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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 $(NH_4)_2SO_4$ 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_2O_2) or TMB(+) substrate (DAKO, Kyoto, Japan) was added. The reaction was stopped by adding 2N H_2SO_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 formaldehyde-inactivated virus.

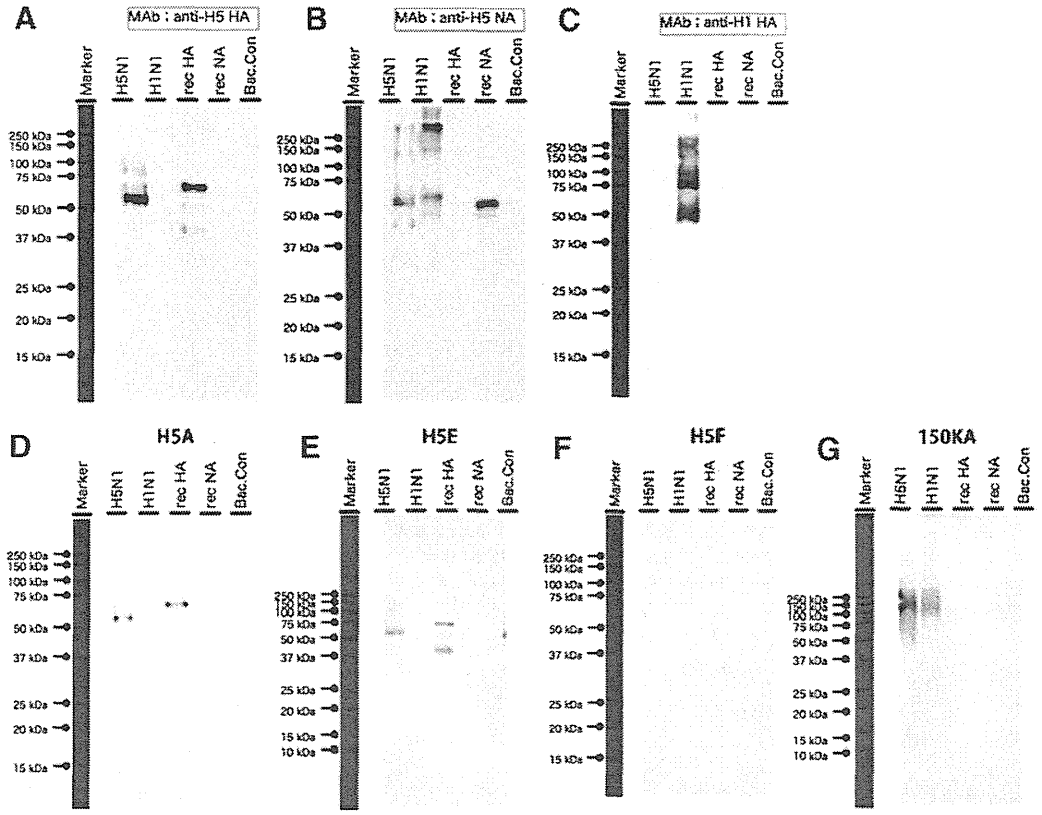

Fig. 1. Detection of influenza virus proteins in Western-blot analysis. Purified influenza virus proteins (0.5 μg/lane) were subjected to SDS-PAGE under reducing conditions. After blotting on a PVDF membrane, the proteins were detected by incubation with the eight monoclonal antibodies (mAbs), followed 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 subfragment 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

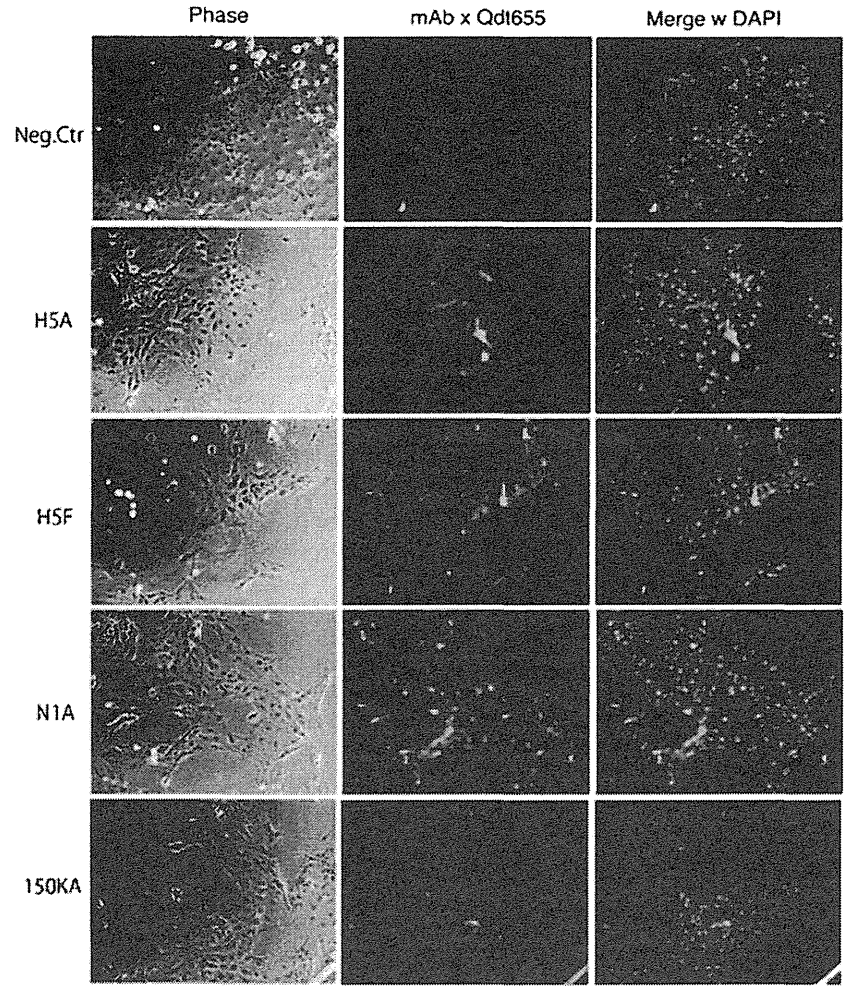

Fig. 2. Fluorescent immunostaining of H5N1 virus-infected MDCK cells with monoclonal antibodies (mAbs). Paraformaldehyde-fixed, H5N1 virus-infected MDCK cells were permeabilized by TBS-Tween and incubated with biotinylated mAbs. The mAbs were detected with Qdot655-conjugated streptavidin (red). Shown 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 $\times$ 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-

