Cross-protective potential of anti-nucleoprotein human monoclonal antibodies against lethal influenza A virus infection

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The nucleoprotein (NP) possesses regions that are highly conserved among influenza A viruses, and has therefore been one of the target viral proteins for development of a universal influenza vaccine. It has been expected that human or humanized antibodies will be made available for the prophylaxis, pre-emptive and acute treatment of viral infection. However, it is still unclear whether anti-NP human antibody can confer protection against influenza virus infection. In this study, we generated transgenic mice expressing anti-NP human mAbs derived from lymphocytes of a patient infected with H5N1 highly pathogenic avian influenza (HPAI) virus, and experimental infections were conducted to examine antiviral effects of the anti-NP antibodies against H5N1 HPAI viral infections with a high fatality rate in mammals. Transgenic mouse lines expressing the anti-NP human mAbs at more than 1 mg ml$^{-1}$ showed marked resistance to H5N1 virus infections. In addition, resistance to infection with an H1N1 subtype that shows strong pathogenicity to mice was also confirmed. Although the anti-NP mAbs expressed in the transgenic mice did not neutralize the virus, the mAbs could bind to NP located on the surface of infected cells. These results suggested a possibility that the non-neutralizing anti-NP human mAbs could induce indirect antiviral effects, such as antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. Taken together, these results demonstrated that anti-NP human mAbs play an important role in heterosubtypic protection against lethal influenza virus infections in vivo.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) viruses of subtype H5N1 cause severe pneumonia and multiple organ dysfunction syndrome, and mortality of infected patients is more than 60% (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Neutralizing antibodies binding to the globular head of haemagglutinin (HA) are effective for the inhibition of viral cell entry and subsequent viral replication. However, the antigenicity of the globular head region is highly variable,
even among homosubtypic HA proteins. Recently, highly conserved regions, which have been found in each ectodomain of external viral proteins, including HA, neuraminidase (NA) and matrix protein 2 (M2), have become attractive targets for broadly cross-reactive antibodies (Gerhard et al., 2006). In experimental infections, these antibodies have been shown to protect mice from lethal influenza infection (Doyle et al., 2013; Fu et al., 2009; Grandea et al., 2010; Heaton et al., 2013; Tan et al., 2012) and are therefore expected to have applications in vaccines and antiviral therapy in the future. However, because amino acid substitutions in the external proteins of influenza viruses occur with high frequency, for effective disease control it is necessary to prepare different types of antiviral antibodies that bind to a variety of epitopes.

In contrast to external viral proteins, the amino acid sequence of nucleoprotein (NP), which is one of the internal viral proteins, is over 90% conserved among influenza A viruses (Shu et al., 1993). Although antibodies against NP do not neutralize the viral infection, unlike those against HA, NP has been one of the target proteins in developing broad-spectrum vaccine inducing cellular immunity (Chen et al., 2000; Epstein et al., 2005; Fu et al., 1997, 1999). Despite tremendous research efforts, such a vaccine has not been developed. Recent studies demonstrated that passive transfer of immune serum against NP antigens inhibited viral replication in lungs of mice infected with an influenza H1N1 virus at sublethal dose (Carragher et al., 2008; Lamere et al., 2011). However, the antiviral potential of these antibodies against severe infections has been uncertain because those animal experiments were conducted under limited infective doses.

It has been reported that some neutralizing mAbs of human origin can suppress viral replication in animal models experimentally infected with influenza viruses (Chen et al., 2015; Kashyap et al., 2010; Simmons et al., 2007). These human mAbs may have potential for the development of effective vaccine and for use in the treatment of influenza infection. We previously collected peripheral blood from a volunteer who had recovered from H5N1 HPAI virus infection in northern Vietnam, and two Fab clones against influenza virus NP were selected by phage display method (unpublished data). In the present study, to investigate the antiviral effects of anti-NP human mAbs, we generated transgenic mouse lines expressing the anti-NP human mAbs. The classical approach to determine the protective activities of non-neutralizing antibodies in vivo is to transfer passively immune sera or mAbs to a naive animal, challenge with virus and observe the outcome (Burton, 2002). However, it is difficult to determine antiviral effects of antibodies unless a precise amount of antibodies is transferred passively into each naive mouse. Experimental infections showed that transgenic mice expressing the human mAbs were highly resistant to lethal infection with H5N1 HPAI viruses and H1N1 influenza A virus, demonstrating that the anti-NP mAbs play an important role in cross-protection against lethal infections with influenza A viruses.

**RESULTS**

**Generation of transgenic mice expressing human mAbs**

Genes of two Fab clones against influenza A virus NP, named 5C and 6C, were obtained from peripheral blood of a volunteer who had recovered from H5N1 HPAI virus infection, as described in Methods. In putative amino acid sequence analyses of 5C and 6C antibodies, only two amino acid differences were found in regions other than the complementarity-determining region of heavy chains. In contrast, there were no amino acid differences in light chains.

