Relationship of the quaternary structure of human secretory IgA to neutralization of influenza virus

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Secretory IgA (S-IgA) antibodies, the major contributors to humoral mucosal immunity to influenza virus infection, are polymeric Igs present in many external secretions. In the present study, the quaternary structures of human S-IgA induced in nasal mucosa after administration of intranasal inactivated influenza vaccines were characterized in relation to neutralization potency against influenza A viruses. Human nasal IgA antibodies have been shown to contain at least five quaternary structures. Direct and real-time visualization of S-IgA using high-speed atomic force microscopy (AFM) demonstrated that trimeric and tetrameric S-IgA had six and eight antigen-binding sites, respectively, and that these structures exhibited large-scale asynchronous conformational changes while capturing influenza HA antigens in solution. Furthermore, trimeric, tetrameric, and larger polymeric structures, which are minor fractions in human nasal IgA, displayed increased neutralizing potency against influenza A viruses compared with dimeric S-IgA, suggesting that the larger polymeric than dimeric forms of S-IgA play some important roles in protection against influenza A virus infection in the human upper respiratory tract.

secretory IgA | influenza virus | intranasal inactivated influenza vaccine | high-speed atomic force microscopy

Antibodies in respiratory mucosa are primary mediators of protective immunity against influenza. Notably, preexisting secretory IgA (S-IgA) antibodies can provide immediate immunity by eliminating a pathogen before the virus passes the mucosal barrier (1–3). Parenteral vaccination induces serum IgG but not S-IgA, so vaccine efficacy is limited. In contrast, intranasal administration of an inactivated influenza vaccine elicits both S-IgA and IgG responses, thus improving the protective efficacy of current vaccination procedures (4–8).

IgA in human serum exists predominantly in the form of monomers, whereas the majority of IgA in external secretions is present in the form of polymers. These polymeric IgA forms are associated with the extracellular portion of the polymeric Ig receptor, generating a complex (receptor + polymeric IgA) called S-IgA (9). S-IgA primarily corresponds to dimeric IgA, although low levels of some larger polymeric forms, particularly tetramers, are also present (9–15). Polymeric S-IgA has been shown (both in vitro and in experimental animal models) to be more effective than monomeric IgA or IgG for the neutralization of influenza viruses (16–19). However, little is known of the quaternary structures and neutralizing potencies in viral infection of the various forms of polymeric S-IgA in the human nasal mucosa. In this study, the quaternary structures and neutralizing potencies of nasal antibodies against influenza virus were examined using nasal wash samples from healthy adults who had received intranasally administered inactivated influenza vaccines. These nasal wash samples, containing variously sized Igs, were separated by gel filtration chromatography (GFC) and assessed for neutralization activity against influenza virus. The quaternary structures of the nasal IgA induced by intranasally administered inactivated influenza vaccines then were determined using biochemical techniques and high-speed atomic force microscopy (AFM). We found that human nasal IgA comprised at least five quaternary structures, including monomer, dimer, trimer, and tetramer structures, as well as a polymeric form larger than the tetramer structure. Among these forms, the polymeric structure demonstrated higher neutralizing potency against seasonal influenza viruses (H3N2) and highly pathogenic avian influenza virus (H5N1) compared with the dimeric form, suggesting that large polymeric S-IgA antibodies play crucial roles in protective immunity against influenza virus infection of the human upper respiratory tract.

Results

Influenza Virus Neutralizing Potency of Each GFC Fractionation of Human Nasal Wash Samples. Nasal wash samples from five healthy adult volunteers, who were vaccinated five times intranasally with inactivated A/Victoria/210/2009 (H3N2; Victoria)-like virus whole-virion vaccines, were collected as described in *Materials and Methods* (also Fig. S1). It was confirmed that these volunteers showed relatively high serum neutralizing antibody responses against Victoria virus after the second vaccination.

Samples were separated by GFC, and the quantities of IgA, IgG, and IgM in each fraction were estimated by ELISA. The ELISA showed that IgM and IgG were detected primarily in fractions 21–24 and 30–38, respectively, whereas IgA were detected in a wider range of fractions (21–36) (Fig. 1 *A* and *B*). These observations suggested that nasal IgA possessed striking size heterogeneity.

Significance

Preexisting secretory IgA (S-IgA) antibodies can provide immediate immunity via their unique capability to eliminate a pathogen before it passes the mucosal barrier. Several clinical trials have reported that S-IgA against influenza virus was induced by intranasal administration of an inactivated influenza vaccine. S-IgA in mucosa consists predominantly of dimers rather than tetramers, as revealed by ultracentrifugation almost 50 years ago. However, direct evidence concerning the quaternary architectures and the physiological function of polymers larger than dimers has not been reported in healthy humans. The present study revealed, for the first time to our knowledge, the existence of large polymeric IgA in the healthy human upper respiratory mucosa, as well as the physiological functions of these molecules in protecting against viral infection in humans.

