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.

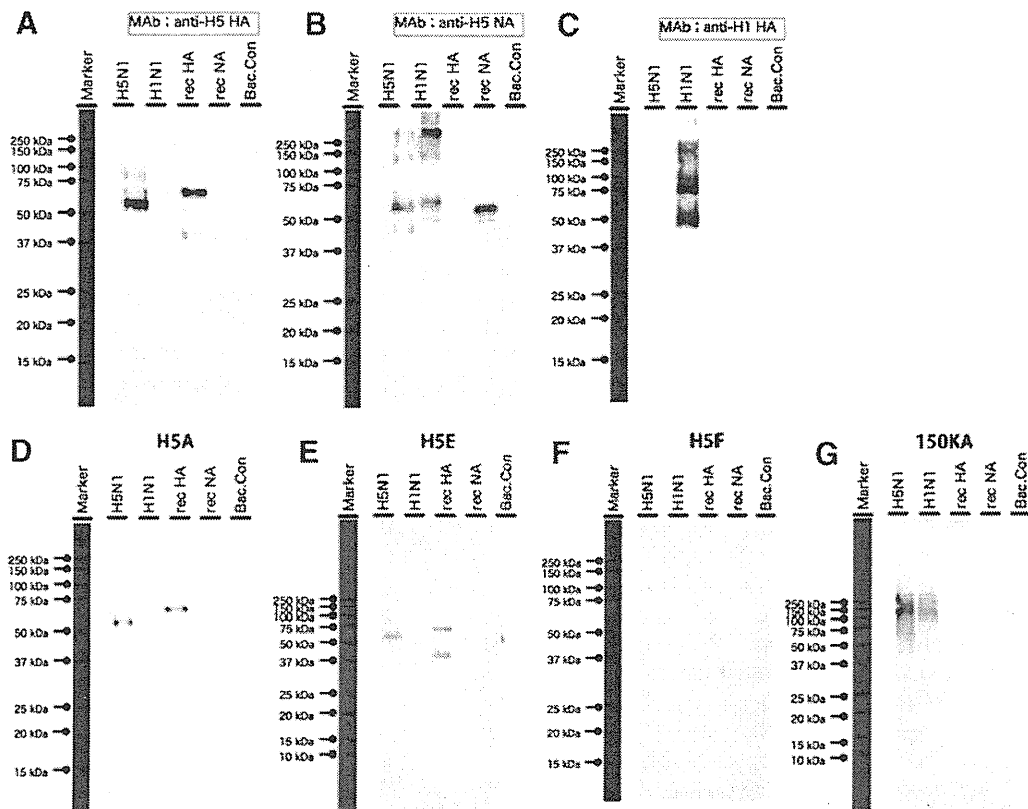

Fig. 1. Detection of influenza virus proteins in Western-blot analysis. Purified influenza virus proteins (0.5 μg/lane) were subjected to SDS-PAGE under reducing conditions. After blotting on a PVDF membrane, the proteins were detected by incubation with the eight monoclonal antibodies (mAbs), followed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

