Induction of Anti-influenza Immunity by Modified Green Fluorescent Protein (GFP) Carrying Hemagglutinin-derived Epitope Structure

Yuji Inoue†1, Ritsuko Kubota-Koketsu†2, Akifumi Yamashita3, Mitsuhiro Nishimura†, Shoji Ideno†, Ken-ichiro Ono*, Yoshihoro Okuno†, and Kazuyoshi Ikuta†

From the†Department of Virology, Research Center for Infectious Diseases Control, Research Institute of Microbial Diseases, and the†Department of Genome Informatics, Genome Information Research Center, Research Institute of Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan, the§Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, 768-0061, Japan, the†Infectious Pathogen Research Group, Osaka Laboratory, Benesis Corporation, Osaka 532-8505, Japan, and the**Ina Laboratory, Medical and Biological Laboratories Corporation, Ltd., Ina, Nagano, 396-0002, Japan

Background: Rapidly producible vaccines are desired for forthcoming influenza pandemics.
Results: Anti-influenza immunity was induced by modified GFP exposing residues from a conserved β-sheet-type hemagglutinin epitope.
Conclusion: A novel strategy for immunization targeting conformational epitopes was established by mimicking the epitope structure.
Significance: Because of its instant producibility, this type of immunogen meets the requirement for next-generation influenza vaccines.

The development of vaccination methods that can overcome the emergence of new types of influenza strains caused by escape mutations is desirable to avoid future pandemics. Here, a novel type of immunogen was designed that targeted the conformation of a highly conserved region of influenza A virus hemagglutinin (HA) composed of two separate sequences that associate to form an anti-parallel β-sheet structure. Our previous study identified this β-sheet region as the structural core in the epitope of a characteristic antibody (B-1) that strongly neutralizes a wide variety of strains within the H3N2 serotype, and therefore this β-sheet region was considered a good target to induce broadly reactive immunity against the influenza A virus. To design the immunogen, residues derived from the B-1 epitope were introduced directly onto a part of enhanced green fluorescent protein (EGFP), whose surface is mostly composed of β-sheets. Through site-directed mutagenesis, several modified EGFPs with an epitope-mimicking structure embedded in their surface were prepared. Two EGFP variants, differing from wild-type (parental) EGFP by only five and nine residues, induced mice to produce antibodies that specifically bind to H3-type HA and neutralize H3N2 virus. Moreover, three of five mice immunized with each of these EGFP variants followed by a booster with equivalent mCherry variants acquired anti-viral immunity against challenge with H3N2 virus at a lethal dosage. In contrast to conventional methods, such as split HA vaccine, preparation of this type of immunogen requires less time and is therefore expected to be quickly responsive to newly emerged influenza viral strains.

Humankind has encountered a number of influenza pandemics. In addition, frequently emerging minor mutations of a vaccine target may also interrupt our efforts to prevent influenza. Generally, influenza vaccine manufacturing takes months (1), approximately half a year for egg-grown virus and at least 2 months for cell culture-grown virus. Such latencies may cause severe problems, especially in pandemics. For example, in the case of the most recent swine-origin pandemic influenza A in 2009 (H1N1pdm) (2), serious concerns arose in countries in the southern hemisphere, such as Australia, because the outbreak occurred immediately before the influenza season (3). To overcome such limitations of conventional vaccination methods, there is a need to develop new methods that can be instantly updatable to newly emerged viral strains and/or induce viral neutralization antibodies that target highly conserved regions.

In a previous study, we succeeded in isolating several clones of human-type monoclonal antibodies produced by hybridoma established from peripheral blood mononuclear cells of influenza-vaccinated volunteers (4). Among them, one notable antibody clone, named B-1, demonstrated high neutralization titers against a wide variety of influenza A viral strains belonging to the H3N2 serotype, including strains isolated more than 40 years ago. Through an epitope-mapping study using synthetic oligopeptides, B-1 antibody was shown to recognize a region of hemagglutinin (HA) protein composed of two separate sequences spanning residues 167–187 and 225–241 (supplemental Fig. S1). According to the known crystal structure of HA, these two subregions appear to associate with each other through an anti-parallel β-sheet structure to form one epitope region located beneath the receptor-binding site (supplemental...
Anti-influenza Immunity by GFP Carrying HA-derived Structure

Fig. S1). Phylogenetic analysis indicated that these sequences and their corresponding sequences in other HA serotypes, including H1N1pdm, are highly conserved in a serotype-dependent manner, suggesting that their broad neutralizing properties have arisen from this high conservation (5). Particularly, the β-sheet portion composed of two sequences spanning residues 175–184 and 229–238 (called the “upper” and “lower” subregions, respectively) appears to be highly conserved; DKLYL-WGVHH occupies 97.8% of the upper subregion, and RISI-YWTIVK occupies 94.5% of the lower subregion among 12,879 HA sequences of H3N2 strains isolated from human within the past 43 years.