To assess the antiviral potential of anti-NP antibody in vivo, we attempted to generate transgenic mice stably expressing whole human mAbs, including each Fab of 5C and 6C. The heavy chain gene of each Fab was inserted into pFab CMV-dhfr2H7 plasmid to fuse the gene of human IgG1 Fc portion, and then both the chimeric cDNA of the full-length heavy chain and the cDNA of the light chain were cloned into pCXN2 plasmid (Fig. S1, available in the online Supplementary Material). The transgenes expressing whole human IgG were extracted from the constructed pCXN2 plasmids as described in Methods, and microinjected into the nuclei of C57BL/6 mouse eggs. Four transgenic mouse lines expressing 5C mAb (lines 2495 and 2497) and 6C mAb (lines 2647 and 2660) were generated. Western blot analysis revealed that the heavy and light chains of human

![Fig. 1. Expression of whole human IgG in sera of transgenic mice. The human mAbs in serum samples from transgenic mice were bound by heavy-chain-specific protein G Sepharose (G) or light-chain-specific protein L Sepharose (L), and were analysed by immunoblotting using anti-human IgG antibody. Quantification of expression level of human mAb in serum samples was done by ELISA. The mean concentrations (mg ml⁻¹) ± SD of human mAbs from serum samples of five mice per transgenic line are shown.](http://jgv.microbiologyresearch.org)
IgG were expressed in sera of all founder transgenic mice (Fig. 1). Antibody concentrations in serum samples from five transgenic mice per line were 0.2±0.1 mg ml⁻¹ for line 5C (2497), 0.4±0.1 mg ml⁻¹ for line 5C (2495), 1.6±0.2 mg ml⁻¹ for line 6C (2647), and 1.2±0.2 mg ml⁻¹ for line 6C (2660), indicating that differences in expression levels of whole human IgG are insignificantly small between transgenic mouse lines.

Characterization of anti-NP human mAbs expressed in transgenic mice

In immunofluorescence analyses using mouse serum from line 6C (2660), we observed positive staining of Madin-Darby canine kidney (MDCK) cells infected with all tested subtypes (H1 to H13) of influenza A viruses isolated from human and avian species, but not with influenza B virus (Fig. 2). Similar results were observed in sera from other transgenic lines. Furthermore, ELISA using glutathione-S-transferase (GST) tagged NP as an antigen was performed to examine the binding properties of whole human 5C and 6C mAbs expressed in transgenic mice. The results showed that there were no significant differences in the binding properties of these mAbs (Fig. 3). To determine the epitope region recognized by 5C and 6C mAbs, C-terminal-truncated NPs expressed in transfected HEK293T cells were precipitated with mouse sera from transgenic lines 5C (2497) and 6C (2660), and examined by immunoblotting. Both antibodies were able to react with full-length NP (1–498) and truncated NP 1–350, but not with truncated NP 1–300 (Fig. 4a). To further investigate the epitope region, immunoprecipitation using cell lysates containing four kinds of truncated NPs 1–310, 1–320, 1–330 and 1–340 were conducted. Western blot analyses revealed that both antibodies reacted with truncated NPs 1–320, 1–330 and 1–340, but not with truncated NP 1–310 (Fig. 4b). These results indicated that the epitope recognized by 5C and 6C mAbs was located within amino acid residues 311–320 of NP. To identify the core sequence of the epitope, an immunofluorescence assay was conducted using HEK293 cells expressing full-length NP mutants in which residues at 306–310, 311–315 or 316–320 were replaced by alanine. Both 5C and 6C mAbs reacted with alanine mutants 306–310 and 316–320, but not with the mutant 311–315 (Fig. 4c), demonstrating that the epitope recognized by 5C and 6C mAbs was within residues 311–315 of NP. A previous study reported that a highly conserved residue at position 313 typically bears an aromatic side chain of tyrosine (Y313) for human, and phenylalanine (F313) for avian and swine influenza viruses (Pan et al., 2010). According to the report, the authors also confirmed that residue 313 of NP of human isolates was tyrosine and that of avian isolates was phenylalanine in viruses used the present study (Fig. 4d). Immunofluorescence assay and epitope analyses in the present study showed that 5C and 6C mAbs can bind to NP of broad subtypes of influenza A viruses, regardless of the amino acid residue at position 313 of NP. Taken together, these results showed that 5C and 6C mAbs expressed in transgenic mouse lines display similar characteristics in their reactivity to NP.

It is known that almost all synthesized NPs are localized in the nucleus and cytoplasm in infected cells (Bullido et al., 2000). In contrast, only a few NPs are transiently located on the cell surface during the early stage of infection (Patterson et al., 1988; Prokudina & Semenova, 1991; Stitz et al., 1990; Virelizier et al., 1977; Yewdell et al., 1981); therefore, we investigated whether the anti-NP 5C and 6C mAbs bind to NP expressed on the surface of infected cells. MDCK cells were infected with A/whistling swan/Shimane/499/1983 (WS/499, H5N3 subtype) at an m.o.i. of 10, and at 5 h post-infection (p.i.) the cells were subjected to immunofluorescence staining using mouse serum from line 6C (2660). In infected cells under non-permeabilizing conditions, signals of 6C mAb were predominantly observed on the edges of cell surfaces (Fig. 5a). In contrast, those signals were not observed in mock-infected cells (Fig. 5b). As expected, in infected cells under permeabilizing conditions, strong signals were observed in the nuclei and cytoplasm (Fig. 5c). Similar results were observed in sera from other transgenic lines. These results indicated that both 5C and 6C mAbs could bind to NPs located on the surface of virus-infected cells.

