2005 (Indo5/PR-8-RG2), A/Turkey/1/2005 (NIBRG-23), A/Anhui/01/2005 (Anhui01/PR8-RG5) were also obtained from NIBSC. All non-H5N1 strains were obtained from a stockpile of seed vaccines of the Influenza Virus Research Center of the National Institute of Infectious Diseases. The live virus was manipulated in a BSL2 laboratory. To produce and purify the virion, the NIBRG-14 and PR8 viruses were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs and purified through a 10–50% discontinuous sucrose gradient by ultracentrifugation (13). The viruses were then resuspended in phosphate-buffered saline (PBS) and inactivated by ultraviolet (UV) irradiation or by treatment with 0.05% formalin at 4°C for 2 weeks. These preparations were served as the inactivated H5N1 virus fraction. These conditions have been previously shown to completely inactivate H5N1 viruses.

Production of mAbs: Nine-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were immunized subcutaneously with 20 μg of UV- or formaldehyde-inactivated NIBRG-14 (H5N1) virus using Freund's Complete Adjuvant (Sigma, St. Louis, Mo., USA). Two weeks later, the mice were boosted with a subcutaneous injection of 5 μg of the inactivated virus emulsified with Freund's Incomplete Adjuvant (Sigma). Three days after the boost, sera from the mice were tested by ELISA to determine the antibody titer against the NIBRG-14 virus. The three mice with the highest antibody titers were given an additional boost 14 days after the first boost by intravenous injection of 5 μg of the inactivated virus. Three days later, the spleens of these three mice were excised, and the spleen cells were fused with Sp2/O-Ag14 myeloma cells using the polyethylene glycol method of Kozbor and Roder (14). The fused cells were cultured on twenty 96-well plates and selected with hypoxiantine-aminopterin-thymidine (HAT) medium. The first screening was conducted by ELISA using formalin-inactivated purified NIBRG-14 (H5N1) and PR-8 (H1N1) virions, which were lysed with 1% Triton X100. The lysates (1 mg/ml) were diluted 2,000-fold with ELISA-coating buffer (50 mM sodium bicarbonate, pH 9.6), and the ELISA plates (Dynatech, Chantily, Va., USA) were coated at 4°C overnight. After blocking with 1% ovalbumin in PBS-Tween (10 mM phosphate buffer, 140 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h, the culture supernatants of the HAT-selected hybridomas were added and incubated for 1 h. After washing with PBS-Tween, the bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse IgG (1:2,000; Zymed, South San Francisco, Calif., USA) and *p*-nitrophenyl phosphate, which served as a substrate. In this first screening, hybridomas that reacted to the H5N1 virus (NIBRG-14) but not to the H1N1 virus (PR-8) were selected.

Baculoviral expression of recombinant HA and NA: Recombinant HA (rHA) and NA (rNA) proteins were produced as previously described (13). Briefly, the HA- and NA-coding genes of NIBRG-14 were amplified by PCR to attach a 6x-His tag to the C terminus of HA and to the N terminus of NA. The amplified DNAs were then cloned into pBacPAK8 (Clontech, Mountain View, Calif., USA) and transfected into Sf-21 (*Spodoptera frugiperda*) insect cells. Recombinant baculoviruses containing the rHA and rNA genes were isolated were used to infect Sf-21 cells. The recombinant proteins tagged with 6x-His were purified with TALON columns (Clontech) according to the manufacturer's protocol.

Neutralization assay: For the neutralization assay, 100 TCID$_{50}$ of H5N1 virus, a standard tissue culture infectious dose for such assays, was incubated for 30 min at 37°C in the presence or absence of the purified mAbs, which had been serially diluted twofold. The viruses

were then added to MDCK cell cultures that had been grown to confluence in a 96-well microtiter plate. The virus strains used were A/Vietnam/1194/2004 (NIBRG-14) (H5N1) (clade 1), A/Indonesia/05/2005 (Ind05/PR8-RG2) (H5N1) (clade 2.1), A/Turkey/1/2005 (NIBRG-23) (H5N1) (clade 2.2), and A/Anhui/01/2005 (Anhui01/PR8-RG5) (H5N1) (clade 2.3). After 3–5 days, the cells were fixed with 10% formaldehyde and stained with crystal violet to visualize the cytopathic effects induced by the virus (15). Neutralization antibody titers were expressed as the minimum concentration of purified immunoglobulin that inhibited a cytopathic effect.

Western blot analysis: UV-inactivated purified H5N1 virus (0.5 μg/lane) was loaded on SDS-PAGE gels under reducing conditions. The proteins were then transferred to a PVDF membrane (Genetics, Tokyo, Japan). After blocking with BlockAce reagent (Snow Brand Milk Products Co., Tokyo, Japan), the membranes were detected with the mAbs or diluted sera (1:1,000) that had been obtained from mice immunized with UV-irradiated H5N1 virus. After washing, the membrane was reacted with the peroxidase-conjugated F(ab')$_2$ fragment of anti-mouse IgG (H + L) (1:20,000; Jackson ImmunoResearch, West Grove, Pa., USA), and the bands were visualized on X-ray film (Kodak, Rochester, N.Y., USA) with chemiluminescent reagents (Amersham Biosciences, Piscataway, N.J., USA).

