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Heterosubtypic antiviral activity of IgA antibodies against influenza A virus hemagglutinins

(A型インフルエンザウイルスヘマグルチニン特異IgA抗体の亜型間交差抗ウイルス活性)

Mieko MURAMATSU

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Abbreviations

CTL	cytotoxic T lymphocyte
EC_{50}	50% effective concentration
ELISA	enzyme-linked immunosorbent assay
HA	hemagglutinin
HEK	human embryonic kidney
HI	hemagglutination inhibition
IC_{50}	50% inhibitory concentration
IgA	immunoglobulin A
IgG	immunoglobulin G
IgY	immunoglobulin Y
kD	kilodalton
MAb	monoclonal antibody
MDCK	Madin-Darby canine kidney
MEM	minimal essential medium
MOI	multiplicity of infection
NA	neuraminidase
NI	neuraminidase inhibition
NW	nasal wash
O.D.	optical density
PBS	phosphate-buffered saline
PBST	PBS containing 0.05% Tween 20
PCR	polymerase chain reaction

pfu	plaque-forming units
p-IgA	polymeric IgA
RDE	receptor destroying enzyme
rHA	recombinant HA
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
TMB	3,3',5,5',-tetramethylbenzidine
TW	trachea-lung wash
w/o	without
2-ME	2-mercaptoethanol

Preface

Influenza A viruses are divided into subtypes based on the antigenicity of two envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). To date, H1-H16 HA and N1-N9 NA subtypes have been identified in viruses isolated from wild aquatic birds, the natural reservoir of influenza A viruses [1-3]. It is known that HA is the major target of neutralizing antibodies against influenza A viruses [4], and HA-specific antibodies are principally subtype-specific. Therefore, the currently used inactivated influenza vaccines, which rely on the induction of serum neutralizing antibodies, are not effective against viruses whose HA antigenicities are different from those of the vaccine strains [5]. However, infection with influenza A virus usually affords some protection against reinfection with viruses having different subtypes [6]. It has been believed that this heterosubtypic protection is mainly mediated by memory cytotoxic T lymphocytes (CTL) recognizing conserved epitopes of viral internal proteins presented with major histocompatibility complex class I on the surfaces of infected cells [7,8]. Therefore, the contribution of virus-specific antibodies to the heterosubtypic immunity has been thought to be limited and has not been evaluated properly.

On the other hand, it has been reported that heterosubtypic immunity was induced by intranasal immunization of mice with formalin-inactivated influenza A viruses, whereas subcutaneous immunization only protected mice from homologous viruses [6,9,10].

Interestingly, this cross-protection was dependent on B-cell, but not on CTL activities [9]. It has been supposed that Immunoglobulin A (IgA) might have major role in this cross-protection, since IgA antibodies are predominantly present in mucosal tissues and well induced by mucosal immunization. However, cross-neutralizing activity of antibodies was not detected in the sera and respiratory secretions of intranasally or subcutaneously immunized mice [10].

IgA antibodies are well documented to have unique properties in mucosa. Polymeric IgA (p-IgA) antibodies are selectively transported to the mucosal surface with a secretory component and resistant to proteolysis in mucosal secretions [11-16], and p-IgA antibodies crossing through epithelial cells via transcytosis are believed to inhibit viral protein functions intracellularly [11,13,15,17-19]. In addition, p-IgA does not induce an inflammatory reaction in mucosa [11,13,16]. Therefore, it has been suggested that induction of the mucosal immune response is more desirable to prevent respiratory infection by influenza A viruses [20-23].

On the other hand, there have been some reports showing HA-specific monoclonal antibodies (MAbs) that have cross-neutralizing activity against multiple HA subtypes of influenza A virus strains [24-31]. Biological and structural analyses indicated that these antibodies had the potential for either of the known neutralization mechanisms, preventing viral attachment to host cells or conformational change/proteolytic cleavage of HA, both of which are essential for virus entry into host cells. Although it might be difficult to induce high levels of antibodies with heterosubtypic

neutralizing activities since these antibodies are thought to recognize minor epitopes, recent studies have suggested that such antibodies are indeed produced in some individuals [32,33]. Thus cross-reactive antibodies could be considered to play important roles in antibody-mediated cross-protection against influenza A viruses of multiple HA subtypes.

In chapter I, I analyzed the cross-reactivities of IgA and Immunoglobulin G (IgG) antibodies induced by intranasal or subcutaneous immunization. In chapter II, I compared the heterosubtypic antiviral activity of IgA and IgG using MAbs recognizing the same HA epitope, which have heterosubtypic neutralizing activity against multiple influenza A viruses.

Chapter I

Heterosubtypic antiviral activity of hemagglutinin-specific antibodies induced by intranasal immunization with inactivated viruses in mice

Introduction

Recent reports demonstrated that heterosubtypic immunity against influenza A viruses was induced by intranasal immunization of mice with formalin-inactivated influenza A viruses, whereas subcutaneous immunization only protected mice from homologous viruses [6,9,10]. Interestingly, this cross-protection was dependent on B-cell, but not on CTL activity [9]. In some of these studies, antibodies induced in mice by immunization showed cross-binding ability to viruses or recombinant HAs (rHAs) of subtypes different from virus used for immunization. However, cross-neutralizing activity of those antibodies could not be detected *in vitro*. On the other hand, there have been some reports about HA-specific MAbs that had cross-neutralizing activity against multiple HA subtypes of influenza A virus strains [24-31], though it may be difficult to induce high levels of such antibodies since these antibodies are thought to recognize minor epitopes. These studies led to the hypothesis that HA-specific antibodies, including nonneutralizing antibodies, might also play important roles in heterosubtypic immunity against influenza A viruses.

In this study, I found that intranasal immunization of mice with inactivated viruses induced high levels of IgA and IgG antibodies in mice

that bound to HAs of multiple subtypes. On the other hand, though subcutaneous immunization similarly induced high levels of cross-reactive IgG, IgA antibodies were detected at very low levels. Though the cross-neutralizing activity of these antibodies was not detected, I could find reduced plaque formations of viruses with heterologous subtypes in the presence of cross-reactive IgA antibodies. Here I discuss a possible role of HA-specific nonneutralizing IgA antibodies in the heterosubtypic immunity against influenza A viruses.

Materials and methods

Viruses and cells

Influenza A virus strains, A/Puerto Rico/8/1934 (H1N1), A/Adachi/2/1957 (H2N2), A/Aichi/2/1968 (H3N2), A/duck/Czechoslovakia/1956 (H4N6), A/rg Viet Nam ΔHA/1194/2004 (H5N1) [31], A/shearwater/Australia/1/1972 (H6N5), A/seal/Massachusetts/1/1980 (H7N7), A/turkey/Ontario/6118/1968 (H8N4), A/Hong Kong/1073/1999 (H9N2), A/chicken/Germany/N/1949 (H10N7), A/duck/England/1/1956 (H11N6), A/duck/Alberta/60/1976 (H12N5), A/gull/Maryland/704/1977 (H13N6), A/mallard/Astrakhan/263/1982 (H14N5), A/duck/Australia/341/1983 (H15N8), and A/black-headed gull/Sweden/5/1999 (H16N3) were kindly provided by Dr. H. Kida, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan and used for immunization of mice, construction of plasmid expressing recombinant HAs, and plaque-reduction assays using Madin-Darby canine kidney (MDCK) cells. MDCK cells were maintained in Eagle's minimal essential medium (MEM) (GIBCO) supplemented with 10% calf serum. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Immunogens

Virus strains of H1, H3, H5, H7, H9, and H13 HA subtypes were propagated in the allantoic cavity in 10-day-old embryonated chicken eggs at

35°C for 48 hours, then the infectious allantoic fluid was collected. Virus particles were concentrated and purified by high-speed centrifugation of the allantoic fluid passed through a 10-50% sucrose density gradient. Purified viruses were resuspended in phosphate-buffered saline (PBS). The protein concentration of each purified virus was measured based on the optical density (O.D.) at 280 nm. All protein concentrations were standardized for each immunogen as relative to each other based on O.D. at 280 nm. The purified viruses were treated with 0.3% formalin at the concentration of 20 mg viral proteins per 1 ml at 4°C for one week. Inactivation of these viruses was confirmed by the absence of detectable hemagglutination activity following inoculation of the treated materials into embryonated eggs. Each inactivated virus was diluted to adequate concentrations with PBS before immunization of mice.

Immunization and sample collection

Six-week-old female BALB/c mice (Japan SLC, Inc.) were inoculated intranasally with inactivated viruses (100 μg in 50 μl) or 50 μl of PBS under anesthesia with isoflurane, or injected subcutaneously with inactivated viruses (100 μg in 200 μl) or 200 μl of PBS (Figure 1). Five or ten mice were used for each group. Mice were immunized three times at three-week intervals. One week after the last immunization, mice were euthanized with isoflurane and serum, trachea-lung wash (TW), and nasal wash (NW) samples were collected as described previously [34]. These samples were stored at -80°C until use. Samples from each group were pooled in equal

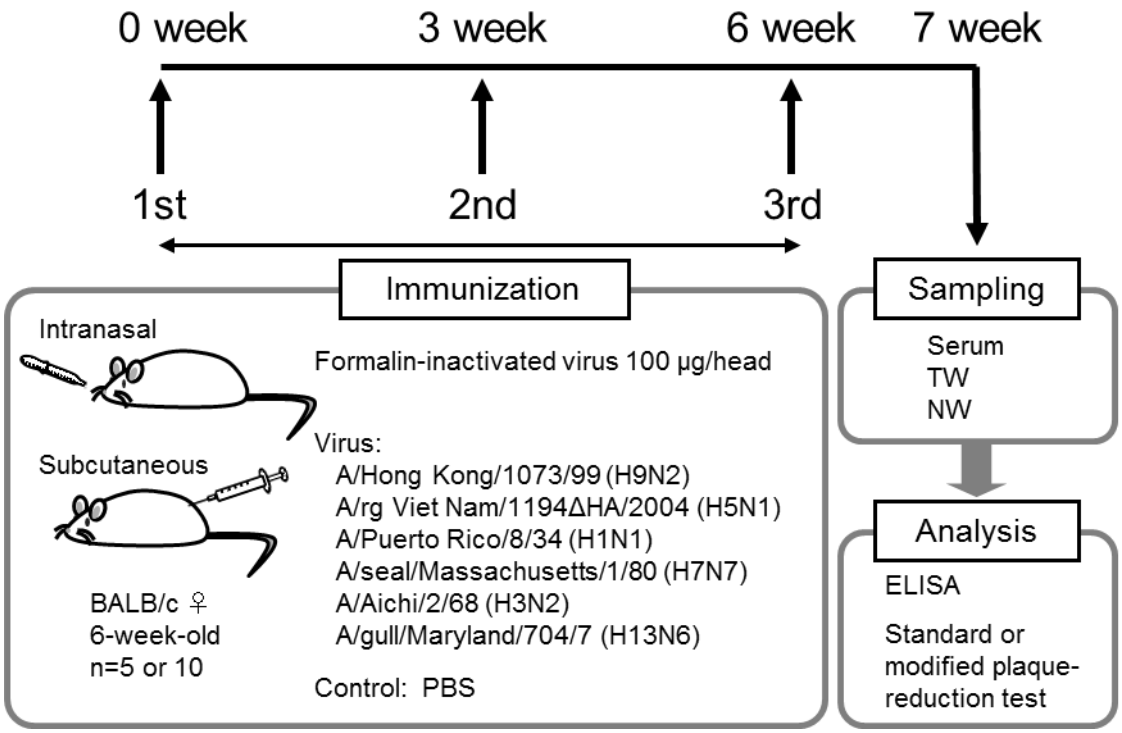


Figure 1. Immunization of mice with formalin-inactivated virus and sampling. Six-week-old female BALB/c mice were inoculated with formalin-inactivated virus (100 μg/head) intranasally or subcutaneously. Five or ten mice were used for each group. Mice were immunized three times at three-week intervals. One week after the last immunization, mice were euthanized with isoflurane and serum, TW, NW samples were collected.

volumes and used for the antibody assays described below. For plaque-reduction neutralization tests, serum samples were pretreated with receptor destroying enzyme (RDE), (RDE II, "SEIKEN") (Denka Seiken Ltd..) according to the manufacturer's instructions. The TW and NW samples were also pretreated with one-tenth volume of RDE. Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan [35]. The protocol was approved by the Hokkaido University Animal Care and Use Committee. Three independent experiments were conducted using 5 or 10 mice/each group/experiments for immunization with H9N2, and in each of these experiment the serum antibodies obtained from mice showed the same spectrum of cross-binding ability to HAs (data not shown).

Expression of recombinant HA and NA

Viral RNAs were extracted using a QIAamp Viral RNA Mini Kit (QIAGEN). After reverse transcription with Moloney murine leukaemia virus reverse transcriptase (Invitrogen) using Uni12 primer (5'-AGCAAAAGCAGG), HA and NA genes were amplified by polymerase chain reaction (PCR) using gene-specific primer sets [36]. PCR products were purified with a Wizard SV Gel PCR Clean-up system (Promega) and cloned into pCAGGS, the mammalian expression plasmid. HEK293T cells (2×10^6) were plated on 10-cm dish, and 24 hours later, cells were transfected with pCAGGS expressing the recombinant HA or NA of each subtype using TransIT-LT1 transfection reagent (Minus). At 48 hours after

transfection, the recombinant HAs or NAs were extracted using a eukaryotic membrane protein extraction reagent kit (Thermo Fisher Scientific), according to the manufacturer's protocol. The extracted membrane proteins were appropriately diluted (1:2000-1:8000) with PBS to give the highest O.D. values at 450 nm for antisera or monoclonal antibodies (repository of our laboratory) specific to the respective HA or NA subtypes and used as antigens for enzyme-linked immunosorbent assay (ELISA).

ELISA

IgG and IgA antibodies in the serum, TW, and NW samples were measured by ELISA as described previously [37]. Briefly, ELISA plates (Nunc Maxisorp) were coated with the HA (H1-H16) or NA (N2 and N5) antigens, and washed with PBS containing 0.05% Tween 20 (PBST), followed by blocking with 3% skim milk in PBS. Serum, TW, and NW samples were diluted at 1:100, 1:3.5, and 1:3.5, respectively, in PBST containing 1% skim milk. The bound antibodies were detected using goat anti-mouse IgA (α) and goat anti-mouse IgG (γ) antibodies conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc.) diluted in PBST containing 1% skim milk. The reaction was visualized by adding 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich) and the O.D. at 450 nm was measured.

NA inhibition (NI) assay

Serum NI activities of H9N2 virus-immunized mice were measured by a standard colorimetric assay using fetuin (Calbiochem) as substrate [38].

The absorbance was measured at 549 nm. Endpoint NI titers were determined as the reciprocal of the highest serum dilution causing 50% inhibition of neuraminidase activity given in the absence of the serum. NI assay was conducted for H9N2, H12N5, and H3N2 viruses.