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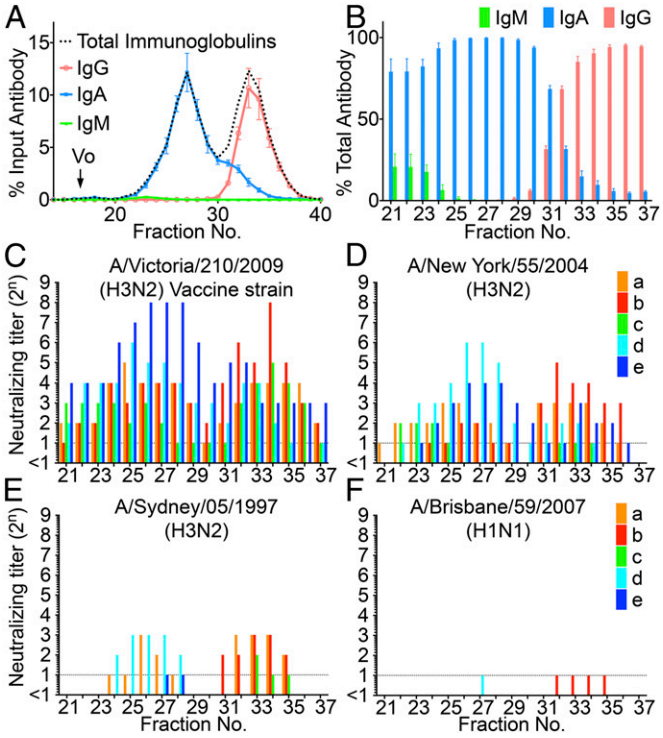

Fig. 1. Size distribution of Igs in the nasal wash samples separated by GFC and estimation of the neutralizing potency of each fraction. (*A*) Fractionation pattern of nasal wash samples on Superose 6 GFC. The amounts of IgM, IgG, and IgA (wt/wt) in each fraction were estimated using ELISA. Values plotted are means, and error bars represent SEM. Vo, void volume. (*B*) Proportions of the amounts of IgM, IgG, and IgA (wt/wt) to the total amounts of antibodies in each fraction (IgM + IgG + IgA, weight) were estimated. Values plotted are means, and error bars represent SEM. (*C*) Neutralization titer against the vaccine strain Victoria virus was determined in each fraction separated by GFC of nasal wash samples collected from five participants (*a*, *b*, *c*, *d*, and *e* aged 33, 23, 27, 24, and 34 years, respectively, at the time of sample collection). (*D*–*F*) Neutralization titer of each fraction against NY virus, Sydney virus, and Brisbane virus also was determined for the same nasal samples collected from the five participants (*a*–*e*).

Each fraction also was assayed for neutralization potency against the Victoria virus (which had been used for vaccination) and against other influenza viruses, including A/New York/55/2004 (H3N2; NY) virus (HA of 96.6% amino acid similarity to Victoria HA), A/Sydney/05/1997 (H3N2; Sydney) virus (HA of 92.9% amino acid similarity to Victoria HA), and A/Brisbane/59/2007 (H1N1; Brisbane) virus (HA of 41.8% amino acid similarity to Victoria HA) (Fig. 1 *C*–*F*). Profiles of neutralization titers against Victoria virus from the five volunteers showed two distinct peaks of neutralization, comprising fractions 21–30 (which contained mainly polymeric IgA) and fractions 31–37 (which contained mainly IgG).

The peak titers of neutralization activity were found to be attenuated when tested using genetically divergent viruses, and the degree of attenuation was consistent with the degree of divergence. Specifically, apparent titers were ranked as Victoria > NY > Sydney > Brisbane viruses, an order that corresponds to decreasing HA amino acid similarity among these strains. This observation suggested that the neutralization activities in fractionated nasal wash samples reflected the presence of virus-specific antibodies (notably, anti-HA antibodies), rather than nonspecific antiviral activities in the nasal mucus (Fig. 1 *C*–*F*). In addition, the profiles varied slightly among volunteers (Fig. 1 *C*–*F*). The profiles against NY virus for nasal wash samples from some volunteers (Fig. 1*D*, *a* and *b*) showed higher neutralization titers in IgG fractions and lower neutralization titers in polymeric IgA fractions, whereas the profiles from other volunteers (Fig. 1*D*, *c*–*e*) showed lower neutralization titers in IgG fractions and higher neutralization titers in polymeric IgA fractions. The profile against Sydney virus showed that the nasal wash sample from one volunteer provided neutralization titers only in the polymeric IgA fractions (Fig. 1*E*, *d*). Meanwhile, neutralization activity against Brisbane virus was not detected in any of the volunteers (Fig. 1*F*). The higher neutralization titers seen in polymeric IgA fractions (compared with IgG fractions) suggested greater cross-reactivity of polymeric IgA to variant H3N2 viruses than of IgG. The higher neutralizing titers of polymeric IgA fractions against influenza virus were further confirmed in additional studies using H5N1 intranasally vaccinated subjects and H5N1 viruses (Fig. S2).