Purification and biotinylation of mAbs: Hybridomas were grown in Hybridoma-SFM medium (Invitrogen, Carlsbad, Calif., USA) supplemented with recombinant IL-6, penicillin (100 U/mL), and streptomycin (100 μg/mL) (16). The culture supernatants were harvested, and 1/100 volume of 1 M Tris-HCl (pH 7.4) and 1/500 volume of 10% NaN$_3$ were applied directly on a Protein G-Sepharose 6B column (Amersham Biosciences). The column was washed with PBS and eluted with glycine/HCl (pH 2.8). After measuring the OD$_{280}$ of the fractions, the protein-containing fractions were pooled, and an equal volume of saturated (NH$_4$)$_2$SO$_4$ was added. The precipitated proteins were dissolved in PBS, dialyzed against PBS, and stored at $-20\,°$C. The purified antibodies were biotinylated with sulfo-NHS-LC-biotin (Pierce, Rockford, Ill., USA) according to the manufacturer's protocol.

Antigen-capture ELISA: The purified antigen-capturing mAb was immobilized on a microplate (Immulon 2; Dynatech) by incubating 4 μg/mL of the mAb in 50 mM sodium bicarbonate buffer (pH 8.6) at 4°C overnight. The microplate was blocked with 1% BSA, washed with PBS-Tween, and reacted with serial dilutions of UV-inactivated purified H5N1 virus for 1 h at room temperature. After washing with PBS-Tween, biotinylated probing mAb (0.1 μg/mL) was added to the wells for 1 h at room temperature. After washing, horseradish peroxidase (HRP)-labeled streptavidin (Zymed) was added to the wells for 1 h at room temperature. After washing, 0.4 mg/mL o-phenylenediamine (OPD Sigma P-8412) in OPD Buffer (0.05 M citrate-phosphate buffer pH 5.0, 0.04% H$_2$O$_2$) or TMB(+) substrate (DAKO, Kyoto, Japan) was added. The reaction was stopped by adding 2N H$_2$SO$_4$, and the OD$_{490}$ or OD$_{450}$ was measured using a multi-well plate reader (Flow Laboratories Inc., Inglewood, Calif., USA).

Immunohistochemistry: Lung tissues were harvested from mice infected with A/Vietnam/1194/2004 (NIBRG-14) or A/HongKong/483/97 (HK483). In addition, autopsied lung tissues of patients infected with influenza virus (H1N1 or 2009 H1N1pdm) were used. Formaldehyde- or formalin-fixed paraffin-embedded lung tissue sections were deparaffinized with xylene and graded ethanol and then autoclaved in 0.1 M citrate-buffer (pH 6.0) at 121°C for 10 min to retrieve the antigens. Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide for 30 min at room temperature. After blocking with M.O.M. blocking reagent (Vector laboratories, Burlingame, Calif., USA) or 5% goat serum, the sections were incubated with each of the mouse mAbs or rabbit polyclonal antibody against type A influenza nucleoprotein at 4°C overnight. After washing off the excess antibodies, the sections were incubated with HRP-labeled anti-mouse IgG followed by tyramide signal amplification system (Biotin-free catalyzed amplification system, CSAII; DAKO) or biotinylated anti-rabbit IgG followed by streptavidin/HRP (LSAB kit; DAKO). The labeled peroxidase activity was detected using diaminobenzidine (DAB; Dojin, Kumamoto, Japan) in 0.015% hydrogen peroxide/0.05 M Tris-HCl (pH 7.6). The sections were counterstained with hematoxylin.

RESULTS

Generation of H5N1-specific mAbs: To establish hybridomas that secrete mAbs specific for the H5N1 virus, BALB/c mice were immunized with the whole virion fraction of purified A/Vietnam/1194/2004 (NIBRG-14) virus. The virus had been inactivated by conventional formaldehyde-fixation or by UV-irradiation to avoid possible changes in antigenicity caused by aldehyde fixation. A standard immunization protocol was used, where mice were boosted twice at 2-week intervals with antigen emulsified first in Freund's Complete Adjuvant and then in Freund's Incomplete Adjuvant. Three days after the final boost, a cell suspension was prepared from the spleens of three immunized mice and fused with SP-2/O myeloma using a polyethylene-glycol method. The fused cells were then selected with HAT (14). Hybridoma screening yielded eight hybridoma clones that reacted to NIBRG-14 lysate but not PR-8 lysate in ELISA (Table 1). Of these clones, seven were from mice immunized with UV-inactivated virion, and one was from mice immunized with formaldehyde-inactivated virion. Six clones (Niid_H5A, Niid_H5B, Niid_H5C, Niid_H5D, Niid_H5E, and Niid_H5F) reacted to rHA protein from a H5N1 virus (recHA_H5N1), while one clone (Niid_N1A) reacted to rNA protein from a H5N1 virus (recNA_H5N1). The remaining clone (Niid_150KA) did not react to either recHA_H5N1 or recNA_H5N1 by ELISA but did react to a 150-kDa molecule on Western blot analysis (described below). Interestingly, seven of the eight clones were from the mice immunized with UV-inactivated virus. The eight hybridomas were successfully cloned by a repeated limiting-dilution method and adapted to a serum-free hybridoma culture medium. The purified antibodies from each clone were biotinylated and used for further experiments.