Resistance to lethal infection with H5N1 and H1N1 influenza viruses in transgenic mice

It has been reported that antibodies, which bind to antigens on the cell surface, have the potential to induce antiviral effects, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) (Greenberg et al., 1977, 1978; Hashimoto et al., 1983a, b; Jegaskanda et al., 2013b; O’Brien et al., 2011; Ohta et al., 2011). However, the in vivo antiviral potential of anti-NP antibodies showing this binding characteristic is unknown. Therefore, we examined resistance of transgenic mice against lethal infections with influenza viruses causing severe pathogenicity to mice. The transgenic mice and their littermates were intranasally inoculated with 10 LD₅₀ H5N1 HPAI virus A/mountain hawk-eagle/Kumamoto/1/07 (MHE/Kumamoto/07), which was classified as clade 2.2 of the highly pathogenic phenotype in mammals (Shivakoti et al., 2010). The survival data demonstrated that the transgenic mice of lines 6C (2647) and 6C (2660) showed marked resistance to infection with the H5N1 virus (Fig. 6a). The survival rates of line 6C (2647) was 80 %, that of line 6C (2660) was 92 %, and that of line 5C (2497) was 30 %; in contrast, all of the control littermates and line 5C (2495) mice died within 12 days post-inoculation (Fig. 6a). Because mAbs expressed in all of the transgenic mouse lines were demonstrated to show similar characteristics in the epitope and the binding properties to NP (Figs 2, 3, 4, 5), these survival rates seemed to be correlated with the antibody concentrations in serum samples from transgenic mice [0.2 mg ml⁻¹ for line 5C (2495), 0.4 mg ml⁻¹ for line 5C (2497), 1.6 mg ml⁻¹ for line 6C (2647), and 1.2 mg
ml⁻¹ for line 6C (2660)]. In immunofluorescence assays using MDCK cells infected with WS/499, positive signals of mouse antibodies against viral proteins were observed in all sera collected from surviving transgenic mice, indicating that all of the surviving mice were infected with the H5 HPAI virus. Next, to investigate resistance against further severe infections, transgenic mice of line 6C (2660) were inoculated with 2000 and 20 000 LD₅₀ MHE/Kumamoto/07. Surprisingly, all of the transgenic mice inoculated with even 2000 LD₅₀ survived without significant weight loss (Fig. 6b). Furthermore, 80 % of transgenic mice inoculated with 20 000 LD₅₀ survived (Fig. 6c). These results revealed that the anti-NP human 6C mAb conferred resistance to lethal H5N1 HPAI viral infection in transgenic mice.

5C and 6C mAbs were derived from lymphocytes of a Vietnamese patient who had H5N1 HPAI virus infection in 2007. To confirm resistance of the transgenic mice against infection with the H5N1 HPAI virus of clade 2.3.4, which was the predominant strain in northern Vietnam in 2007 (Takakuwa et al., 2012), transgenic mice of line 6C (2660) were inoculated with 10 LD₅₀ of strain A/duck/Vietnam/G12/2008 (G12). As shown in Fig. 6(d), all transgenic mice survived, whereas over 70 % of control littermates succumbed to the infection within 12 days p.i. These results demonstrated that transgenic mice expressing the anti-NP human mAb showed resistance to infections with different H5HA clades of HPAI viruses.

Furthermore, to investigate resistance of transgenic mice against infection with influenza A virus other than the H5 subtype, a mouse-adapted strain A/Puerto Rico/8/1934 of H1N1 subtype was inoculated into line 6C (2660) mice. All of the transgenic mice survived for 14 days, although a mean weight loss was observed of up to 20 % at 8 days p.i. (Fig. 6e). In contrast, all control littermates died at 7 days p.i. These results demonstrated that the anti-NP human mAb could confer resistance to lethal H1N1 HPAI infections.

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**Fig. 2.** Cross-reactivity of serum of transgenic mouse with various subtypes of influenza A viruses. Immunofluorescence analyses using mouse serum of line 6C (2660) in MDCK cells infected with influenza A viruses of H1–H13 subtypes and an influenza B virus. The nuclei were stained with Hoechst 33342.

**Fig. 3.** Binding properties of anti-NP mAbs expressed in transgenic mice in response to NP-GST protein. ELISA was performed using diluted serum as the primary antibody and alkaline phosphatase-conjugated anti-human IgG as the secondary antibody. Five serum samples from each transgenic line were used, and the mean±SD of the OD are shown.
Fig. 4. Epitope analyses of anti-NP human mAbs expressed in transgenic mouse. (a, b) Reactivity of 5C and 6C mAbs with truncated NPs possessing a 6xHis-tag sequence in the C terminus. The truncated NPs expressed in HEK293T cells were
infections with influenza A viruses of both H5N1 and H1N1 subtypes, indicating the anti-NP human mAb plays an important role in heterosubtypic protection against influenza A viruses.

To demonstrate specificity of the resistance observed in the transgenic mice, experimental infections with an influenza B virus strain B/Lee/1940 were performed because of the different antigenicity of NPs in influenza A and B viruses. As shown in Fig. 6(f), there were no significant differences in survival rates and body weight changes between the transgenic mice and their littermates. Furthermore, we performed experimental infection with a pseudorabies virus as a control DNA virus. Similarly, no significant differences were observed in survival rates between transgenic mice and their littermates (Fig. 6g). Taken together, these results demonstrated that the resistance observed in the transgenic mice was specific for influenza A virus infections.