Standard plaque-reduction test

The inhibition of viral entry into cells was evaluated by the standard procedure of plaque-reduction neutralization tests using MDCK cells. Serial dilutions of the samples (50 μl) were mixed with an equal volume of diluted virus solution containing approximately 100 plaque-forming units (pfu) of virus, and incubated for 1 hour at room temperature. Then the mixture was inoculated onto a monolayer of MDCK cells on a 12-well tissue culture plate. After 1-hour incubation at 35°C, the inoculum was aspirated and cells were washed once with serum-free MEM and overlaid with MEM containing 1% Bacto-agar and trypsin (5 μg/ml) (GIBCO). The plaques were enumerated after incubation at 35°C for 2 days.

Modified plaque-reduction test

To further test the potential of antibodies to inhibit virus replication, plaque-reduction tests were modified. In this test, viruses were first inoculated onto MDCK cells without preincubation with antibodies and then cultured in media containing appropriately diluted samples. MDCK cells cultured in 12-well plates were inoculated with a virus solution (50-100 pfu/well), followed by incubation for 1 hour at 35°C. The inoculum was

replaced with overlay medium (MEM with 1% Bacto-agar) containing the serum, TW, or NW samples at final dilutions of 1:200, 1:5, or 1:5, respectively. A higher concentration (15 μg/ml) of trypsin was used for the medium containing serum samples. After incubation for 48 hours at 35°C, the overlay medium was removed, and the cells were washed 2 times with PBS and fixed with methanol. Plaques were stained with chicken antisera to the respective HA subtypes (kindly provided by Dr. H. Kida), horseradish peroxidase-conjugated rabbit anti-chicken Immunoglobulin Y (IgY) (IgG) (H+L) (Jackson Immuno Research), and 3,3'-diaminobenzidine (Wako). In some experiments, to inhibit the interaction of IgA or IgG with target molecules [39,40], goat anti-mouse IgA or IgG (polyclonal serum) (SouthernBiotech) was added to the NW samples, and reacted for 1 hour at room temperature. Then, the reaction mixture or same volume of PBS was mixed with overlay medium. Anti-mouse IgA or IgG antibodies were used at 1 μg/ml (final concentration in the medium). I confirmed preliminarily that pretreatment with these anti-IgA or IgG antibodies efficiently inhibited neutralizing activities of HA-specific monoclonal IgA or IgG *in vitro* (data not shown). Each experiment was performed at least twice.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

MDCK cells infected with viruses at a multiplicity of infection (MOI) of 1.0 were maintained with MEM containing the sample for 7 hours, and culture supernatants were collected and mixed with SDS-PAGE sample

buffer. After 5-20% SDS-PAGE, separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). Anti-A/turkey/Wisconsin/1/1966 (H9N2), anti-A/duck/Alberta/60/1976 (H12N5), and anti-A/tern/South Africa/1961 (H5N3) hyperimmune chicken sera were used as primary antibodies to detect viral H9, H12, and H5 HA proteins, respectively. The bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgY (IgG) (H+L) (Jackson Immuno Research), followed by visualization with Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore). Band intensities of the HA proteins were analyzed with a VersaDoc Imaging System (Bio-Rad) and Image Lab software (Bio-Rad).

Phylogenetic analysis

Phylogenetic analysis was based on whole amino acid sequences of HAs obtained from GenBank under accession numbers ABO21709.1 (H1), BAG72216.2 (H2), BAF48361.1 (H3), BAF48478.1 (H4), ABP51976.1 (H5), BAF36386.1 (H6), BAF02934.2 (H7), BAF43468.1 (H8), CAB95856.1 (H9), BAF46908.1 (H10), BAF43435.1 (H11), BAF43416.1 (H12), BAF46906.1 (H13), BAF43460.1 (H14), BAF48363.1 (H15), and AAV91217.1 (H16). The sequences were aligned by using GENETYX (Genetyx Corp.) for Windows, version 10. A phylogenetic tree was constructed by using the neighbor-joining method in MEGA 5.1 [41].

Results

Both intranasal and subcutaneous immunizations of mice induced heterosubtypic antibody responses to multiple HAs.

The virus strains of H1, H3, H5, H7, H9, and H13 HA subtypes were selected for immunization as representatives of each cluster in a phylogenetic tree based on HA amino acid sequences (Figure 2A). Mice were immunized intranasally or subcutaneously with these viruses, and HA-specific IgG and IgA antibodies in serum, NW, and TW samples were analyzed by ELISA for the binding activity to HAs of H1-H16 subtypes (Figure 3). In the serum samples of mice immunized subcutaneously with the H1N1 virus, IgG cross-reactive to H2, H3, H5, H7, H8, H9, H10, H11, H12, H14, and H15 HAs was detected (Figure 3A, upper right). Lower levels of IgG were detected in mice immunized intranasally than in mice immunized subcutaneously (Figure 3A, upper left). IgA was not detected remarkably in any samples of mice immunized subcutaneously (Figure 3A, upper left). The HA subtypes to which IgG or IgA showed cross-reactivity were almost the same (i.e., H7, H10, H11, and H12) in all samples of mice immunized intranasally or subcutaneously (Figure 3A). The spectrum of heterosubtypic responses (i.e., the HA subtypes to which induced antibodies showed cross-binding activity) varied depending on the HA subtypes of the viruses used for immunization (Figures 3A-F); HA-specific serum antibodies detected in mice immunized subcutaneously were cross-reactive to H7 in H3N2 virus-immunized mice (Figure 3B), to H1, H2, and H3 in H5N1

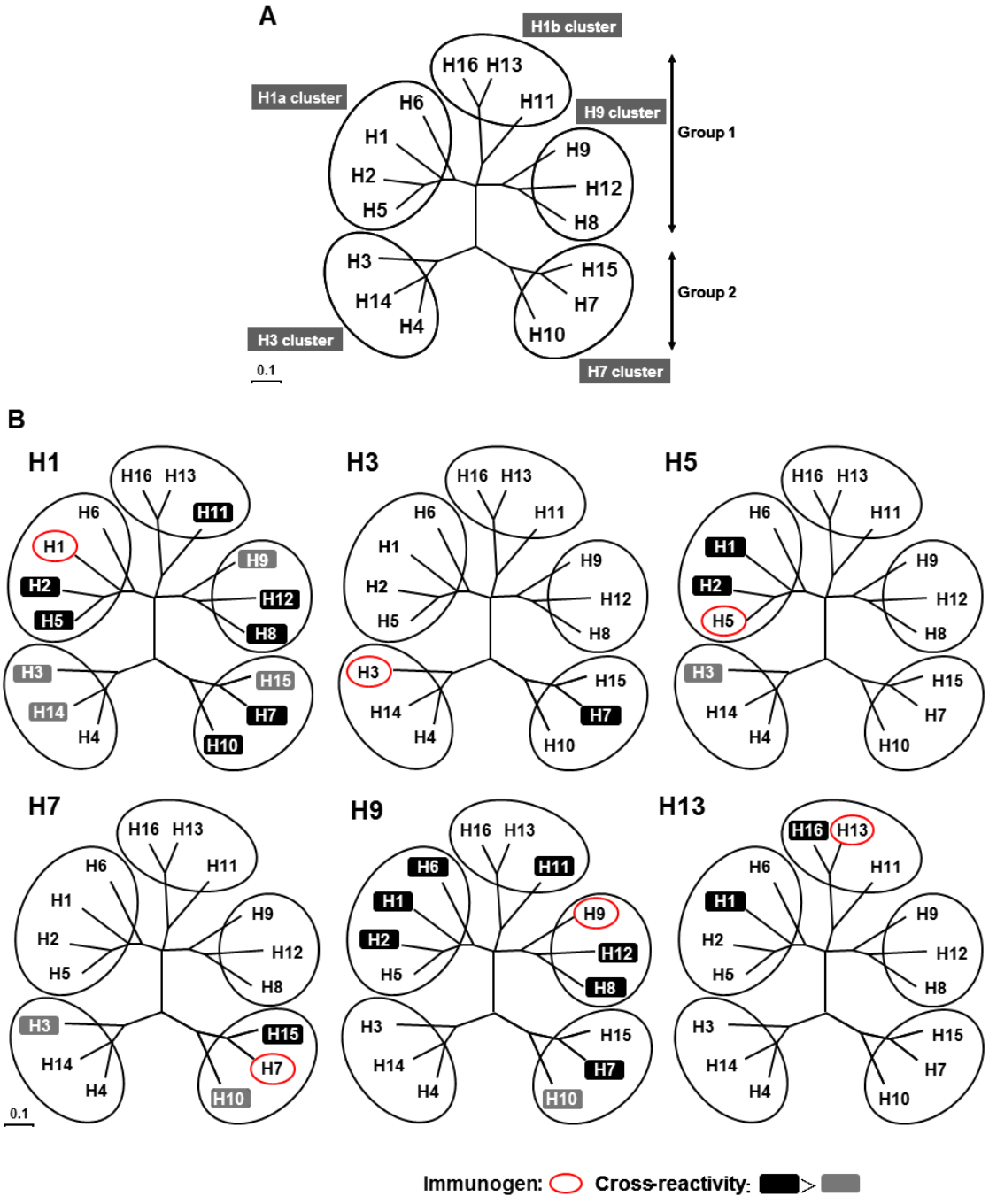
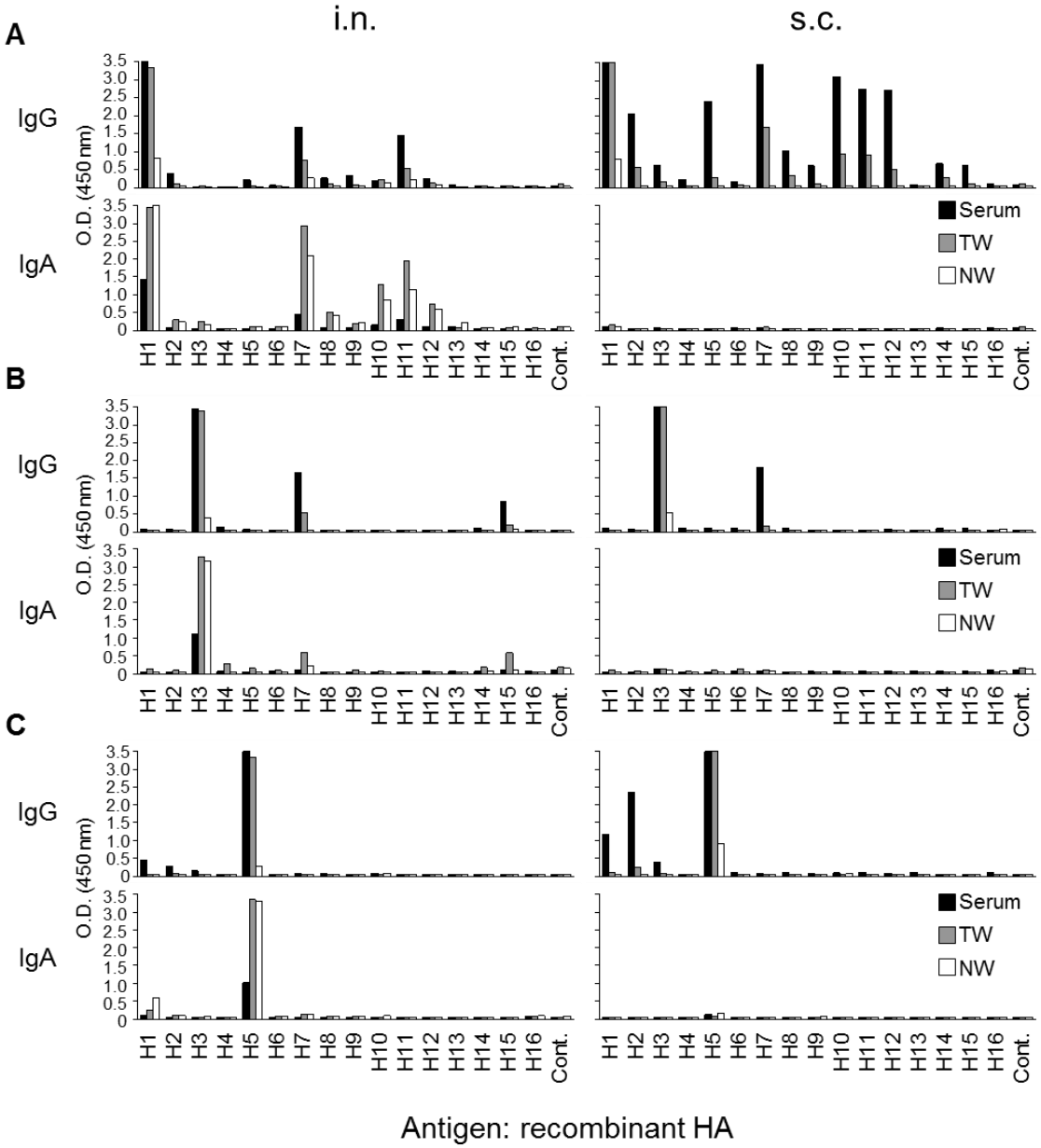


Figure 2. Correlations between cross-binding activities of antibodies and phylogenetic grouping of HA. (A) Phylogenetic relationships among 16 HA subtypes of influenza A viruses based on amino acid sequences. The HA amino acid sequences of the viruses used for ELISA antigens were used. (B) The subtypes of HAs to which serum IgG antibodies of subcutaneously immunized mice showed cross-binding activity (See also Figure 2). In each phylogenetic tree, the HA subtype used for immunization is shown in a red circle. The HA subtypes to which serum antibodies of mice immunized subcutaneously showed cross-binding activity are shown in black (O.D. $\geqq$ 1.0) or gray (1.0 > O.D. $\geqq$0.3) background.