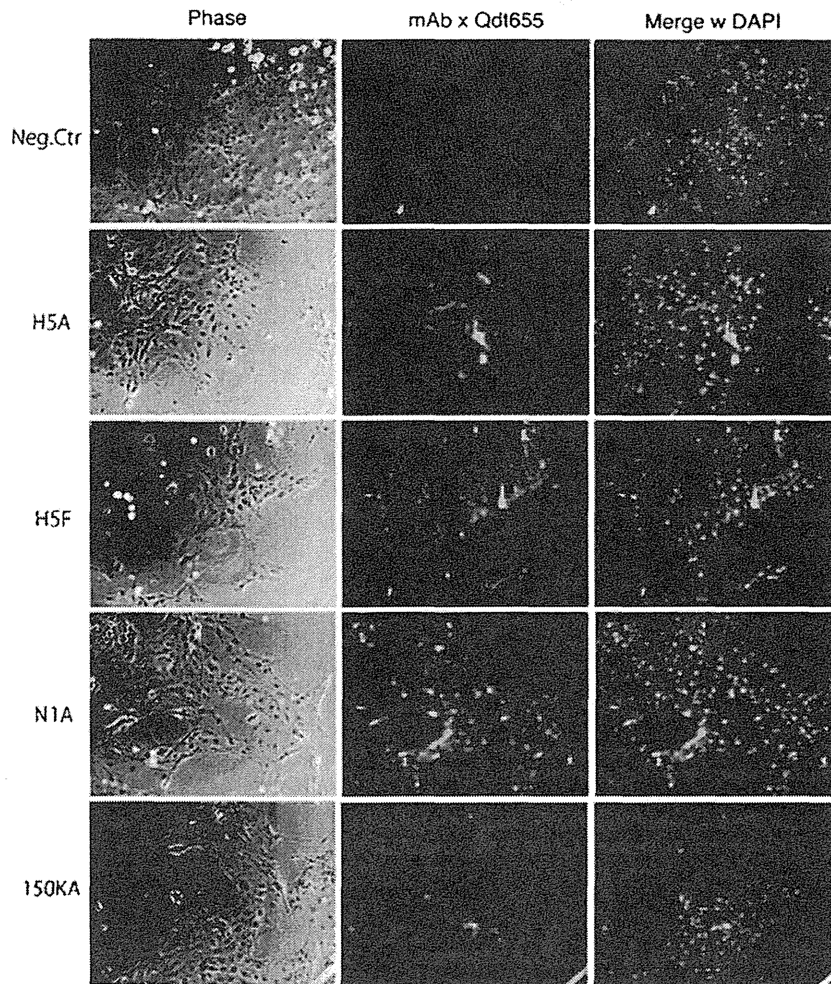

Fig. 2. Fluorescent immunostaining of H5N1 virus-infected MDCK cells with monoclonal antibodies (mAbs). Paraformaldehyde-fixed, H5N1 virus-infected MDCK cells were permeabilized by TBS-Tween and incubated with biotinylated mAbs. The mAbs were detected with Qdot655-conjugated streptavidin (red). Shown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

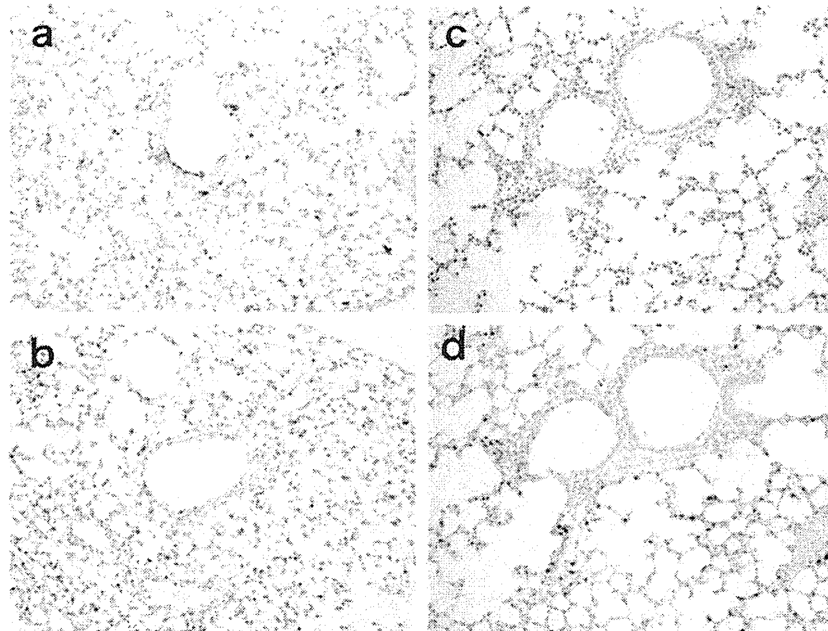

Fig. 3. Immunohistochemical analyses of lung sections from mice infected with A/Vietnam/1194/2004 (NIBRG-14) or A/HongKong/483/97 (HK483) virus. (a, b) Influenza virus antigens were detected in the epithelial cells of the bronchioles and alveoli of the mouse infected with A/Vietnam/1194/2004 (NIBRG-14)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