Binding Activity of Antibodies to Influenza Virus HA in Each GFC Fraction. To determine the isotypes of the Igs responsible for neutralization activity in nasal mucus, HA-specific IgM, IgA, and IgG responses for Victoria HA, NY HA, and Sydney HA were examined using ELISA. HA-specific IgA titers were detected in a broad range of fractions and peaked in fractions 21–30, which

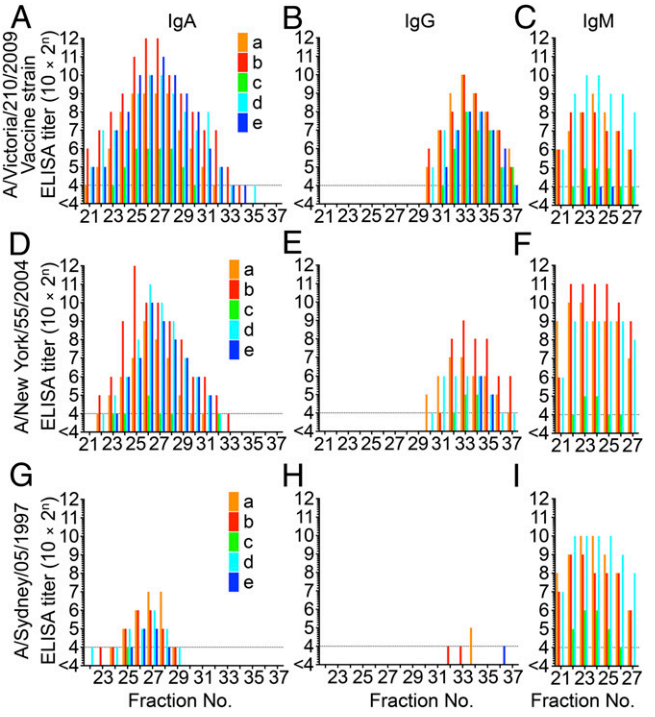

Fig. 2. Determination of binding activity of antibodies to HA molecules in each GFC fraction by ELISA. The IgA ELISA titer (*A*), IgG ELISA titer (*B*), and IgM ELISA titer (*C*) against HA molecules of Victoria virus (H3N2) were determined in each fraction separated by GFC of nasal wash samples collected from five participants (*a*, *b*, *c*, *d*, and *e* aged 33, 23, 27, 24, and 34 years, respectively, at the time of sample collection). HA-specific IgA titers were detected in a broad range of fractions and peaked at fractions 21–30, which corresponded to the first of two peaks of neutralization activity. HA-specific IgG titers peaked at fractions 31–37, which corresponded to the second of two peaks of neutralization activity. The IgA ELISA titer (*D*), IgG ELISA titer (*E*), and IgM ELISA titer (*F*) against HA molecules of NY virus (H3N2) were determined in each fraction separated by GFC of nasal wash samples collected from five participants (*a*–*e*). The IgA ELISA titer (*G*), IgG ELISA titer (*H*), and IgM ELISA titer (*I*) against HA molecules of Sydney virus (H3N2) were determined in each fraction separated by GFC of nasal wash samples collected from five participants (*a*–*e*). Reduction of the peak titers of HA-specific IgA and IgG antibodies against NY HA (which has 96.6% amino acid similarity to Victoria HA) or Sydney HA (which has 92.9% amino acid similarity to Victoria HA) correlated with decreased sequence similarity to Victoria HA.

corresponded to one of the peaks of neutralization activity (Fig. 2 *A*, *D*, and *G*). HA-specific IgG titers peaked in fractions 31–37, which corresponded to the second peak of neutralization activity (Fig. 2 *B*, *E*, and *H*). Furthermore, reduction of the peak titers of HA-specific IgA and IgG against NY HA or Sydney HA coincided with the decrease of neutralization titers in each peak fraction (and, as noted above, correlated with genetic divergence from Victoria HA). Notably, HA-specific antibodies that were cross-reactive to Sydney HA were detected in the polymeric IgA fractions, but not in the IgG fractions (Fig. 2 *G* and *H*). This observation suggested that polymeric IgA possess greater cross-reactivity to variant H3N2 viruses than do IgG antibodies. In contrast, the reduction of the peak titer of HA-specific IgM (correlating with genetic divergence from Victoria HA) was not observed regardless of the detected level of HA-specific IgM (Fig. 2 *C*, *F*, and *I*). These observations demonstrated that IgA and IgG contributed to the neutralization activities detected in the range of fractions 21–30 and fractions 31–37, respectively; IgM did not appear to contribute to these neutralization activities.

