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Chimeric Influenza Virus Hemagglutinin Proteins Containing Large Domains of the *Bacillus anthracis* Protective Antigen: Protein Characterization, Incorporation into Infectious Influenza Viruses, and Antigenicity

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Large polypeptides of the *Bacillus anthracis* protective antigen (PA) were inserted into an influenza A virus hemagglutinin glycoprotein (HA), and the chimeric proteins were functionally characterized and incorporated into infectious influenza viruses. PA domain 1′, the region responsible for binding to the other toxin components, the lethal factor and edema factor, and domain 4, the receptor binding domain (RBD), were inserted at the C-terminal flank of the HA signal peptide and incorporated into the HA1 subunit of HA. The chimeric proteins, designated as LEF/HA (90 amino acid insertion) and RBD/HA (140 amino acid insertion), were initially analyzed following expression using recombinant vaccinia viruses. Both chimeric proteins were shown to display functional phenotypes similar to that of the wild-type HA. They transport to the cell surface, can be cleaved into the HA1 and HA2 subunits by trypsin to activate membrane fusion potential, are able to undergo the low-pH-induced conformational changes required for fusion, and are capable of inducing the fusion process. We were also able to generate recombinant influenza viruses containing the chimeric RBD/HA and LEF/HA genes, and the inserted PA domains were maintained in the HA gene segments following several passages in MDCK cells or embryonated chicken eggs. Furthermore, DNA immunization of mice with plasmids that express the chimeric RBD/HA and LEF/HA proteins, and the recombinant viruses containing them, induced antibody responses against both the HA and PA components of the protein. These approaches may provide useful tools for vaccines against anthrax and other diseases.

Influenza viruses are segmented negative-strand RNA viruses belonging to the family *Orthomyxoviridae*. They are highly effective immunogens capable of inducing both systemic and mucosal antibody responses (35), and influenza vaccines are produced on a large scale on an annual basis. The influenza virus hemagglutinin glycoprotein (HA) is the most important viral antigen with regard to the stimulation of neutralizing antibodies and vaccine design. In nature, 15 antigenic subtypes of HA have been documented. Although only three of these subtypes, i.e., H1, H2, and H3, have circulated extensively in humans over the past century, limited outbreaks of other subtypes raise concerns that antigenically novel viruses with the potential to cause pandemics may soon emerge and spread in humans. Vaccine design and development for influenza viruses continues to be a source of extensive investigation.

Since the advent of reverse genetics techniques with influenza viruses (33), a major area of research has involved the use of influenza viruses as vectors for the expression of foreign protein-antigenic regions for vaccine purposes. The strategies that have been utilized include the incorporation of additional genome segments into viruses, the use of bicistronic genes that contain internal ribosome entry sequences allowing for the translation of a second coding region, the use of gene segments encoding polyproteins containing embedded proteolytic self-cleavage sites for the generation of independent proteins, and the use of chimeric influenza virus proteins that have foreign protein sequences incorporated into their coding regions (15; for reviews, see references 14, 16, 40, and 42). More recently, the capacity to decipher influenza virus genome packaging signals has allowed for gene segment substitutions that allow for foreign protein expression (12, 47, 54).

Some of the approaches involving chimeric proteins have benefited from the structural promiscuity of particular regions of the HA and neuraminidase (NA) glycoproteins. The stalk regions of the NA can vary in length to a significant degree among natural isolates, and inserted foreign protein sequences of as many as 28 to 41 amino acids have been reported (5, 32). High-resolution structural information is available for the influenza virus HAs of prototype viruses representing H1, H3, H5, and H9 subtype viruses (13, 21, 22, 53, 57). Among these, the HA of A/Aichi/2/68 virus, an H3 subtype, has been the most well characterized and has provided one of the best model systems for the study of viral glycoproteins in general. For the Aichi HA, X-ray crystal structures have been determined for the polypeptide precursor (HA0), the cleaved form that exists on the surface of infectious viruses, the conformation that is adopted following the structural rearrangements

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required for membrane fusion, HA complexes with receptor analogs, and HA complexes with neutralizing antibodies (48). The HA contains several flexible loop domains in the well-characterized antigenic regions of the protein designated as sites A, B, C, D, and E, to which neutralizing antibodies are targeted (56). As these domains vary during the antigenic drift of viruses from year to year, they provide attractive targets as sites for the insertion of alternative sequences. Sites B and E have been utilized for epitope swap experiments among HAs of different subtypes and insertion of foreign peptides for immunity studies (16). However, the chimeric HA proteins that have been incorporated into infectious viruses have contained insertions of only 7 to 12 amino acids at sites B and E.