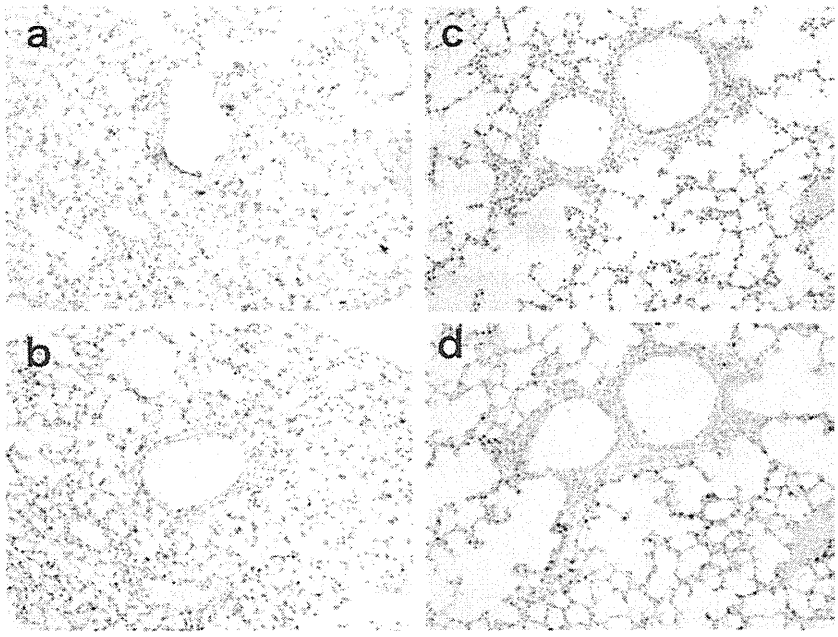

Fig. 3. Immunohistochemical analyses of lung sections from mice infected with A/Vietnam/1194/2004 (NIBRG-14) or A/HongKong/483/97 (HK483) virus. (a, b) Influenza virus antigens were detected in the epithelial cells of the bronchioles and alveoli of the mouse infected with A/Vietnam/1194/2004 (NIBRG-14) 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

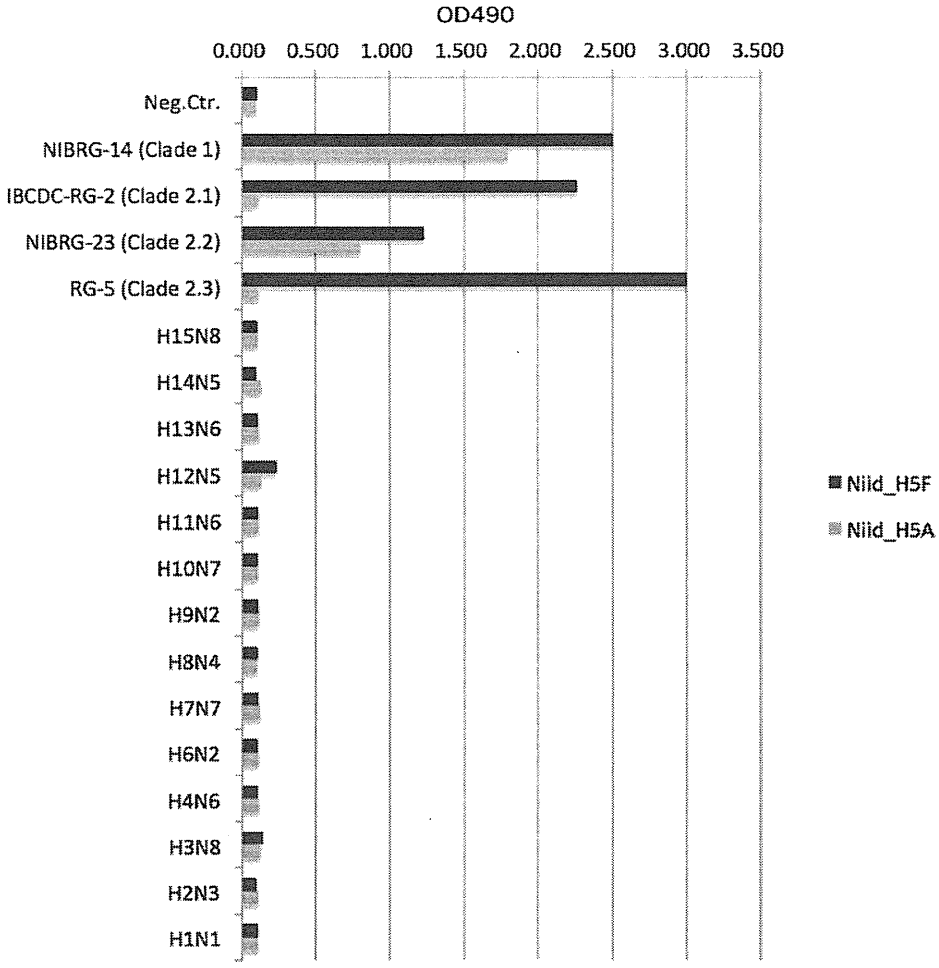

Fig. 4. ELISA reactivity of the Niid_H5A and Niid_H5F monoclonal antibodies (mAbs) to various influenza virus strains. Different influenza virus strains were immobilized on 96-well plates and incubated with biotinylated Niid_H5A or Niid_H5F mAbs followed by peroxidase-labeled streptavidin. The binding 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-