Quaternary Structures of Nasal IgA. To determine the quaternary structures of human nasal IgA, purified nasal IgA was obtained by affinity chromatography using pooled (among the five volunteers) nasal wash samples, and the purified IgA molecules were then applied to GFC fractionation (Fig. 3*A*). Blue native PAGE of fractionated samples showed that nasal IgA antibodies were composed of at least five distinct quaternary structures (Fig. 3*B*). SDS/PAGE revealed that the IgA antibodies in fractions 23–30 were composed of α-heavy chains, light chains, secretory component (SC), and J chain (JC) (Fig. 3*C*). In contrast, the IgA in fraction 32 lacked an SC or JC, suggesting that the fraction 32 IgA was monomeric (Fig. 3*C*). Western blotting using several antibodies revealed that the subclass proportions (of IgA1 and IgA2) varied with the quaternary structure (Fig. 3*D*). To determine more accurately the molecular size of these antibodies, high-mass MALDI-TOF MS was used. For analysis of monomeric IgA, serum IgA was used. Serum IgA yielded one major peak with $MH^+ = 151.379 \pm 0.174$ kDa (mean ± SD), a size that is consistent with monomeric IgA (Fig. 3*E*); fraction 28 of nasal IgA yielded one major peak with $MH^+ = 406.649 \pm 0.389$ kDa, a size that corresponds to dimeric S-IgA (two monomers, one SC, and one JC) (Fig. 3*F*). Meanwhile, three major peaks with $MH^+ = 406.519 \pm 0.422$ kDa, $MH^+ = 562.161 \pm 0.576$ kDa, and $MH^+ = 722.342 \pm 0.844$ kDa were detected in the mixture of fractions 24–26 of nasal IgA, indicating that fractions 24–26 consisted of dimeric, trimeric (three monomers, one SC, and one JC), and tetrameric (four monomers, one SC, and one or two JCs) S-IgA (Fig. 3*G*). These results suggested that nasal IgA comprised a total of at least five quaternary structures: monomer, dimer, trimer, and tetramer structures, as well as a polymer larger than the tetramer.

Visualized Quaternary Structures and Dynamics of Nasal IgA. To analyze the quaternary architectures of these oligomeric complexes, nasal IgA was visualized using high-speed AFM. Separate preparations of nasal and serum IgA were subjected to AFM imaging under liquid conditions. Each IgA population comprised a homogeneous mix of particle sizes (Fig. 4 *A*–*C*). However, nasal IgA (of both fractions 27–28 and fractions 24–25) particles were significantly larger ($P < 0.0001$) than particles of serum monomeric IgA (678.6 ± 328.9 nm^3, mean ± SD). Furthermore, nasal IgA particles in fractions 24–25 (2,456 ± 865.1 nm^3) were also significantly larger ($P < 0.0001$) than particles in fractions 27–28 (1,738 ± 431.4 nm^3). High-resolution observations revealed that molecules of serum IgA, which were monomeric, were nearly all triangular with acute angles, apparently consisting of two Fab regions and one Fc region (Fig. 4*D*). Furthermore, molecules of nasal IgA in fractions 27–28 appeared to consist of two IgA monomers arranged end-to-end (Fig. 4*E*). In addition, nasal IgA molecules in fractions 24–25, which were composed of trimers and tetramers, appeared as asterisk- or four-leaf clover–shaped complexes (Fig. 4*F*). To observe the structural dynamics of tetrameric IgA, successive images were captured. We observed that each radial