A similar broadly neutralizing antibody clone, named D-1, has also been established from another donor’s peripheral blood mononuclear cells and, interestingly, recognizes a region almost identical to that of B-1 (4). Currently, vaccinations with synthetic oligopeptide(s) corresponding to highly conserved epitopes are widely applied to produce antibodies that protect against a broad range of viral strains (6, 7). Because B-1 and D-1 were the strongest and the broadest neutralizing antibodies among those isolated from the peripheral blood mononuclear cell population of each vaccinee, the B-1 (D-1) epitope, which has not to our knowledge been previously mapped, was thought to be an attractive candidate target for peptide-based vaccines. Thus, we tested the ability of synthetic oligopeptides corresponding to the epitope sequences to induce the production of neutralizing antibodies against influenza virus. When peripheral blood mononuclear cells from human vaccinees were stimulated by the oligopeptide(s) ex vivo, we observed production of antibodies with viral neutralizing activity,2 indicating that oligopeptide stimulation may be effective for individuals that already have certain memories. However, our attempt to immunize naïve animals with the synthetic oligopeptide(s) to induce production of virus-neutralizing antibodies appeared less successful,3 probably because the antigenicity for primary immunity was highly dependent on the conformation of the epitope. In addition, according to previously determined crystal structure models, this epitope region appears mostly buried in the stable HA trimer (supplemental Fig. S1), and therefore immunization with intact HA would not be so effective to target this region, although B-1 and D-1 were probably raised against HA in vaccines.

In this study, to induce antibodies specifically recognizing this region, we designed immunogens using a novel strategy focusing on secondary structure. The basic strategy was to build an epitope-mimicking structure directly on the surface of an epitope carrier protein that originally exposes anti-parallel β-sheet structure. The model carrier proteins we selected here are two fluorescent proteins: enhanced green fluorescent protein (EGFP)4 and mCherry (8). Despite the rather low overall similarity between their sequences, their overall structures are quite similar, with 11 β-strands on the surface surrounding the fluorophore to form a barrel-like structure. Utilizing this scaffold, the epitope-derived residues were introduced directly onto the surface of EGFP and mCherry by site-directed mutagenesis. We prepared four variants for each carrier protein. By using these as immunogens, we tested the induction of anti-HA antibody production and the acquisition of anti-influenza immunity. Using monoclonal antibodies isolated from mice immunized with the modified EGFPs, we also evaluated their viral neutralization activities.

EXPERIMENTAL PROCEDURES

Reagents—As a positive control for immunization, a seasonal influenza vaccine, Biken HA (Research Foundation for Microbial Diseases of Osaka University, Kanonji, Japan), containing a mixture of HA-enriched extracts from A/Brisbane/59/2007 (H1N1), A/Uruguay/716/2007 (H3N2), and B/Brisbane/60/2008 strains, was used at 100 μl/body. As an ELISA antigen to test anti-HA reactivity, HA-enriched split vaccine prepared from A/Hiroshima/52/2005 (H3N2) virus was used. As a probe for cell infection by influenza A virus, anti-influenza-A NP antibody C43 (Abcam) was used (9).

Design of Variant EGFPs—The β-strands of EGFP to which HA-derived residues were introduced were determined mainly through the evaluation of the structural similarity between α-carbons (Cα) from HA and EGFP in their crystal structure (Protein Data Bank accession numbers 2VIU and 1EMA, respectively), which was estimated by the root mean square value calculated using PyMOL software (DeLano Scientific).

Mutagenesis—The oligonucleotides used for mutagenesis are summarized in supplemental Table S1. All PCRs were performed with KOD Plus DNA polymerase (Toyobo). The ORFs of pEGFP-N and pmCherry-N (Clontech) were previously subcloned onto pET-28a (+) plasmid vector (Merck Millipore) to produce pET-EGFP and pET-mCherry, respectively. Using each plasmid as a template, fragments containing the sequences corresponding to N-terminal and C-terminal halves of EGFP or mCherry were amplified by PCR with primer pairs P1/P4 to produce pET-EGFP and pET-mCherry, respectively. Using each plasmid as a template, fragments containing the sequences corresponding to N-terminal and C-terminal halves of EGFP or mCherry were amplified by PCR with primer pairs P1/P2 and P3/P4, respectively. In the course of this PCR step, mutations were introduced by primers P2 and P3. These two fragments were combined by PCR with the P1/P4 primer pair. The combined fragment was subcloned onto the pET28a vector (Novagen) using NdeI and EcoRI restriction sites. U1 variants were produced by introducing L1 mutations into the U1 variants.