Table 1. Summary of the eight H5N1-specific mAbs generated in this study

| Clone name | Old name | Ig-subclass | ELISA | | | | Western blot | IFA | Histology | Neutralization (µg/mL) | Hemagglutination inhibition |
			H5N1 NIBRG-14	H1N1 PR-8	recHA H5N1	recNA H5N1					
Niid_H5A[1]	YH-1A1	IgG2a	+++	−	+	−	57 kDa	++		1.5 (Clade-dep)	−
Niid_H5B[1]	YH-2F11	IgG2a	+++	−	+++	−	57 kDa			25	+
Niid_H5C[1]	OM-A	IgG2a	+++	−	++	−	57 kDa		+(mo/hu)	12	
Niid_H5D[1]	OM-B	IgG2a	+++	−	++	−	57 kDa		+(mo)	12	
Niid_H5E[1]	OM-C	IgG2a	+++	−	++	−	57 kDa			12 (Clade-dep)	
Niid_H5F	AY-2C2	IgG1	+++	−	++	−	ND	++		6	−
Niid_N1A[1]	YH-2D3	IgG2a	+++	−	−	+	ND	++			
Niid_150KA[1]	OM-D	IgG1	+++	−	−	−	150 kDa	++			−

[1]: Clones derived from mice immunized with UV-inactivated virus. The remaining clone is derived from a mouse immunized with formalde-hyde-inactivated virus.
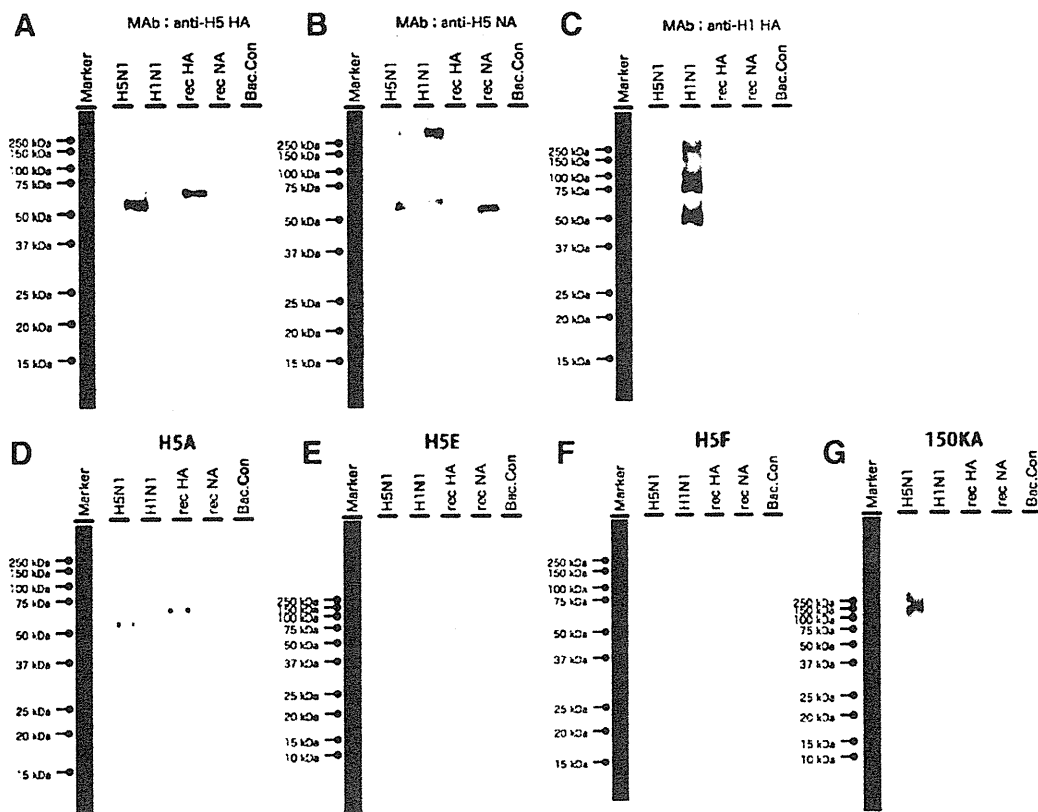

Fig. 1. Detection of influenza virus proteins in Western-blot analysis. Purified influenza virus proteins (0.5 µg/lane) were subjected to SDS-PAGE under reducing conditions. After blotting on a PVDF membrane, the proteins were detected by incubation with the eight monoclonal antibodies (mAbs), followed by incubation with the peroxidase-labeled F(ab')₂ fragment of donkey anti-mouse IgG. The mAbs were then visualized by chemiluminescent reaction. A, authentic anti-H5_hemagglutinin mAb; B, authentic anti-H5_neuraminidase mAb; C, authentic anti-H1_ hemagglutinin mAb; D, Niid_H5A; E, Niid_H5E; F, Niid_H5F; G, Niid_150KA. The molecular weight markers are shown on the left.