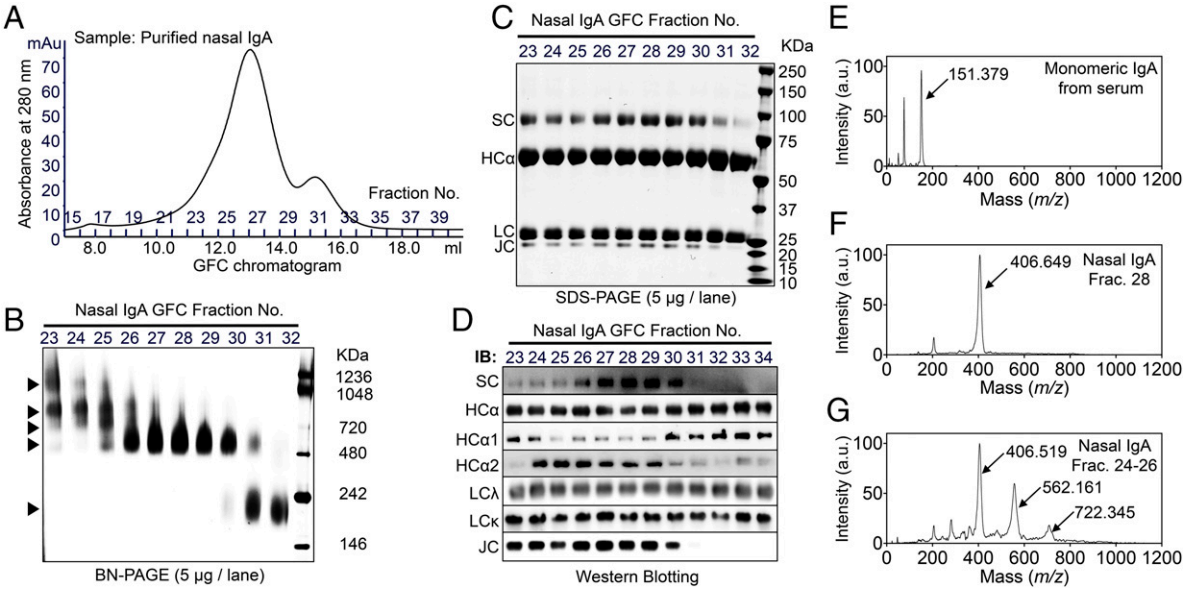

Fig. 3. Biochemical characterization of the quaternary structures of nasal IgA. (*A*) Purified nasal IgA was subjected to GFC separation according to quaternary structures. (*B*) Blue native (BN)/PAGE analysis of each fraction of gel-filtered nasal IgA. Nasal IgA samples were composed of at least five distinct quaternary structures (arrowheads). (*C*) SDS/PAGE analysis of each fraction of gel-filtered nasal IgA under reducing conditions. (*D*) Western blotting analysis of each fraction of gel-filtered nasal IgA using anti-SC, anti-human IgA α-heavy chain (HCα), anti-human IgA1 subclass-specific (HCα1), anti-human IgA2 subclass-specific (HCα2), anti-human light chain λ (LCλ), anti-human light chain κ (LCκ), and anti-JC antibodies. IB, immunoblot. High-mass MALDI-TOF analysis of serum IgA (*E*), fraction 28 of nasal IgA (*F*), and fractions 24–26 of nasal IgA (*G*). (*E*) With serum IgA, one major peak (arrow) was detected. (*F*) With fraction 28 (Frac.) of nasal IgA, one major peak (arrow) was detected. (*G*) With mixtures of fractions 24, 25, and 26 of nasal IgA antibodies, three major peaks (arrows) were detected. a.u., arbitrary unit; Frac., fraction.