Protein Expression—Escherichia coli Rosetta (DE3) pLysS (EMD Millipore) cells were transformed by each plasmid prepared as described above. Transformed cells were cultured in LB (plus kanamycin) medium at 37 °C. When the optical density at 600 nm reached 0.5, protein expression was induced by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. Importantly, the culturing temperature should be changed to 18 °C immediately after the isopropyl-1-thio-β-D-galactopyranoside addition because the maturation of several variants of EGFP or mCherry was found to be inefficient with standard culture at 37 °C. Cell culture was continued overnight. If EGFP or mCherry and their variants were expressed successfully, the collected cell pellet would already appear green or purple-red in color, respectively.

2 R. Kubota-Koketsu and K. Ikuta, unpublished data.
3 S. Ideno, K. Sakai, M. Yunoki, R. Kubota-Koketsu, Y. Inoue, S. Nakamura, T. Yasunaga, and K. Ikuta, submitted for publication.
4 The abbreviations used are: EGFP, enhanced green fluorescent protein; FCA, Freund’s complete adjuvant; FRNT, focus reduction neutralization test; proK, protease K; RDE, receptor-destroying enzyme; MEM, minimum essential medium.
**Protein Purification**—After the protein expression, collected *E. coli* cells suspended in PBS containing 0.1 mg/ml lysozyme (Seikagaku Corp.) and 1 mM phenylmethylsulfonyl fluoride (Sigma) were incubated for 30 min on ice and lysed by sonication. After removal of the insoluble fraction by centrifugation, the cell extract was applied onto a nickel-Sepharose Fast Flow (GE Healthcare) column equilibrated with PBS containing 5 mM imidazole. Washing the column with PBS containing 40 mM imidazole, bound protein was eluted by PBS containing 300 mM imidazole. Colored fractions were dialyzed against 20 mM Tris-HCl (pH 8.0) and applied onto a Q Sepharose (GE Healthcare) column equilibrated with 20 mM Tris-HCl (pH 8.0). After washing the column, bound protein was eluted by a 0–200 mM NaCl gradient.

The purity of each fraction was checked with Coomassie Brilliant Blue staining after SDS-PAGE. The EGFP- and mCherry-enriched fractions were collected and dialyzed against PBS. If necessary, protein was concentrated by Centriprep 10k (Millipore). In SDS-PAGE, each variant protein was found to retain its fluorescence in the gel but had a unique mobility when boiling was omitted prior to sample injection (Fig. 1C), whereas almost equal mobility was observed when samples were boiled in SDS (Fig. 2C, without proteinase K treatment). Protein concentration was quantified by the Pierce 660-nm protein assay (Thermo Scientific). In SDS-PAGE of mCherry or its variants purified as described above and boiled in SDS, some extra bands appeared below the full-length protein, whereas very little additional banding appeared in the non-boiled condition. This observation indicates that these samples contain some degraded fragments that cannot be fully separated by column under native conditions because they have been integrated to form the mature mCherry structure.

**Circular Dichroism (CD)**—Purified wild-type (parental) EGFP and its UL1 and UL2 variants were adjusted to 100 μM in 20 mM Tris-HCl (pH 8.0). Far-UV CD spectra were measured in a 0.1-mm path length cell from 260 to 190 nm using a CD spectrometer (model 202, Aviv Biomedical). All measurements were performed at room temperature under a nitrogen flow.

**Protease Resistance and Thermostability Tests**—To test protease resistance, EGFP and its variants, each at a final concentration of 100 nM, were incubated with the indicated concentration of proteinase K (proK) for the indicated time at 37°C. After the incubation, fluorescence was measured with excitation and emission wavelengths at 470 and 507 nm, respectively. To test thermostability, each EGFP or its variant at a final concentration of 100 nM was incubated in the absence or presence of 0.1% SDS for 5 min at the indicated temperature. Fluorescence was measured as described above. Denaturation temperature (T_d) was estimated by fitting the plot to a sigmoidal function, \( F = F_{\text{max}}/(1 + \exp(p(T_d - T))) \), where \( F \) is the fluorescence after heat treatment at \( T \)°C, and \( F_{\text{max}} \) is the fluorescence without heat treatment. \( T_d \) indicates the temperature at which the fluorescence \( F = 0.5 \times F_{\text{max}} \).