A
B
C
i.n.
s.c.
IgG
IgA
O.D. (450 nm)
Serum
TW
NW
H1 H2 H3 H4 H5 H6 H7 H8 H9 H10 H11 H12 H13 H14 H15 H16 Cont.
Antigen: recombinant HA

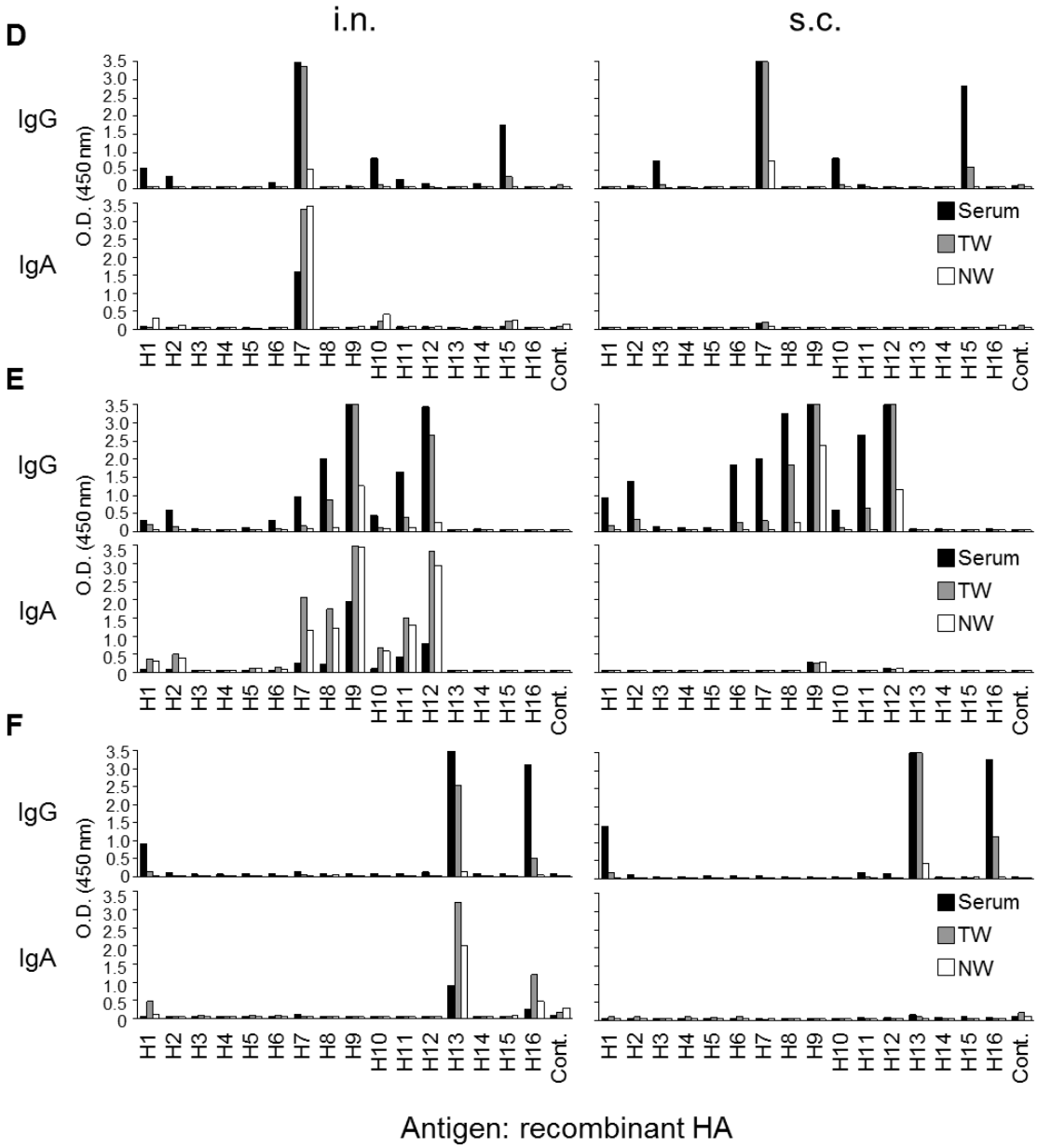


Figure 3. Cross-binding activities of HA-specific antibodies. Mice were immunized intranasally (i.n.) or subcutaneously (s.c.) with formalin-inactivated H1N1 (A), H3N2 (B), H5N1 (C), H7N7 (D), H9N2 (E), or H13N6 (F) viruses. Serum, TW, and NW samples were collected 7 days after the last immunization. Samples from each group were pooled and diluted at 1:100 (serum), 1:3.5 (TW and NW) with 1% skim milk in PBST. HA-specific IgG and IgA antibodies were detected by ELISA as described in the Materials and Methods section.

virus-immunized mice (Figure 3C), to H3, H10, and H15 in H7N7 virus-immunized mice (Figure 3D), to H1, H2, H6, H7, H8, H10, H11, and H12 in H9N2 virus-immunized mice (Figure 3E), and to H1 and H16 HAs in H13N6 virus-immunized mice (Figure 3F). As expected, there was a common observation, irrespective of the subtype used for immunization, that subcutaneous immunization induced IgG, but only slight IgA responses, whereas intranasal immunization induced both IgG and IgA responses, but IgG responses were generally lower than those of mice immunized subcutaneously.

The spectrum of cross-reactive antibodies correlated with phylogenetic classification of HAs.

Influenza virus HAs are phylogenetically divided into two groups (groups 1 and 2) consisting of 5 clusters (H1a, H1b, H9, H3, and H7 clusters) based on their amino acid sequences [42,43] (Figure 2A). On the phylogenetic tree, I mapped the HA subtypes that were recognized by cross-reactive antibodies induced by each immunogen (Figure 2B). I found that the spectrum of the HA-specific cross-reactive antibodies was directed to particular HA subtypes that were closely related to the viruses used for immunization. However, it was noted that broader cross-reactivity beyond the clusters and groups was found for the antibodies of H1N1 and H9N2 virus-immunized mice. For example, in mice immunized subcutaneously with H9N2 virus, the serum IgG was cross-reactive to H12 and H8 HAs, both of which belonged to the same cluster, but also to other HAs such as H6, H7,

and H11 which belonged to different clusters or groups (Figure 2B). On the other hand, it was also noteworthy that the cross-reactivity did not necessarily cover all subtypes belonging to the same clusters to which each immunogen belonged (e.g., H1-, H3-, and H13-induced antibodies did not bind to H6, H4, and H11 HAs, respectively).

Cross-reactive antibodies showed no heterosubtypic neutralizing activity in a standard plaque-reduction test.

To evaluate whether the induced cross-reactive antibodies had the ability to inhibit virus entry into cells (i.e., so called neutralizing activity), I focused on H9-induced antibodies that showed remarkable cross-reactivity (Figures 2B and 3E), and a standard plaque-reduction test was carried out using the serum, TW, and NW samples of H9N2 virus-immunized mice. In this test, viruses were mixed with antibodies prior to inoculation onto MDCK cells and the reduction of infectivity was estimated by plaque counts. Viruses having H1, H2, H7, H8, H11, and H12 HAs, to which H9N2 virus-induced antibodies bound, H9N2 as a positive control, and H5N1 as a negative control to which H9N2 virus-induced antibodies are little cross-reactive in ELISA, were selected as challenge viruses. The heterosubtypic neutralizing activity of antibodies was not detected in any samples even at the lowest sample dilutions tested, although the neutralizing activity against the homologous virus (i.e., H9) was clearly detected in the serum, TW and/or NW samples of immunized mice (Figure 4A). It was noted that the TW and NW samples of subcutaneously

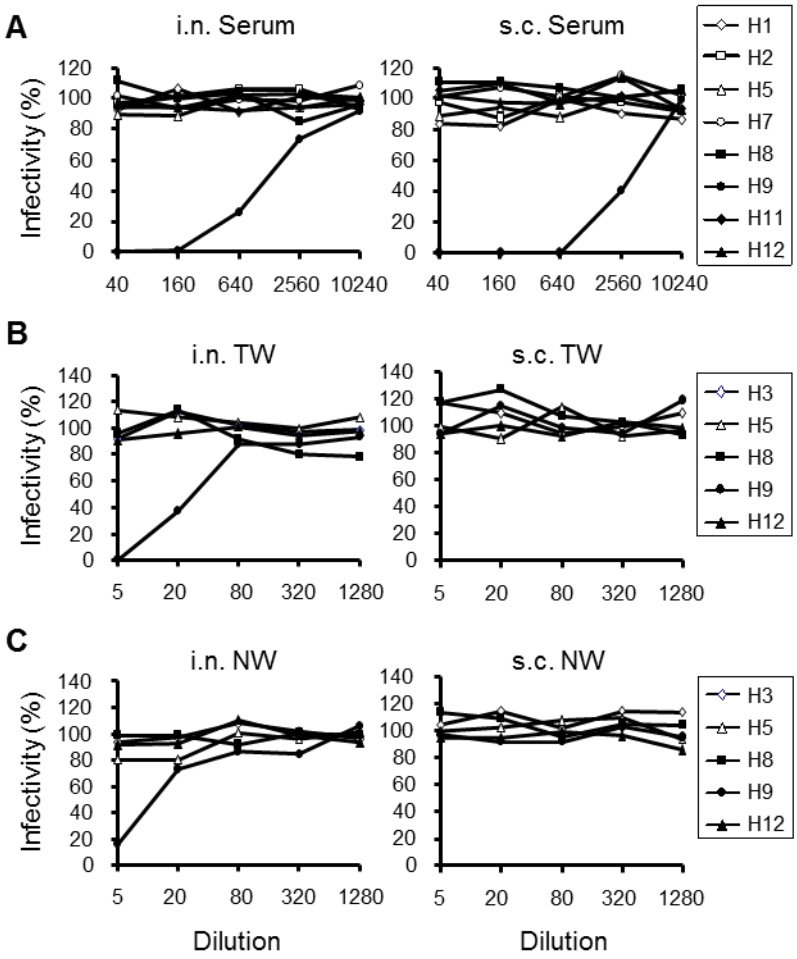


Figure 4. Neutralizing activities of the samples of mice immunized with the H9N2 virus in a standard plaque-reduction test. Appropriately diluted viruses were mixed at indicated dilutions with the serum (A), TW (B), or NW (C) sample of mice immunized with H9N2 intranasally (i.n.) or subcutaneously (s.c.). Neutralizing activities were evaluated by counting the number of plaques formed on MDCK cells.

immunized mice did not show neutralizing activity even against the homologous virus though these samples appeared to contain high levels of anti-H9 IgG (Figure 3E, upper).

Plaque formation of H12N5 virus was reduced in the presence of H9N2 virus-induced cross-reactive IgA antibodies.

To further examine the antiviral activities of the cross-reactive antibodies, I modified the procedure of the plaque-reduction test. In the assay applied here, viruses were first inoculated onto MDCK cells without preincubation with antibodies and then cultured in media containing appropriately diluted samples. This assay might enable us to detect antibodies that inhibit budding or extracellular release of virus particles from infected cells, even if antibodies do not inhibit HA-mediated entry of viruses into host cells [44,45]. I selected viruses of 4 different subtypes as challenge viruses for this assay; H9N2 as a positive control, H12N5, which was most effectively bound by H9-induced cross-reactive antibodies in ELISA (Figure 3E), H5N1 as a negative control to which H9N2 virus-induced antibodies are little cross-reactive, and H3N2, whose NA subtype is the same as that of the virus used for immunization (H9N2) in order to test the inhibitory effects of NA-specific antibodies. In the presence of serum antibodies of mice immunized intranasally or subcutaneously with H9N2, the homologous virus formed no visible plaques (Figures 5A and D). Interestingly, plaque sizes and numbers of H12N5 were reduced to some extent in the presence of serum antibodies containing both IgG and IgA, of

mice immunized intranasally, but the reduction was minimally found in the presence of high levels of serum IgG of mice immunized subcutaneously (Figures 5A and D). Antibodies in the TW and NW samples exhibited more substantial differences in heterosubtypic plaque-reduction activity between intranasally and subcutaneously immunized mice. The TW and NW samples of mice immunized intranasally that contained cross-reactive IgG and IgA remarkably reduced the number and/or size of plaques of the H12N5 virus (NW samples showed plaque size reduction, however, there were still small plaques), whereas the samples of subcutaneously immunized mice did not show such inhibitory effects (Figures 5B, C, and D). It was noted that the TW and NW samples of subcutaneously immunized mice contained high levels of IgG (Figure 2E), but gave much less plaque reduction of even the homologous virus (i.e., H9) than those of intranasally immunized mice (Figures 5B and D). Actually, the extent of plaque reduction seemed to be associated with the presence of cross-reactive anti-HA IgA in the samples. No apparent plaque reduction was observed for H3N2 and H5N1 viruses in the presence of H9N2-induced antibodies, regardless of the sample origin (i.e., serum, TW, or NW). To confirm the role of IgA antibodies in heterosubtypic plaque reduction, I pretreated the NW samples of H9N2 virus-immunized mice with goat anti-mouse IgA or anti-IgG antibodies and mixed with overlay medium in the modified plaque reduction test. As expected, the heterosubtypic plaque reduction of H12 virus was interfered with anti-mouse IgA but not anti-IgG antibodies (Figure 6).

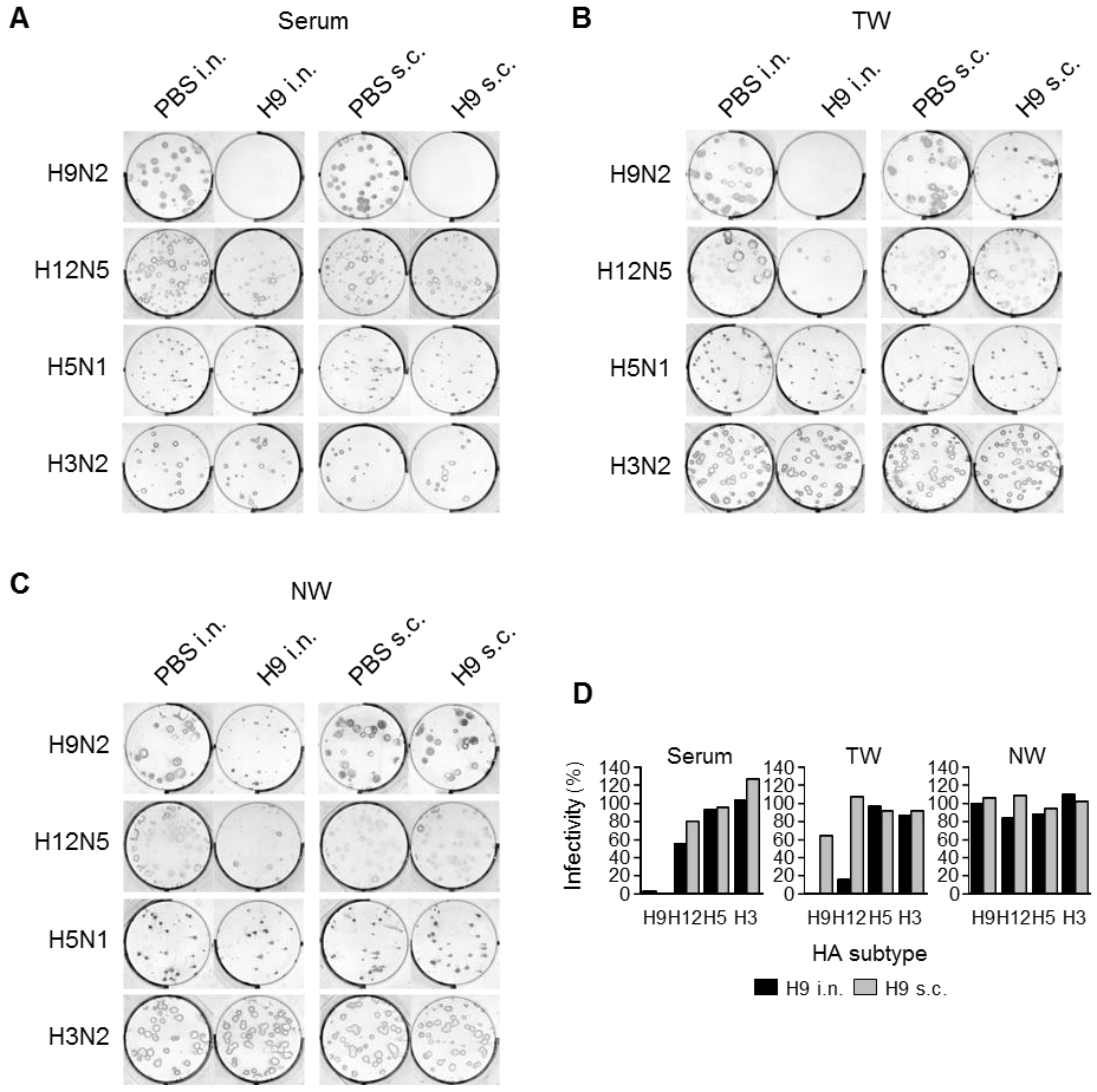


Figure 5. Reduced plaque formation in the presence of antibodies of mice immunized with H9N2. After being inoculated with H9N2, H12N5, H5N1, or H3N2, MDCK cells were cultured in the presence of antibodies in the serum (1:200) (A), TW (1:5) (B), or NW (1:5) (C) samples of mice immunized intranasally (i.n.) or subcutaneously (s.c.). Numbers of plaques were counted and the infectivity was calculated relative to the number of plaques formed in the presence of the samples of mice given PBS intranasally or subcutaneously for i.n. or s.c. immunized mice, respectively (D).