Western blot analyses with the mAbs: Five mAbs (Niid_H5A, Niid_H5B, Niid_H5C, Niid_H5D, Niid_H5E) detected the 57-kDa H5_H1 protein by Western blot analysis, which suggests that the antibodies detected the linear epitope(s) of a HA1 fragment of H5_HA (Table 1 and Fig. 1). These antibodies also detected the 60-kDa recombinant H5-HA containing the His-tag. One of these clones, Niid_H5E, detected a 40-kDa sub-fragment of recombinant HA1, which suggests that the antigenic footprint detected by the mAb differs from

that of the other four clones (Fig. 1). Niid-H5F, which reacted strongly to NIBRG-14 and rHA (H5) in ELISA, did not react to any proteins by Western blot analysis, presumably because the mAb detects a conformational epitope of H5-HA. The remaining clone, Niid_150KA, detected an unknown high molecular weight protein of approximately 150 kDa.

IFA with mAbs: Upon IFA, the HA-specific mAbs Niid-H5A and Niid_H5F, the NA-specific mAb Niid-N1A, and the Niid_150KA mAb that detects an
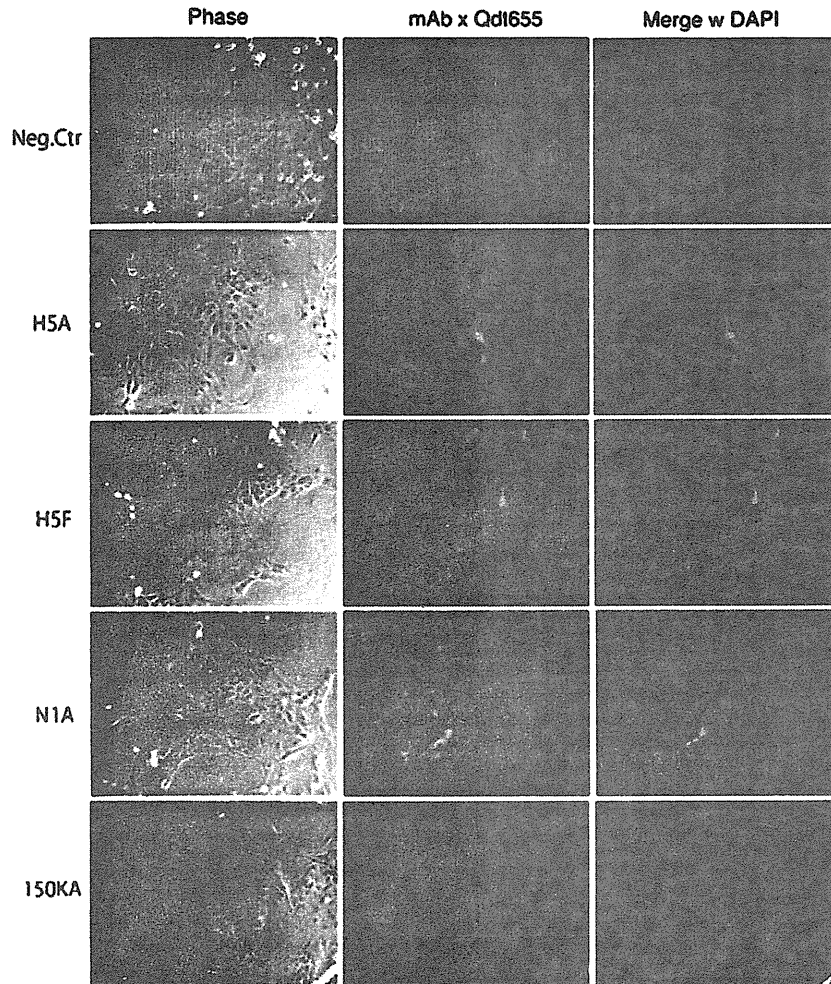
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Fig. 2. Fluorescent immunostaining of H5N1 virus-infected MDCK cells with monoclonal antibodies (mAbs). Paraformaldehyde-fixed, H5N1 virus-infected MDCK cells were permeabilized by TBS-Tween and incubated with biotinylated mAbs. The mAbs were detected with Qdot655-conjugated streptavidin (red). Shown are representative staining patterns with Niid_H5A, Niid_H5F, Niid_N1A, and Niid_150KA. The negative control staining without mAb is shown on top. The nuclei were counterstained with DAPI (blue).

unknown 150-kDa protein bound to NIBRG-14-infected MDCK cells (Fig. 2). With the exception of Niid_H5F, these mAbs detected both the perinuclear region and the cell surface of NIBRG-14-infected MDCK cells. Niid_H5F did not detect the perinuclear region (presumably the Golgi body), which suggests that the antigenic footprint detected by this mAb differs from those of the other mAbs.