**Immunization**—To prepare immunogens, each purified variant of EGFP or mCherry was adjusted to the appropriate concentration in PBS and mixed with an equal volume of Freund’s complete adjuvant (FCA) or Freund’s incomplete adjuvant (Wako) into an emulsion. Emulsion can be formed efficiently by plunging back and forth in two 2.5-ml syringes linked with a three-way stopcock. Immunogen prepared as described above containing 7.5 mg/ml EGFP variant with FCA was inoculated intraperitoneally into female 6-week-old BALB/c mice at 200 μl (1.5 mg)/body. After 3 weeks, booster immunization was performed with the same immunogen. Mice immunized with seasonal influenza vaccine at 100 μl/body were used as a positive control group, and mice immunized with PBS/FCA emulsion were used as a negative control group. After 3 weeks, blood was collected from the tail, and serum was used for tests. Hybridomas described below were prepared from mice immunized in that manner. For virus challenge, immunogen containing 750 or 75 μg/ml EGFP variants was prepared as emulsion with FCA and inoculated into female 5-week-old BALB/c mice at 200 μl (150 or 15 μg)/body. After 2 weeks, antigens prepared with the same concentration of equivalent mCherry variants and Freund’s incomplete adjuvant were inoculated intraperitoneally at 200 μl/body as a booster. After a further 2 weeks, influenza A/Guizhou/54/1989 × Puerto Rico/8/1934 (H3N2) mouse-adapted reassortant virus was infected intranasally at 6 × 10^5 focus-forming units/g body weight.

**ELISA**—Fifty microliters of 2 μg/ml antigen solution were adsorbed on the bottom of 96 Maxisorp Nunc-Immuno Plates (Thermo Scientific) and incubated overnight at 4°C. After blocking with PBS containing 25% (v/v) Blocking One (Nacalai Tesque), the plate was reacted with serum or purified antibody appropriately diluted in PBS containing 5% (v/v) Blocking One. After five washes with PBS containing 0.02% Tween 20 (PBS-T), the plate was reacted with HRP-conjugated secondary antibody at a 1:5,000 dilution. After five washes with PBS-T, 50 μl of SureBlue TMB 1-component Microwell Peroxidase Substrate (KPL) was added and incubated at room temperature. Color development was stopped by the addition of 50 μl of 1 N H_2SO_4, and optical density at 450 nm was measured.

**Hybridoma Preparation**—Mice that exhibited sera with a high content of HA-reactive antibodies and high viral neutralization titers were finally boosted by intraperitoneal inoculation with the equivalent variant of mCherry without adjuvant at 0.2 mg/body. After 3 days, splenocytes were collected and fused with mouse myeloma PA1 cells by a standard polyethylene glycol fusion method. Fused cells were cultured on 5 × 96-well plates at 100 μl/well. After forming colonies, culture supernatants were tested by ELISA against either HA vaccine prepared from A/Hiroshima/52/2005 (H3N2) virus or EGFP. Cells containing HA-reactive clones were diluted to 10–50 cells/ml and mixed with fresh splenocytes from a naïve mouse (~10^6 cells/ml) and then cultured on a 96-well plate at 100 μl/well. Finally, through a second screening by ELISA, HA-reactive clones were stocked. To prepare antibodies produced by these clones, cultured hybridomas were inoculated intraperitoneally into female BALB/c mice previously stimulated with pristane (Sigma) at 7 days prior to hybridoma inoculation. Antibodies were purified by protein G columns from the ascites fluids accumulated in hybridoma-inoculated mice.

**In Vitro Viral Neutralization Assay**—Viral neutralization by antibodies was tested in *vitro* by focus reduction neutralization test (FRNT) (10). Sera collected from immunized animals were treated with three volumes of receptor-destroying enzyme.
Anti-influenza Immunity by GFP Carrying HA-derived Structure

(RDE) (RDE(II) “SEIKEN,” Denka Seiken) for 16 h at 37 °C, and the enzyme was inactivated by incubation for 30 min at 56 °C. In the case of purified antibodies, this treatment was omitted. After adjusting to the appropriate concentrations, the RDE-treated sera or antibodies were mixed with an equal volume of MEM containing 2,000 focus-forming units of either influenza A/Hiroshima/52/2005 (H3N2), A/New Caledonia/20/1999 (H1N1), or A/duck/Czechoslovakia/1/1956 (H4N6) virus. After incubation for 1 h at 37 °C, 100 μl of the complexes were applied to 90% confluent Madin-Darby canine kidney cells in a 96-well plate. After incubation for 1 h at 37 °C, medium was changed to MEM containing 10% FBS, and incubation was continued overnight. Infected cells were visualized by an immunofluorescence assay as described below and counted. The changes in number of infected cells were represented as percentages calculated as 100 × (N− − N+/N−), where N+ is the number of infected cells in the presence of antibody, and N− is the average number of infected cells in the absence of antibody.