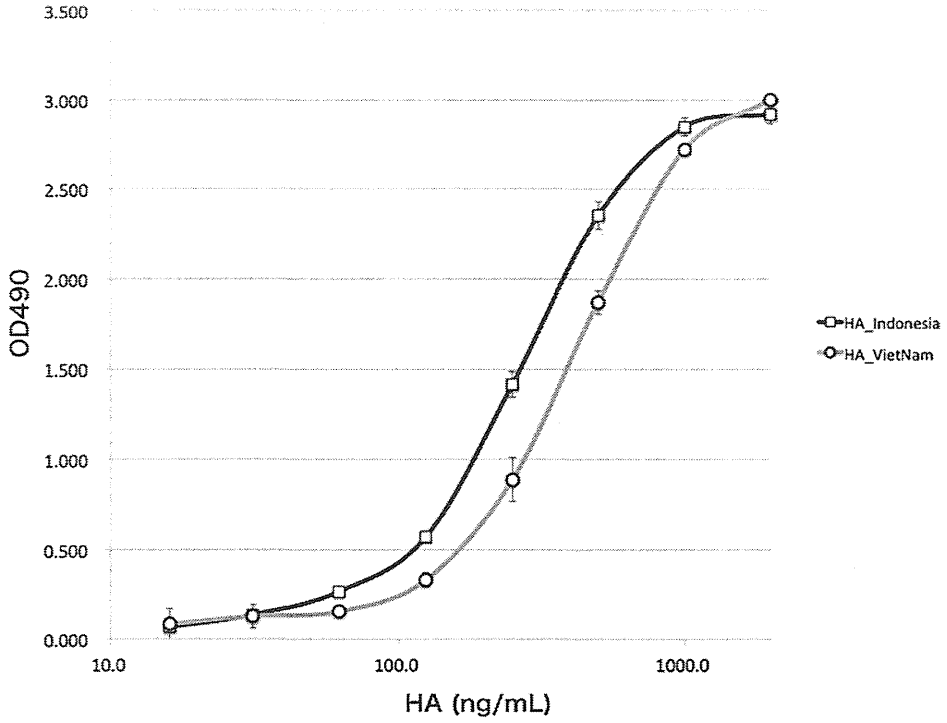

Fig. 5. Antigen-capture ELISA reactivity of monoclonal antibodies (mAbs) to H5N1 and H1N1 virus strains. The anti-H5 mAb Niid_H5F was immobilized on 96-well plates and reacted with serially-titrated purified H5N1 virus fractions for 1 h at room temperature. The bound virus proteins were detected by incubation 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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Lack of antibody response to Guillain–Barré syndrome-related gangliosides in mice and men after novel flu vaccination

During a mass vaccination campaign in the USA in 1976, there was a statistically significant increased risk of developing Guillain–Barré syndrome (GBS) following receipt of the A/NJ/1976/H1N1 'swine flu' vaccine.[1] Because the currently circulating pandemic A (H1N1) flu virus is partially of swine origin, there has been concern about a similar association of GBS with the novel flu A (H1N1) vaccine. Preliminary analysis showed an elevated, statistically significant association between 2009 H1N1 vaccination and GBS.[2] If confirmed, the increased risk of GBS associated with 2009 H1N1 vaccine of 0.8 cases per 1 million vaccinations would be comparable with the risk described previously for some trivalent seasonal flu vaccine formulations.

GBS is divided into two major subtypes, acute inflammatory demyelinating polyradiculoneuropathy (AIDP) and acute motor axonal neuropathy (AMAN).[3] AMAN, but not AIDP, is significantly associated with IgG antibodies against GM1, GM1b, GD1a, GalNAc-GD1a and GD1b. It is not known if the 1976 flu vaccine was associated with AIDP or AMAN. A recent report, however, demonstrated that the 1976 swine flu vaccines, seasonal flu vaccines from 1991–1992 and 2004–2005, and recombinant haemagglutinin proteins derived from high pathogenic avian H5N1 viruses A/HK/156/97 and A/Vietnam/1203/04 induced IgM and IgG anti-GM1 antibodies in mice.[4] Here, we report our assessment of the pandemic 2009 A (H1N1) and H5N1 vaccines' ability to induce antiganglioside antibodies in mice and humans, providing information as to the possible risk of developing AMAN following these vaccinations.

Inactivated A/H1N1pdm split vaccines (without adjuvant) used during the Japan 2009–2010 vaccination programme (A/California/7/2009 NYMC X-179A) were supplied by Kitasato Institute (Tokyo, Japan) and Denka Seiken (Tokyo, Japan). Inactivated, aluminium hydroxide-adjuvant H5N1 whole vaccines (A/Indo/05/2005-PR8-IBCDC-RG2, A/Anhui/01/2005-PR8-IBCDC-RG5 and A/Viet Nam/1194/2004-NIBRG-14) were provided by Research Foundation for Microbial Diseases of Osaka University (Biken) (Kagawa, Japan) and Kitasato Institute (Tokyo, Japan). Trivalent seasonal split vaccine (without adjuvant) of the Japan 2008–2009 vaccination programme (A/Brisbane/59/2007, A/Uruguay/716/2007 and B/Florida/4/2006) was supplied by Denka Seiken (Tokyo, Japan). Additional trivalent vaccine preparation used during the US 2004–2005 vaccination programme (A/New Caledonia/20/99, A/Wyoming/03/2003 and B/Jiangsu/10/2003) was kindly provided by Irving Nachamkin (University of Pennsylvania School of Medicine, Philadelphia). The 1976 swine flu vaccines were not available to study.

Mice lacking the functional gene for (*N*-acetylneuraminyl)-galactosylglucosylceramide *N*-acetylgalactosaminyltransferase ($GalNAcT^{-/-}$ mice) do not express complex gangliosides and are naïve hosts against ganglioside. In these mice, ganglioside-like lipo-oligosaccharide of a *Campylobacter jejuni* strain from AMAN elicits high titres of antiganglioside antibodies.[5] As previously described,[4] 7- to 10-week-old $GalNAcT^{-/-}$ mice were intramuscularly injected with the recommended adult human dose (0.5 ml, equivalent to 15 μg of haemagglutinin) of vaccine 3 weeks apart, whereas 9-week-old C3H/HeN mice were subcutaneously injected (box 1). Serum samples were obtained before each immunisation and 2 weeks after the second immunisation. Experimental protocols were approved by Animal Care and Use Committees.

Neither IgM nor IgG antibodies against GM1, GM1b, GD1a, GalNAc-GD1a, GD1b, GT1a and GQ1b were detected in $GalNAcT^{-/-}$ mice vaccinated with A/H1N1pdm vaccine (Denka Seiken) (n=10), H5N1 vaccines (A/Indo/05/2005-PR8-IBCDC-RG2 (n=5), A/Anhui/01/2005-PR8-IBCDC-RG5 (n=5) and A/Viet Nam/1194/2004-NIBRG-14 (n=5)), trivalent seasonal vaccines from Japan 2008–2009 (n=5) and the US 2004–2005 vaccination programmes (n=5). No antiganglioside antibodies were induced in C3H/HeN mice inoculated with A/H1N1pdm vaccine (Denka Seiken) (n=5), H5N1 vaccine (A/Indo/05/2005-PR8-IBCDC-RG2) (n=5) or trivalent seasonal vaccines from Japan 2008–2009 vaccination programme (n=5), whereas serum haemagglutination inhibition titres increased from <10 to 80±49 after inoculation of A/H1N1pdm vaccine. Despite the use of the same seasonal 2004–2005 flu vaccine and C3H/HeN mice,[4] we could not confirm earlier observations that these flu vaccines elicit an antiganglioside antibody response. Moreover, no antiganglioside antibodies were induced in the naïve mice. The previous study did not describe whether optical densities of GM1-free wells were subtracted from densities of GM1-coated wells,[4] raising the possibility that non-specific IgM and IgG responses were shown.