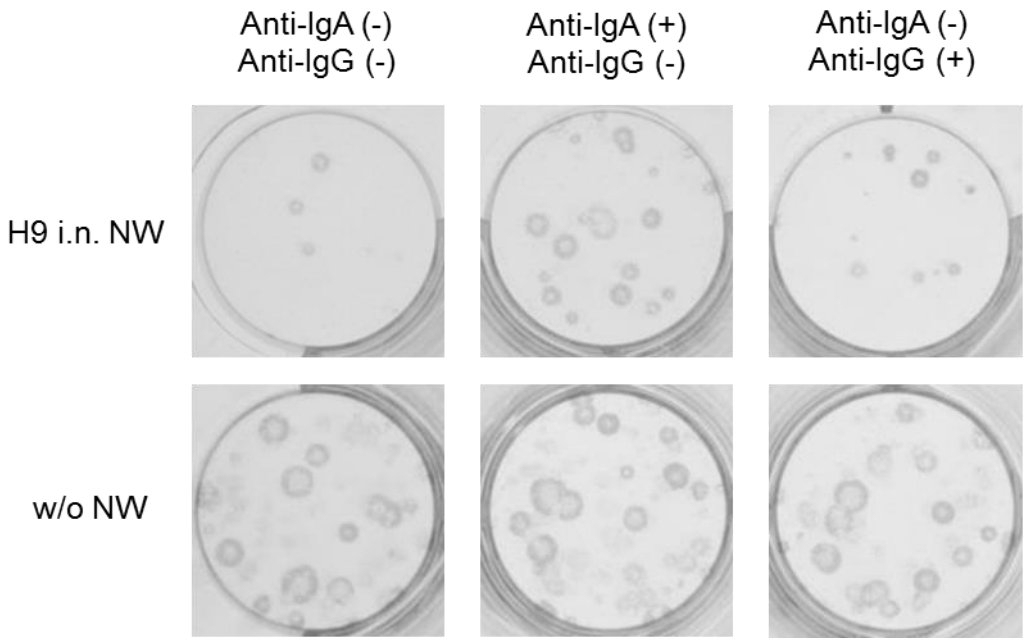


Figure 6. Heterosubtypic plaque reduction interfered by anti-mouse IgA antibodies. NW samples of mice immunized with H9N2 virus intranasally (i.n.) were pretreated with anti-mouse IgA or anti-mouse IgG antiserum (1 μg/ml). MDCK cells infected with H12N5 virus were incubated in the presence of or without (w/o) the NW samples treated or nontreated with the antiserum.

Budding and release of H12N5 from infected cells were impeded in the presence of H9N2 virus-induced cross-reactive antibodies.

To gain insight into the mechanism of antibody-mediated inhibitory effects on the plaque formation, I examined extracellular release of virus particles in the presence of antibodies. MDCK cells infected with H9N2, H12N5, or H5N1 were cultured with the TW of mice immunized with H9N2 virus intranasally or subcutaneously, and then the amounts of virus particles released into the culture supernatants were estimated by detecting viral HA proteins in Western blotting with anti-H9, H12, or H5 chicken serum, respectively (Figure 7). I found that significantly lower amounts of HAs of H9N2 and H12N5 were detected in the supernatants of infected cells cultured with TW antibodies of mice immunized intranasally with H9N2, compared with those seen in samples from subcutaneously immunized mice. No remarkable decrease of the viral particle formation was appreciable for H5N1, regardless of the immunization route. Taken together, these data suggested that cross-reactive nonneutralizing antibodies, most likely IgA, induced by H9N2 effectively inhibited the budding and release of H12N5 virus particles from infected cells.

No cross-reactive NI activity was detected in the serum of H9N2-virus immunized mice.

To test the possibility that the cross-reactive NI activity was correlated with the reduced viral replication and budding of the H9N2 virus, I examined NI activity in the serum samples of H9N2 virus-immunized mice

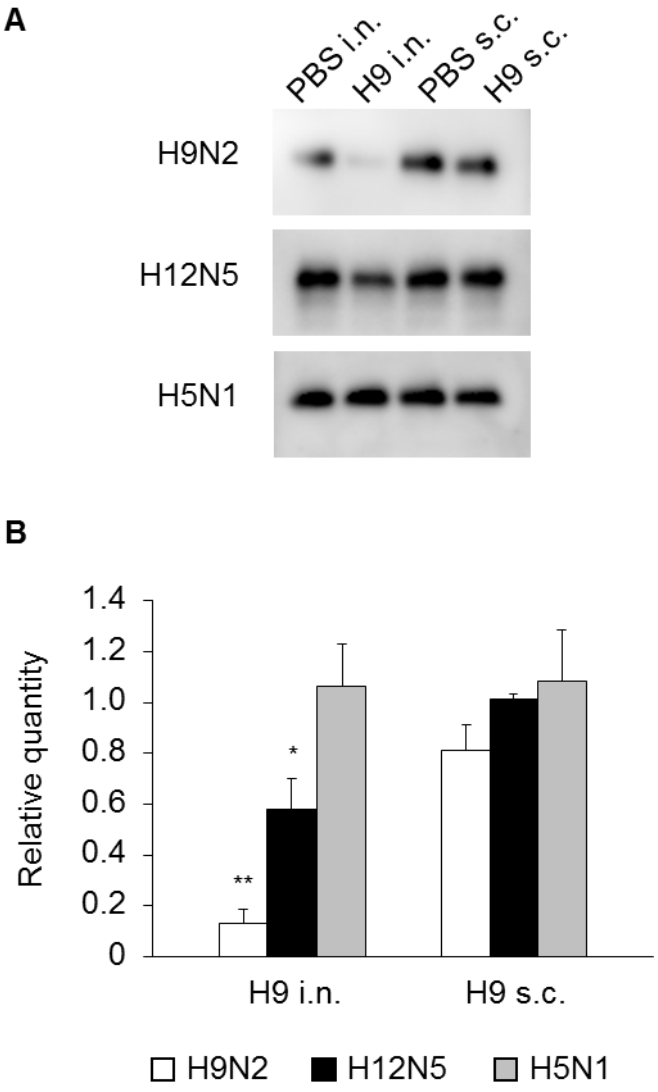


Figure 7. Detection of viral proteins in the supernatants of cells infected with H9N2, H12N5, or H5N1. MDCK cells were infected with viruses and cultured with the TW samples of intranasally (i.n.) or subcutaneously (s.c.) immunized mice as described in the legend of Figure 6. Supernatants were collected 7 hours after infection, and virus particles released into the supernatants was detected by Western blotting (A). The relative intensity of the HA bands compared to each of the control (PBS i.n. and s.c.) samples was obtained using Image Lab version 3.0 (BIO RAD) (B). Experiments were performed 3 times, and averages and standard deviations are shown. Student *t*-test was used for statistical analysis. Asterisks indicate statistically significant differences in band densities compared to each of the control (PBS i.n. and s.c.) (**$p<0.01$, *$p<0.05$).

against H12N5 and H3N2 viruses (Table 1). Serum of mice immunized intranasally or subcutaneously with H9N2 showed high NI titers against the homologous H9N2 virus, while there was no detectable inhibition against H12N5. A slight NI activity was detected in the serum of mice immunized intranasally with the H3N2 virus.

Table 1. Serum NI activities of H9N2 virus-immunized mice against H9N2, H12N5, and H3N2 viruses.

Virus	Immunization	NI titer
H9N2	Subcutaneous	320
	Intranasal	320
H12N5	Subcutaneous	<20
	Intranasal	<20
H3N2	Subcutaneous	<20
	Intranasal	20

Discussion

It has been shown that intranasal immunization with an inactivated virus confers heterosubtypic protection from influenza A viruses in mice, whereas subcutaneous immunization is only effective against viruses with homologous HA subtypes [9,10,21,46]. Such heterosubtypic protection was shown to be B-cell-dependent [9]. In these previous studies, cross-neutralizing antibodies were consistently undetectable in a standard neutralization test, while HA-specific antibodies with cross-binding activity were often induced [47]. Thus, this study aimed to clarify whether intranasal immunization of mice with an inactivated influenza virus generally induces HA-specific nonneutralizing antibodies reactive to multiple HA subtypes and to elucidate the potential roles of these antibodies in the heterosubtypic immunity against influenza A viruses.

I first analyzed cross-binding activities of HA-specific antibodies induced by intranasal or subcutaneous immunization with viruses having different HA subtypes (i.e., H1, H3, H5, H7, H9, or H13), and found that substantial amounts of cross-reactive IgG antibodies to multiple HA subtypes, which were predominantly related to each immunogen phylogenetically, were indeed detectable in the sera and respiratory secretions of immunized mice. There was no fundamental difference in the overall spectrum of the cross-reactivity between the antibodies produced by intranasal and subcutaneous immunizations. Importantly, however, both IgA and IgG antibodies, which are principal isotypes of mucosal immunity

and systemic immunity, respectively [11,39], were produced by intranasal immunization; whereas only the IgG antibody response was induced by subcutaneous immunization. These results suggest that the IgA antibodies may play a major role in the B-cell-dependent heterosubtypic immunity induced by intranasal immunization of mice.

In a standard neutralization test, neutralizing activities of the antibodies induced in H9N2 virus-immunized mice were only targeted to the homologous virus, regardless of the immunization route and sample origin, which was consistent with previous studies [9,10,21,46]. This results indicates that majority of the cross-reactive antibodies detected in ELISA do not recognize the so-called neutralizing epitopes that are typically located on functionally important sites of HA (e.g., the receptor binding sites). It was also noted that antibodies in the TW or NW samples of mice immunized subcutaneously did not neutralize even the homologous H9N2 virus (Figures 4B and C), while these samples contained similar or even higher levels of IgG than in those from intranasally immunized mice (Figure 3E). Instead, neutralizing activities of the TW and NW samples were associated with the presence of IgA, suggesting that IgA antibodies neutralized virus infectivity more efficiently than IgG.

In the modified plaque-reduction test, the plaque formation of H12N5, but not H5N1 and H3N2, was suppressed in the presence of antibodies in the serum, TW, and NW samples from mice immunized with H9N2 intranasally (Figure 5), although these antibodies did not display “classical” neutralizing activity against the H12N5 virus (Figure 4). No plaque reduction of H12

virus was seen in the presence of serum or TW samples of negative control and H13N6 virus-immunized mice (data not shown), in which antibodies cross-reactive to H12 HA were not detected, suggesting that unlikelihood that the plaque reduction of H12 virus was due to nonspecific inhibitors in the samples. The extent of plaque reduction indeed seemed to reflect the concentration of cross-binding anti-HA IgA in each sample (Figure 3E). By contrast, no significant plaque reduction of H12N5 was observed in the serum samples of mice immunized with H9N2 subcutaneously. It is noteworthy that the serum, TW, and NW samples from mice immunized subcutaneously with H9N2 contained higher amounts of IgG antibodies cross-reactive to H12 HA than those of intranasally immunized mice (Figure 3E), but did not show inhibitory activity to reduce the plaque number and size of the H12N5 virus (Figure 5). Furthermore, the plaque reduction activity of the samples of mice immunized with H9 virus intranasally was decreased when the samples were pretreated with anti-mouse IgA antibodies but not anti-mouse IgG antibodies (Figure 6). Taken together, these results suggested that cross-reactive IgA antibodies had the ability to reduce plaque formation, likely by preventing the budding or extracellular release of virus particles from infected cells (Figure 7), whereas these antibodies did not block HA-mediated entry of viruses into host cells (Figure 4).

It is known that nonneutralizing antibodies against NA or M2 protein, which are the other envelope proteins of influenza A viruses, inhibit the extracellular release of progeny viruses from host cells, and thus play a role in protective immunity [48]. These antibodies do not prevent virus

entry into host cells, but significantly inhibit plaque formation of influenza viruses when infected cells are cultured in the presence of the antibodies [49,50]. Such antibodies are thought to produce a crosslink between virus-associated NA or M2 and those expressed on the cell surface, leading to reduced budding of the virus [44,51-53]. In this study, antibodies induced by immunization with H9N2 did not reduce the plaque formation of H3N2, despite having the same NA subtype. In a neuraminidase inhibition test, the serum samples of mice immunized intranasally with H9N2 showed only slight inhibition of H3N2 but not H12N5 viruses (Table 1). Furthermore, in ELISA using recombinant NA antigens, cross-reactive antibodies to N5 were undetectable in the serum of H9N2-immunized mice (data not shown). These data suggest that NA-specific antibodies did not mainly contribute to the heterosubtypic plaque reduction observed in this study. M2-specific antibodies were also unlikely to be involved in the heterosubtypic plaque reduction seen in this study since no plaque reduction by H9-induced antibodies was observed for H3N2 and H5N1, despite the highly conserved antigenicity of the M2 protein among influenza A viruses [48]. Overall, our data suggest that some of the cross-reactive nonneutralizing anti-HA IgA antibodies induced by intranasal immunization may have similar inhibitory effects on the virus particle formation and play a role in the heterosubtypic immunity against influenza A viruses. Accordingly, it may be reasonable to assume that subcutaneous immunization induces only subtype-specific immunity since it does not induce sufficient amounts of IgA.