Immunohistochemistry: The Niid_H5C and Niid_H5D mAbs detected influenza virus antigens in the epithelial cells of the bronchioles and alveoli of 4% formaldehyde-fixed, paraffin-embedded lung tissue sections from mice infected with A/Vietnam/1194/2004 (NIBRG-14) (Fig. 3a). However, none of the mAbs detected influenza virus antigen in lung tissue sections from mice infected with A/HongKong/483/97 (HK483) (Fig. 3). In contrast, a polyclonal antibody against type A influenza nucleoprotein detected type A influenza virus nucleoprotein in the tissue sections from both the NIBRG-14- and HK483-infected mice (Fig. 3b, d). Thus, Niid_H5C and Niid_H5D specifically detected the HA antigen of A/Vietnam/1194/2004 (NIBRG-14). The specificity of these mAbs was then examined by using autopsied lung tissue sections from patients infected with seasonal influenza virus (H1N1) or 2009 pandemic influenza virus (2009H1N1pdm). Niid_H5C did not exhibit any crossreactivity, but the Niid_H5D mAb did show non-specific staining with the human lung section. Two other mAbs, Niid_H5B and Niid_N1A, were also subjected to such immunohistochemical analysis but did not show any reaction.

Neutralization assay with mAbs: The ability of the mAbs to neutralize several H5N1 influenza strains was then tested (Table 2). The four purified H5N1 virus strains, NIBRG-14, Indo-RG2, NIBRG-23, and Anhui-RG5, were diluted to 2-3 × 10^2 TCID$_{50}$/0.05 mL (Table 2, lower panel) and incubated with titrated amounts of anti-H5_HA mAbs. The remaining infectivity was then noted (Table 2, upper panel). Niid_H5A most potently neutralized the NIBRG-14 strain; it completely neutralized influenza virus infectivity at a concentration of 78 ng/mL. However, Niid_H5A was less potent in neutralizing the Indo-RG2 and Anhui-RG5 strains, which indicates that the neutralizing ability of this mAb was clade-dependent. In contrast, Niid_H5F and Niid_H5D exhibited relatively broad neutralizing abilities, since they neutralized all of the strains that were tested. Niid_H5C and Niid_H5E also showed characteristic clade-
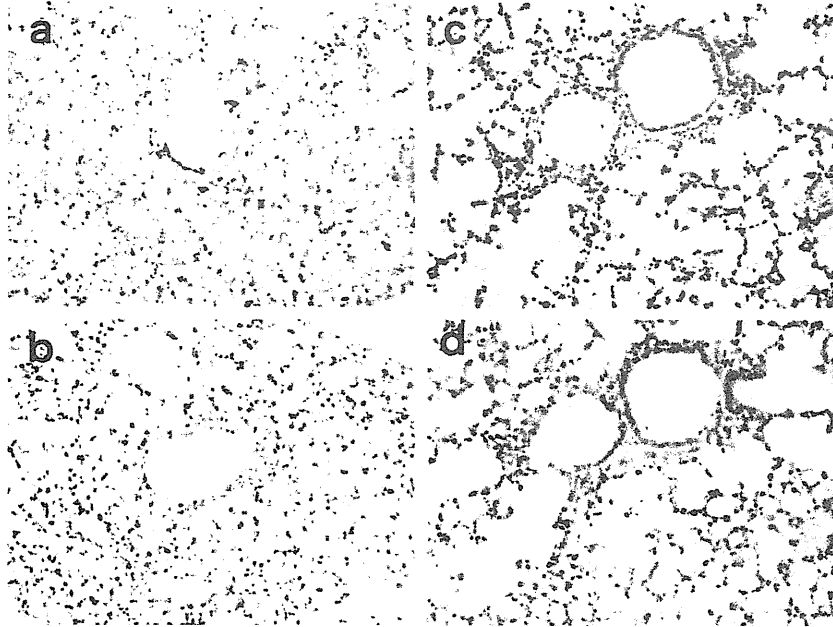
Fig. 3. Immunohistochemical analyses of lung sections from mice infected with A/Vietnam/1194/2004 (NIBRG-14) or A/HongKong/483/97 (HK483) virus. (a, b) Influenza virus antigens were detected in the epithelial cells of the bronchioles and alveoli of the mouse infected with A/Vietnam/1194/2004 (NIBRG-14) by the Niid_H5C clone (a) and polyclonal antibody against type A influenza nucleoprotein (b). (c, d) Virus antigens were not detected in the lung tissue section of the mouse infected with A/HongKong/483/97 (HK483) when Niid_H5C was used (c). However, virus antigens were detected in this section when a polyclonal antibody against type A influenza nucleoprotein was employed (d).