A total of 200 eligible subjects underwent randomisation to receive 15 μg of haemagglutinin antigen (A/H1N1pdm split vaccine, Kitasato Institute) subcutaneously or 30 μg intramuscularly. They had previously received two doses of the assigned vaccine 3 weeks apart in 2009 (box 1). A total of 121 eligible subjects were administered 15 μg of haemagglutinin antigen (whole H5N1 vaccines; A/Viet Nam/1194/2004-NIBRG-14, Biken or A/Anhui/01/2005-PR8-IBCDC-RG5A, Kitasato Institute) adjuvanted with alum, who previously received two subcutaneousdoses of the assigned vaccine 3 weeks apart in 2008. Serum samples were obtained from each subject before each vaccination, 3 weeks after the second vaccination or 6 months after the first vaccination. A total of 137 eligible subjects were administered 5 or 15 μg of haemagglutinin antigen (whole H5N1 vaccine; A/Indo/05/2005-PR8-IBCDC-RG2, Biken) intramuscularly 3 weeks apart in 2006, then received 15 μg of haemagglutinin antigen (whole H5N1 vaccines; A/Viet Nam/1194/2004-NIBRG-14 or A/Anhui/01/2005-PR8-IBCDC-RG5A) in 2008. Serum samples were obtained before the second vaccination, and 1 and 3 weeks after the second vaccination. Informed written consent was obtained from each subject.

Box 1

(A) Mouse immunisation
- $GalNAcT^{-/-}$ mice 15 μg im, 3 weeks apart
- C3H/HeN mice 15 μg sc, 3 weeks apart.

(B) Human vaccination
- A/H1N1pdm split vaccines.
  - 15 μg sc, two doses, 3 weeks apart in 2009 (n=100).
  - 30 μg im, two doses, 3 weeks apart in 2009 (n=100).
- Whole H5N1 vaccines.
  - 15 μg sc, two doses, 3 weeks apart in 2008 (n=121).
  - 5 or 15 μg im, two doses 3 weeks apart in 2006 and 15 μg im, one dose in 2008 (n=137).

IgM anti-GM1 antibodies and low-affinity IgG anti-GM1 antibodies are induced in non-diseased rabbits sensitised with GM1 or in AMAN rabbits before the onset.[3] This raises a possibility that such low-affinity anti-GM1 antibodies were induced in some of human subjects, although none of those developed GBS. Both IgM and IgG antibodies against GM1, GM1b, GD1a, GalNAc-GD1a, GD1b, GT1a and GQ1b were undetectable in sera from the 200 subjects who received A/H1N1pdm vaccine twice, whereas they obtained high titres of neutralising antibodies against the vaccine-strain flu virus as well as haemaglutination inhibition (http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou04/inful_iken-koukan1111.html). The aforementioned antiganglioside antibodies were also not induced in 258 subjects who received H5N1 vaccine, although the neutralising antibodies and haemaglutination inhibition activities against the vaccine virus were present (Ihara, Ito, Kobayashi and Kamiya, in preparation).

The flu vaccines studied here elicited no antiganglioside antibody response in mice (n=50) and men (n=458). A thin-layer

chromatogram with resorcinol staining did not detect gangliosides in any of the vaccines. Previous haemagglutinin inhibition findings suggested the presence of a GM1 epitope on flu viruses,[4] but our immunoblot results failed to detect haemagglutinin-associated GM1. In conclusion, our results suggest that the flu vaccines are unlikely to induce the production of antiganglioside antibodies associated with AMAN.

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Patient consent Obtained.

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Mechanical thrombectomy in severe acute stroke: preliminary results of the Solitaire stent

INTRODUCTION

Intravenous recombinant tissue plasminogen activator (rTPA) therapy has limited recanalisation-rates in large artery occlusions (nadir of 5.9% in Carotid-T-Occlusions).[1] Therefore, we prospectively evaluated the Solitaire stent (versions AB and FR, ev3 Inc., Plymouth, Minnesota, USA) in mechanical thrombectomy in acute ischaemic stroke.

MATERIALS AND METHODS

Acute stroke patients were triaged on admission for potential mechanical thrombectomy.

Inclusion criteria

- Age ≤80
- NIHSS score ≥10, less if symptoms were fluctuating
- Onset-to-treatment-time ≤4.5 h or secondary worsening (increase in NIHSS score ≥4). When symptom onset was unclear, patients were eligible if there was mismatch between symptoms and CT-scan
- Any brainstem syndrome.

Exclusion criteria

- Cerebral haemorrhage. Acute infarction >1/3 of middle cerebral artery (MCA) territory on CT-scan.
- Prestroke modified Rankin Scale (mRS) score ≥4

Eligible patients had immediate CT-angiography without delaying intravenous rTPA-thrombolysis if applicable according to the guidelines of the German Neurological Society (DGN). In case of occlusion of either the internal carotid artery (ICA), the MCA-M1-segment or the basilar artery (BA) mechanical thrombectomy was carried out. Up to four clot extraction maneuvers were performed. Any preceding stenosis was a priori stented. These patients received intravenous eptifibatide for 24 h to prevent in-stent-thrombosis; its short half-life would allow for emergency decompressive craniectomy. Combination of rTPA and eptifibatide is safe.[2]

NIHSS and mRS scores were assessed on admission and discharge. mRS ≤2 on discharge was defined as good functional outcome. 'Thrombolysis in Myocardial Infarction' (TIMI) scores of 2 or 3 were defined as successful recanalisation.

RESULTS

Twenty-six patients were eligible for mechanical thrombectomy with the Solitaire stent since October 2009. In 22/26 cases (85%), the moment of symptom onset was clear, averaging 64 min till arrival (table 1). The average NIHSS score on arrival amounted to 16, ranging from 7 to 31. Ninety-two per cent had a NIHSS score ≥10 and 96% had a mRS score ≥4. Prior to mechanical thrombectomy 19/26 patients (73%) received intravenous rTPA. Acute a priori stenting of the ICA was necessary in 12 cases (46%). The revascularisation rate was 88%; the optimum (TIMI 3) was reached in 69%. In 50%, the first attempt led to recanalisation. Five of six carotid-T-occlusions reached TIMI 3.