Indeed, I found that the budding and extracellular release of H12N5

virus particles were inhibited in the presence of cross-reactive IgA induced in H9N2 virus-immunized mice (Figure 7). Thus, the most plausible explanation for the mechanism underlying the inhibitory effects of cross-reactive nonneutralizing IgA antibodies on virus particle budding is that the antibodies crosslink multiple HA molecules expressed on the infected cell surface before initiation of the budding process or on the budded virus and, consequently, this intricate cross-linkage via antibodies and HA molecules interferes with the virus pinch-off of virus particles or release of budded particles. Accordingly, the accumulation of unreleased virus particles on the cell surface was partially observed in electron microscopy, although a quantitative analysis could not be carried out (data not shown).

As mentioned above, the viral budding process is an important target for some antibodies that have protective properties. In addition, IgA antibodies have been shown to bind newly synthesized viral proteins in infected cells and to inhibit virus protein functions intracellularly [17-19,54]. This mechanism may allow cross-reactive anti-HA IgA antibodies to interfere with the maturation of HA (e.g., glycosylation, molecular folding, and proteolytic processing) in infected cells. It might also be possible that IgA antibodies contribute to the antibody-dependent cell-mediated cytotoxicity, which may reduce the production of progeny viruses by readily clearing infected cells. These results emphasize the idea that the "classical" neutralizing activity is not the only indicator of antibody function contributing to heterosubtypic immunity. A detailed analysis of the precise mechanism by which IgA antibodies interfere with the virus replication may

provide new insights into the development of universal mucosal vaccines against multiple subtypes of influenza viruses.

Summary

Influenza A virus subtypes are classified on the basis of the antigenicity of their envelope glycoproteins, HA and NA. Since HA-specific neutralizing antibodies are predominantly specific for a single HA subtype, the contribution of antibodies to the heterosubtypic immunity is not fully understood. In this study, mice were immunized intranasally or subcutaneously with viruses having the H1, H3, H5, H7, H9, or H13 HA subtype, and cross-reactivities of induced IgG and IgA antibodies to recombinant HAs of the H1-H16 subtypes were analyzed. I found that both subcutaneous and intranasal immunizations induced antibody responses to multiple HAs of different subtypes, though IgA was detected remarkably only in mice immunized intranasally. Using serum, NW, and TW samples of H9 virus-immunized mice, cross-antiviral activities of antibodies were then evaluated. No heterosubtypic neutralizing activity was detected by a standard neutralization test. Interestingly, however, the extracellular release of the H12 virus, to which H9-induced antibodies showed cross-binding activity, was remarkably surpressed when infected cells were cultured in the presence of HA-specific cross-reactive IgA. These results suggest that the majority of HA-specific cross-reactive IgG and IgA antibodies produced by immunization do not block cellular entry of viruses, but cross-reactive IgA may have the potential to inhibit viral egress from infected cells and thus to play a role in heterosubtypic immunity against influenza A viruses.

Chapter II

Comparison of antiviral activity between IgA and IgG specific to influenza virus hemagglutinin: Increased potential of IgA for heterosubtypic immunity

Introduction

It is known that both IgA and IgG antibodies play important roles in protection against influenza virus infection [11,39]. While IgG is the major isotype of antibodies important for systemic immunity, IgA is predominantly present in mucosal tissues, including the upper respiratory tract, providing the first line of defense with unique properties in mucosal immunity at the primary site of influenza virus infection. p-IgA antibodies bind to polymeric immunoglobulin receptors expressed at the basolateral surface of epithelial cells, and are selectively transported to the mucosal surface. While crossing through epithelial cells, p-IgAs are believed to inhibit viral protein functions intracellularly. Following transcytosis, p-IgAs are released with a secretory component to mucosal surface, and are resistant to proteolysis in mucosal secretions [11-19]. In addition, p-IgA does not induce an inflammatory reaction in mucosa [11,13,16]. Therefore, it has been suggested that induction of the mucosal immune response is more desirable to prevent respiratory infection by influenza A viruses [20-23].

Since HA-specific IgG antibodies induced by subcutaneous or intramuscular injection with inactivated influenza vaccines are principally subtype-specific, protective effects are limited to viruses whose antigenicity

is closely related to those of the vaccine strains [5,55]. However, previous studies experimentally demonstrated that B-cell-dependent heterosubtypic immunity was induced by intranasal immunization of mice with formalin-inactivated viruses, whereas systemic immunization only protected mice from viruses with homologous HA subtypes [6,9,10].

In chapter I, I showed that both subcutaneous and intranasal immunization of mice with inactivated viruses induced antibodies that bound to HAs of multiple subtypes, but IgA antibodies might show greater ability than IgG antibodies to reduce plaque formation of viruses with heterologous subtypes [56], suggesting different antiviral potentials for IgA and IgG antibodies in heterosubtypic immunity. Yoshida *et al.* (2009) previously reported an HA-specific MAb S139/1 (IgG) that originated from mice immunized with a virus of subtype H3 showed broad cross-reactivity to viruses with multiple HA subtypes, including H1, H2, H3, and H13 [31,57]. MAb S139/1 binds to a conformational epitope located on the globular head of the HA, adjacent to the receptor-binding domain, and shared among a variety of HA subtypes. In this study, a cell line producing p-IgA was subcloned from an S139/1 IgG-producing hybridoma with spontaneous class switching *in vitro*. By using these S139/1 MAbs recognizing the same epitope, I compared antiviral activities of IgA and IgG antibodies against influenza A viruses of the H1, H2, H3, and H13 HA subtypes *in vitro*.

Materials and methods

Viruses and cells

Influenza A virus strains, A/Aichi/2/1968 (H3N2) (Aichi/H3), A/WSN/1933 (H1N1) (WSN/H1), A/Adachi/2/1957 (H2N2) (Adachi/H2), and A/gull/Maryland/704/1977 (H13N6) (Maryland/H13), were kindly provided by Dr. H. Kida, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan. These viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 35˚C for 48 hours. Virus particles were concentrated and purified by high-speed centrifugation of the allantoic fluid passed through a 10-50% sucrose density gradient. Purified viruses were disrupted with 50 mM Tris-HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl, and used as antigens for ELISA [31,58]. MDCK cells were maintained in MEM (GIBCO) supplemented with 10% calf serum. Hybridoma cells producing MAb S139/1 IgG, which were previously generated by immunization of BALB/c mice with formalin-inactivated purified Aichi/H3, were used [31]. Hybridoma cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum.

Generation of hybridoma variants producing p-IgA

To obtain hybridoma variants producing p-IgA antibodies recognizing the same epitope as S139/1 IgG, we modified a sib selection/ELISA method that relied on spontaneous class-switching of the cells [59,60]. Hybridoma

cells were first grown in 96-well microplates at 1,000 cells per well and incubated at 37˚C in the presence of 5% CO_2. At 70% to 90% confluence, culture supernatants were screened for IgA production by sandwich ELISA using immunoplates previously coated with goat anti-mouse IgA (BETHYL). Cells from positive wells were harvested and replated at 10-100 cells per well. Screening for IgA and replating were repeated several times and IgA-producing cells were finally established from a single cell clone. Messenger RNA was extracted from the variant hybridoma clone using an RNeasy Mini Kit (QIAGEN). The variable region gene was amplified by reverse transcription and PCR using a One-Step PCR Kit (QIAGEN) with primers 5'-GAT GGT GGG ATT TCT CGC AGA CTC-3' and 5'- SAR GTN MAG CTG SAG SAG TC-3' for the heavy chain, and 5'- GGA TAC AGT TGG TGC AGC ATC-3' and 5'- GAY ATT GTG MTS ACM CAR WCT MCA-3' for the light chain, followed by direct sequencing. I confirmed that the amino acid sequence of the variable region of S139/1-derived IgA was identical to that of the original S139/1 IgG [57]. S139/1 IgA and IgG interfered each other in a competitive antibody binding assay (Figure 8) and did not show hemagglutination-inhibition (HI) activities against WSN/H1 and Aichi/H3 escape mutants obtained previously [31], confirming the same epitope recognition of S139/1 IgA and IgG.

Purification of MAbs

Hybridoma cells producing S139/1 IgA and IgG antibodies were cultured in BD Cell MAb Medium (BD Bioscience), Serum Free using

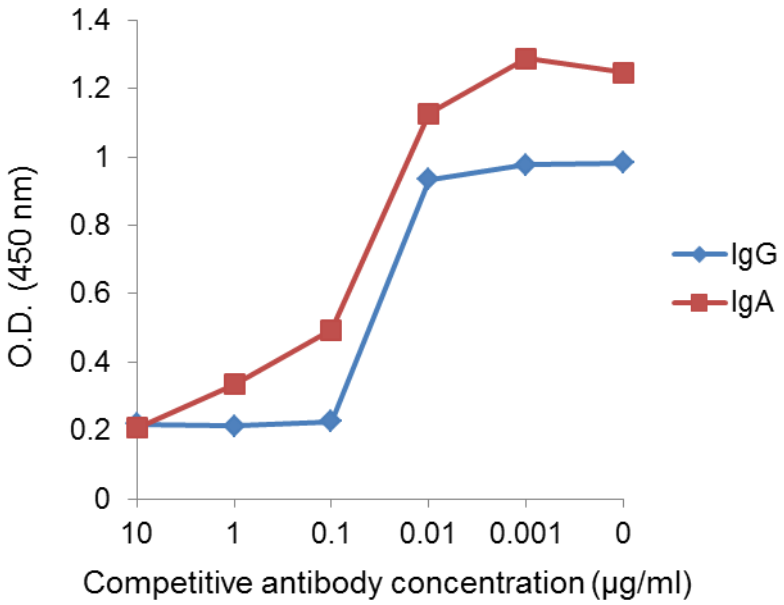


Figure 8. Competitive antibody binding assay using S139/1 IgA and IgG. ELISA plates were coated with the disrupted virus antigens (Aichi/H3), followed by blocking with 3% skim milk in PBS. Tenfold serially diluted S139/1 IgG and IgA were plated as competitive antibodies, followed by incubation with S139/1 IgA and IgG (1 ng/ml), respectively. Bound IgA and IgG were detected using goat anti-mouse IgA (α) and goat anti-mouse IgG (γ) antibodies conjugated to horseradish peroxidase. The reaction was visualized by adding TMB and the absorbance at 450 nm was measured.

CELLine Flask (BD Bioscience). S139/1 IgA and IgG antibodies were purified from culture supernatants using KAPTIV-AE (TECNOGEN) and Protein G Sepharose 4 Fast Flow (GE Healthcare), respectively, according to the manufacturers' instructions. MAb concentrations were measured by O.D. at 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Purified MAbs were analyzed by SDS-PAGE under reducing or nonreducing conditions. For reducing conditions, MAbs were mixed with SDS-PAGE sample buffer with 2-mercaptoethanol (2-ME) (Wako) and heated at 98˚C for 5 minutes. For nonreducing conditions, MAbs were mixed with SDS-PAGE sample buffer without 2-ME and incubated at room temperature for 30 minutes. Protein bands stained with Quick-CBB PLUS (Wako) were quantified using a VersaDoc Imaging System (Bio-Rad) and Image Lab software (Bio-Rad).

Binding assay

Binding of MAbs was measured by ELISA as described previously [37]. Briefly, ELISA plates (Nunc Maxisorp) were coated with the disrupted influenza A virus antigens (Aichi/H3, WSN/H1, Adachi/H2, and Maryland/H13), and washed with PBS containing 0.05% Tween 20 (PBST), followed by blocking with 3% skim milk in PBS. Fourfold serially diluted MAbs in PBST containing 1% skim milk were plated in duplicate, and bound MAbs were detected using goat anti-mouse IgA (α) and goat anti-mouse IgG (γ) antibodies conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc.) diluted in PBST containing 1% skim milk. The reaction

was visualized by adding TMB (Sigma-Aldrich) and the absorbance at 450 nm was measured. MAb concentrations required to give half-maximal binding (50% effective concentration: EC_{50}) to influenza A virus antigens were determined using SoftMax Pro 6.2.1 software (Molecular Devices) [61,62].

HI and neutralization tests

HI activities of the purified MAbs were tested by the standard method using 0.5% chicken erythrocytes. Neutralizing activities of MAbs were evaluated by the standard procedure of plaque-reduction tests using MDCK cells. Fourfold serial dilutions of MAbs (50 µl) were mixed with an equal volume of diluted virus solution (approximately 50-100 plaque-forming units), and incubated for 1 hour at room temperature. Then the mixture was inoculated onto a monolayer of MDCK cells on 12-well tissue culture plates. After 1-hour incubation at 35°C, the inoculum was aspirated and cells were washed once with serum-free MEM and overlaid with MEM containing 1% Bacto-agar and trypsin (5 µg/ml) (GIBCO). Plaques were counted after incubation at 35°C for 1 day (WSN/H1 and Adachi/H2), 2 days (Aichi/H3), or 3 days (Maryland/H13).

Viral release inhibition assay

MDCK cells on 24-well plates were first inoculated with viruses at an MOI of 1-2, followed by incubation for 1 hour at 35°C. The inoculum was aspirated, and the cells were washed three times and cultured with MEM

containing S139/1 IgG or IgA antibodies (0.1 or 1 μg/ml) for 12 hours. Culture supernatants were collected at 0, 6, and 12 hours after infection, and mixed with SDS-PAGE sample buffer with 2-ME and treated at 98˚C for 5 minutes. After 10% SDS-PAGE, separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). Chicken antiserum to Aichi/H3 was used as the primary antibody to detect viral proteins. The bound antibody was detected with peroxidase-conjugated rabbit anti-chicken IgY (IgG) (H+L) (Jackson Immuno Research), followed by visualization with Immobilon Western (Millipore). Band intensities of the M1 protein were analyzed with a VersaDoc Imaging System (Bio-Rad) and Image Lab software (Bio-Rad).

Electron microscopy

Transmission electron microscopy (TEM) was carried out as described previously [63]. For ultrathin sections, MDCK cells infected with viruses at an MOI of 1-2 were cultured with MEM containing S139/1 IgA or IgG antibodies (1 μg/ml) for 8 or 12 hours and fixed for 20 minutes with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The cells were scraped off the plate, pelleted by centrifugation, and then fixed for 30 minutes with the same fixative. Small pieces of the fixed pellet were washed with cacodylate buffer, postfixed with 2% osmium tetroxide in the cacodylate buffer for 1 hour at 4°C, dehydrated with a series of ethanol gradients followed by propylene oxide, embedded in Epon 812 Resin mixture (TAAB Laboratories Equipment Ltd.), and polymerized at 60°C for 2 days. Ultrathin

sections (70 nm) were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7650 electron microscope at 80 kV.