Sequence alignment of the HA subtypes shows that the N-terminal regions of HA1 can differ in length by as many as 15 residues (44), indicating that there may be structural flexibility in these regions and that the molecule may be able to accommodate extra polypeptide segments at the N terminus without interfering with essential HA functions. Hatziioannou and colleagues (24) used an H7 subtype HA to generate several different HA mutants with insertions between the HA signal peptide and ectodomain sequences at the HA1 N terminus. These chimeric proteins contained large polypeptide domains derived from epidermal growth factor, single-chain antibodies, and an immunoglobulin G (IgG) Fc-binding domain, and they ranged in size from 53 to 246 amino acids. They were also shown to incorporate into pseudotyped retroviruses. Haynes and Schnierle (25) made two epidermal growth factor-HA proteins with insertions in this domain bearing point mutations in the receptor region, and although these were shown to incorporate into retroviral particles, no fusion activity was detected. Despite the latter observation, these two studies indicated that it might be possible to insert reasonably large domains into functional HAs, incorporate them into infectious viruses, and utilize these for vaccine purposes.

One pathogen for which vaccine development has proven problematic is *Bacillus anthracis*, the causative agent of anthrax, a disease that can lead to high fatality rates in humans. Spores of this bacterium currently constitute one of the major potential threats for acts of bioterrorism and were employed as such in late 2001, when spores were mailed to various locations, including the Senate Office Building, through the U.S. Postal Service. These unresolved incidents caused five fatalities and resulted in the closure of several places of work. The vaccine that is currently utilized requires multiple injections over an 18-month period and therefore is not optimal should incidents of bioterrorism occur (11). For this reason, we initiated studies using the *Bacillus anthracis* protective antigen (PA) as a model protein for large-domain polypeptide insertions into the HA for functional analyses of such chimeric proteins and for determinations of the capacities of these to incorporate into influenza viruses and induce protective immune responses against both influenza and anthrax disease.

Anthrax disease is caused by a bacterial toxin which is composed of three subunits, i.e., the PA, lethal factor (LF), and edema factor (EF), but it is antibodies to domain 1′ and domain 4 of the PA that mediate the neutralization of toxin activity and confer protection (10, 30, 34). The structure of PA (Fig. 1B) suggests that individual domains may be able to fold independently and that it may be possible to incorporate them into recombinant molecules as vaccine components. Therefore, we attempted to insert the PA domain 1′ (90 amino acids) and domain 4 (140 amino acids) polypeptides into HA to generate functional chimeric proteins and infectious viruses for immunogenicity studies. The chimeric proteins were found to express on the cell surface and induce membrane fusion to levels that were indistinguishable from that of wild-type (WT) HA. Plasmids expressing these chimeric proteins were shown to induce immune responses in mice to a greater degree than plasmids that express the PA domains alone do, suggesting an adjuvant-like effect when PA polypeptides are presented as components of HA-PA chimeric proteins. Furthermore, the chimeric proteins were able to incorporate into infectious influenza virus particles using reverse genetics, which also produced specific immune responses against both PA and HA following the inoculation of mice. The viruses containing chimeric HAs replicated at levels approaching that of the WT virus, and the inserts were stably incorporated following several serial passages of the virus. This demonstrates that DNAs, expressed proteins, or recombinant influenza viruses may prove useful as immunogens for the prevention of anthrax and that recombinant viruses may be even more useful. The technological aspects of our studies may extend to other pathogens for which vaccine design has proven problematic.