Outcome

Twenty-four of 26 patients survived the stroke, averaging 7.4 on the NIHSS on discharge. Two patients died and were completely excluded from all NIHSS statistics. Ten patients (38%) had a good clinical outcome; mean hospitalisation time was 19 days. Five patients (19%) had no residual symptoms at all. Eighteen patients (69%) showed improvement. Two of 23 patients with anterior circulation stroke died; the 21 survivors had a mean initial NIHSS score of 15.4 and 7.6 on discharge. Nine patients (39%) had a good outcome. Six patients (26%) presented with carotid T occlusion; 3 of them reached a good outcome although 4/6 suffered from tandem stenosis. Acute ICA stenting proximal to the occlusion site did not significantly influence the outcome. Of three occlusions in the posterior circulation (2 BA, 1 VA) only one had a good outcome. Two of the 19 bridging-patients died at the hospital. The remaining 17 bridging-patients had a mean NIHSS score of 15.3 on admission and 5.8 on discharge (62% reduction). Good outcome was reached by 8/19 bridging-patients (42%). Seven patients ineligible for bridging had an average base NIHSS score of 13.7 and 9.6 on discharge (30% reduction); 2/7 reached a good outcome (29%). NIHSS score reduction in the bridging group was significantly better (p=0.045; Mann–Whitney U test).

Overall mean time from symptom onset to mechanical revascularisation (OTR) was 327 min (5:27 h), including four patients with only last well time known. For the

特集I　B細胞の分化と機能発現

B細胞内因性TLRシグナルによるB細胞応答の制御機構*

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小林和夫**
高橋宜聖**

Key Words：B cells, antibody response, Toll-like-receptors, viral infection

はじめに

ウイルスやバクテリアなどの病原体の侵入を感知すると，生体内では自然免疫系につづいて獲得免疫系が始動し，それらが協調して速やかに病原体の排除に働く．自然免疫系は，病原体成分の共通パターンを認識するパターン認識レセプター(pattern recognition receptor；PRR)を用いて病原体の識別を行うのに対し，獲得免疫系では，遺伝子再構成により多様性を獲得した抗原受容体が，病原体識別の役割を担う．B細胞は，PRRの一つであるToll様受容体(Toll-like receptor；TLR)と，B細胞抗原受容体(BCR)を同時に発現し，これら2種類の抗原受容体を利用して病原体構造を多角的に認識できる点が大きな特徴である．これまで，BCRシグナルのB細胞分化に果たす重要性が明らかにされてきた反面，B細胞内因性のTLRシグナルがB細胞の分化，活性化に果たす役割は，用いる抗原構造の不均一性を主な原因として，統一した見解は得られていない．本稿では，B細胞内因性のTLRシグナルが，ウイルス粒子に対する一次免疫/二次免疫応答でのB細胞分化に果たす機能的役割について，最新の知見を含めて議論したい．

B細胞におけるTLRの発現

B細胞が病原体を直接認識した際，もたらされるTLRシグナルは病原体の種類により多種多様である．TLRはヒト，マウスとも13種類(TLR1～TLR13)が同定されているが，細胞内発現部位において2つのグループに大別される．1つは細胞表面に発現しているグループであり，多くは病原体表面成分であるリポ蛋白質やリポ多糖を認識し，TLR2やTLR4などがこれに含まれる．2つ目はエンドソーム内に発現するものが相当し，病原体内部に存在するRNAもしくはDNAを認識するTLR3, TLR7, TLR8, TLR9などが含まれる．

B細胞におけるTLRの発現レベルは，①B細胞サブセットの種類，②B細胞が生着する組織の種類，③ヒト/マウス(動物種)，のような複数の要因により大きな影響を受けることが知られている．また，これらのパラメーターが同じB細胞を解析した場合でも，研究グループごとに実験結果が異なる例も報告されており，これ以外の別の要因がB細胞のTLR発現レベルに影響を与える可能性も否定できない．これまで得られている統一的な見解をまとめると，ヒトの場合ではナイーブB細胞($CD19^+CD27^-$)と記憶B細胞($CD19^+CD27^+$)の双方でTLR1, TLR6～10の発現が認められている[1)~5)](表1)．

* Control of B-cell responses by B-cell intrinsic TLR signaling.

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表1　ヒトおよびマウスB細胞におけるTLRの発現

	ヒト		マウス			
TLR	ナイーブB細胞	記憶B細胞	FO	MZ	B1	Memory
TLR1	＋	＋＋	＋＋	＋＋	＋	＋＋
TLR2	＋/－	＋/－	＋	＋＋	＋＋	＋
TLR3	＋/－	＋/－	＋/－	＋/－	＋/－	＋＋
TLR4	＋/－	＋/－	＋＋	＋＋	＋＋	＋＋
TLR5	＋/－	＋/－	＋/－	＋/－	＋/－	＋/－
TLR6	＋	＋＋＋	＋/－	＋/－	＋	＋＋
TLR7	＋	＋＋＋	＋＋	＋＋＋	＋＋＋＋	＋＋
TLR8	＋	＋＋	(＋/－	＋/－	＋	＋/－)*
TLR9	＋	＋＋＋＋	＋＋＋	＋＋＋	＋＋＋＋	＋＋＋
TLR10	＋	＋＋＋＋	(ND	ND	ND	ND)*
TLR11	ND	ND	ND	ND	ND	ND
TLR12	ND	ND	ND	ND	ND	ND
TLR13	ND	ND	ND	ND	ND	ND

B細胞サブセットの種類，ヒトまたマウスの種によりTLRの発現は異なる．ヒト，マウスともに記憶B細胞ではナイーブB細胞に比べいくつかのTLRの発現が上昇している．
＋：低発現，＋＋：中程度の発現，＋＋＋：高発現，＋/－：発現の有無は懐疑的．FO：濾胞B細胞，MZ：辺縁帯B細胞，B1：B1B細胞，Memory：記憶B細胞，ND：not determined
*マウスにおいてTLR8, 10は機能的な蛋白質として発現しない．

さらに，記憶B細胞ではナイーブB細胞に比べて大部分のTLRでその発現レベルが上昇し，in vitroでのTLRアゴニスト刺激に対する応答性も高いことが報告されている[1)5)6)]．この記憶B細胞のTLRアゴニストへの高い感受性は，記憶B細胞のBCRを架橋できない別の病原体が侵入した場合でも，TLRシグナルを介して，ヒト記憶B細胞が抗原非依存的に長期維持されるというモデル(bystanderモデル)を支持する根拠の一つとなっている[7)]．