Table 2. Neutralizing ability of the eight mAbs generated in this study

Clone	Neutralizing antibody titer (ng/mL)			
	NIBRG-14 (clade 1)	Indo-RG2 (clade 2.1)	NIBRG-23 (clade 2.2)	Anhui-RG5 (clade 2.3)
Niid_H5A	78	>10,000	625	>10,000
Niid_H5C	625	625	313	>10,000
Niid_H5D	625	625	313	5,000
Niid_H5E	625	>10,000	>10,000	>10,000
Niid_H5F	313	313	156	2,500

Test no.	Virus infection index (Log_{10} $TCID_{50}/0.05$ mL)			
	NIBRG-14	Indo-RG2	NIBRG-23	Anhui-RG5
1	2.5	3.1	2.4	2.1
2	2.0	NT	2.0	2.4

The in vitro neutralization assay examined the ability of the mAbs to neutralize H5N1 virus infection of cultured MDCK cells. Briefly, purified H5N1 virus was diluted to 2–3 × 10^2 $TCID_{50}/0.05$ mL (the quantities are shown in the lower table) and incubated with serially-titrated purified mAbs for 1 h at 37°C. The samples were then placed into 96-well plates in which MDCK cells had been grown to 90% confluence. After 48 h, the cytotoxicity of the mAb-treated viruses was visualized by staining the cells with crystal violet. NT, not tested.

dependency, suggesting that the epitopes of these mAbs differ. Interestingly, the mAbs were least able to neutralize Anhui-RG5. This may reflect the genetic distance between Anhui-RG5 (clade 2.3) and NIBRG-14 (clade 1).

Antigen-capture ELISA: To quantitatively detect H5N1 virus, we constructed a sandwich ELISA-based virus antigen-capture detection system. Preliminary experiments tested all combinations of two mAbs from the eight mAbs; Niid_H5F had the highest detection sensitivity for purified H5N1 virion and reacted broadly to the H5_HA of viruses belonging to clades 1, 2.1, 2.2, and 2.3. Therefore, Niid_H5F was selected as the antigen-capturing mAb. The antigen-capture ELISA was constructed by immobilizing Niid_H5F (and/or Niid_H5C) on the ELISA plate and using biotinylated Niid_H5D as the detection mAb, since this combination gave the best results (data not shown). Since the eight mAbs
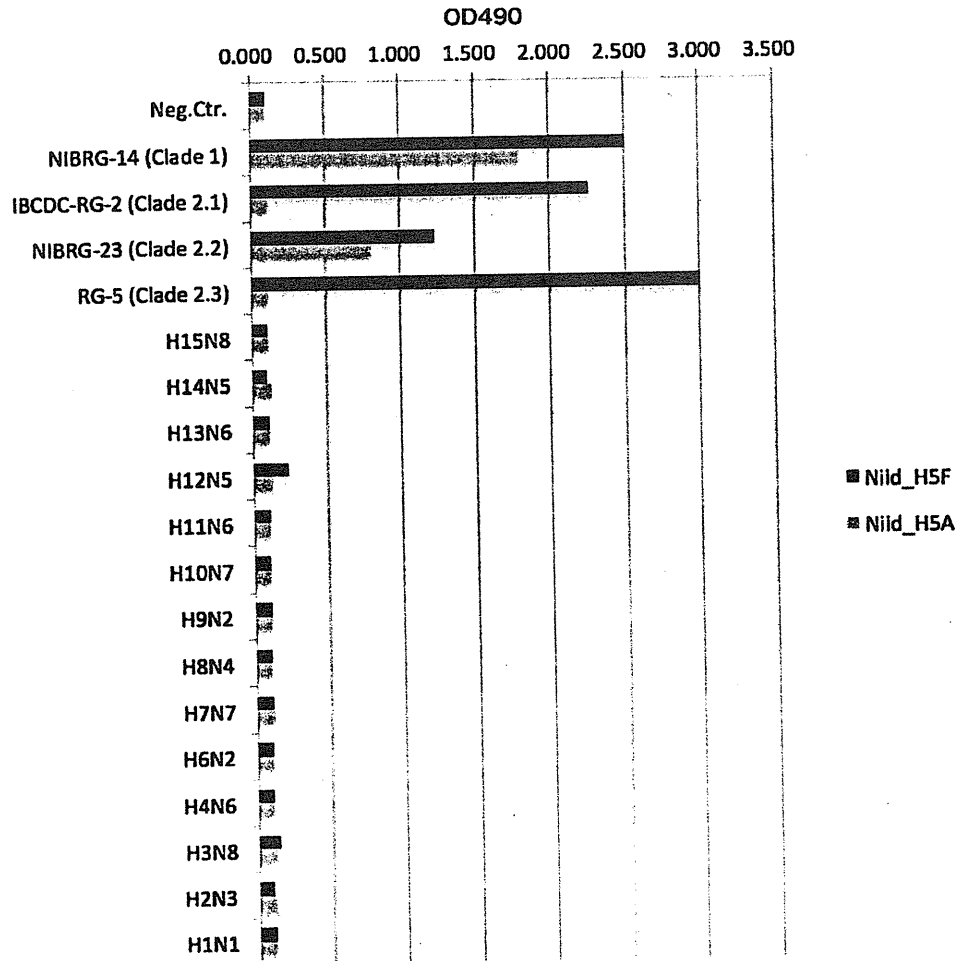
OD490

Fig. 4. ELISA reactivity of the Niid_H5A and Niid_H5F monoclonal antibodies (mAbs) to various influenza virus strains. Different influenza virus strains were immobilized on 96-well plates and incubated with biotinylated Niid_ H5A or Niid_H5F mAbs followed by peroxidase-labeled streptavidin. The binding of the mAbs was then quantitated by a colorimetric assay using TMB as a substrate.

were originally raised against the H5N1 virus strain A/Vietnam/1194/2004 (NIBRG-14), the validity of this system with other strains of H5N1 virus was also examined. As shown in Fig. 4, this system could detect the A/Indonesia/05/2005 (Indo5/PR-8-RG2), A/Turkey/ 1/2005 (NIBRG-23), and A/Anhui/01/2005 (Anhui01/ PR8-RG5) strains but none of the non-H5N1 strains. The sandwich ELISA could detect H5N1 virus protein at concentrations as low as 50 ng/mL HA, namely, >3 SD of negative samples (Fig. 5).