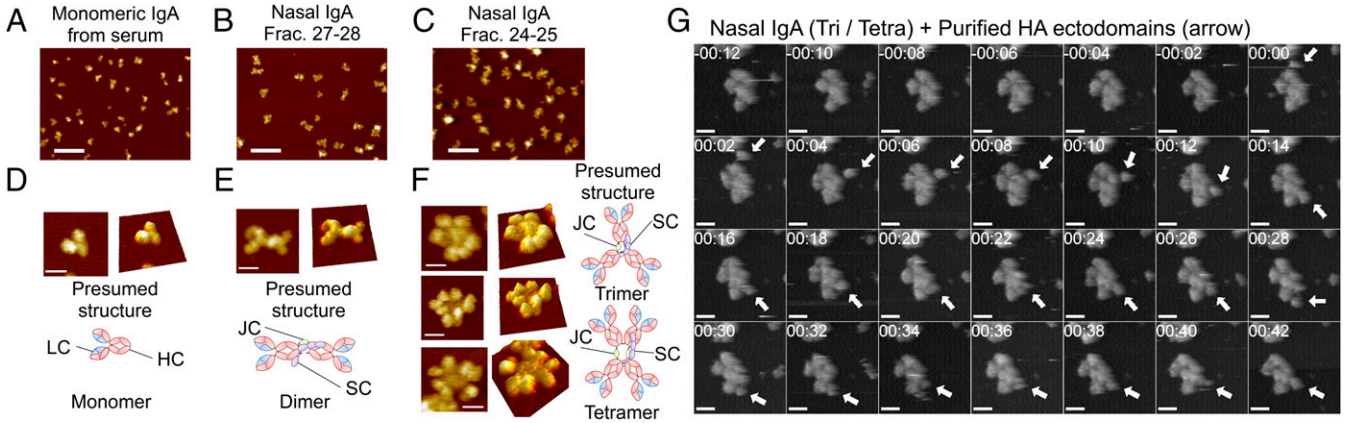

Fig. 4. AFM revealed the quaternary molecular structures of nasal IgA. AFM images of serum IgA (*A*), fractions 27–28 of nasal IgA (*B*), and fractions 24–25 of nasal IgA (*C*) antibodies at low resolution. (Scale bars, 100 nm.) AFM images of serum IgA (*D*), fractions 27–28 of nasal IgA (*E*), and fractions 24–25 of nasal IgA (*F*) at the single particle level. (Scale bars, 20 nm.) Schematic diagrams next to or under the AFM images show presumed structures of monomeric, dimeric, trimeric, and tetrameric IgA. (*G*) Successive AFM images of the interaction between nasal trimeric/tetrameric IgA and HA ectodomains of influenza virus (arrows). The time stamp is given as min:sec. The frame rate is 0.5 frames per second. (Scale bars, 20 nm.) (Also Movie S3.) Tetra, tetramer; Tri, trimer.

arm rocked asynchronously and appeared to bend out of the plane defined by the central portion of the complex (Fig. S3 *A* and *B* and Movies S1 and S2). To address whether these radial arms were capable of capturing antigens, specific HA antigens were added during the AFM observations. Imaging revealed that the round-shaped HA antigens (Fig. S4 *A* and *B*) associated with the radial regions of nasal IgA and moved discontinuously along with the edge of the IgA, suggesting that the radial arms of trimeric and tetrameric IgA were reversibly binding to the antigens (Fig. 4*G*, Fig. S4*C*, and Movie S3).

Relationship Between Ig Structure and Neutralizing Potency. To determine whether there were molecular size-related differences in neutralizing potency, the minimum neutralizing concentration (MNC) of antibody was calculated for each GFC fraction of the nasal wash sample in each subject. For this purpose, the neutralization titer of each fraction was defined as the lowest concentration at which neutralization was detected. The MNC then was expressed as the ratio of total antibody concentration to neutralization titer. MNCs were compared between groups as classified by the quaternary molecular architectures of the Igs. The MNC of antibody (against Victoria virus) in each fraction progressively decreased as molecular size increased (Fig. 5*A*). To estimate quaternary structure-related differences in neutralizing activity, each GFC fraction was classified according to the quaternary structure of the Igs. The antibodies in each fraction consisted of various proportions of Ig isotypes, including IgM, IgG, and IgA. Monomer fractions consisted mainly of IgG, with a minor proportion of IgA. In contrast, the polymeric forms larger than the tetramer fraction were composed primarily of IgA, and the remaining portion was composed of IgM. Furthermore, dimer and trimer/tetramer fractions were composed of IgA exclusively (Fig. 5*B*), suggesting that polymeric Igs in nasal mucus consist primarily of IgA. The MNCs of each Ig structure also were determined. The MNCs of trimeric/tetrameric and larger polymeric Igs against Victoria virus were significantly decreased compared with the MNCs of the monomeric and dimeric Igs (Fig. 5*C*). Furthermore, the MNCs of the larger polymeric Igs against NY virus were significantly decreased compared with the MNCs of the monomeric, dimeric, and trimeric/tetrameric Igs (Fig. 5*D*). These results indicated that polymeric nasal mucus Igs (including the trimer, tetramer, and larger Igs) had significantly higher neutralizing activity against H3N2 influenza virus than the monomeric and dimeric nasal mucus Igs. The higher neutralizing potencies of larger polymeric Igs against influenza virus were further confirmed in additional studies using H5N1 intranasally vaccinated subjects and H5N1 viruses (Fig. 5*E* and Fig. S5).

Nasal IgA antibodies appeared to be composed of 60% dimer, 20% monomer, 15% trimer/tetramer, and 5% larger polymers (Fig. 5*F*). To confirm whether these quaternary structure-related differences in IgA antibody size were evident, the minimum binding concentration (MBC) of Victoria HA-specific IgA antibodies in each GFC fraction of the nasal wash sample also was estimated as the lowest concentration at which binding was detectable at or above a minimum binding threshold. The MBC of Victoria HA-specific IgA in each fraction progressively decreased as molecular size increased (Fig. 5*G*), and the MBCs of Victoria HA-specific dimeric, trimeric/tetrameric, and larger polymeric IgA antibodies were significantly decreased in comparison to the MBC of monomeric IgA antibodies (Fig. 5*H*). Furthermore, the MBC of Victoria HA-specific larger polymeric IgA antibodies also was significantly decreased in comparison to the MBC of dimeric IgA antibodies (Fig. 5*H*). In addition, the MBCs of trimeric/tetrameric and larger polymeric IgA antibodies against NY HA were significantly decreased in comparison to the MBC of monomeric IgA antibodies (Fig. 5*I*). These observations suggested that polymeric IgA antibodies, including the dimer, trimer, tetramer, and larger forms, contained significantly higher amounts and affinities of HA-specific antibodies.