マウスナイーブB細胞は3種類(濾泡B細胞，辺縁帯B細胞，B1細胞)に大別され，すべてのB細胞サブセットはTLR1, 2, 7, 9に加え，ヒトナイーブB細胞には検出されないTLR4を発現する[8)~10)](表1)．また，記憶B細胞におけるそれぞれのTLRの発現に関してはDNA tipによる解析から，その発現は濾胞B細胞とほぼ同様の発現パターンが認められている．なかでも，TLR3やTLR6などのいくつかのTLRでその発現がナイーブB細胞に比べて上昇しており，ヒトとマウスの種によらず記憶B細胞ではTLRの発現が上昇していると考えられる[11)]．ただし，B細胞が実際の病原体からTLRシグナルを受け取る際に，あらかじめB細胞はサイトカインやBCRで刺激されていると推定されるが，これらの刺激はB細胞のTLR発現レベルを修飾することが確認されている[1)2)12)13)]．そのため，より生体内での条件に近づけるためには，これらの刺激の存在下でのTLR発現レベルの検証が必要となるかもしれない．また，TLR発現レベルを調べた多くの実験がmRNAを指標にしているが，蛋白レベルとの相関性に乏しい点や[4)]，TLR蛋白の細胞内局在部位が機能発現に重要であると推察されることなどから[14)]，今後はmRNAレベルでの解析に加え，蛋白レベルでの細胞内分布に基づいた議論が待たれるところである．

抗原の形状による B細胞内因性TLRの 機能的役割の相違

TLR3を除くすべてのTLRは共通のアダプター蛋白質であるmyeloid differentiation primary response protein 88(MyD88)を介することによりNF-κBに至るシグナルカスケードを動かし細胞内シグナルを伝達する[15)16)]．2005年にMedzhitovらは，細胞移入系により作製したB細胞選択的なMyD88欠損マウスを解析した結果，B細胞内因性のTLRが一次抗体産生応答に必須であることを報告した[17)]．しかし，その後ハプテン/キャリア蛋白にアジュバントとしてTLRアゴニスト

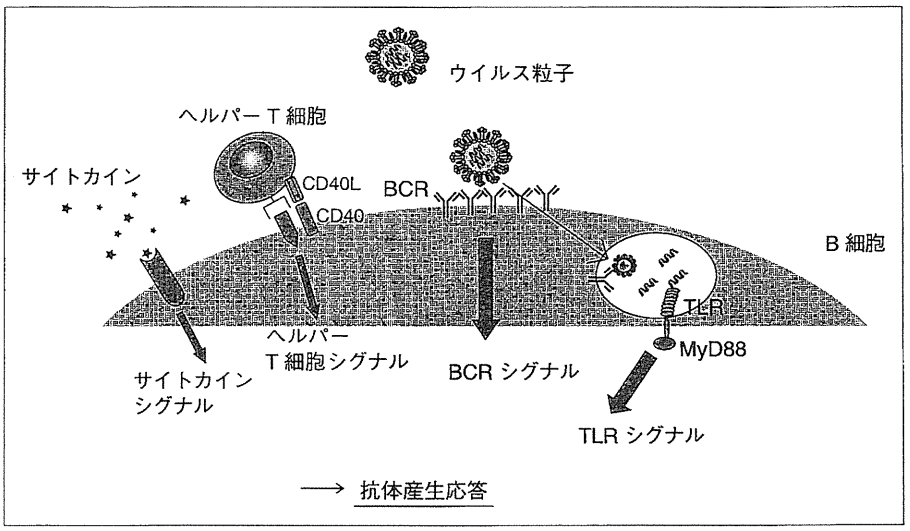

図1 抗体産生応答に寄与するB細胞内因性に供給されるシグナル
ウイルス粒子を認識したB細胞は，ウイルス表面の規則的に配置された高密度なエピトープをBCRで認識すると同時に，そこに含まれるTLRアゴニストをB細胞内因的に認識することで，ヘルパーT細胞やサイトカインからのシグナルと協調して抗体産生応答を誘導する．

を添加し行った実験では，抗体産生応答に変化が認められないか，認められたとしても，ある限られた条件下でのみ認められたことから，B細胞内因性TLRシグナルの抗体産生応答への関与について，疑問が投げかけられた[18)19)]．しかし，近年B細胞特異的なMyD88欠損マウスを用いた解析により，抗体産生応答におけるB細胞内因性のTLRシグナルへの依存性が抗原とTLRアゴニストの形状に依存していることが示された[20)]．つまり，TLRアゴニストを内包させたウイルス様粒子(virus like particle；VLP)やインフルエンザウイルス粒子に対するIgG抗体産生応答はB細胞内因性のTLRシグナルに依存する一方で，蛋白抗原とTLRアゴニストの混合物，あるいは単にこれらを架橋した抗原への抗体産生応答は，B細胞内因性のTLRに依存しないことが判明したのである．同様の現象は，ウイルス粒子を模倣したナノサイズの人工ポリマーにTLRアゴニストを内包させたワクチンでも観察されている[21)]．多くのウイルス粒子は，直径約数100nmの粒子表面にB細胞エピトープを発現し，粒子内部にTLRアゴニストを内包するという特徴的な構造を有している．前述の結果は，このウイルス粒子に特徴的な構造を有することが，B細胞内因性のTLRシグナルへの依存性を高める重要な要素となる可能性を強く示唆する．このように，BCRとTLRという2つの抗原レセプターを用いて，B細胞がウイルス粒子構造を認識することが，抗体産生応答の制御には重要である(図1)．

ウイルス粒子に対する一次B細胞応答へのTLRシグナルの役割

1．一次抗体産生応答とTLRシグナル

感染初期に病原体排除に働く抗体は辺縁帯B細胞やB1 B細胞などのB細胞から産生されるT細胞非依存的な抗体産生応答に大きく依存している．このT細胞非依存的な抗体産生応答は抗原への結合親和性が低く，その多くはIgM型であるが，T細胞との相互作用の必要がないため，迅速に産生され，感染初期の病原体の広がりを抑える．実際，急性インフルエンザウイルス感染に対する一次免疫応答においてT細胞非依存的な抗体産生応答が誘導され，迅速な中和抗体の産生を経て感染防御にかかわることが示されている[22)]．特に，急性感染症など病原体の増殖が著しく病状の進行速度が速い感染症の場

