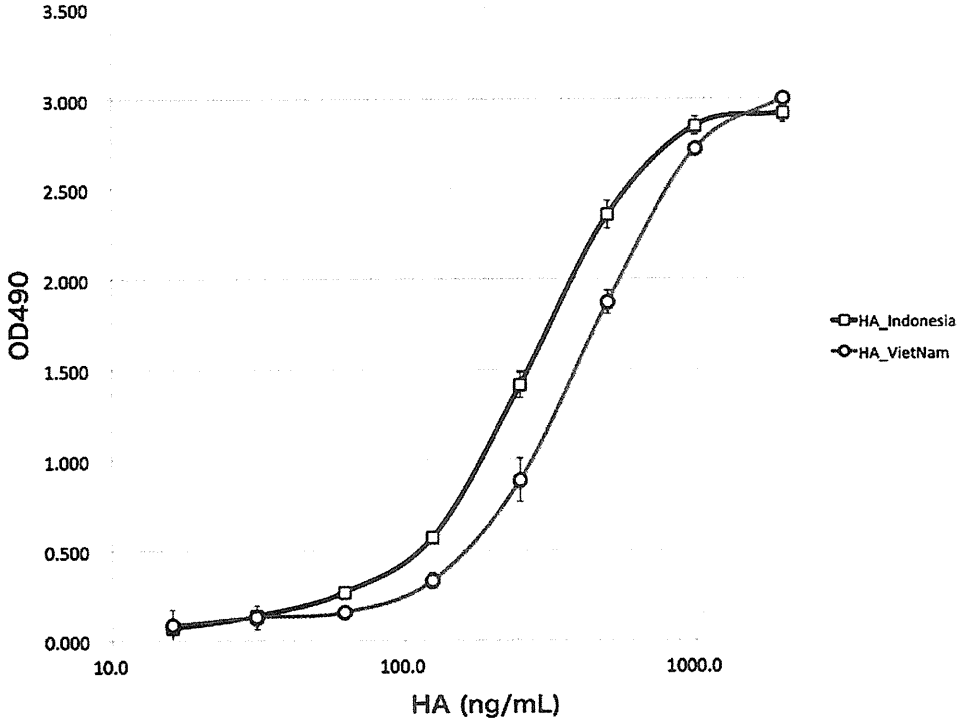

Fig. 5. Antigen-capture ELISA reactivity of monoclonal antibodies (mAbs) to H5N1 and H1N1 virus strains. The anti-H5 mAb Niid_H5F was immobilized on 96-well plates and reacted with serially-titrated purified H5N1 virus fractions for 1 h at room temperature. The bound virus proteins were detected by incubation 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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Conflict of interest None to declare.

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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.

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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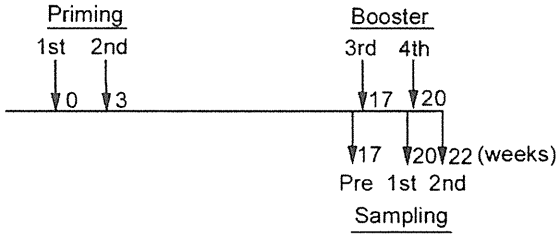

Fig. 1. Vaccination and blood sampling schedule of Table 1.

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 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).