Discussion

In this study, quaternary structures and influenza virus neutralization potency of S-IgA in nasal wash samples were characterized. These nasal washes were obtained from healthy adults immunized intranasally with inactivated influenza vaccine.

Previous studies on polymeric IgA have been performed using samples obtained from the sera of patients with multiple myeloma (20) or external secretions, such as colostrum and saliva (10–12). IgA myeloma sera have been shown to contain monomeric, dimeric, trimeric, and tetrameric IgA in various proportions (20). In contrast, IgA antibodies in physiological external secretions are represented by S-IgA and consist predominantly of dimers (sedimentation constant 11 S) rather than tetramers (15.5 S), as revealed by ultracentrifugation (9, 13–15). However, reliable information concerning the presence of polymers larger than tetramers in nasal mucus has not been reported. The present study using nasal wash samples revealed, for the first time to our knowledge, the existence of larger polymer IgA in the healthy human upper respiratory tract, as well as the physiological functions of these molecules in protecting against viral infection.

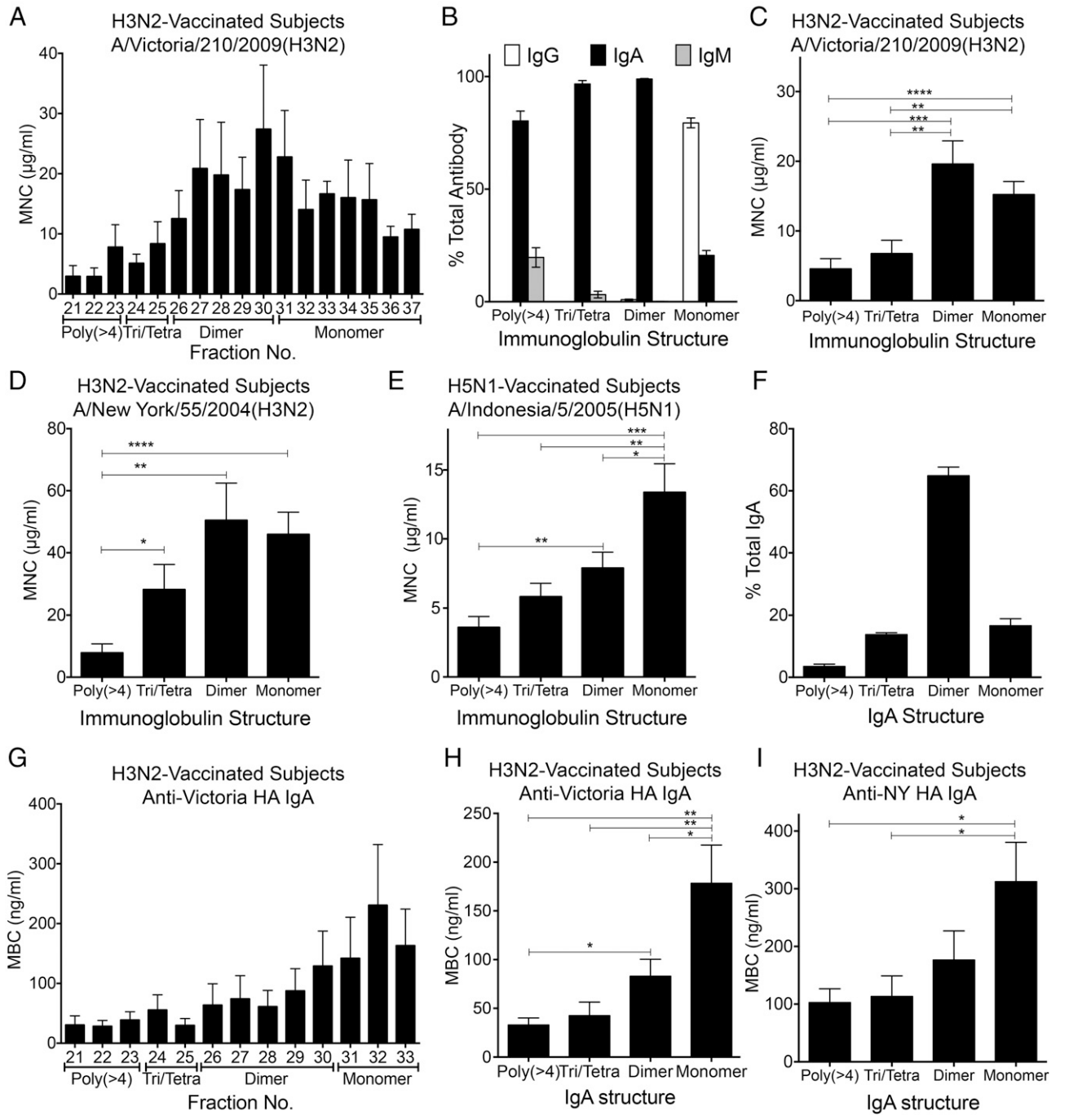

Fig. 5. Comparison of neutralizing potency between Igs with different quaternary structures. (*A*) Neutralizing potency of various-sized antibodies against Victoria virus was defined as the MNC of antibodies (IgM + IgG + IgA) in each fraction. (*B*) Proportions of IgM, IgG, and IgA quantity (wt/wt) in each quaternary structure to total antibody quantity in each quaternary structure (IgM + IgG + IgA, weight). The mean MNC of each quaternary structure of antibody against Victoria virus (*C*) and NY virus (*D*) is shown. (*E*) Mean MNC of each quaternary structure of antibody against Indonesia virus (H5N1) from subjects vaccinated with H5N1 vaccines. (*F*) Proportion of IgA quantity (wt/wt) in each quaternary structure to total nasal IgA (weight). (*G*) Binding activities of IgA with various quaternary structures specific for HA of Victoria virus were defined as the MBC of IgA in each fraction. The mean MBC of each quaternary structure of IgA specific for HA of Victoria virus (*H*) and NY virus (*I*) is shown. All error bars are SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. Poly, polymer; Tetra, tetramer; Tri, trimer.

As we have shown here, the relationship between influenza virus neutralization potency and the quaternary structures of Igs demonstrated that multiple antigen-binding sites on each Ig polymer contributed to effective neutralization of influenza virus. These polymeric antibodies contained two isotypes, IgA and IgM. However, HA-specific ELISA experiments demonstrated that HA-specific IgM titers were not correlated with neutralization titers, indicating that polymeric IgA (not IgM) had the highest neutralization potencies. HA-specific IgA ELISA also demonstrated that the MBCs of HA-specific polymeric IgA were significantly lower than the MBCs of monomeric IgA. We propose three possible reasons as to why polymeric IgA is involved in the efficient