When antibody treatment was performed after viral adsorption, Madin-Darby canine kidney cells were previously incubated with virus in cold MEM without antibody for 1 h at 4 °C. After the removal of supernatant, cells were incubated with cold MEM containing the appropriate concentration of antibodies for 30 min at 4 °C followed by further incubation for 30 min at 37 °C. After changing the medium to MEM (plus 10% FBS), the assay was continued as described above.

Immunofluorescence Assay—Infected cells were treated with 4% (w/v) paraformaldehyde for 20 min and with 0.1% (v/v) Triton X-100 for 5 min at room temperature. Cells were then treated with antibody appropriately diluted in PBS for 1 h. After washing with PBS, cells were treated with secondary antibody labeled with Alexa Fluor 488 at 1:5,000 dilution for 1 h. After washing with PBS, cells were observed by fluorescence microscopy, and if necessary, cells with fluorescent focus were counted.

RESULTS

Design and Preparation of Modified EGFPs and mCherries Carrying Epitope-like Structure on Their Molecular Surfaces—

The anti-parallel β-sheet region in HA focused on in the present study is composed of two separate sequences: residues 175–184 (upper) and 229–238 (lower). EGFP variants were first constructed by introducing substitutions at several positions that take part in its β-barrel scaffold. Corresponding to the side of the β-sheet in the epitope, two types of variants were possible: one type mimics the side facing the central axis of the HA trimer, and the other mimics the side facing laterally (Fig. 1, A and B). To design the former variant, named UL1, amino acids at EGFP residue positions 94, 96, 98, 100, 102, 179, 181, (183), 185, and 187 were substituted to Lys, Tyr, Trp, Val, His, Arg, Ser, (Tyr), Thr, and Val, respectively, which were originally located at residue positions 176, 178, 180, 182, 184, 229, 231, (233), 235, and 237 in HA1 of the major H3N2 strain. Upper region-only and lower region-only versions, named U1 and L1 respectively, were also constructed. Likewise, the latter variant, named UL2, was designed by introducing Asp–175, (Leu–177), Ile–179, Gly–181, His–183, Ile–230, Ile–232, Trp–234, Ile–236, and Lys–238 of HA1 onto EGFP at residue positions 177, (179), 181, 183, 185, 163, 165, 167, 169, and 171 (Fig. 1, A and B). Conformational similarity between the mutated region on EGFP and the corresponding region of the B-1 epitope was evaluated as the root mean square between Ca atoms (Table S2). No calculated root mean square value exceeded 2 Å. The relevance of these conformational gaps is discussed collectively with the results from immunization tests (see “Discussion”). Each EGFP variant was expressed in E. coli and purified. Fluorescence of the expressed protein was used as a helpful indicator of whether it has been folded successfully or not because the fluorophore formation of fluorescent proteins generally depends on folding accuracy of the entire molecule, including the β-barrel (11). Several variants were hardly expressed with the correct fluorescent conformations under culture at 37 °C, but by culturing at a lower temperature, such as 18 °C, every variant was expressed successfully. Every purified EGFP variant retained its fluorescence and distinct mobility after SDS-PAGE under non-boiling conditions (Fig. 1C). Equivalent variants of mCherry were prepared similarly to the EGFP variants.

Although the overall β-barrel scaffold of fluorescent proteins is nearly assured by their fluorescence (12, 13), we confirmed the structural relevance of EGFP UL1 and UL2 variants using far-UV CD spectra. Both variants demonstrated spectra similar to that of wild-type EGFP, indicating practically no change in secondary structure (supplemental Fig. S2).

Stability of EGFP Variants against Degradation and Denaturation—GFP and its derivatives are known to demonstrate high stability against protease digestion, detergent, and heat treatment (12), which are favorable for application as a scaffold for our immunogen carrier. We tested the protease resistance and thermostability of EGFP variants used here. Upon proK treatment, wild-type EGFP showed no significant decrease in fluorescence for up to 2 days at 37 °C. This finding indicates the maintenance of overall structure, because fluorescent proteins should generally lose their fluorescence by destruction of the β-barrel structure, even after the formation of their fluorophores (12, 13). Similarly, every variant used here maintained its fluorescence under proK treatment (Fig. 2, A and B). SDS-PAGE under non-boiling conditions revealed a fluorescent band for every proK-treated variant positioned slightly lower than that of variants with no proK treatment, whereas only proteolytic fragments appeared as a ladder when samples were boiled in SDS prior to electrophoresis (Fig. 2C). Upon heat treatment for 1 min at 80 °C, wild-type EGFP demonstrated no significant decrease in fluorescence (Fig. 2D). In the presence of 0.1% SDS, wild-type EGFP showed a sigmoidal decrease in fluorescence as temperature increased, with a denaturation temperature (Td) of 62 °C (Fig. 2E). Each variant EGFP also demonstrated high thermostability comparable with wild-type EGFP in the absence of detergent (Fig. 2D). In the presence of 0.1% SDS, each variant EGFP demonstrated a distinct Td value ranging from 58 to 76 °C (Fig. 2E). Similar to wild-type EGFP (14, 15), every EGFP variant showed a decrease in fluorescence at low pH. Wild-type and variants except UL2 showed 50% fluorescence at pH 6.0 (pH 6.4 for UL2) compared with the fluorescence at pH 7.2, but the change in fluorescence was fully reversible at values as low as pH 3.0 (data not shown). Collec-
tively, we concluded that the EGFP variants used here were as stable as wild-type EGFP under physiological conditions and could be used equivalently for further examinations.