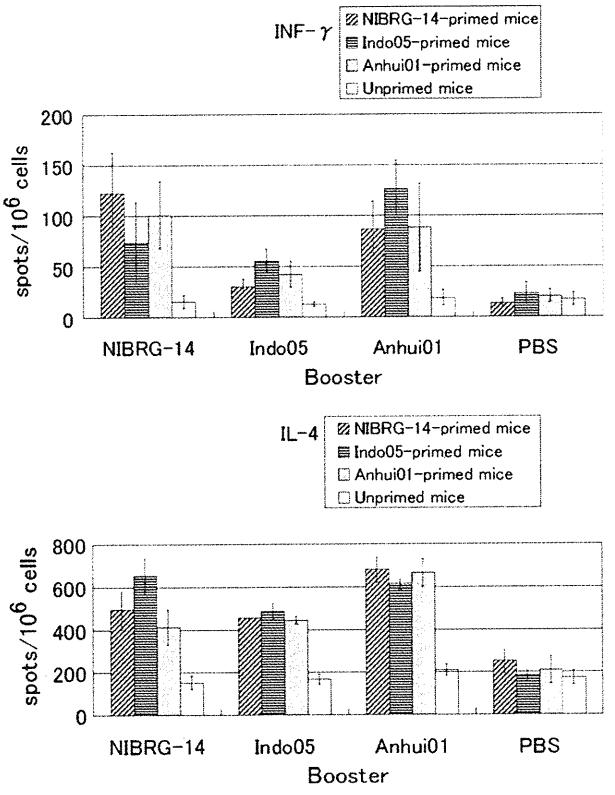

Fig. 2. BALB/c mice were injected twice intramuscularly with 0.2 μg HA/dose of an H5N1 strain plus 0.03 mg of alum adjuvant at a 3-week interval. As a control, mice were injected with alum-containing PBS alone. Spleen cells were harvested at 14 weeks after the second injection, and dispensed into polyvinylidene difluoride-coated 96-well plates with anti-IFN-γ or anti-IL4 at 3×10^5 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/10^6 cells, 95% CL).

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	NIBRG-14	**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	NIBRG-14	**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	Indo05	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	Indo05	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	Anhui01	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	Anhui01	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	NIBRG-14	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	Indo05	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	Anhui01	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	Qinhai1A	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	Qinhai1A	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	Qinhai1A	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.

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Letter to the Editor

Posterior reversible encephalopathy syndrome in a child with post-transplant HHV-6B encephalitis

Running title: PRES and HHV-6 encephalitis

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Abstract

An 11-year-old Japanese boy underwent mismatched unrelated donor bone marrow transplant for relapsed acute lymphoblastic leukemia. On day 20, the patient had stupor, confusion, and disorientation. Blood pressure ranged from 130–170/70–90 mmHg. T2-weighted and fluid-attenuated inversion recovery magnetic resonance imaging on day 22 post-transplant showed high intensity areas in both cerebellar hemispheres and the left occipital lobe. The patient was diagnosed with posterior reversible encephalopathy syndrome (PRES) based on clinical and neuroimaging findings. As high levels of HHV-6B DNA were detected in the patient's serum and cerebrospinal fluid (CSF), HHV-6B was thought to be associated with PRES. Therefore, ganciclovir was initiated on day 24 post-transplant, with gradual improvement in his neurological symptoms. The kinetics of viral DNA in serially collected serum and CSF samples were analyzed. High copy numbers of HHV-6B DNA were detected in the serum starting three days before the onset of neurological symptoms, and HHV-6B DNA decreased to undetectable levels in the serum while high copy of HHV-6B DNA was detected in the CSF on day 24 post-transplant. Although IL-1β concentrations were similar in the serum and CSF on day 24 post-transplant, no obvious increases in other biomarkers were observed in the CSF.

Key words: HHV-6, encephalitis, PRES, cytokine

Human herpesvirus 6B (HHV-6B) is the etiologic agent of exanthem subitum, a common febrile illness in infants and young children (1). Similar to other human herpesviruses, the virus remains latent in hosts after primary HHV-6B infection and reactivates in immunocompromised patients, including hematopoietic stem cell transplant (HSCT) recipients. Based on *in vitro* and *in vivo* investigations, HHV-6B is thought to have neurovirulence (2, 3). Our recent data suggested that the pathogenesis and clinical features of HHV-6B encephalitis are different in patients with primary infection and HSCT recipients with viral reactivation (4). Various types of clinical manifestations including acute encephalopathy with biphasic seizures and late reduced diffusion, acute necrotizing encephalopathy, and hemorragic shock and encephalopathy syndrome have been reported in patients with primary HHV-6B encephalitis (5). It is also well known that HHV-6B is the most important pathogen in post-transplant acute limbic encephalitis (6).

Posterior reversible encephalopathy syndrome (PRES) was first reported by Hinchey *et al.* as a syndrome of headaches, confusion, seizures, and visual disturbances associated with transient, predominantly posterior white matter changes on neuroimaging studies (7). PRES has been associated with a number of medical conditions, including hypertensive encephalopathy, eclampsia, and the use of cytotoxic and immunosuppressive drugs. PRES is also seen in solid organ transplant recipients and HSCT recipients. Although several infectious agents are thought to be involved in its pathophysiology (8), no cases of PRES associated with HHV-6B have been reported to date. Herein we report the first case of PRES with HHV-6B reactivation after HSCT.