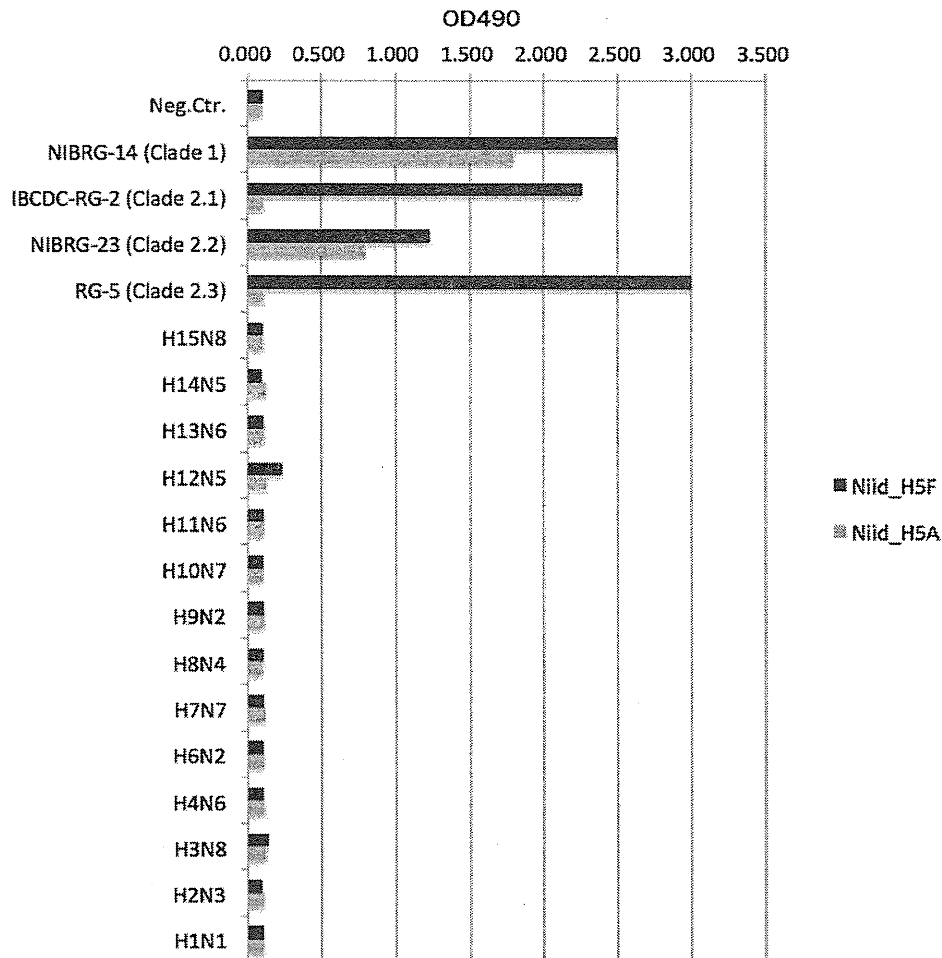

Fig. 4. ELISA reactivity of the Niid_H5A and Niid_H5F monoclonal antibodies (mAbs) to various influenza virus strains. Different influenza virus strains were immobilized on 96-well plates and incubated with biotinylated Niid_H5A or Niid_H5F mAbs followed by peroxidase-labeled streptavidin. The binding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