Results

Polymeric structure of S139/1 IgA

I first analyzed the purity and the molecular weight of purified S139/1 IgA and IgG antibodies by SDS-PAGE (Figure 9). Two protein bands corresponding to the light and heavy chains were exclusively visible in reducing conditions, thus confirming that the presence of impurities was negligible in the MAb preparations. In nonreducing conditions, while monomeric IgG antibodies of approximately 150 kilodaltons (kD) [13] were predominant, only a small amount of the monomeric form of IgA, which was expected to be an approximately 170-kD protein [64], was visible. Instead, three bands with molecular weights corresponding to dimeric, trimeric, and tetrameric IgA antibodies, were observed. Accordingly, these bands were also detected by immunoblotting with anti-mouse IgA (data not shown). Based on the quantified band intensities, I estimated that the relative amounts of monomeric, dimeric, trimeric, and tetrameric forms of IgA were approximately 1, 5, 1, and 3, respectively. These data indicated that the majority of S139/1 IgA produced from the hybridoma cells were considered to be p-IgA antibodies.

Binding activities of S139/1 IgA and IgG antibodies to HAs of multiple subtypes

I then investigated the binding activities of S139/1 IgA and IgG antibodies to the homologous HA antigen (Aichi/H3, which was used for

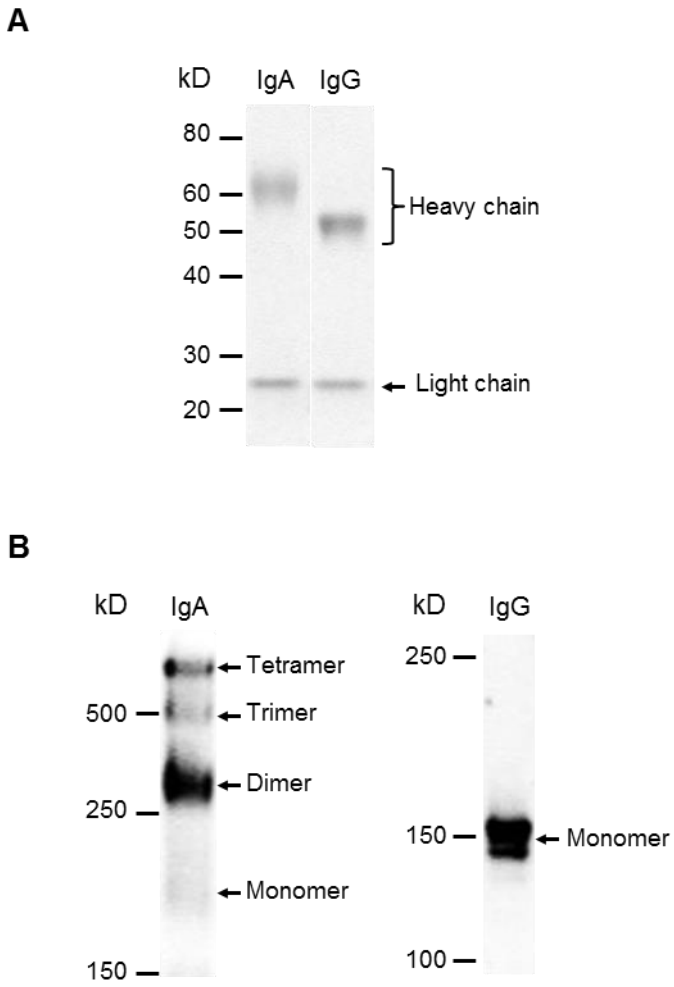


Figure 9. SDS-PAGE analysis of purified S139/1 IgA and IgG antibodies. Equal amounts (2.5 µg) of purified S139/1 IgA and IgG antibodies were analyzed by SDS-PAGE under reducing conditions (5%-20% gradient gel) (A). Polymeric forms of purified S139/1 IgA and IgG antibodies (5 µg) were analyzed under nonreducing conditions (3%-10% gradient gel) (B).

production of this MAb) and heterologous HA antigens (WSN/H1, Adachi/H2, and Maryland/H13) by ELISA (Figure 10), and determined the EC_{50} values (Table 2). EC_{50} is generally used as a reasonable approximation of the dissociation constant (Kd) [61,62]. I found that both S139/1 IgA and IgG antibodies showed similar binding curves for the Aichi/H3 antigen and that there was no significant difference in their EC_{50} values (0.0013 μg/ml and 0.0017 μg/ml, respectively). In contrast, there were remarkable differences between S139/1 IgA and IgG antibodies in reactivities to the WSN/H1, Adachi/H2, and Maryland/H13 antigens. IgA bound to these heterologous antigens at an extent similar to that with the homologous H3 antigen, and the EC_{50} values for WSN/H1, Adachi/H2, and Maryland/H13 were only 2- to 5-fold lower than that for the Aichi/H3 antigen. However, IgG reactivities to the heterologous HAs were uniformly much lower than that to the H3 antigen as indicated by the considerably larger EC_{50} values for WSN/H1, Adachi/H2, and Maryland/H13 (0.0777, 0.144, and 0.670 μg/ml, respectively) than for Aichi/H3 (0.0017 μg/ml).

HI activities of S139/1 IgA and IgG antibodies against influenza A viruses of different HA subtypes

I next compared HI activities of S139/1 IgA and IgG antibodies (Table 3). Consistent with their binding activities shown in ELISA (Figure 10), both S139/1 IgA and IgG antibodies exhibited the highest HI activity to Aichi/H3 and their endpoint concentrations were only slightly different. In contrast, although IgA showed lower HI activities to WSN/H1, Adachi/H2,

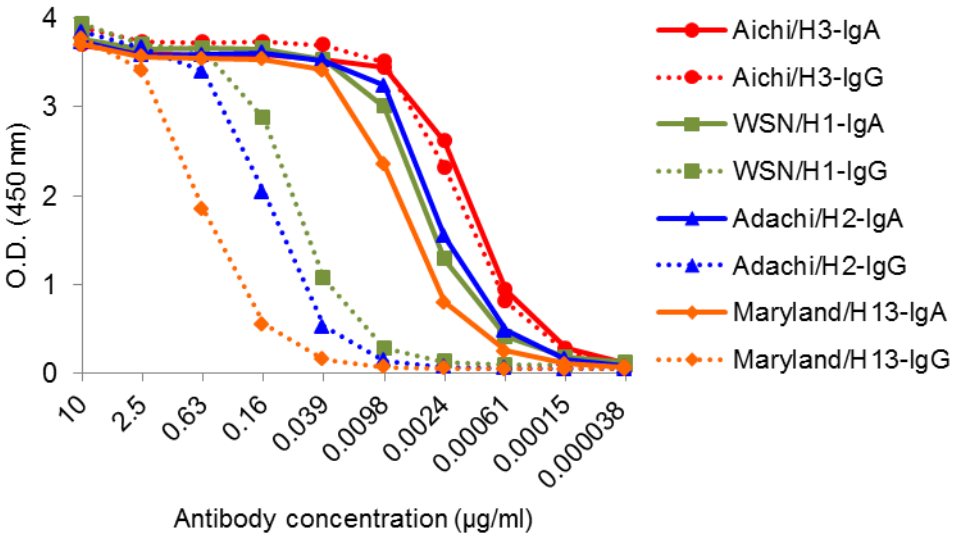


Figure 10. Comparison of binding activities of S139/1 IgA and IgG antibodies. Binding activities of S139/1 IgA (continuous lines) and IgG (dashed lines) were tested in ELISA. Disrupted viral particles of Aichi/H3, WSN/H1, Adachi/H2, and Maryland/H13 were used as antigens. Data are mean values of duplicate wells. EC_{50} values calculated based on the ELISA data are shown in Table 2.

Table 2. Comparison of binding abilities of S139/1 IgA and IgG to influenza A viruses.

Virus	EC_{50} (μg/ml)*		IgG/IgA
	IgA	IgG	
Aichi/H3	0.0013	0.0017	1.3
WSN/H1	0.0039	0.0777	19.9
Adachi/H2	0.0029	0.144	49.7
Maryland/H13	0.0063	0.67	106.4

*The concentrations of MAbs giving 50% of maximal binding (EC_{50}) to influenza A viruses were determined according to regression curves obtained from ELISA. The assays were performed three times and the representative data are shown.

and Maryland/H13 than to Aichi/H3, endpoint concentrations of IgA were much lower than those of IgG. As clearly indicated by the IgG/IgA ratios of endpoint concentrations, IgA showed significantly higher HI activity against heterologous viruses than IgG.

Neutralizing activities of S139/1 IgA and IgG antibodies against influenza A viruses of different HA subtypes

I further compared the neutralizing activities of S139/1 IgA and IgG antibodies by the standard plaque reduction test (Figure 11) and the 50% inhibitory concentrations (IC_{50}) of S139/1 IgA and IgG were determined (Table 4). Consistent with the similar binding and HI activities to Aichi/H3 (Tables 2-3 and Figure 10), both S139/1 IgA and IgG antibodies showed similar neutralization curves for this H3 virus and their IC_{50} values were almost indistinguishable. As was the case with HI activities, IgA showed remarkably higher heterosubtypic neutralizing activities to WSN/H1, Adachi/H2, and Maryland/H13 than IgG, as indicated by 4- to 23-fold differences of IC_{50} values (i.e., IgG/IgA), although both S139/1 IgA and IgG antibodies exhibited less neutralizing activity for heterologous viruses than for Aichi/H3.

Different potentials of S139/1 IgA and IgG antibodies to inhibit viral release from infected cells

To compare the abilities of S139/1 IgA and IgG antibodies to suppress the extracellular release of virus particles from infected cells [56], MDCK

Table 3. Comparison of HI activities of S139/1 IgA and IgG antibodies.

Virus	HI endpoint (μg/ml)*		IgG/IgA
	IgA	IgG	
Aichi/H3	0.0244	0.0488	2
WSN/H1	0.0488	0.1953	4
Adachi/H2	0.1953	3.125	16
Maryland/H13	0.1953	6.25	32

*The lowest MAb concentration that completely inhibited hemagglutination of each virus is shown. The assays were performed three times and the representative data are shown.

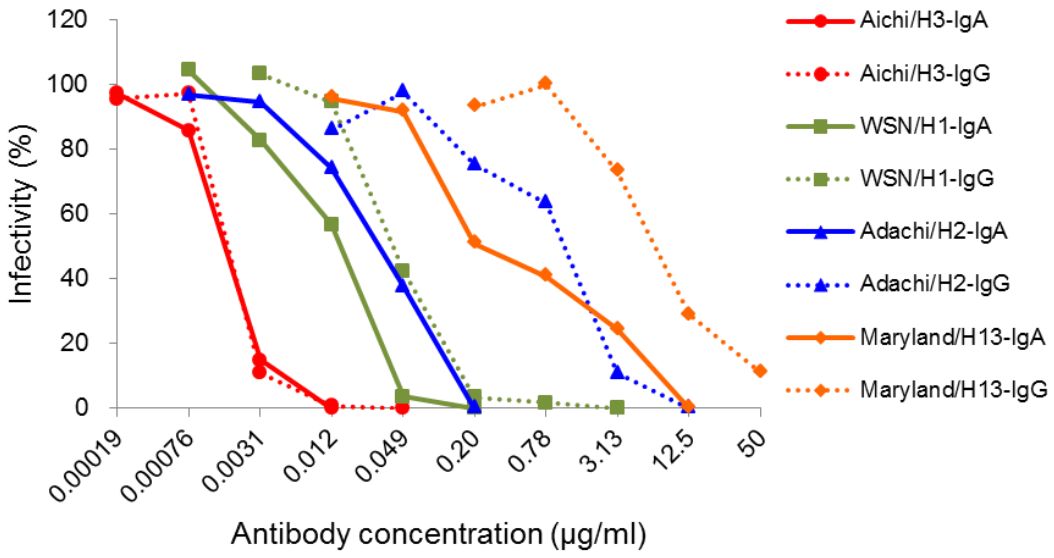


Figure 11. Comparison of neutralizing activities of S139/1 IgA and IgG antibodies. Appropriately diluted viruses were mixed with S139/1 IgA (continuous lines) or IgG (dashed lines) at the indicated concentrations. Neutralizing activities were evaluated by counting the number of plaques formed on MDCK cells. IC_{50} values calculated based on the neutralization curves are shown in Table 4.

Table 4. Comparison of neutralizing activities of S139/1 IgA and IgG antibodies.

Virus	IC_{50} (μg/ml)*		IgG/IgA
	IgA	IgG	
Aichi/H3	0.0015	0.0016	1.1
WSN/H1	0.0113	0.0445	3.9
Adachi/H2	0.0305	0.7117	23.3
Maryland/H13	0.4461	6.1585	13.8

*IC_{50} values were calculated according to the neutralization curves shown in Figure 3. The assays were performed three times and the representative data are shown.

cells infected with Aichi/H3, WSN/H1, Adachi/H2, or Maryland/H13 were subsequently cultured in the presence of S139/1 IgA or IgG antibodies, and the amounts of virus particles released into the culture supernatants were estimated by detecting the viral matrix (M1) protein in Western blotting (Figures 12A-D). I detected significantly lower amounts of the M1 protein of Aichi/H3 in the supernatants of infected cells cultured with S139/1 IgA (1 μg/ml) at both 6 and 12 hours after infection, whereas IgG had a smaller inhibitory effect only at 6 hours after infection (Figure 12A). At a lower concentration (0.1 μg/ml) of S139/1 IgA and IgG antibodies, no significant reduction was observed. Next, I performed the same assay for WSN/H1, Adachi/H2, and Maryland/H13 at the MAb concentration of 1 μg/ml and found that S139/1 IgA significantly suppressed the extracellular release of all tested viruses both at 6 and 12 hours after infection (Figures 12C and D). On the other hand, IgG showed limited activity only against WSN/H1 at 6 hours after infection. These data suggested that S139/1 IgA had greater potential to inhibit viral release from infected cells than IgG, which might be particularly important for the heterosubtypic antiviral potential.