Reactivity and Viral Neutralization Activity of Sera from Modified EGFP-immunized Mice against H3-type HA—To test these EGFP variants as immunogens to induce production of anti-HA antibodies, we inoculated mice intraperitoneally with each variant as an emulsion with FCA. After a booster with the same immunogens, we collected serum from each mouse and tested the reactivity against either EGFP or HA by ELISA. As expected, every serum sample from wild-type and variant EGFP-immunized mice demonstrated major reactivity against EGFP (data not shown). We found sera from several mice immunized with EGFP U1 and UL1 variant that demonstrated significant reactivity with H3-type HA in vaccine solution (Fig. 3A). We also tested the in vitro viral neutralizing activity of each serum. FRNT (10) with the antisera revealed viral neutralizing activities almost parallel to immunoreactivities against HA (Fig. 3B). These results indicate that immunogenicity depends mainly on the inner side of the upper region.

Recognition of Cells Infected with H3N2-type Influenza Virus by Monoclonal Antibodies Raised against Modified EGFP—Of the mice immunized with either EGFP U1 or UL1 variant, two mice in each group whose sera showed positive reactivity with HA were finally boosted with the equivalent variant constructed on the mCherry scaffold. Hybridomas were prepared from their splenocytes. Culture supernatants of cell clones were screened by ELISA against either HA vaccine or wild-type EGFP. Although most clones demonstrated EGFP-specific reactivity as expected, several clones demonstrated HA-specific reactivity (Fig. 3C). HA-specific hybridoma clones were selectively isolated, and the monoclonal antibody secreted from each clone was purified. Reactivities of purified antibodies against HA were confirmed by ELISA (Fig. 3D). Unexpectedly, in the immunofluorescence assay, five of eight clones demonstrated very weak reactivity against Madin-Darby canine kidney cells infected with H3N2 virus (supplemental Fig. S3).

Viral Neutralization by Anti-variant-EGFP Monoclonal Antibody—We tested the in vitro viral neutralizing activity of the monoclonal antibodies isolated above against H3N2
virus by FRNT. In the antibody concentration range tested here, one U1-derived clone and four UL1-derived clones demonstrated positive neutralizing activity. Notably, all FRNT-positive clones except 1E12 were nearly negative in the immunofluorescence assay, as described above (supplementary Fig. S3). Two FRNT-positive clones, 3H11 and 5E11, were further tested after the adsorption of virus (supplementary Fig. S4). In our experimental conditions, as many as 60% of virions would have already started to enter the cells during incubation at 4 °C without antibody. When B-1 antibody was used as a positive control, ~40% of viruses at maximum were protected to infect after the adsorption onto cells. We found that both 3H11 and 5E11 also showed 40% protection at maximum after the viral adsorption. This observation may indicate that these antibodies recognize a state of HA after adsorption, in which the conformation may be changed to expose the epitope region (see “Discussion”). We also tested the viral neutralization of 3H11 and 5E11 against non-H3 strains H1N1 and H4N6. H1-HA was categorized as group 1, whereas H3-HA and H4-HA have been assigned to group 2.
Residues corresponding to those we introduced on the UL1 variant are highly similar in H4-HA but not less similar in H1-HA (nine and six of 10 residues conserved, respectively). We found that neither 3H11 nor 5E11 demonstrated significant neutralization activity against H1N1 infection. Unexpectedly, H4N6 also showed no significant focus reduction by treatment with either 3H11 or 5E11. Because the differing residue between H3-HA and H4-HA is positioned in the upper region, this finding, together with the result shown in Fig. 3B, may also reflect the significance of the upper region in immunogenicity. Collectively, antibodies raised against the EGFP UL1 variant were able to neutralize virus in a serotype-dependent manner.