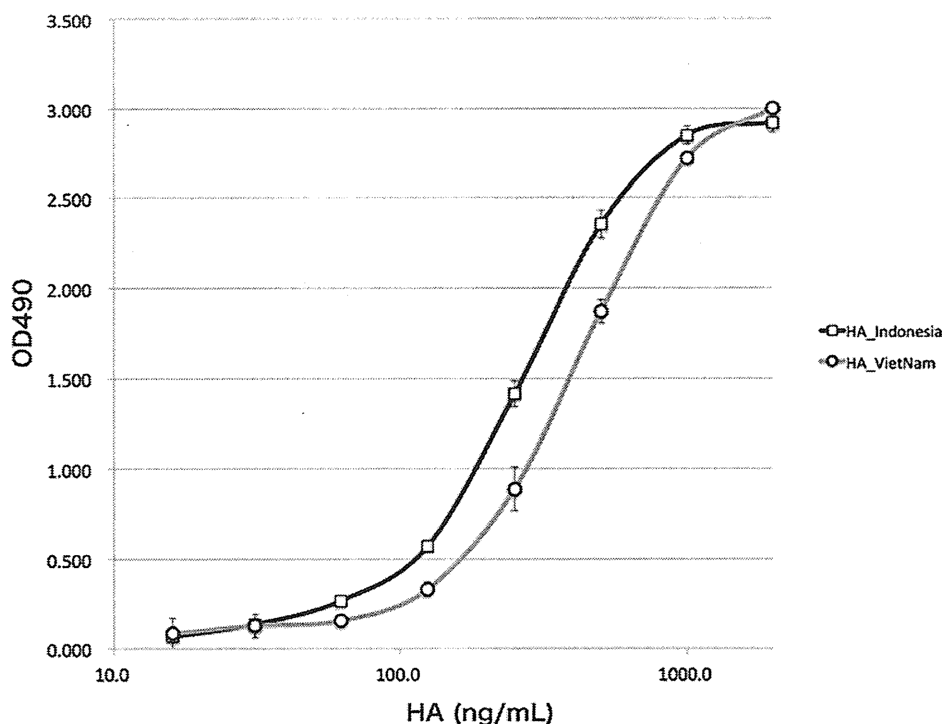

Fig. 5. Antigen-capture ELISA reactivity of monoclonal antibodies (mAbs) to H5N1 and H1N1 virus strains. The anti-H5 mAb Niid_H5F was immobilized on 96-well plates and reacted with serially-titrated purified H5N1 virus fractions for 1 h at room temperature. The bound virus proteins were detected by incubation 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.[5] 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

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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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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

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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**
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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

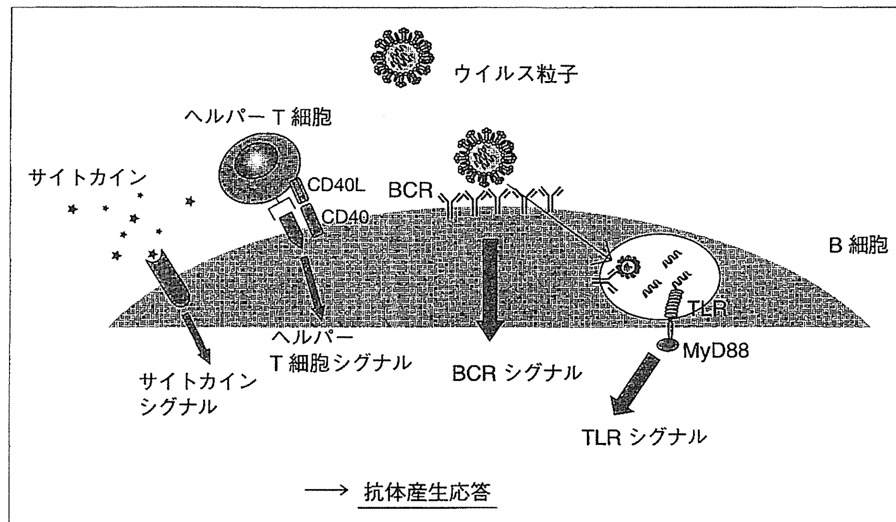

図1　抗体産生応答に寄与するB細胞内因性に供給されるシグナル
ウイルス粒子を認識したB細胞は，ウイルス表面の規則的に配置された高密度なエピトープをBCRで認識すると同時に，そこに含まれるTLRアゴニストをB細胞内因的に認識することで，ヘルパーT細胞やサイトカインからのシグナルと協調して抗体産生応答を誘導する．