**Suppression of viral replication in tissues of transgenic mice**

To investigate viral replication in tissues of transgenic mice, 6C (2660) mice and their littermates were intranasally inoculated with 10 LD₅₀ of MHE/Kumamoto/07. At 1, 2, 3 and 5 days p.i., lungs, brains, spleens, kidneys and livers were collected from the mice, and viral titres in these organs were measured. The results are shown in Fig. 7. During days 1–5 p.i., viral titres in lungs of transgenic mice were 10–1000-fold lower than those of their littermates. At 5 days p.i., the viruses were isolated from two of three brains in control mice, but not in transgenic mice. Moreover, the viruses were recovered from spleens of all control mice at 3 days p.i., and two of three spleens of control mice at 5 days p.i. In contrast, the virus was recovered from only one spleen in transgenic mice at 3 days p.i. Viruses were not recovered from kidneys and livers of infected mice. These results suggested that suppression of viral replication in tissues of transgenic mice may be correlated with high survival rates as observed in Fig 6(a–c).

**Histopathological analyses in tissues of infected mice**

After 1 day p.i. with MHE/Kumamoto/07, the degeneration of bronchial epithelium and peribronchial infiltration of inflammatory cells were not observed in both transgenic mice of line 6C (2660) and non-transgenic littermates (Fig. 8a). At 3 days p.i., peribronchial and alveolar inflammation with interstitial thickening were observed in lungs of non-transgenic littermates (Fig. 8a). Compared with the non-transgenic control, these pathological changes were milder and limited to the peribronchial areas in transgenic lungs. At 5 days p.i., severe inflammatory cell infiltration and lung interstitial oedema were observed in non-transgenic lungs (Fig. 8a, asterisk), in contrast, the lesion was confined to focal areas in peribronchial alveoli of transgenic lungs (Fig. 8a, hash symbol). To investigate lung damage after the viral infection, podoplanin staining was performed. Podoplanin is a known marker (brown colour) for type I alveolar epithelial cells and has been documented in lung injury models to demonstrate loss of these cells (McElroy et al., 1995). As shown in Fig. 8b, it was observed that podoplanin staining was apparently reduced in broad areas of lungs of the non-transgenic control as compared with transgenic mice, indicating that lung injury of transgenic mouse was limited.

Specific staining for anti-NP human mAb was observed on the surface of bronchiolar and alveolar epithelia in the lungs of transgenic mice, but not in the lungs of non-transgenic littermates (Fig. 8c). In particular, the antibody was strongly expressed in cytoplasm of alveolar epithelium cells. Furthermore, to demonstrate the distribution of viral antigens in lungs of transgenic mice and their littermates, lung tissues were immunohistochemically stained with rabbit anti-WS/499 (H5N3) hyperimmune serum (Fig. 8d). In both transgenic mice and non-transgenic littermates at 1 day p.i., a small number of viral antigens were detected in the bronchiolar epithelium. At 3 and 5 days p.i., the distribution of viral antigens was confined to the bronchi and peribronchial areas in the transgenic lungs (Fig. 8d). In contrast, intense

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**Fig. 5.** Binding of anti-NP human mAbs to NPs on the surface of infected cells. MDCK cells infected with strain WS/499 at 5 h p.i. were stained with mouse serum of line 6C (2660) and Dyelight-488-conjugated anti-human IgG antibody (green). The nuclei were stained with Hoechst 33342 (blue). The cells shown in panels (a) and (b) were not permeabilized. The cells shown in panel (c) were permeabilized. The image was taken at ×40 magnification. Bars, 20 µm.
signals of viral antigens were observed in extensive areas of the lung interstitium of control mice with inflammation and tissue destruction (Fig. 8d). These results suggest that distribution of viral antigens in infected lungs was related to the viral replication and the damage in the tissues. In double immunofluorescence, we observed that signals of viral antigen-positive cells were frequently co-localized with those of anti-NP mAb-positive secretions in the transgenic bronchi.
that transgenic embryonic fibroblasts themselves did not
infection with strain WS/499. These results demonstrated
and 24 h p.i. (Fig. S2). Similar results were observed after
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infected with PR8 at m.o.i. values of 10, 1 and 0.1, viral
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To investigate whether expression of anti-NP human mAbs
expression of the intracellular anti-NP mAbs.
(Fig. 8e). Quantitative analysis in four different fields of the
bronchi areas showed that the co-localization ratio of anti-
gen-positive signals with cells secreting the mAb were 10.8
±1.3 % for 1 day p.i., 14.8±1.7 % for 3 days p.i. and 11.0
±2.4 % for 5 days p.i.

**Viral replication in transgenic embryonic fibroblasts**

To investigate whether expression of anti-NP human mAbs
involves an effect on the viral replication in transgenic fibroblasts, primary cultured embryonic fibroblasts from
line 6C (2660) and control littermates were infected with
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and 24 h p.i. (Fig. S2). Similar results were observed after
infection with strain WS/499. These results demonstrated
that transgenic embryonic fibroblasts themselves did not
show any resistance to infection with influenza A viruses *in vitro*, and, in addition, the viral replication was not affected
by expression of the intracellular anti-NP mAbs.