**Acquisition of Immunity against Influenza Virus by Immunization with EGFP/mCherry Variants**—We finally tested whether the mice inoculated with the designed immunogens acquired immunity against the influenza virus. Mice were first immunized with either 150 or 15 μg/body of wild type, U1, or UL1 variant of EGFP. Mice were boosted by equivalent variants of mCherry. These mice were then infected intranasally with reassortant mouse-adapted H3N2 virus. Approximately 6 days after infection, four of five mice in the positive control group that was immunized with seasonal influenza vaccine demonstrated recovery of body weight, with one mouse dead. Similar survival rates were observed in the EGFP/mCherry U1- and UL1-infected groups at high dosage (150 μg/body) (Fig. 4A). The effect
FIGURE 4. Acquisition of anti-influenza immunity by modified EGFPs and mCherries. Mice were immunized with either EGFP or its U1 or UL1 variant at 15 and 150 μg/body. After boosting with the equivalent variant of mCherry, mice were intranasally infected with influenza A H3N2 mouse-adapted virus. Mice immunized with seasonal vaccine were used as positive controls, and mice immunized with PBS mixed with adjuvant were used as negative controls. After infection, survival rate (A) and change in body weight (B) were recorded daily for 3 weeks. DPI, days postinfection.
of immunization with high dose U1 or UL1 variant was more apparent in recovery of body weight. In each group, one of five mice began to recover from 7 days after infection. Although the recovery rates were slower than those of the positive control group, four and three mice in the high dose U1- and UL1-infected groups, respectively, recovered body weight, whereas few signs of recovery were observed in either the PBS-immunized group or wild-type EGFP/mCherry-immunized groups within 3 weeks (Fig. 4B).

**DISCUSSION**

We have demonstrated a novel approach to induce immunity specifically against conformational epitopes characterized by β-sheet structure, proposing one form for the next generation of influenza vaccines. Although fluorescent proteins, such as those used here, may be unsuitable epitope carrier molecules for practical uses, they were sufficient to evaluate the validity of our strategy, and their fluorescence was quite helpful in confirming retention of the β-barrel structure in each variant, which is the most important point in this study. A similar approach can be found in recent investigations of amyloidosis, in which the β-sheet conformation of the pathogenic polypeptide is characteristic to its toxicity; several modified GFPs in which residues corresponding to the β-sheet region of the altered form of amyloid-β peptide were embedded in its surface β-barrel have been shown to interfere with the formation of toxic amyloid-β oligomer (18, 19). Conversely, our results suggest that proteins designed to carry a mimic of the surface structure of a toxic form of amyloid protein, such as amyloid-β or prion protein, may be used as vaccines against amyloidoses like Alzheimer disease and prion disease.

EGFP UL1 variant differs from wild-type EGFP by only nine residues. More strikingly, the U1 variant carrying just a five-residue difference was able to induce the production of anti-HA antibodies and anti-influenza immunity, although the isolated antibodies demonstrated weaker reactivity against HA and virus neutralization titers than those against the UL1 variant. The structural gaps represented as average root mean square values between the backbone Cα atoms of the mutated region of EGFP variants and that of the corresponding region of HA were ~1.4 Å for U1 and 1.7 Å for the UL1 variant. Our results indicate that such degrees of structural gap are allowable to induce primary immunity against a conformational epitope.

Because the target region focused on here seems mostly buried within the stable trimeric HA complex corresponding to the previously determined crystal structure models, questions remain concerning how it could induce antibodies, such as B-1, and how some antibodies raised against modified GFPs, in which an epitope mimicking the structure of this region is exposed. Recall that the FRNT-positive antibody clones isolated here demonstrated nearly negative results against H3N2-infected cells in the immunofluorescence assay. This finding may indicate that these antibodies attack HA in a limited conformation. Our observation that 3H11 and 5E11 antibodies demonstrated viral neutralization even after viral adsorption onto the cell surface also supports this model (supplemental Fig. S4).

In this study, we could not obtain any antibodies with neutralization titers as high as that of B-1 (supplemental Fig. S4). In Western blotting analysis, B-1 demonstrated only weak reactivity against U1, L1, and UL1 variants (data not shown), probably because the upper/lower-like structure presented on the surface of EGFP does not cover enough of the B-1 epitope. Although this type of immunogen may not be sufficient to induce the production of strong neutralizing antibodies like B-1, it has the advantage of rapid production. Especially in the case of outbreak of a newly emerged viral strain that is resistant to seasonal vaccines available at the time, vaccines that can be produced instantaneously must be more helpful than conventional vaccines that require several months to be supplied (1). Recombinant immunogens produced according to the strategy proposed here require less effort for strain selection because they target a highly conserved region. Even in the case of an emergence of strains carrying mutations at the target region, updated immunogens can be produced quickly through a simple redesign and expression in an appropriate system. One of the most concerning current threats is the possible adaptation of highly pathogenic avian influenza A H5N1 virus into humans to cause serious pandemics (22). We have already confirmed that EGFP variants corresponding to residues from H5-HA as well as H1-1HA can be prepared successfully. After steps of improvement, we expect this type of designed immunogen to be a highly valuable backup strategy for forthcoming pandemics.