neutralization of influenza virus in the respiratory mucosa. The first is that the proportion of HA-specific antibodies in polymeric IgA is higher than the proportion of HA-specific antibodies in monomeric IgA. The second is that the avidity of polymeric IgA increases with the increased number of antigen-binding sites (compared with the avidity of monomeric IgA). The third is that HA-bound polymeric antibodies contribute to the inhibition of infection due to their large molecular size. In this regard, we note our finding (using nasal wash samples from volunteers vaccinated intranasally with an inactivated whole-virion H5N1 virus vaccine; Figs. S2 and S5) that the neutralizing potency of polymeric Igs against influenza virus was higher than the neutralizing potency of monomeric Igs.

Our results are in agreement with results obtained in previous experiments using mouse mAbs, in which the polymeric IgA mAb had an enhanced antivirus immune response (17–19). In the present work, we demonstrated (for polyclonal antibodies) that polymeric IgA has significantly greater neutralization potency than either dimeric or monomeric IgA; this difference was consistent with the absolute neutralization activity in nasal mucus. These results suggest that polymeric IgA has a critical in vivo role in protection against influenza A virus infection.

In conclusion, we have demonstrated that large polymeric S-IgA has a critical in vivo role in protection from influenza virus infection in humans, and intranasal administration of inactivated influenza vaccines resulted in the induction of multiple molecular forms of neutralizing antibodies in the human nasal mucosa. Moreover, the characteristic flower-shaped quaternary structure of the polymeric complex appears to play a pivotal role in protecting the human upper respiratory tract from viral infection.

Materials and Methods

Detailed materials and methods are provided in *SI Materials and Methods*.

Human Participants, Vaccines, and Nasal Wash Sample Collection. For human studies, the protocol and other relevant study documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases. Written informed consent was obtained from each participant using an ethics committee-approved form. All vaccines were supplied by the Research Foundation for Microbial Disease of Osaka University (Kanonji, Kagawa, Japan). Nasal wash samples were collected as described by Ainai et al. (5).

Purification of IgA and Analysis of Purified Igs. Nasal IgA antibodies were purified from pooled nasal wash samples by affinity chromatography using CaptureSelect IgA (Invitrogen) according to the manufacturer's instructions.

High-Speed AFM. The AFM experiments were performed using high-speed AFM (Nano Live Vision and Nano Explorer, Research Institute of Biomolecule Metrology Co., Ltd.).

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