**DISCUSSION**

Recently, some of the anti-HA human antibodies isolated
by screening human combinatorial antibody libraries and
immortalized memory B-cells from patients infected with
influenza A viruses have been shown to exhibit neutralizing
activity and can protect from viral infection in experimentally
infected animals (Chen et al., 2015; Kashyap et al.,
2010; Simmons et al., 2007). These reports indicate human
mAbs derived from recovered patients can provide significant
advantages in prophylactic and therapeutic strategies
against influenza virus infections. Anti-NP antibodies have
been detected in sera of patients infected with influenza A
viruses, as well as anti-HA and anti-NA antibodies (Sukeno
et al., 1979). However, the antiviral potential of anti-NP antibodies of human origin has not been defined. In this
study, we generated transgenic mice expressing anti-NP human mAbs derived from a patient who recovered from
H5N1 HPAI infection, and experimental infections with
H5N1 HPAI and H1N1 viruses of lethal doses were con-
ducted. In experimental infections with two different HA
classes of H5N1 HPAI strains, marked resistance was
observed in transgenic mice, and the antiviral effects was
dependent on the expression level of the anti-NP mAbs
(Fig. 6a). Furthermore, 6C (2660) mice expressing high
amounts of the mAb in sera showed a complete resistance
to lethal infections with the H1N1 mouse-adapted PR8 strain (Fig. 6e). In contrast, there were no significant differ-
ces in survival rates between 6C (2660) mice and their
non-transgenic littermates in experimental infections with
influenza B virus and pseudorabies virus (Fig. 6f, g). These
results demonstrated that the anti-NP human mAbs used in
this study could confer efficient resistance to infections with
several types of influenza A viruses *in vivo*.

Resistance of transgenic mice to influenza A virus infection
was irrelevant to both the neutralizing effect by anti-HA antibody and the immunological memory because the mice
were unimmunized and unexposed to the viruses before the
challenge. In addition, sera of mice from all the transgenic
lines failed to neutralize the virus *in vitro* (data not shown),
demonstrating that the anti-NP mAbs did not themselves
have strong antiviral activity. Furthermore, the viral replication
properties were comparable between transgenic mouse
embryonic fibroblasts expressing the anti-NP mAbs and
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cell-based ELISA (Stitz et al., 1990), and radioimmunoassays (Prokudina & Semenova, 1991; Yewdell et al., 1981), although the mechanism responsible for the penetration of synthesized NP into the lipid bilayer membrane remains unknown. Several anti-NP mAbs that could bind NP expressed on the infected-cell surface were used in the previous studies (Patterson et al., 1988; Prokudina & Semenova, 1991; Stitz et al., 1990; Yewdell et al., 1981); however, there have been no reports on antiviral effects of those antibodies in vivo. We demonstrated that the anti-NP 5C and 6C mAbs could bind to cell-associated NP in vitro by immunofluorescence analysis (Fig. 5), and the mAbs were frequently co-localized with viral antigens on infected cells of the bronchi and peribronchial areas of transgenic mice (Fig. 8e). Based on the above results, the present study further demonstrated that anti-NP antibody that binds cell-associated NP on the surface of the infected cells can play important roles in viral clearance and consequent protection from the influenza A virus infection. Although the precise protective mechanism observed in the transgenic mice remains unclear, the following hypothesis is conceivable. Primary influenza pneumonia that causes difficulty in breathing is the most common finding in severe cases and a frequent cause of death (WHO, 2009). In these severe cases, patients generally show respiratory deterioration around 3 to 5 days p.i. and subsequently progress to respiratory failure within 24 h (WHO, 2009). In the transgenic lungs at 3 and 5 days p.i., histopathological lesions were confined to bronchi and peribronchiolar areas and pneumonia was also moderate; in contrast, severe pathological changes were observed in large areas of non-transgenic lungs (Fig. 8a, d). These findings indicated that virus-infected cells in upper respiratory organs were eliminated at early phase of infection, which was induced by ADCC or CDC activities with anti-NP mAbs that bind to cell-associated NP antigens. Overdijk et al. (2012) reported that the Fc portion of human IgG1 contained in the anti-NP 5C and 6C mAbs was an important mediator of ADCC and CDC in mice as well as in humans. Jegaskanda et al. (2013a) showed that

**Fig. 8.** Histopathological analyses. Transgenic mice of line 6C (2660) and non-transgenic littermates were inoculated intranasally with 10 LD$_{50}$ of H5N1 HPAI virus strain MHE/Kumamoto/07. (a) On days 1, 3 and 5 p.i., lungs were collected from the infected mice, and sections were stained with haematoxylin and eosin. Bars, 200 µm. (b, c) Immunohistochemical staining for podoplanin (b) and anti-NP mAb (c). Bars, 200 µm. (d) On days 1, 3 and 5 p.i., lungs were collected from the infected mice and sections were immunohistochemically stained with anti-WS/499 (H5N3) hyperimmune serum. Bars, 100 µm. (e) Double immunofluorescence staining for viral antigen (green) and anti-NP mAb (red). Arrow indicates co-localization between viral antigens and anti-NP mAbs. Arrowheads indicate viral antigens surrounded by anti-NP mAbs. Bars, 20 µm.
anti-NP antibodies inducing ADCC were detectable in plasma of macaques infected with influenza A virus. Establishment of transgenic mice expressing whole antibodies consisting of human IgG1 Fc and Fab's of those antibodies that were confirmed to induce ADCC in vitro would be useful for studying antiviral effects of non-neutralizing anti-NP antibodies. Furthermore, several amino acid mutations leading to changes in ADCC or CDC activities have been found in the Fc region of human IgG1 (Armour et al., 1999; Idusogie et al., 2001; Shields et al., 2001). These reports provide useful information concerning ADCC and CDC activities of 5C and 6C mAbs. Further investigations are needed to elucidate the relation between in vivo resistance to viral infections and the contribution of ADCC and/or CDC induced by the anti-NP mAbs.