DISCUSSION

In the present study, mAbs against H5N1 influenza virus were established. These mAbs could detect the virus when used in Western blot analyses, IFA, immunohistochemical analyses, neutralization assays, and antigen-capture ELISA. The characteristics of the mAbs are summarized in Table 1.

Of the eight mAb clones that reacted to H5N1 virus in ELISAs, six reacted to rHA. Only one clone reacted to NA protein. Another clone detected an unknown 150-kDa molecule upon Western blot analysis. A hybridoma that secreted a mAb that could detecte the nuclear protein or other protein components of H5N1 virus was

not detected, presumably because the first screening step identified H5 specificity. These results indicate that the HA protein is a dominant target in the antibody response of HA-subtype specificity, as suggested by other studies (17,18). There is accumulating evidence that the influenza strain-specific epitopes are often localized on the HA1 region, whereas the epitopes that are conserved among various strains are localized on the HA2 region (19-22). It has been reported that the immune response elicited by H1N1pdm yields a high frequency of HA2-specific mAbs (23,24). In the present study, none of the established clones detected the HA2 fragment of H5HA, presumably because this study focused on H5-specific clones.

The mAbs isolated in the present study were assessed for their ability to detect H5N1 virus-infected MDCK cells in IFA. Indeed, the anti-HA and anti-NA mAbs detected the cytoplasmic Golgi-rich region and the cell surface membrane. This reflects the common assembly process of influenza virus (25).

In general, a single diagnostic test is not reliable because of the potential for false positives and negatives. Considering the restricted availability of RNA detection systems (26,27), serological screening systems other than those that detect antibodies are currently being ex-
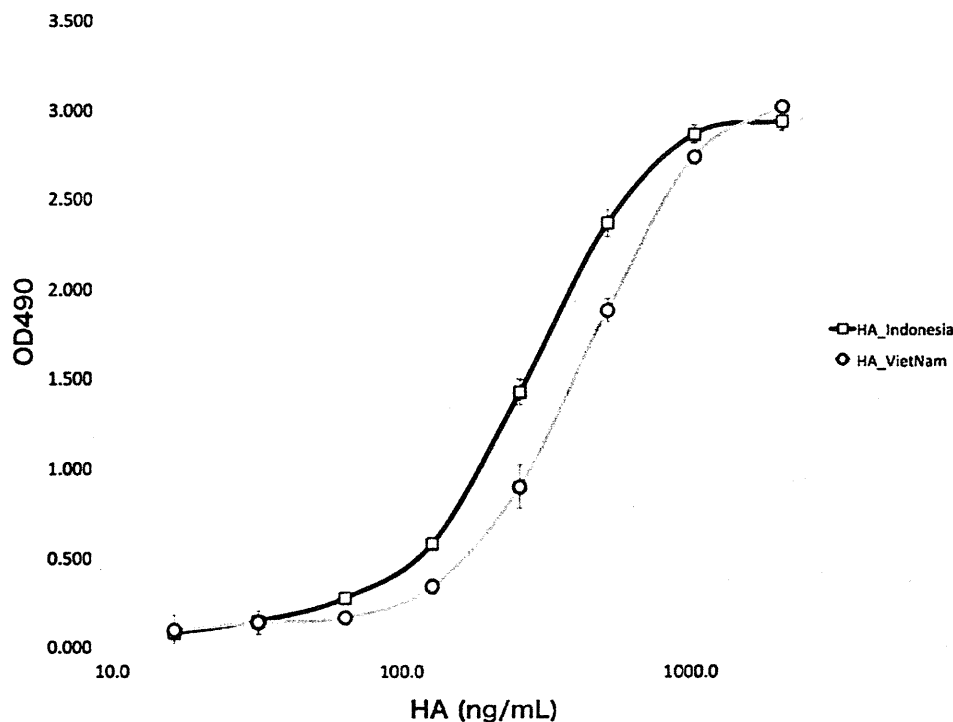
3.500

3.000

2.500

OD490

2.000

1.500

1.000

0.500

0.000

10.0   100.0   1000.0

HA (ng/mL)

HA_Indonesia
HA_VietNam

Fig. 5. Antigen-capture ELISA reactivity of monoclonal antibodies (mAbs) to H5N1 and H1N1 virus strains. The anti-H5 mAb Niid_H5F was immobilized on 96-well plates and reacted with serially-titrated purified H5N1 virus fractions for 1 h at room temperature. The bound virus proteins were detected by incubation with biotinylated Niid_H5D (anti-H5) antibody followed by peroxidase-labeled streptavidin. The binding was quantitated by a colorimetric assay that used TMB as a substrate. Abscissa, concentration of purified H5N1 virus proteins. Ordinate, absorbance unit (OD490).

amined. ELISA-based antigen-capture assays offer high specificity and reproducibility and have been used to diagnose and monitor many diseases. The present study describes the development of an antigen-capture ELISA system that detects purified H5N1 virus virion at levels as low as 50 ng/mL. The sensitivity of this system, which comprises three anti-HA mAbs, appears sufficiently high to detect virus protein in patient sera, particularly since a recently reported antigen-capture ELISA system detects 50 ng/mL of purified recombinant HA1 protein (28). At present, the sensitivity of the system is being improved, and its usefulness in diagnosing and monitoring H5N1 virus infections is being validated.