Aggregation of unreleased virus particles on the surface of infected cells cultured in the presence of S139/1 IgA

To gain insight into the mechanism of IgA-mediated inhibitory effects on the viral release from infected cells, I used electron microscopy to examine Aichi/H3-infected MDCK cells cultured with S139/1 IgA or IgG. After 8-hour incubation, tight aggregation and abnormal accumulation of

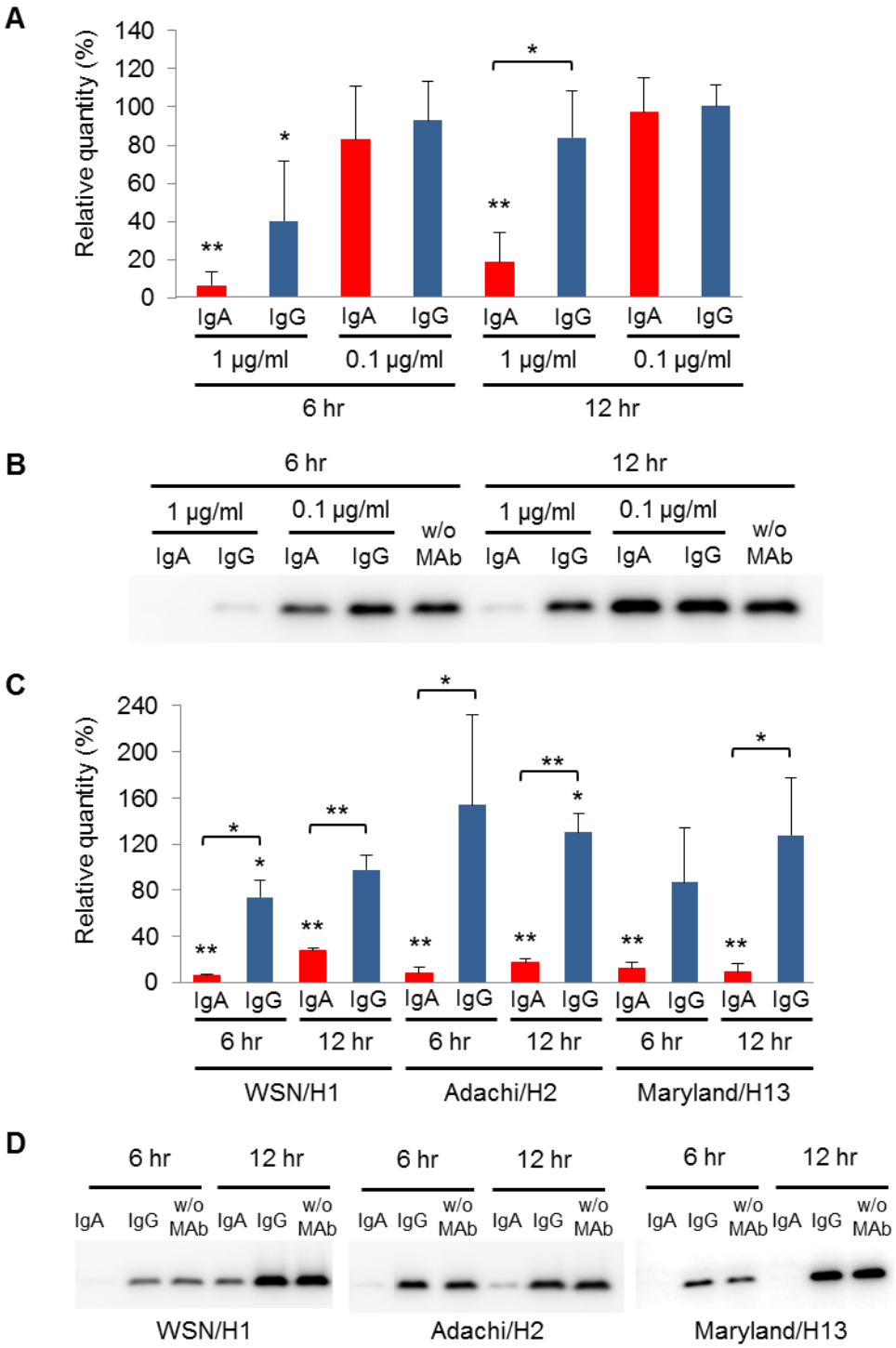


Figure 12. Viral release from infected cells cultured in the presence of S139/1 IgA or IgG antibodies. After inoculation with Aichi/H3 (A and B), WSN/H1, Adachi/H2, or Maryland/H13 (C and D), MDCK cells were cultured in the presence of S139/1 IgA or IgG antibodies at the concentrations of 1.0 or 0.1 μg/ml (A and B) and 1.0 μg/ml (C and D). Supernatants were collected 6 and 12 hours after infection, and M1 protein of influenza viruses released into the supernatants were detected by Western blotting (B and D). The relative quantity of the M1 protein was calculated based on the band intensity by using Image Lab version 3.0 (Bio-Rad) (A and C). The intensity of the M1 protein bands detected in the control supernatants collected from infected cells cultured without a MAb (w/o MAb) was set to 100%. Experiments were performed 3 times, and averages and standard deviations are shown (A and C). Statistical significance was analyzed by Student's *t*-test (**p < 0.01, *p < 0.05). Asterisks placed directly above the bars indicate significant differences compared to respective controls, and asterisks placed between the bars show significant differences between S139/1 IgA and IgG antibodies.

unreleased virus particles were found on the virus-infected cells cultured in the presence of S139/1 IgA (Figures 13A and D), which was quite similar to the well-known phenomenon for the effect of dysfunction of the NA activity [65-68]. On the other hand, lower numbers of virus particles in less proximity were found on the infected cells cultured with IgG (Figures 13B and E). Only limited numbers of virus particles were seen on the surfaces of cells incubated without MAbs, suggesting efficient virus release from infected cells (Figures 13C and F). These data indicated that S139/1 IgA deposited newly produced viral particles on the cell surface more efficiently than S139/1 IgG. Interestingly, I observed that heavily accumulated and aggregated virus particles were likely incorporated into cytoplasmic vesicles in the cells incubated with S139/1 IgA at 12 hours after infection (Figures 14A and B). A similar observation was reported for MDCK cells infected with an NA-deficient influenza A virus [66]. The accumulation of densely aggregated virus particles in the cellular vesicles was hardly seen in infected cells cultured with S139/1 IgG (Figures 14C and D).

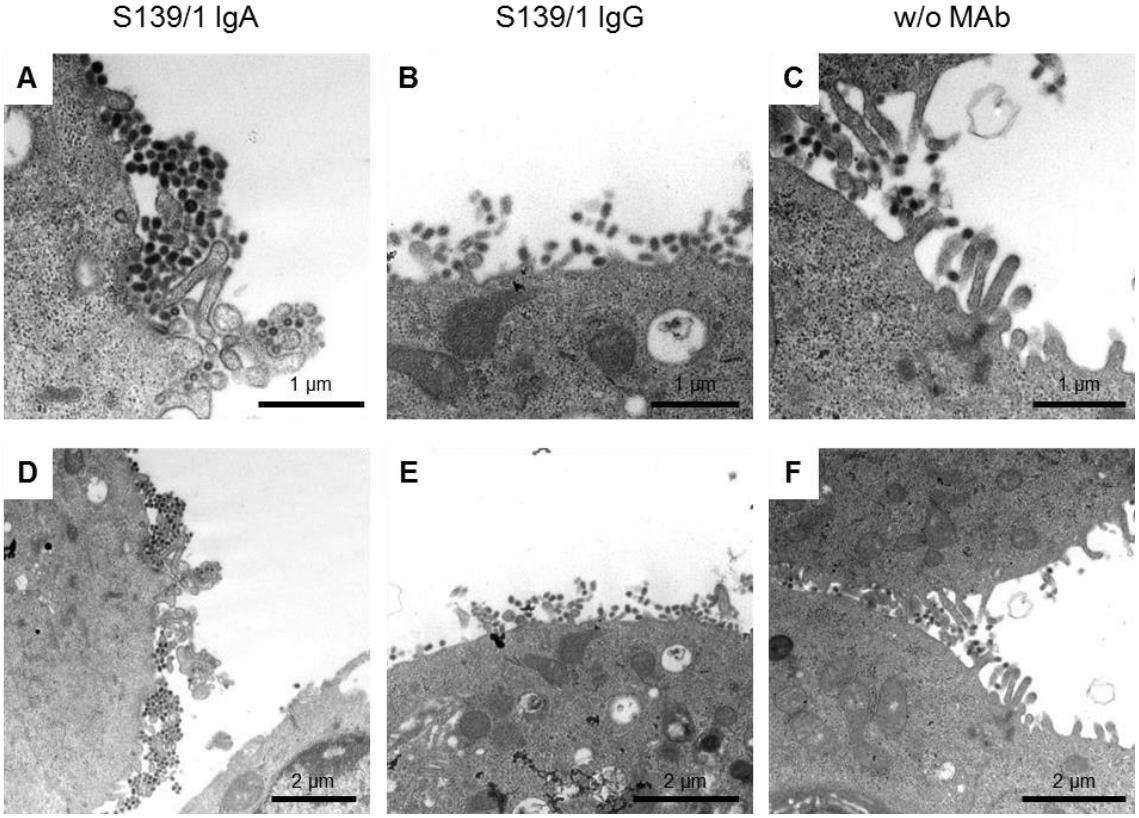


Figure 13. Viral particles deposited on the surface of infected cells cultured with S139/1 IgA. MDCK cells were infected with Aichi/H3 and incubated for 8 hours in the presence of S139/1 IgA (A and D), IgG (B and E), or in the absence of antibodies (w/o MAb) (C and F). TEM images of the cell surface are shown at high (A to C) and low (D to F) magnifications. Scale bars represent 1 µm (A to C) and 2 µm (D to F).

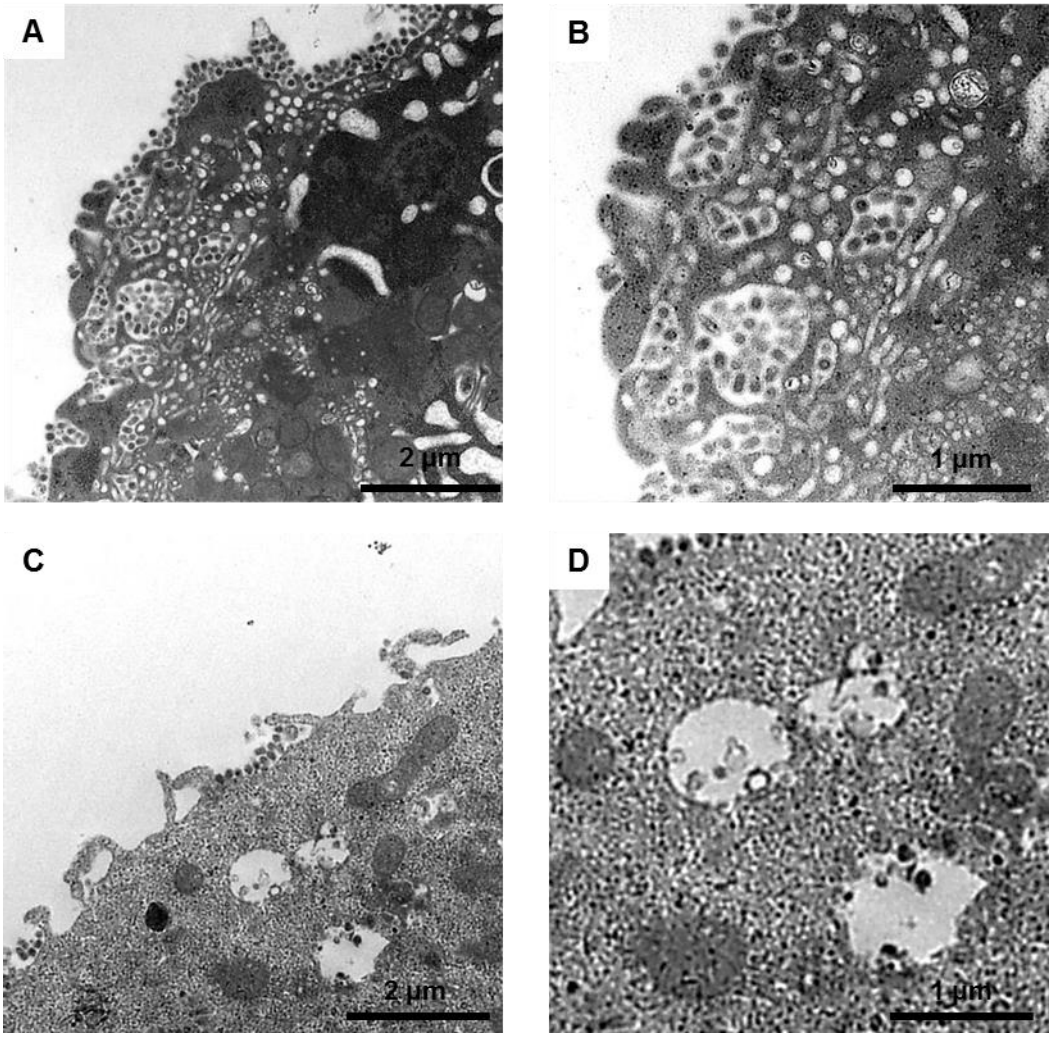


Figure 14. TEM images of Aichi/H3-infected MDCK cells cultured in the presence of MAb S139/1 IgA. MDCK cells infected with Aichi/H3 at a multiplicity of infection of 1-2 were incubated for 12 hours in the presence of S139/1 IgA (A and B) or IgG (C and D). Ultrathin sections were examined by TEM. TEM images are shown at low (A and C) and high (B and D) magnifications.

Discussion

It has been reported that intranasal immunization induces a more efficient cross-protective immune response against influenza virus than systemic immunization, and IgA antibodies are suggested to play a major role in antibody-mediated heterosubtypic immunity [9,10,20-22,69]. On the other hand, the currently used inactivated influenza vaccines, which rely on the induction of serum IgG antibodies, are believed to be effective only against viruses whose HA antigenicities are closely related to those of the vaccine strains. In this study, I directly demonstrated distinct differences in the heterosubtypic antiviral activities of IgA and IgG antibodies by using cross-reactive S139/1 IgA and IgG antibodies that had originally been produced against Aichi/H3 but recognized the same epitope shared among multiple HA subtypes (e.g., H1, H2, H3, and H13) [31,57].

In ELISA, while S139/1 IgG bound strongly to the homologous Aichi/H3 HA, its cross-binding activity to heterologous HAs (i.e., WSN/H1, Adachi/H2, and Maryland/H13) was appreciably lower, in accord with previous studies [31,57]. Interestingly, however, the difference between the S139/1 IgA reactivity to Aichi/H3 and to the heterologous viruses tested was much less prominent. The different antiviral activities of S139/1 IgA and IgG antibodies were directly confirmed by standard HI and plaque reduction neutralizing tests. Consistent with the binding assay, S139/1 IgA had much higher HI and neutralizing activities against WSN/H1, Adachi/H2, and Maryland/H13 than S139/1 IgG, whereas S139/1 IgA and IgG antibodies

showed similar activities against Aichi/H3 virus. These results may suggest the increased avidity conferred by the polymeric form of S139/1 IgA due to its multivalent binding. Since bivalent binding of the whole S139/1 IgG molecule was shown to be important for its avidity to HAs other than the H3 subtype [57], this might be an important feature for heterosubtypic reactivities of S139/1 IgA and IgG antibodies. Alternatively, it might also be possible that class-switching from IgG to IgA itself might enhance the affinity of monomeric antibody molecules (i.e., the Fab fragment) to a single epitope by altering the flexibility of constant heavy chain of IgA, as suggested by a previous study by others [70].