One of the practical uses of human mAbs against NP could be therapeutic administration to a patient infected with an influenza A virus of undefined subtype or prophylactic administration to workers who might get exposed to animals and animal products suspected to be infected. It is important that development of human mAbs can lead to reduced immunogenicity in patients. Although efficacy studies of antibodies in mouse models are an essential step in preclinical development, the antiviral effects identified in the animal model are not always translated to human trials. In particular, evaluation of antiviral effects of non-neutralizing antibodies inducing cell-mediated cytotoxicity is difficult, because there is a significant variation in the affinity of IgG Fc isotypes for individual Fc receptors (Overdijk et al., 2012). A recent report showed that cell-mediated cytotoxicity induced by human IgG1 is the strongest activity among all human Fc isotypes in both mouse models and humans (Overdijk et al., 2012). Therefore, we established transgenic mice expressing anti-NP human mAbs carrying the IgG1 Fc domain, and experimental infections were conducted. Our present study provides a new strategy to obtain precise knowledge of antiviral effects of non-neutralizing human mAbs against influenza virus and their extrapolation to the human system.

CD8⁺ T-cells recognize the short peptides of viral antigens presented by MHC class I molecules on the surface of virus-infected cells and trigger the lysis of the infected cells. Several NP peptides located in regions conserved between influenza A viruses have been shown to generate CD8⁺ cytotoxic T-lymphocyte (CTL) responses and protect mice from the infection (Epstein et al., 2005; Fu et al., 1997, 1999). The epitope recognized by both 5C and 6C mAbs was found within amino acid residues 311–315 of NP; however, these residues did not include the CTL epitopes, as shown previously (Chen et al., 2000; Epstein et al., 2005; Fu et al., 1997, 1999; Heiny et al., 2007; Quiñones-Parra et al., 2014; Röttschke et al., 1990; Ulmer et al., 1993). Utilization of a combination of CTL epitopes and the epitope of the anti-NP mAbs may facilitate the development of a much more powerful universal vaccine. Furthermore, recent studies have reported that transfer via the intraperitoneal route of NP-hyperimmune serum (200 µl) or anti-NP mouse IgG (300 µg) to recipient mice can inhibit viral replication in the lungs before challenge with sublethal doses [0.2–0.25 minimum LD₅₀ (MLD₅₀)] of PR8 (Carragher et al., 2008; LaMere et al., 2011). In our preliminary data, mice receiving 6C (2660) mouse serum via the intraperitoneal route (300 µl day⁻¹) 1 day before challenge with 10 MLD₅₀ PR8 had increased mean survival time (9.4 days) as compared with mice receiving PBS (8.2 days). These results suggested that the anti-NP mAbs used in this study may be suitable for development of preventive and therapeutic strategies against viral infection. Effective protection of broadly cross-reactive anti-NP antibodies against influenza A virus infections will promote the improvement of strategies to control influenza epidemics and pandemics in the future.

**METHODS**

**Viruses and cells.** HPAI viruses of the H5N1 subtype [A/mountain hawk-eagle/Kumamoto/1/2007 (Shivakoti et al., 2010) and A/duck/Vietnam/G12/2008 (Takakuwa et al., 2012)], low-pathogenic influenza viruses [A/Puerto Rico/8/1934 (H1N1), A/swan/Shimane/1997 (H3N3), A/Aichi/2/1968 (H3N2), A/duck/Czechoslovakia/1956 (H4N6), A/whistling swan/Shimane/499/1983 (H5N3), A/turkey/Massachusetts/3740/1963 (H6N2), A/swan/Shimane/42/1980 (H7N7), A/turkey/Ontario/6118/1968 (H8N4), A/turkey/Wisconsin/1966 (H9N2), A/chicken/Germany/N/1949 (H10N7), A/duck/Vietnam/G32/2008 (H11N9), A/duck/Alberta/60/1976 (H12N5) and A/gull/Maryland/704/1977 (H13N3)] and an influenza B virus (B/Lee/1940) were used in this study. These viruses were propagated in 10-day-old embryonated chicken eggs at 37°C for 2 days and stored at ~80°C until use. Pseudorabies virus strain YS-81 was propagated in Vero cells. MDCK cells, HEK293 cells, HEK293T cells and mouse embryonic fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) containing 10% FBS at 37°C in an atmosphere containing 5% CO₂.