An 11-year-old Japanese boy who was seropositive for HHV-6B underwent mismatched unrelated donor bone marrow transplant for relapsed acute lymphoblastic leukemia. The conditioning regimen included total body irradiation (12 Gy) and melphalan (180 mg/m^2). Tacrolimus was administered initially as 0.02 mg/kg intravenously daily, starting one day before transplant. Methotrexate was administered on day 1 (15 mg/m^2 of body surface area), and day 3, 6, and 11 (10 mg/m^2) post-transplant for graft-versus-host disease (GVHD) prophylaxis. The patient had fever and rash on day 7 and was given methylprednisolone 2mg/kg daily starting on day 9 for engraftment syndrome. The fever and rash promptly resolved. On day 20 he developed a high fever and a generalized clonic seizure. Subsequently, he also developed stupor, confusion, and disorientation. No other abnormal findings were observed on neurological examinations. Blood pressure ranged from 130–170/70–90 mmHg. Hematological examinations revealed mild thrombocytopenia (85,000/μL). The serum concentration of tacrolimus was within target limits (6.1 ng/mL). Additionally, cerebrospinal fluid (CSF) collected on day 24 post-transplant showed increased protein (42 mg/dL) and no pleocytosis. T2-weighted and fluid-attenuated inversion recovery (FLAIR) magnetic resonance imaging (MRI) on day 22 post-transplant showed high intensity areas in both cerebellar hemispheres and the left occipital lobe (Figure 1). The patient was diagnosed with PRES based on clinical and neuroimaging findings. He was treated with a calcium channel blocker to lower blood pressure starting on day 22 post-transplant.

Neither bacteria nor fungus was isolated from the patient. Real-time polymerase chain

reaction (RT-PCR) did not detect cytomegalovirus DNA in the serum. However, as high levels of HHV-6B DNA were detected in the patient's serum (29,600 copies/mL; day 17 post-transplant) and CSF (311,000 copies/mL, day 24 post-transplant), HHV-6B was thought to be associated with PRES. Therefore, ganciclovir was initiated on day 24 post-transplant, with gradual improvement in his neurological symptoms. Although his neurological symptoms completely resolved, the patient died on day 105 post-transplant due to GVHD.

In order to elucidate the pathophysiology of HHV-6B-associated PRES, the kinetics of viral DNA and biomarkers in serially collected serum and CSF samples were analyzed. HHV-6B DNA copy number was measured by RT-PCR, and six biomarkers were measured by the Cytometric Bead Array system (BD Biosciences, San Jose, California, USA). As shown in Table 1, high copy numbers of HHV-6B DNA were detected in the serum starting three days before the onset of neurological symptoms, and HHV-6B DNA decreased to undetectable levels in the serum while high copy of HHV-6B DNA was detected in the CSF on day 24 post-transplant. Serum levels of three inflammatory cytokines (interleukin (IL)-1β, IL-6, and tumor necrosis factor-α) and IL-8 were increased in the serum sample collected after onset of PRES. Although IL-1β concentrations were similar in the serum and CSF on day 24 post-transplant, no obvious increases in other biomarkers were observed in the CSF.

Based on the typical clinical course and neuroimaging findings, the patient was diagnosed with PRES. Although the precise mechanism of PRES remains unclear, hypertension and

94 immunosuppressive drugs such as tacrolimus have been proposed as risk factors (9). As the patient
95 had hypertension and received tacrolimus, these two factors might play important roles in the
96 pathogenesis of PRES. In addition, systemic and central nervous system (CNS) reactivation of
97 HHV-6B occurred concurrently with the onset of PRES. Furthermore, ganciclovir, which has
98 antiviral activity against HHV-6B, in combination with a calcium channel blocker, appeared to be
99 effective in resolving the patient's neurological symptoms. Thus, these present findings strongly
100 suggest that HHV-6B might be a new infectious agent associated with PRES. To our knowledge, this
101 is the first patient with PRES associated with HHV-6B reactivation after HSCT. It has been
102 suggested that post-transplant HHV-6B encephalitis generally manifests as limbic encephalitis (2).
103 Among HSCT recipients, patients with limbic encephalitis and PRES should be checked for HHV-6B
104 infection.

105 Although it is difficult to determine the precise role of HHV-6B in the pathogenesis of
106 PRES based on one case, the virological and biomarkers analysis provides interesting observations.
107 Systemic HHV-6B reactivation preceding the onset of neurological symptoms and followed by
108 up-regulation of inflammatory cytokines and IL-8 may be a trigger for vasogenic edema in the brain.
109 Moreover, although HHV-6B reactivation occurred in the CNS during the acute phase, no obvious
110 up-regulation of cytokines except for IL-1β was seen in the CSF. Thus, it is likely that antiviral drugs
111 that inhibit HHV-6B reactivation in the CNS are more useful than anti-cytokine treatments for
112 treating PRES. Although this patient had a complete recovery from PRES, severe neurological

113 sequelae have been reported in some PRES patients (10). Thus, the development of reliable treatment
114 strategies for HHV-6B associated PRES is necessary to improve prognosis. Further analysis of a
115 larger number of cases should be carried out to elucidate the precise pathophysiology and the best
116 treatment strategy for HHV-6B associated PRES.