**REFERENCES**

1. Gerdil, C. (2003) The annual production cycle for influenza vaccine. *Vaccine* 21, 1776–1779
2. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Da-wood, F. S., Jain, S., Finelli, L., Shaw, M. W., Lindstrom, S., Garten, R. J., Gubareva, L. V., Xu, X., Bridges, C. R., and Uyeki, T. M. (2009) Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N. Engl. J. Med.* 360, 2605–2615
3. Bishop, J. F., Murnane, M. P., and Owen, R. (2009) Australia’s winter with the 2009 pandemic influenza A (H1N1) virus. *N. Engl. J. Med.* 361, 2591–2594
4. Kubota-Koketsu, R., Mizuta, H., Oshita, M., Ideno, S., Yunoki, M., Kuhara, M., Inoue, F., Fujii, and K. Ikuta, unpublished data.
M., Yamamoto, N., Okuno, Y., and Ikuta, K. (2009) Broad neutralizing human monoclonal antibodies against influenza virus from vaccinated healthy donors. *Biochem. Biophys. Res. Commun.* **387**, 180–185

5. Yamashita, A., Kawashita, N., Kubota-Koketsu, R., Inoue, Y., Watanabe, Y., Ibrahim, M. S., Ideno, S., Yunoki, M., Okuno, Y., Takagi, T., Yasunaga, T., and Ikuta, K. (2010) Highly conserved sequences for human neutralization epitope on hemagglutinin of influenza A viruses H3N2, H1N1 and H5N1. Implication for human monoclonal antibody recognition. *Biochem. Biophys. Res. Commun.* **393**, 614–618

6. Ben-Yedidia, T., and Arnon, R. (2007) Epitope-based vaccine against influenza. *Expert Rev. Vaccines* **6**, 939–948

7. Stanekovič, Z., and Varečková, E. (2010) Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. *Virol. J.* **7**, 351

8. Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004) Improved monomeric red, orange, and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567–1572

9. Okuno, Y., Isegawa, Y., Sasao, F., and Ueda, S. (1993) A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J. Virol.* **67**, 2552–2558

10. Okuno, Y., Tanaka, K., Baba, K., Maeda, A., Kunita, N., and Ueda, S. (1990) Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. *J. Clin. Microbiol.* **28**, 1308–1313

11. Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G., and Ward, W. W. (1993) Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. *Biochemistry* **32**, 1212–1218

12. Bokman, S. H., and Ward, W. W. (1981) Renaturation of *Aequorea* green-fluorescent protein. *Biochem. Biophys. Res. Commun.* **101**, 1372–1380

13. Ward, W. W., and Bokman, S. H. (1982) Reversible denaturation of *Aequorea* green-fluorescent protein. Physical separation and characterization of the renatured protein. *Biochemistry* **21**, 4535–4540

14. Wilson, I. A., Niman, H. L., Houghten, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984) The structure of an antigenic determinant in a protein. *Cell* **37**, 767–778

15. Ward, W. W., Prentice, H. J., Roth, A. F., Cody, C. W., and Reeves, S. C. (1982) *Photochem. Photobiol.* **35**, 803–808

16. Nobusawa, E., Aoyama, T., Kato, H., Suzuki, Y., Tateno, Y., and Nakajima, K. (1991) Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology* **182**, 475–485

17. Air, G. M. (1981) Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7639–7643

18. Takahashi, T., Ohta, K., and Mihara, H. (2007) Embedding the amyloid β-peptide sequence in green fluorescent protein inhibits Aβ oligomerization. *Chembiochem* **8**, 985–988

19. Takahashi, T., Ohta, K., and Mihara, H. (2010) Rational design of amyloid β peptide-binding proteins. Pseudo-Aβ β-sheet surface presented in green fluorescent protein binds tightly and preferentially to structured Aβ. *Proteins* **78**, 336–347

20. Eigen, M., and Rigler, R. (1994) Sorting single molecules. Application to diagnostics and evolutionary biotechnology. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5740–5747

21. Kettling, U., Koltermann, A., Schwille, P., and Eigen, M. (1998) Real-time enzyme kinetics monitored by dual-color fluorescence cross-correlation spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1416–1420

22. Amendola, A., Ranghiero, A., Zanetti, A., and Pariani, E. (2011) Is avian influenza virus A (H5N1) a real threat to human health? *J. Prev. Med. Hyg.* **52**, 107–110

23. Emsley, P., and Cowtan, K. (2004) Coot. Model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132