MATERIALS AND METHODS

Cell lines. HeLa, Madin-Darby canine kidney (MDCK), and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For CV1 and BHK21 cells, 5% fetal calf serum was utilized. All media were supplemented with 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Construction of plasmids. To make the chimeric LEF/HA and RBD/HA constructs, the Aichi/2/68 HA gene was used, and the *B. anthracis* domain 1′ and domain 4 regions of the PA gene were constructed by single-chain oligonucleotide extension with codons optimized for mammalian usage (23). Recombinant PCR was performed with different primers (primer sequences are available upon request). The full-length WT Aichi HA gene was cloned into pRB21 (2) for protein expression and functional studies, and sequences of the entire coding regions were verified. The sequence encoding the receptor binding domain (RBD) of PA and the lethal factor and edema factor binding domain (LEF) were inserted between the 3′ end of the HA signal peptide sequence and the nucleotide sequences encoding the N-terminal domain of the HA1 ectodomain by use of a strategy similar to that employed by Hatziioannou et al. (24). To enhance the possibilities that the inserted domains could fold as individual units, sequences encoding Gly-Gly-Gly-Gly-Ser linkers were inserted at the 3′ flanks of the PA domains in the genome sense with the chimeric proteins. For convenience, NheI and PacI sites were introduced into 5′- and 3′-flanking areas of the RBD coding region when the RBD/HA construct was made. One extra T nucleotide was added after the PacI site to avoid causing a change in the reading frame. For the DNA immunization study, the open reading frames (ORFs) of the RBD, LEF, WT HA, RBD/HA, and LEF/HA proteins were cloned into plasmid vector pCAGGS (kindly provided by Y. Kawaoka) under the control of a chicken β-actin promoter.

Expression and characterization of PA/HA chimeric proteins. Recombinant vaccinia viruses were generated by the method developed by Blasco and Moss (2). HA-expressing recombinant viruses were plaque purified twice prior to the generation of stock viruses utilized in experiments. Trypsin cleavage experiments for the analysis of protein expression were carried out as described in the work of Cross et al. (7). Cell surface expression, as well as conformational change assays, was also analyzed by enzyme-linked immunosorbent assay (ELISA) using vaccinia virus-infected HA-expressing HeLa cells as described previously (52). Heterokaryon formation assays were carried out using recombinant vaccinia virus-infected BHK21 cells as described previously by Steinhauer et al. (52).

Reverse genetics and analysis of mutants. The PA/HA chimeric genes were introduced into the RNA expression plasmid pPolI Aichi HA, and the gene segment sequences were verified in entirety. Infectious influenza viruses were then generated from plasmid cDNAs essentially as described by Neumann et al.