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

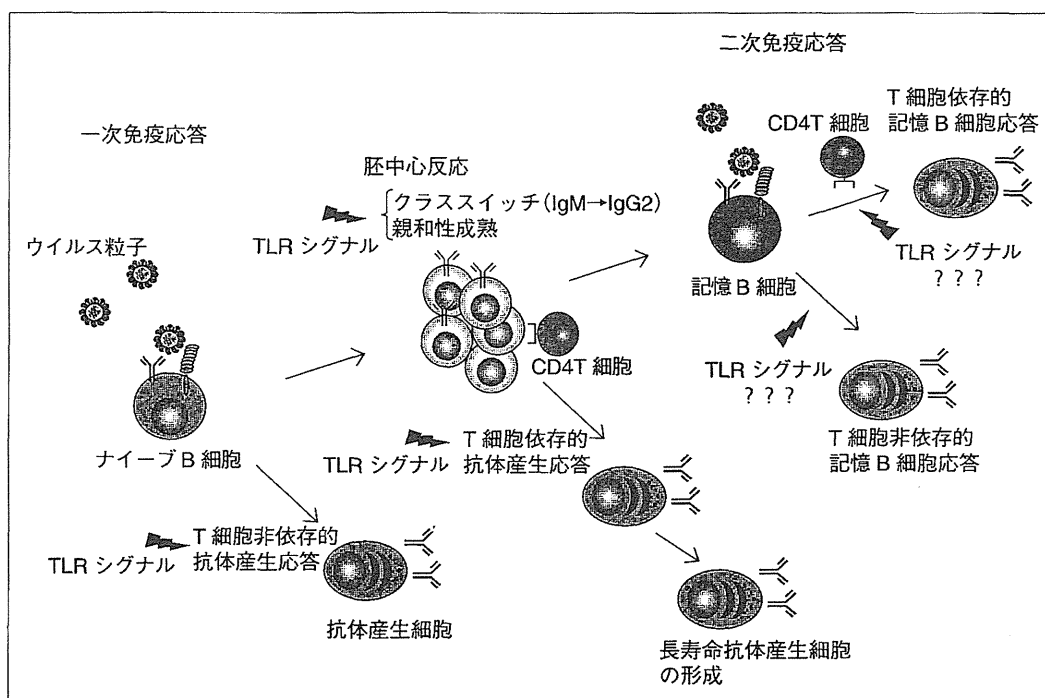

図2　B 細胞内因性のTLRシグナルによる抗体産生応答の制御モデル

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こところである．

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細胞濾泡に存在し[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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先天性サイトメガロウイルス(CMV)感染症の病態・診断・治療・予防(後方視的診断も含め)
脳性麻痺

庵原俊昭*

先天性サイトメガロウイルス(CMV)感染は妊婦の0.3〜1%に認める比較的頻度の高い先天性ウイルス感染である。抗体陰性妊婦が妊娠早期に感染すると重篤な症状が児に出現し，予後不良である。出生時CMV感染症の症状がなくても先天性CMV感染児の10%に遅発性難聴と知的障害が出現する。わが国では生後3週以降に先天性CMV感染が疑われたときの診断に，乾燥臍帯を用いた核酸検出法が行われている。CMV感染母体に抗CMV抗体高力価免疫グロブリンを投与すると児の感染予防に有効である。わが国では先天性CMV感染スクリーニング検査は行われていないが，CMV抗体陰性妊婦への生活指導の介入は胎児への感染予防に有効である。

はじめに

サイトメガロウイルス（cytomegalovirus；CMV）はヘルペスウイルス科βヘルペス亜科に属するエンベロープを持つ大型のDNAウイルスである。免疫健常者では多くは不顕性感染であるが，移植レシピエント，がん患者，ヒト免疫不全ウイルス(HIV)感染者などの免疫不全者に感染すると，肺炎，脳炎，網膜炎，腸炎などの多彩な臨床症状を呈してくる。

ヘルペスウイルス科のウイルスの特徴は潜伏感染することである。CMVも初感染後骨髄単球系細胞や上皮細胞に潜伏感染し，宿主の免疫状態に応じて再活性化する[1)2)]。CMV抗体陽性者は周期的に唾液や腟分泌液にウイルスを排出している。また，CMVに対する中和抗体は，すべてのCMVを中和することができない部分防御抗体であるため，抗体陽性であっても再感染することがある[3)4)]。なお，初感染したあと，成人ではCMVは数週から数カ月間，乳幼児では数カ月から数年間尿中や唾液中にウイルスを排出している[1)]。免疫低下状態にあるCMV抗体陽性妊婦は，周産期は産道に，出産後は母乳中にウイルスを排出している。先天性CMV感染症の病態，診断，治療，予防について解説する。

1．CMVの感染

CMVは抗体陽性者の唾液や尿を介して感染する(水平感染)。保育施設に通園している乳幼児は通園し始めると水平感染し，20〜40%の園児は尿や唾液に長期間CMVを排出している[1)]。保育施設に働く職員や通園している乳幼児を診察する機会の多い小児医療機関職員は，CMV感染を受けるハイリスク者である。おむつを替えたあとや唾液で汚れたおもちゃなどに触れたあとの手洗いが大切である。