**Construction of plasmids expressing whole human IgG.** Peripheral blood of a volunteer who had recovered from H5N1 HPAI virus infection in northern Vietnam in 2007 was collected previously. Collection of the human material was conducted according to protocols approved by the Institutional Review Boards of the National Institute of Hygiene and Epidemiology, Vietnam. From the collected sample, two Fab clones against influenza virus NP, named 5C and 6C, were selected by phage display method (unpublished data), and nucleotide sequences of kappa light chains and gamma-1 heavy chains of the Fab fragments were determined (GenBank accession nos: BAK48745 and BAK48746 for 5C and 6C light chains, respectively; BAK48747 and BAK48748 for 5C and 6C heavy chains, respectively). In order to construct cDNAs of heavy chains of whole human IgG1, the amplified DNA fragments encoding the variable region of heavy chains were inserted into pFab CMV-dhfr2H7 vector. cDNAs of the heavy and light chains were amplified by PCR using the constructed pFab CMV-dhfr2H7 and the cDNA of Fab fragments as templates for heavy and light chains, respectively, and specific primers sets (5’-GCATGTTGATCTATCCTACCATGAA TGAGGC-3’ and 5’-GCATGTTGATCTCTGAGATATACTACC- 3’ for heavy chain; 5’-GCATGTTGATCTCTCAGGACGAA CAGG-3’ and 5’-GCATGTTGATCTCTGAGATATACTACC-3’ for light chain), and digested by BglII and BanII. Each digested cDNA was inserted into the BglII site of the pCXN2 expression vector (Niwa et al., 1991), and constructs were designated pCXN2/HC and pCXN2/LC for heavy and light chains, respectively. To construct cDNAs of heavy chains of whole human IgG1, the amplified DNA fragments encoding the variable region of heavy chains were inserted into pFab CMV-dhfr2H7 vector. cDNAs of the heavy and light chains were amplified by PCR using the constructed pFab CMV-dhfr2H7 and the cDNA of Fab fragments as templates for heavy and light chains, respectively, and specific primers sets (5’-GCATGTTGATCTATCCTACCATGAA TGAGGC-3’ and 5’-GCATGTTGATCTCTGAGATATACTACC- 3’ for heavy chain; 5’-GCATGTTGATCTCTCAGGACGAA CAGG-3’ and 5’-GCATGTTGATCTCTGAGATATACTACC-3’ for light chain), and digested by BglII and BanII. Each digested cDNA was inserted into the BglII site of the pCXN2 expression vector (Niwa et al., 1991), and constructs were designated pCXN2/HC and pCXN2/LC for heavy and light chains, respectively. The Sall fragment containing the CAG promoter, the light chain gene and the rabbit beta-globin poly(A) signal isolated from pCXN2/LC was inserted into one of the Sall sites of pCXN2/HC. The resulting plasmid contained the heavy and light chain genes under the control of the CAG promoter in parallel (Fig. S1).
Construction of plasmids expressing different forms of NP. Viral RNA was extracted from allantoic fluid infected with A/duck/Hong Kong/278/1978 (H2N9) (Kobasa et al., 2001) using a QiAamp Viral RNA Mini kit (Qiagen), and reverse transcription (RT) was then performed using a PrimeScript Reverse Transcription kit (TaKaRa Bio) with Uni-12 primer (Hoffmann et al., 2001). To construct plasmids expressing full-length or truncated NPs possessing the 6×His-tag sequence in the C terminus, cDNA of the viral NP gene was amplified by PCR using the RT product as a template, and a forward primer 5'-CCGCTGAGCTACTAGTTGATGGTGATGATGTTCTTCAAATGCTGCAGAA-3' and one of the following reverse primers: 5'-CCGCTGAGCTACTAGTTGATGGTGATGATGATGGTCTTC-3' for the amplification of truncated NP (aa 1-498) cDNA, 5'-CCGCTGAGCTACTAGTTGATGGTGATGATGATGGTCTTC-3' for the amplification of truncated NP (aa 1-350) cDNA, 5'-CCGCTGAGCTACTAGTTGATGGTGATGATGATGGTCTTC-3' for the amplification of truncated NP (aa 1-330) cDNA, 5'-CCGCTGAGCTACTAGTTGATGGTGATGATGATGGTCTTC-3' for the amplification of truncated NP (aa 1-310) cDNA and 5'-CCGCCGCTACTAGTTGATGGTGATGATGATGGTCTTC-3' for the amplification of truncated NP (aa 1-310) cDNA, 5'-CCGCCGCTACTAGTTGATGGTGATGATGATGATGGTCTTC-3' for the amplification of truncated NP (aa 1-310) cDNA and 5'-CCGCCGCTACTAGTTGATGGTGATGATGATGATGGTCTTC-3' for the amplification of truncated NP (aa 1-310) cDNA. All of the amplified cDNAs were inserted into the EcoRI and XhoI site of the pCAGGS vector (Niwa et al., 1991).

pCAGGS plasmids expressing alanine mutants of NP were constructed using a PrimeSTAR Mutagenesis Basal kit (TaKaRa Bio) and the following primer sets: 5'-CCGCCGCTACTAGTTGATGGTGATGATGATGATGGTCTTC-3' for alanine substitutions at position 311 and 5'-GCAGCTGAGCTACTAGTTGATGGTGATGATGATGATGGTCTTC-3' for alanine substitutions at position 311-315, and 5'-GCAGCTGAGCTACTAGTTGATGGTGATGATGATGATGGTCTTC-3' for alanine substitutions at position 316-320. The pCAGGS plasmid expressing full-length NP was used as a template for mutagenic chain reaction.

To construct a plasmid expressing a fusion NP protein with GST on the N-terminal side, cDNA of the viral NP gene was amplified by PCR using primers 5'-GGGGAGTCTGACGACGACGAGAAGATCTATTCC-3' for alanine substitutions at position 306-310, 5'-GCAGCTGACGACGACGAGAAGATCTATTCC-3' for alanine substitutions at position 311-315, and 5'-GCAGCTGACGACGACGAGAAGATCTATTCC-3' for alanine substitutions at position 316-320. The pCAGGS plasmid expressing full-length NP was used as a template for mutagenic chain reaction.