Figure legend

Fluid-attenuated inversion recovery (FLAIR) magnetic resonance imaging (MRI) on day 22 post-transplant demonstrated high intensity areas in the left occipital lobe (A) and in both cerebellar hemispheres (B).

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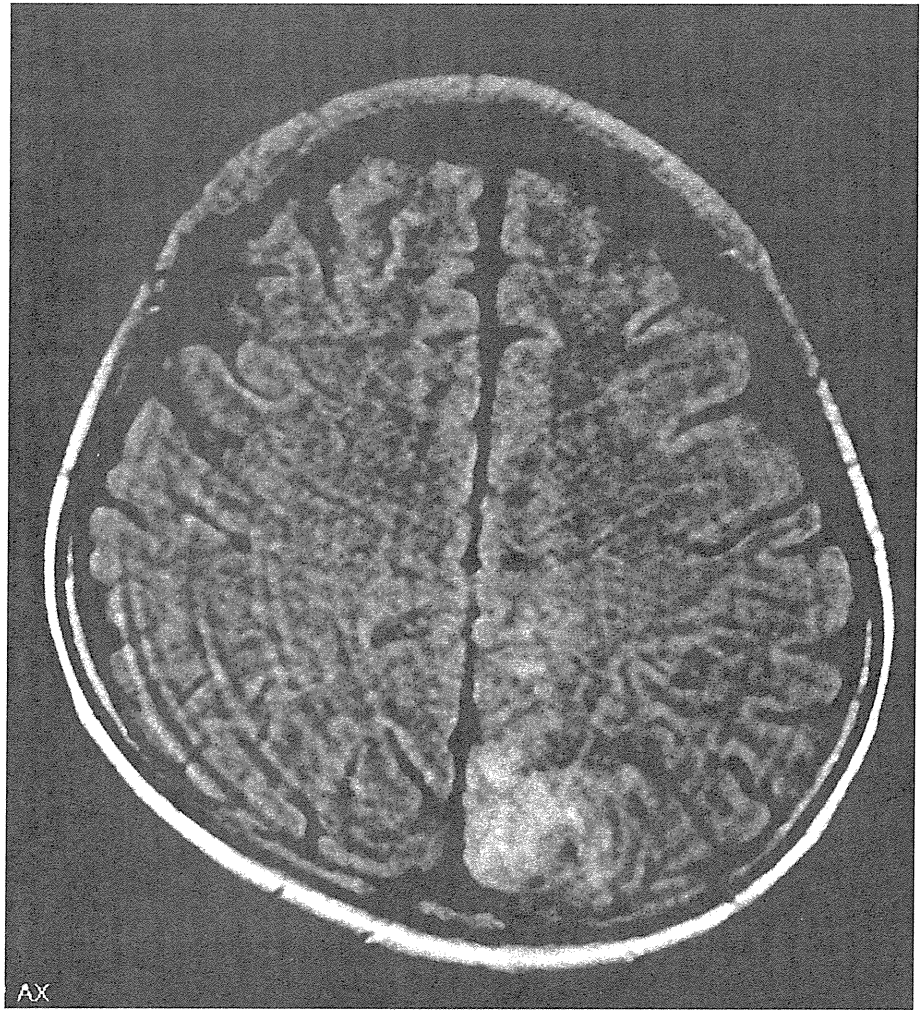
Table 1 HHV-6B DNA and cytokines levels in serum and CSF

Days post-transplant		3	10	17	24	24	32	38
Specimen		serum	serum	serum	serum	CSF	serum	serum
HHV-6B DNA	(copies/mL)	0	0	29,600	100	311,000	0	50
IL-1β	(pg/mL)	0	0	8	58	54	63	12
IL-6	(pg/mL)	26	31	6	45	0	187	342
IL-8	(pg/mL)	27	410	208	1906	4	717	1943
IL-10	(pg/mL)	0	53	13	6	3	14	13
IL-12p70	(pg/mL)	0	0	0	0	0	0	7
TNF-α	(pg/mL)	0	0	0	15	0	35	6

CSF; cerebrospinal fluid

Figure 1

A



B