保育園に通園している乳幼児の母親もCMV感染のハイリスク者である。特に母親が妊娠している場合，抗体陰性であれば初感染，抗体陽

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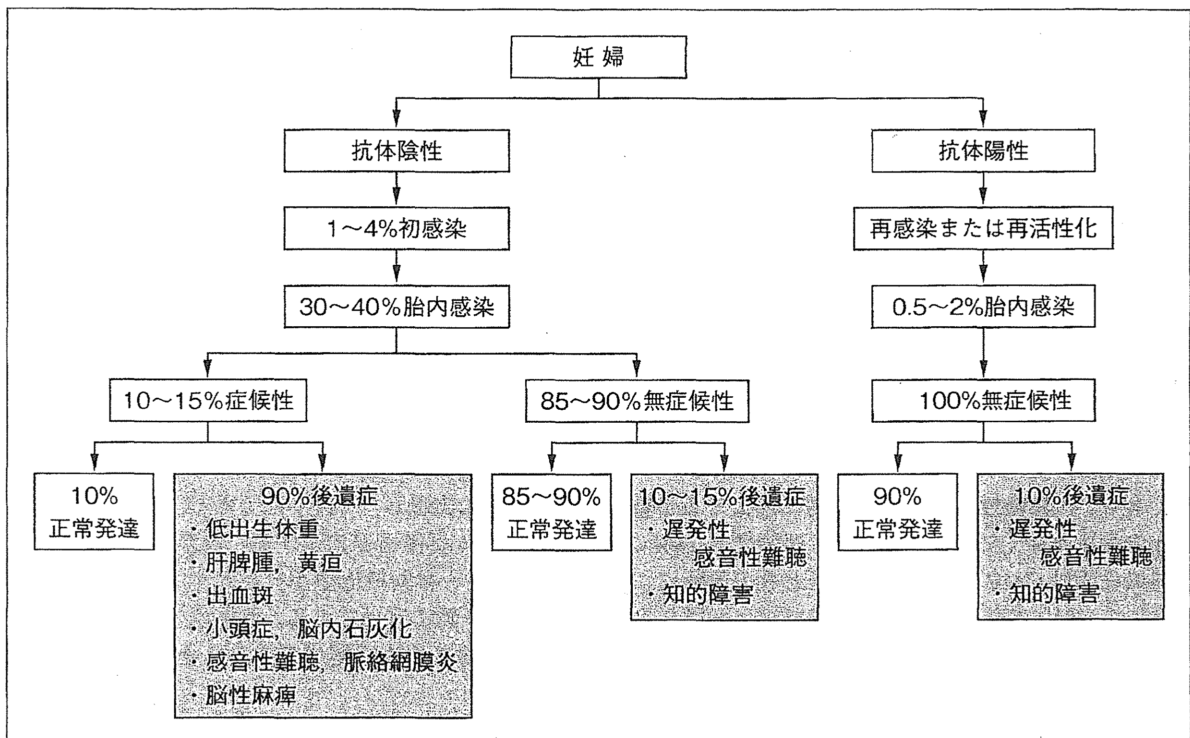

図1 CMV胎内感染と児の臨床症状

性であったとしても再感染によりCMVが感染し，胎児に影響を及ぼすリスクがある。保育施設職員と同様に，抗体陰性の妊婦はおむつ交換後や子どものおもちゃに触れたあとの手洗いが大切である[5]。成人がCMVの初感染を受けても多くは不顕性感染であるが，5%の人は伝染性単核球症様の症状(発熱，リンパ節腫大，肝脾腫，肝機能異常，白血球数増多など)が出現する。

ほかのCMV感染ルートとして性行為による感染や輸血を介しての感染がある。CMV抗体陽性者では腟分泌液や唾液にウイルスを周期的に排出している。輸血のドナーが抗体陽性の場合，輸血により骨髄単球系細胞に潜伏しているCMVがレシピエントに感染する。移植患者ではドナーの臓器に潜伏しているCMVの感染により，肺炎，肝炎，腸炎などを発症する。

CMVは，妊娠中の胎内感染（垂直感染），出産時の産道感染および出産後の母乳による感染の3ルートで母子感染する。母乳中のウイルス量のピークは生後2週〜2カ月である[1]。母乳中のウイルス量が多いほど，児への感染リスクが高率である。周産期にCMVに感染した児は出生後22日〜3カ月以内にCMVを排出し始める。

成熟児では産道感染および母乳による感染を受けても多くは不顕性感染か軽症に経過する。症状出現に曝露されたウイルス量と移行抗体の多寡が関与している。一方，移行抗体量が少ない未熟児では，輸血や母乳を介して周産期にCMVが感染すると，肝炎や間質性肺炎を発症することがある。未熟児に輸血する場合は，必要に応じ白血球を除いた製剤を使用する。また，母乳は凍結融解することでCMVの感染リスクは軽減する。