The five selected anti-HA mAbs exhibited significant neutralization activity against several viral strains in a clade-dependent manner (Table 2). Of these, Niid_H5F showed the broadest spectrum of neutralization activity, but it neutralized NIBRG-23 (clade 2.2) more efficiently than the original immunogen NIBRG-14 (clade 1). It would be of interest to determine the features that determine this clade-dependency of mAb recognition. It is also possible that these mAbs have therapeutic potential, if humanized by means of complementarity determining region grafting or mouse-human chimerism.

In conclusion, eight new H5N1-specific mAbs were generated from A/Vietnam/1194/2004 (NIBRG-14)-hyperimmunized mice, six of which were HA-specific. These mAbs were useful in Western blot analyses, IFA, and immunohistology and had in vitro neutralization activity against H5N1 viruses. These mAbs also perform well in a highly sensitive antigen-capture sandwich

ELISA system. As such, these mAbs may be useful for the rapid and specific diagnosis of H5N1 subtype influenza virus and may have therapeutic potential.

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Epitope mapping of neutralizing monoclonal antibody in avian influenza A H5N1 virus hemagglutinin

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ARTICLE INFO

ABSTRACT

The global spread of highly pathogenic avian influenza A H5N1 viruses raises concerns about more widespread infection in the human population. Pre-pandemic vaccine for H5N1 clade 1 influenza viruses has been produced from the A/Viet Nam/1194/2004 strain (VN1194), but recent prevalent avian H5N1 viruses have been categorized into the clade 2 strains, which are antigenically distinct from the pre-pandemic vaccine. To understand the antigenicity of H5N1 hemagglutinin (HA), we produced a neutralizing monoclonal antibody (mAb12–1G6) using the pre-pandemic vaccine. Analysis with chimeric and point mutant HAs revealed that mAb12–1G6 bound to the loop (amino acid positions 140–145) corresponding to an antigenic site A in the H3 HA. mAb12–1G6 failed to bind to the mutant VN1194 HA when only 3 residues were substituted with the corresponding residues of the clade 2.1.3.2 A/Indonesia/5/05 strain (amino acid substitutions at positions Q142L, K144S, and S145P), suggesting that these amino acids are critical for binding of mAb12–1G6. Escape mutants of VN1194 selected with mAb12–1G6 carried a S145P mutation. Interestingly, mAb12–1G6 cross-neutralized clade 1 and clade 2.2.1 but not clade 2.1.3.2 or clade 2.3.4 of the H5N1 virus. We discuss the cross-reactivity, based on the amino acid sequence of the epitope.

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1. Introduction

The highly pathogenic avian influenza (HPAI) H5N1 virus is a highly contagious and fatal pathogen in poultry and has been transmitted to humans with high mortality. It has raised concerns of evolving to bring about the next human influenza pandemic. Since 2003, numerous clinical cases have been reported in humans who live in close contact with infected birds in Southeast Asia [1,4]. Subsequently, a second clade of H5N1 viruses, genetically and antigenically distinct from clade 1 viruses, has been found in Indonesia and has become endemic with several subclades [3,14,15].

The influenza virus hemagglutinin (HA) is a virion surface glycoprotein and the primary target for neutralizing antibodies (Ab). HA is initially synthesized as precursor HA0 and is cleaved into

HA1, variable external subunit and HA2, relatively conserved transmembranous subunit. The major part of HA1 forms the globular head domain containing the binding pocket to sialic acid expressed on host cells.

Elicitation of neutralizing Ab by vaccination is the most effective prophylaxis against influenza virus at present. Numerous studies have shown that Ab binding adjacent to the receptor-binding pocket in HA1 blocks virus attachment to the sialic receptors on host cells [7,18]. The three-dimensional structure of HA has been resolved initially for H3, and five antigenic sites (sites A, B, C, D, and E) have been mapped within the molecule [16,17]. Their corresponding antigenic sites have been mapped in the HAs of H1 and H2 subtypes [2,13]. The structure of the H5 HA molecule has recently been resolved by crystallography [6], and the sites corresponding to sites A and B of H3, have been mapped using virus escape mutants and designated as sites II and I, respectively [9] and also as sites 5 and 1, respectively in another work [10]. However, epitope mapping studies using escape mutants indicated that sites 5 and 1 were closely located in H5 HA when compared with sites A and B in H3 HA [8], and some monoclonal Abs indeed