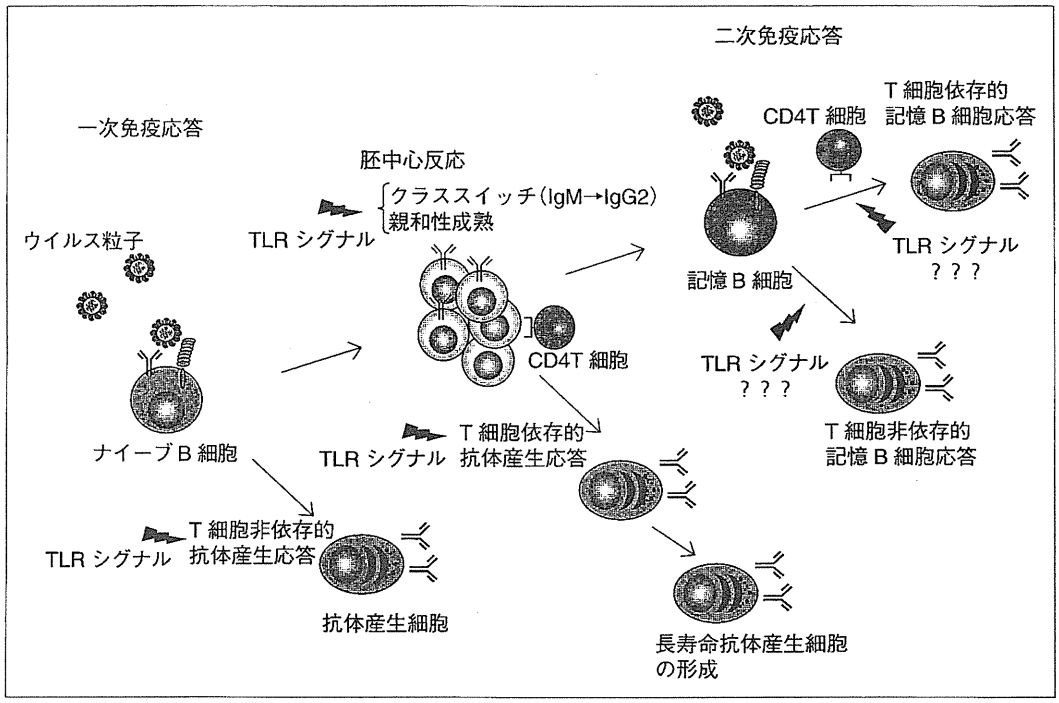

図2　B細胞内因性のTLRシグナルによる抗体産生応答の制御モデル
ナイーブB細胞はウイルス粒子などの病原体を直接認識することによりBCRシグナルと同時にB細胞内因性のTLRシグナルが供給される．一次免疫応答においてB細胞内因性のTLRシグナルはT細胞非依存的な抗体産生応答，胚中心反応によるクラススイッチや抗体の親和性成熟，T細胞依存的な抗体産生応答，また長寿命形質細胞の形成に影響を及ぼす．一方，記憶B細胞応答におけるB細胞内因性TLRシグナルの作用は不明な点が多く詳細な解析が必要である．

合には，これらT細胞に依存しない迅速な抗体産生応答の寄与は大きい．いくつかの細菌感染の実験システムにおいて，TLRシグナル依存的にT細胞非依存的な抗体産生応答が促進されることから[23)24)]，ウイルス感染に対するT細胞非依存的な抗体産生応答へのTLRシグナルの関与についても同様な解析が待たれるところである．

2．胚中心反応とTLRシグナル

通常，この早期に誘導されたT細胞非依存性応答の後にT細胞依存性の抗体産生応答が起動し，胚中心反応を経てクラススイッチと親和性成熟を果たした質の高い抗体産生が行われ，より効率的な抗原排除に貢献する．この胚中心反応は，polyoma virus感染やインフルエンザウイルスVLPに対してMyD88欠損マウスにおいても正常に形成されることが報告されている一方で[25)26)]，TLRアゴニストを含むバクテリオファージ由来のVLPや[20)]，Friend virus(FV)感染に対する免疫応答ではB細胞内因性のTLRシグナルに依存することが報告されている[27)]．そのため，少なくともいくつかの条件下ではB細胞内因性のTLRシグナルが胚中心反応の誘導/維持に寄与すると考えられるが，それ以上の詳細は現時点で不明である．

また，病原体に対する免疫応答では，B細胞はクラススイッチにより抗体のサブクラスを変えることで，サブクラスに応じた新たなエフェクター機能を抗体に付与する．なかでも，IgG2(ヒト)，IgG2a/2c(マウス)クラスの抗体は補体の活性化能が高いことなどから，病原体の排除により有効なサブクラスとして知られている．しかし，MyD88欠損マウスでは，インフルエンザウイルスやpolyomaウイルスに対してIgG1クラスの抗体産生応答は正常なのに対し，IgG2a/2cクラスの抗体産生応答が非常に減弱してお

り[25)26)28)~30)]，さらにB細胞内因性のMyD88のみが欠損した条件でも認められることから[20)31)]，B細胞内因性のTLRシグナルがIgG2クラススイッチに必須な役割を果たしていることが伺える（図2）．

ウイルス粒子に対する二次B細胞応答

同じ病原体に再び曝露された場合，一次免疫応答の際に形成/維持された長寿性抗体産生細胞由来の中和抗体が再感染した病原体の排除に重要な役割を果たす．しかし，この中和抗体価が十分でない場合，長寿性抗体産生細胞と同様に胚中心で誘導された記憶B細胞がバックアップにあたると考えられてきたが，実際，インフルエンザウイルスのような急性感染の場合に，記憶B細胞が感染防御に寄与できるほど，迅速に中和抗体を産生できるかどうか不明であった．最近，われわれはインフルエンザウイルスへの気道感染により，これまで報告されてきた二次リンパ器官や末梢血に加え，感染局所である肺にもクラススイッチした記憶B細胞が長期間維持されることを見出した．さらに，同じインフルエンザウイルスで再感染した場合，持続性抗体によるバリアーシステムを乗り越えたウイルスにより肺記憶B細胞が再活性し，速やかに気道粘膜に中和抗体を供給し，ウイルス感染防御に寄与することを明らかにしている[32)]．