As evidenced by the well-known protective efficacy of influenza virus NA inhibitors, the viral budding/release process is a promising target for antiviral development [65,67,71]. Kajihara et al. previously reported that some antibodies to viral surface proteins inhibited the budding or release of virus particles from infected cells *in vitro* [45,56]. In chapter I, the extracellular release of influenza A viruses from infected cells was suppressed in the presence of cross-reactive IgA, but not IgG antibodies, induced by immunization of mice [56]. To directly confirm the differential abilities of IgA and IgG antibodies to inhibit influenza virus release from infected cells, I used anti-HA monoclonal IgA and IgG antibodies (i.e., S139/1 IgA and IgG) recognizing the same epitope in this study. I found that S139/1 IgA efficiently inhibited the release of progeny viruses of all tested subtypes (i.e., H1, H2, H3, and H13), but the effects of S139/1 IgG were significantly weaker than those of IgA and limited to the homologous subtype

(i.e., H3) (Figure 12). It was also noted that, unlike HI and neutralizing activities against the H3 virus that were similar for S139/1 IgA and IgG antibodies, appreciable differences in the inhibition of viral release were observed between S139/1 IgA and IgG antibodies even against Aichi/H3. Electron microscopy also revealed a notable difference between S139/1 IgA and IgG antibodies in the ability to trap newly produced virus particles on the cell surface (Figures 13A, B, D, E, and 14). Taken together, these results suggest that the polymeric structure of IgA is particularly important for this virus release inhibitory effect, since cross-linking and tethering activities are likely required for depositing virus particles on the cell surface [11,45,72,73]. The proposed underlying mechanisms of these antiviral activities are shown in Figure 15.

Based on this hypothesis, the epitope may also be an important factor, since efficient crosslinking is likely done by antibodies that target the globular head region of HA. Accordingly, S139/1 recognizes highly conserved residues in the receptor binding site of the HA molecule, and thus has strong neutralizing activity [31,57]. However, I assume that the neutralizing activity that blocks viral entry into cells is not necessarily required for the inhibition of viral release from infected cells. Interestingly, in chapter I, it was suggested that HA-specific non-neutralizing IgA antibodies induced by intranasal immunization of mice might have such potential [56]. Conversely, it is also conceivable that not all neutralizing antibodies have cross-linking and tethering activities if their epitope locations do not fit the required conditions. Thus, the “classical”

neutralizing activity is not the only indicator of a protective antibody that may play a role in heterosubtypic immunity against influenza A viruses.

The present study demonstrates that anti-HA S139/1 IgA has greater antiviral potential against influenza A virus infection *in vitro* than IgG, and the advantage of IgA for interaction with viruses is more prominent in heterosubtypic cross-reactivity. The polymeric structure might be important for the enhanced ability of IgA antibodies. In addition, our data suggest that tethering and depositing newly budded virus particles on the infected cell surface may generally be one of the antibody-mediated protective mechanisms against enveloped viruses and emphasize the importance of p-IgA in mucosal immunity.

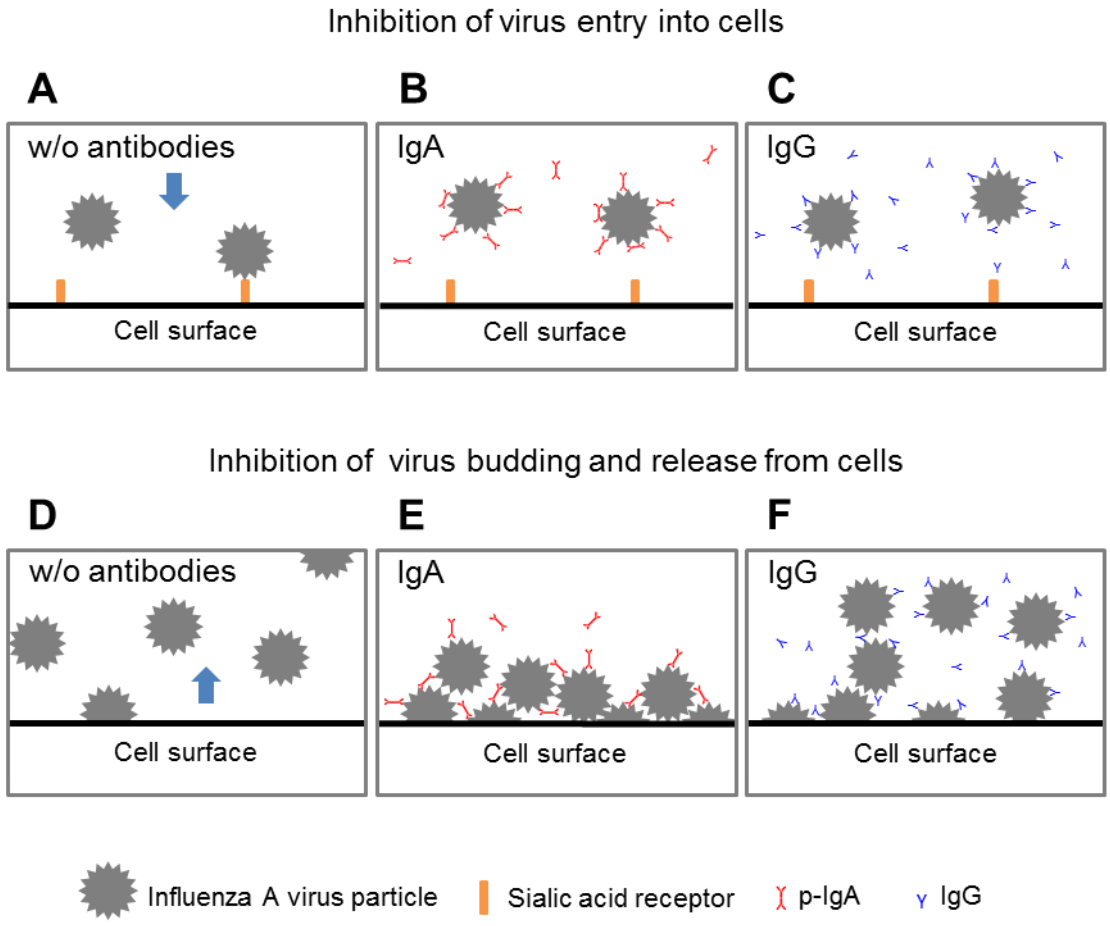


Figure 15. Proposed mechanisms of antiviral activities of HA specific p-IgA antibodies. Influenza A viruses attach sialic acid receptors on cell surface and enter into cells (A). In HI and neutralization tests, IgA (B) and IgG (C) antibodies bind to HAs of virus and inhibit virus attachment to the receptors or entry into cells. After the replication in host cells, newly produced viral particles are released from cell surface (D). In viral release inhibition assay, p-IgA (E) but not IgG antibodies (F) are considered to bind to HAs and form cross-linkage efficiently with neighboring virus particles.

Summary

Both IgA and IgG antibodies are known to play important roles in protection against influenza virus infection. Although IgA antibodies are believed to have unique advantages in mucosal immunity, information on comparisons of the *in vitro* antiviral activities of IgA and IgG antibodies is limited. In this study, I demonstrated differences in antiviral activities between these isotypes using MAb S139/1 IgA and IgG antibodies, which recognize the same epitope on H3 HA, and have cross-neutralizing activities against the H1, H2, and H13 subtypes. I found that, to the homologous H3 virus, both antibodies showed strong binding activity in ELISA, and there were no significant differences in their HI and neutralizing activities. In contrast, S139/1 IgA showed remarkably higher cross-binding to and antiviral activities against H1, H2, and H13 viruses than S139/1 IgG. It was also noted that S139/1 IgA, but not IgG, drastically suppressed the extracellular release of the viruses from infected cells. Electron microscopy revealed that S139/1 IgA deposited newly produced viral particles on the cell surface, most likely by tethering the particles. These results suggest that anti-HA IgA has greater potential to prevent influenza A virus infection than IgG antibodies, likely due to increased avidity conferred by its multivalency, and that this advantage may be particularly important for heterosubtypic immunity.

Conclusion

Both IgA and IgG antibodies are known to play important roles in protection against influenza A viruses. In this research, I analyzed the properties of IgA and IgG antibodies cross-reactive to influenza A viruses of multiple HA subtypes and discussed the potential roles of IgA antibodies in heterosubtypic immunity.

In chapter I, I found that both intranasal and subcutaneous immunization induced IgA and IgG antibodies cross-reactive to multiple HA subtypes, whereas IgA was not detected remarkably in mice immunized subcutaneously. There was no fundamental difference in the overall spectrum of cross-reactivity between the antibodies produced by intranasal and subcutaneous immunization. Though cross-neutralizing activities of those antibodies were not detected, a remarkable inhibition of virus budding or release from infected cells was observed in the presence of cross-reactive IgA antibodies. These results suggest that the majority of HA-specific cross-reactive antibodies do not block cellular entry of viruses, but non-neutralizing IgA may have the potential to inhibit viral release from infected cells.

In chapter II, I demonstrated the difference in antiviral activity between IgA and IgG, using MAbs S139/1 IgA and IgG which recognized the same epitope on HA. These MAbs were originated from mice immunized with a virus of H3 subtype, and had cross-neutralizing activity against viruses of H1, H2, and H13 subtypes. Against the homologous H3 virus,

S139/1 IgA and IgG antibodies similarly showed strong binding activities and there was no significant difference in their HI and neutralizing activities. On the other hand, S139/1 IgA showed higher cross-binding, HI, and neutralizing activities against the heterologous H1, H2, or H13 viruses than S139/1 IgG. Furthermore, S139/1 IgA significantly suppressed the extracellular release of the heterologous viruses from infected cells, but S139/1 IgG did not. Electron microscopy of cells infected with the H3 virus and then cultured in the presence of S139/1 MAbs, revealed that S139/1 IgA deposited newly produced viral particles on the cell surface, most likely by tethering the particles. The polymeric structure of IgA is considered to be important for this tethering activity.

Taken together, anti-HA IgA showed greater potential in antiviral activities against influenza A viruses than IgG antibodies. The advantage of IgA is likely due to increased avidity and cross-linking activity conferred by its multivalency. Particularly, the results obtained in the present study suggest that tethering and depositing newly budded virus particles on the infected cell surface may be one of the IgA-mediated protective mechanisms important for heterosubtypic immunity against influenza A viruses.

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和文要旨

A 型インフルエンザウイルスはエンベロープ表面に存在する二種類の糖タンパク Hemagglutinin (HA) および Neuraminidase (NA) の抗原性によって多くの亜型に分類される。本ウイルスの自然宿主である野生水禽類からこれまでに分離されたウイルスは H1～H16 の HA 亜型および N1～N9 の NA 亜型に分けられている。A 型インフルエンザウイルスに対する中和抗体の多くは HA を認識するものであり、同一の HA 亜型のウイルスにのみ中和活性を示す。しかしながら、不活化ウイルスを鼻腔内に投与し粘膜免疫を誘導したマウスでは、異なる複数の亜型のウイルスに対する交差感染防御が成立することが報告されている。この亜型間交差感染防御は B 細胞依存性であり、経鼻免疫で誘導される IgA が関与していると考えられている。そこで本研究では、A 型インフルエンザウイルスに対する亜型間交差感染防御免疫における IgA 抗体の役割を解明するために、HA 特異的 IgA 抗体の交差反応性および抗ウイルス活性を解析した。

第一章では、ホルマリン不活化ウイルス（H1、H3、H5、H7、H9またはH13亜型）をBALB/cマウスの鼻腔内または皮下に接種し、血清、気管肺胞洗浄液および鼻腔洗浄液を採材し、これらのサンプル中に含まれるIgAおよびIgG抗体について解析した。異なる亜型のHAへの交差結合性を調べた結果、いずれの亜型のウイルスを免疫原として用いた場合でも、免疫経路および抗体のアイソタイプに関わらず複数の亜型のHAに交差結合する抗体が誘導されることが明らかとなった。交差結合が認められたHA亜型は、HAのアミノ酸配列に基づいた系統樹上で免疫原と近縁な亜型に多い傾向が認められた。IgAは経鼻接種群でのみ高い誘導が認められた。次に、これらのサンプル中の中和抗体の有無を調べたが、

免疫原とは異なる亜型のウイルスに対する交差中和活性は認められなかった。

通常の中和試験で評価される抗体はウイルスの細胞侵入を阻害するものであるが、本研究ではウイルス粒子の感染細胞からの放出過程を抗体が阻害する可能性に着目した。抗体によるウイルス粒子放出阻害を評価するために、予めウイルスに感染させた細胞を各サンプル存在下で培養し、プラック形成が阻害されるか否かを調べた。その結果、交差結合性IgA存在下でプラック形成が阻害されることが明らかとなった。そのプラック形成阻害効率は気管肺胞洗浄液を使用したときに最も高く認められ、サンプル中の交差結合性IgA量と相関が認められた。またプラック形成阻害効果はヤギ抗マウスIgA抗体を添加すると抑制された。以上の結果から、交差結合活性を有するが交差中和活性は持たないIgAが亜型間交差感染防御免疫に重要な役割を担っている可能性が示唆された。

第二章では、モノクローナル抗体を用いて IgA と IgG の抗ウイルス活性を比較した。モノクローナル抗体 S139/1（IgG）は、H3 ウイルスを免疫原として作出した HA 特異的中和抗体であるが、H1、H2 および H13 亜型のウイルスに対して交差中和活性を示す。S139/1（IgG）抗体産生ハイブリドーマを IgA 産生細胞に分化させ、同一のエピトープを認識する IgA（主に二量体）を得た。これらの抗体を用いて、H3 ウイルスおよび交差中和活性の認められた異なる亜型(H1、H2 および H13 亜型）のウイルスに対する抗ウイルス活性を比較した。

抗体の結合活性、赤血球凝集抑制活性および中和活性を調べたところ、免疫原である H3 ウイルスに対してはアイソタイプによる差は認められなかったが、他の亜型のウイルスに対しては IgA が IgG よりも高い活性を示した。さらに、予めウイルスに感染させた MDCK 細胞を IgA 存在下で培養したところ、上清中に放出されるウイルス粒子量は、用いた全てのウイルスにおいて顕著に減少した。一方 IgG 存在下では、H3 ウイルスに対してもウイルス粒子放出阻害効果

は IgA と比較して有意に少なく、異なる亜型のウイルスに対してはほとんど認められなかった。透過型電子顕微鏡観察の結果からは、IgA 存在下では細胞から出芽したウイルス粒子が凝集し、細胞膜表面に留められている様子が確認された。

本研究の結果より、HA 特異的交差反応性 IgA は IgG よりも高い抗インフルエンザウイルス活性を有していることが示された。特に、通常の中和試験では評価されないウイルス粒子の放出阻害活性は IgA 抗体で顕著に認められ、亜型間交差感染防御免疫に寄与しているものと考えられた。抗ウイルス活性における IgA の優位性は、多量体 IgA が一分子あたりの抗原結合部位を多く持っているために affinity（抗原結合部位単独の親和性）が低い抗原に対しても avidity（分子全体の親和性の総和）が強化されること、またその多量体としての架橋能力に大きく起因すると考えられた。

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