Generation of transgenic mice. The transgene fragments containing heavy and light chains of whole human IgG genes (Fig. S1) were isolated from the constructed pCXN2 plasmid by Salt digestion. The DNA fragment was microinjected into nuclei of C57BL/6 mouse eggs to generate transgenic mice as described previously (Ono et al., 2007). To identify transgenic founders, genomic DNA from mouse tails was prepared using DirectPCR Lysis Reagent (Viagen Biotech), and PCR was performed using specific primers for the heavy and light chains as described above. All mice were maintained in the animal facility at each institute, and all animal experiments were carried out according to the Guideline for Proper Conduct of Animal Experiments published by the Science Council of Japan. The ethics committees of Kyushu University approved the experimental protocols.

Epitope mapping of anti-NP human mAbs expressed in transgenic mouse. pCAGGS plasmids encoding the full-length and C-terminal-truncated NP genes were transfected into HEK293T cells seeded into 35 mm diameter dishes with MetaFECTENE PRO (Biontex Laboratories). Forty-eight hours later, the cells were lysed with 0.5 ml RIPA buffer (ATTO). The truncated NPs were then immunoprecipitated by sera of transgenic mice and protein-G sepharose (Sigma). The precipitated samples were analysed by Western blotting using rabbit anti-His-tag serum (1:2000; Medical & Biological Laboratories) and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:4000; Sigma) to detect NP proteins.

pCAGGS plasmids expressing the alanine mutants of NP were transfected into HEK293 cells. Forty-eight hours later, immunofluorescence analyses were performed using sera of transgenic mice and goat Dyelight 488-conjugated anti-human antibodies (1:100; Abcam), or rabbit anti-His-tag serum (1:100) and FITC-conjugated goat anti-rabbit IgG (1:100; Sigma).

Immunofluorescence assay. MDCK cells infected with influenza A viruses were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PH 7.4). The cells were blocked with 10% goat serum and stained with transgenic mouse sera as the primary antibodies and goat Dyelight 488-conjugated anti-human antibodies (1:100) as secondary antibodies. The nuclei were stained with Hoechst 33342 (1:1000; Enzo Life Sciences). Fluorescence was visualized using BZ-9000 (Keyence).

ELISA. Expression levels of human IgG in sera of transgenic mice were determined using a human IgG Ready-Set-Go kit (eBioscience) according to the manufacturer's instructions.

To investigate binding property of antibodies, ELISA was performed using diluted sera from transgenic mice as the primary antibody and alkaline phosphatase-conjugated goat anti-human IgG (1:10000; Sigma) as the secondary antibody. NP-GST protein was purified as described previously (Fujimoto et al., 2013).

Experimental infections in mice. Experimental infections with H5N1 strains MHE/Kumamoto/07 and G12 were carried out in the BSL-3 facility at Hokkaido University and Kyoto Sangyo University, and those with H1N1 strain PR8, B/Lee/40 and pseudorabies viruses were carried out in the BSL-2 facility at the Center of Biomedical Research, Research Center for Human Disease Modeling, Graduate School of Medical Sciences, Kyushu University. The ethics committees of Kyushu University, Hokkaido University and Kyoto Sangyo University approved all experimental protocols. For intranasal infections, mice (6–8 weeks of age) were anaesthetized by intraperitoneal injection of a mixture of ketamine/xylazine (1:1; Sigma) and 0.1 ml 0.9% saline. They were then intranasally inoculated with 40 µl of each virus. The survival and body weights of infected mice were recorded for 14 days. At the end of the infectious experiments with MHE/Kumamoto/07, surviving mice were euthanized and sera were collected. To confirm seroconversion, haemagglutination inhibition tests for MHE/Kumamoto/07, surviving mice were performed; however, titres of almost all sera were under the detection limit (8 haemagglutination-inhibition antibody titres). Therefore, immunofluorescence assays of MDCK cells infected with the WS/499 strain were performed using 10-fold diluted sera as the primary antibodies and goat Dyelight 488-conjugated anti-mouse antibodies (1:100; Abcam) as the secondary antibodies to detect viral protein-specific mouse antibodies.

To determine viral replication in brain, lungs, spleen, kidneys and liver, mice were inoculated with MHE/Kumamoto/07, and three mice from each group were sacrificed on days 1, 2, 3 and 5 p.i. Collected tissues were homogenized, and the viral titre of each tissue sample was tested by determining TCID50 in MDCK cells.

Histopathological analyses. Lung tissues collected from infected mice were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer and embedded in paraffin wax. Sections (4 µm thick) were cut using a Leica microtome and stained with haematoxylin and eosin (HE). In immunohistochemistry, viral antigens, anti-NP mAb, and type I alveolar cells were detected using the following antisera or antibodies: rabbit anti-WS/499 hyperimmune serum (1:10000), goat polyclonal anti-human IgG (1:2000; Sigma), and rabbit polyclonal anti-podoplanin (1:1000; Sino...
Viral growth in mouse embryonic fibroblasts. For the investigation of viral growth in mouse embryonic fibroblasts, the cells were seeded in 35 mm diameter dishes (5 × 10^4 cells dish^-1). PR8 and WS/499 at an m.o.i. of 10, 1 or 0.1 were adsorbed onto the cells at 37°C for 1 h, and the cells were then washed to remove non-adherent virus and cultured in 3 ml DMEM with 5 µg ml^-1 trypsin. Five hundred micro-litres of supernatant was harvested from the infected cells at 8 and 24 h p.i., and TCID₅₀ titration was performed using MDCK cells.

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