このような二次免疫応答の際，クラススイッチした記憶B細胞は辺縁帯や胚中心近傍領域を含めたB細胞濾胞に存在し[33)34)]，T細胞との相互作用を経ることにより抗体産生細胞へ速やかに分化することが，モデル抗原の実験系で証明されてきた[35)36)]．しかし，初回感染の時と同様の状況を考えた場合，侵入してきた病原体を記憶B細胞がそれ単独で認識し，迅速に抗体を産生して病原体排除に働くことは感染防御に有利になると考えられる．実際，サイトメガロウイルス（CMV）[37)]や水泡性口内炎ウイルス（VSV）[38)]に対し，T細胞非存在下においても記憶B細胞から抗体産生応答が誘導され，抗原排除に働いていることが示されており，ウイルス粒子に対する二次抗体産生応答では，記憶B細胞からのT細胞非依存的な抗体産生応答が寄与することが伺える．このT細胞非依存的な記憶B細胞応答は，ウイルス粒子構造に依存しており，その構造を失ったウイルス抗原では抗体産生応答は誘導されないことが示されている[38)39)]．そのため，ウイルス粒子表面に規則的に配置されたエピトープによるBCR架橋の関与が示唆されている一方で，粒子構造によるBCRの架橋のみではT細胞非依存的な記憶B細胞応答を十分に再現できないことも報告されている[40)]．以上の結果から，TLRアゴニストを含むほかのウイルス粒子構造の関与が推察されるが，詳細については今後の解析が待たれるところである．

記憶B細胞がウイルス粒子などの病原体を認識して取り込み，MHC class IIへ抗原提示を行うことで同じ抗原特異性を持つヘルパーT細胞からの補助シグナルを受け，BCRシグナル，TLRシグナルと合わせた協調作用による記憶B細胞の再活性化が誘導される．このT細胞依存的な記憶B細胞の再活性化においてTLRシグナルが持つ意味はどのようなものであろうか．T細胞が正常に存在するMyD88欠損マウスにおいてインフルエンザウイルスのVLPによるboost後のIgG抗体産生応答が非常に減弱していることや，インフルエンザウイルスの再感染に対し脆弱になっていることなどから[26)29)]，MyD88を介したTLRシグナルがT細胞依存的な記憶B細胞の再活性化応答もコントロールしている可能性を示唆するものである．しかし，ここにおけるB細胞内因性のTLRシグナルの寄与に関しては未検索であり，今後のより詳細な解析が必要とされる（図2）．

おわりに

BCRとTLRを介して獲得免疫応答と自然免疫応答の2つを担う能力を同時に兼ね備えたB細胞は病原体の侵入に際し，それらからもたらされる2つシグナルを協調的に処理して適切なアウトプットを行うことで迅速かつ効率よく病原体排除に働いている．近年，ウイルス粒子やTLRアゴニストを内包したVLP，また人工ポリマーワクチンなどに対するB細胞応答の解析により，ウイルス粒子の特徴である規則的に配置された抗

原エピトープによってもらされる効果的なBCR架橋と同時に，B細胞が内在的にTLRシグナルを認識することがいかにB細胞の免疫応答を質的，量的なコントロールに重要であるかが明らかとなってきた．これらは進化の過程上，ウイルスなどの病原体に繰り返し曝露されてきた中で，生体の免疫機構が効率よく免疫応答を誘導し，それらを排除する過程を試行錯誤してきた中で獲得した精緻な免疫制御機構であろう．この免疫制御機構をより深く理解することは，今後新たに克服しなければならない感染症の制圧に貢献するもと考えられる．

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Experience Evidences

麻疹・風疹血清疫学と麻疹風疹混合（MR）ワクチンによる抗体反応からみた今後の麻疹および風疹対策

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抄　録

1歳時に麻疹ワクチン・風疹ワクチンを受けた世代は，自然感染世代よりも麻疹抗体価および風疹抗体価が低値である。麻疹ウイルス野生株・風疹ウイルス野生株の排除を目指すならば，ワクチン世代に麻疹風疹混合（MR）ワクチン追加接種が必要であり，ワクチン接種時の抗体価に応じて効果的な免疫応答が認められる。なお，麻疹ワクチン・風疹ワクチン接種率向上により，麻疹・風疹の血清疫学は変化しており，変化に応じた麻疹および風疹対策の構築が必要である。

キーワード：麻疹，風疹，血清疫学，MRワクチン，麻疹排除

はじめに

麻疹，風疹ともに多くの人が免疫を持つと流行の抑制，さらには野生株の排除が可能な感染症である。現在，フィンランドや米国では麻疹ウイルスと風疹ウイルス野生株が排除されている[1,2]。世界保健機関（WHO）はパンアメリカ地域に続いて，2010年までにヨーロッパ地域と地中海地域で，2012年までに日本が属する西太平洋地域で麻疹ウイルス野生株の排除を目指している[3]。麻疹ウイルス野生株の排除を達成するためには，麻疹の集団免疫率である90～95%を越える高い接種率で麻疹ウイルスを含むワクチン（MCV）を2回接種する必要がある[4,5]。

わが国では，1978年から1歳児を対象に麻疹ワクチン定期接種が開始され，1989年から1994年まで麻疹ムンプス風疹（MMR）混合ワクチンが定期接種として併用され，1994年から1歳児を対象に風疹ワクチン定期接種が開始された。この結果，2008年12月末の時点で，わが国の30歳未満は麻疹ワクチン接種世代であり，18歳未満は風疹ワクチン接種世代である。今回，MRワクチン2回接種の必要性を明らかにするために，麻疹および風疹の血清疫学とMRワクチン接種による抗体反応について検討を行った。

対象および方法

1．成人の麻疹，風疹，ムンプス，水痘の血清疫学の検討

対象は平成19年度，20年度の国立病院機構三重病院の採用者および転勤者のうち，麻疹，風疹，ムンプス，水痘の抗体価測定に同意が得られた65人である。血清抗体は，麻疹はマイクロ中和（mNT：判定基準は100%細胞変性効果抑制）法，風疹は赤血球凝集抑制（HI）法，ムンプスは酵素免疫法（EIA），水痘は免疫付着赤血球凝集（IAHA）法で測定した。なお，血清抗体価は2を底とする対数に変換すると正規分布するため，2を底とする対数に変換後平均抗体価を比較検討した。

2．2期接種対象者，3期接種対象者および思春期（19～23歳）の麻疹，風疹抗体価とMRワクチン接種による抗体反応の検討

対象は2期接種対象者75人，3期接種対象者69人，思春期59人である。保護者または本人の同意後に，MRワクチン（ミールビック®）接種前および接種4週後に血清を採取し，EIA法にて血清抗体価を測定した。

統計学的検討は，ANOVA検定，t検定，クラスカル・ワーリス検定，マン・ホイットニ検定を用いて行った。

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