2．先天性CMV感染の頻度（図1）

妊娠中に母体を介して胎児にCMVが感染した状態が先天性CMV感染であり，臨床症状を伴う場合が先天性CMV感染症である。胎盤は胎児へのウイルス感染防御に働いているため，母体がCMV初感染であっても，胎児に感染するのは40%程度である[6)7)]。母体に感染したCMVは，胎盤に感染し，そこで増殖したあと胎児に感染する。

先天性CMV感染の頻度は0.2〜2%（平均1%），わが国では0.3%である[1)8)]。抗体陰性妊婦にCMVが初感染する頻度は1〜4%であり，胎児に垂直感染する頻度は30〜40%である。垂直感染した胎児のうち，出生時に低出生体重児，肝脾腫，網膜炎，出血斑などの症状(症候性感染，巨細胞封入体症)が出現する頻度は10〜15%である。症候性感染児の90%は聴力障害，視覚障害，知的障害，運動障害などの後遺症が出現する。在胎20週以上の死産例の15%にCMV感染を認めることから，胎内CMV感染が死産の一因と考えられている[9)]。一方，CMV垂直感染を受けても出生時症状を認めなかった乳幼児の10〜15%に，遅れて感音性難聴や知的障害が出現する。生後6カ月時点で聴力障害がない児でも，8年間フォローすると1年に1%の割合で聴力障害児が発見される[10)]。

抗体陽性妊婦では，CMVが再感染または再活性化することで0.5〜2%が胎内感染する。抗体陽性妊婦から生まれたCMV感染児は出生時無症状であるが，その後10%に遅発性感音性難聴が出現する[3)4)11)]。母体の再感染による先天性CMV感染の場合は，症候性CMV感染児と比較すると聴力障害の進行は緩やかで，障害の程度も軽度である。先天性CMV感染は遺伝子異常に伴う難聴に次いで頻度の高い難聴の原因であり，米国では先天性難聴の原因の15〜25%を占めている[10)]。

母体にCMVが感染したとき，胎児にCMVが感染する頻度は妊娠時期にかかわらず30〜50%である。しかし，症候性先天性CMV感染症の頻度は，妊娠5週までは40%，妊娠6〜20週16%，妊娠20週以降0%と，妊娠早期にCMVが感染するほど高率である[7)]。胎児の器官を形成する時期や移行抗体の量が，胎児での先天性CMV感染後の発症に関与している。

3．先天性CMV感染症の症状

症候性先天性CMV感染症はTORCH症候群の一つである。症候性感染の多くはCMV初感染妊婦から生まれた児である[1)]。第1子よりも第2子以降に出生した児のほうが発症頻度は高率である。症状は，低出生体重，肝脾腫，肝機能異常，黄疸，出血斑，血小板減少，小頭症，脳内(脳室周囲)石灰化，難聴，脈絡網膜炎，播種性血管内凝固障害(DIC)など，多彩で重篤である。出生時に正常でも，難聴や神経学的後遺症が遅れて出現する例もある。

表1 妊婦，胎児，新生児におけるCMV感染の診断

1) 妊婦のCMV感染の診断
1. 抗体の陽転化または有意上昇
2. IgM抗体の検出
3. ウイルス分離
・尿，唾液，腟分泌液
4. ウイルス核酸の検出*
・尿，唾液
5. ウイルス抗原血症
2) 胎児のCMV感染の診断
1. ウイルス核酸の検出*
・羊水
3) 先天性CMV感染の診断
〔条件〕
生後3週以内のサンプル（尿，唾液，血液など）を採取する
〔診断〕
1. ウイルス分離
・尿，唾液
2. ウイルス核酸の検出*
・乾燥唾液
・尿濾紙
・ガスリー検査濾紙
・乾燥臍帯
3. ウイルス抗原血症
4. 血清IgM抗体の検出

*：ウイルス核酸の検出にはPCR法，real time PCR法が用いられる。

4．先天性CMV感染の診断（表1）

1 妊婦のCMV感染の診断

CMV初感染の診断には，血清CMV-IgM抗体の検出，CMV-IgG抗体の陽転化または有意上昇が有用である。しかし，CMV抗体陽性者においては，血清抗体価だけでCMVの再感染または再活性化の診断は困難である。尿，唾液，腟分泌液からのウイルス分離またはポリメラー