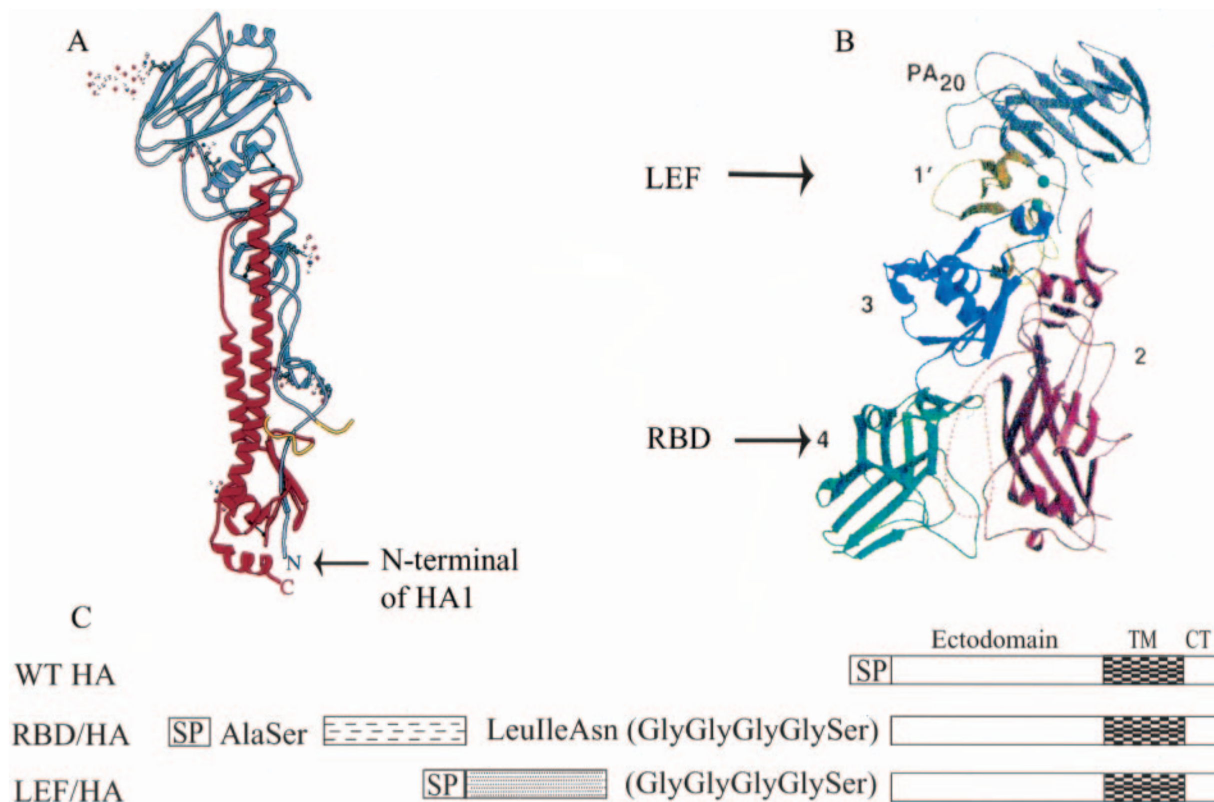

FIG. 1. (A) Structural depiction of the HA monomer showing the site of insertion of the PA domains. (B) The structure of PA indicating domain 1′ (LEF) and domain 4 (RBD) subunits (45). (C) The schematic structure of the constructs used in this study. The proteins RBD and LEF were inserted after the HA signal peptide (SP) followed by a Gly-Gly-Gly-Gly-Ser linker to increase flexibility. NheI (encoding Ala-Ser) and PacI (encoding Leu-Ile-Asn) sites were inserted on the 5′ and 3′ ends of the inserted RBD sequences, so one extra T was added to avoid a frameshift mutation. TM, transmembrane domain; CT, cytoplasmic tail.

(41). The viruses generated were of H3N1 composition, containing the Aichi HA and other gene segments derived from the A/WSN/33 virus. Human 293T cells were transfected with the 17 protein and RNA expression plasmids using either Mirus (Panvera) or Superfect (QIAGEN) transfection reagents following the suppliers' guidelines. At day 3 posttransfection, cell supernatants were plaque purified once and passaged on MDCK cells to generate primary stocks. MDCK cells were also utilized for titrating viruses.

Virus passage in MDCK cells and embryonated chicken eggs. The rescued viruses were plaque purified once in MDCK cells. For the serial passage studies, MDCK cells were infected at multiplicities of infection (MOI) of between 0.01 and 0.001. For experiments with embryonated chicken eggs, 0.2 ml of serial 10-fold dilutions of the viruses were injected into the allantoic cavities and, at 48 h after infection, the allantoic fluids were harvested. Following serial passage of the MDCK-grown viruses and egg-grown viruses, stocks were used to infect MDCK cells in 24-well plates at an MOI of 5. At 7 hours after infection, MDCK cells were treated either in the presence or in the absence of 5 μg/ml trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone [TPCK]-treated; Sigma) for 5 min. The cell lysates were then analyzed by Western blotting using ECL technology (Amersham Biosciences) following sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation of the proteins on 10% polyacrylamide gels. To confirm the genetic stability of the chimeric PA/HA gene sequences of the rescued viruses after serial passage in MDCK cells and embryonated chicken eggs, the viral RNAs were purified using a QIAamp Viral RNA Mini Kit (QIAGEN) following the manufacturer's instructions. Reverse transcription-PCR analyses were performed using a Stratagene kit following the manufacturer's manual, and PCR products were sequenced and confirmed.

Kinetics of virus replication. MDCK cells were infected with wild-type and recombinant viruses at MOI of 0.001. At the indicated times after infection, the supernatant of MDCK cells was harvested. After low-speed centrifugation to remove cells, the virus titers in the supernatant were titrated on the confluent MDCK cells.

DNA immunization. Female BALB/c mice (6 to 8 weeks old) purchased from Jackson Labs were used in the animal experiments. The plasmids were amplified in *Escherichia coli* DH5α and purified with a QIAGEN Endo-Free Megaprep kit. The plasmids were then resuspended at 1 μg/μl in sterile phosphate-buffered saline and stored at −80°C until used for immunization. The mice (6 per group) were immunized with a total of 100 μg DNA per mouse by intramuscular injection with 50 μl DNA in separate sites in both side quadriceps, followed by boosting with the same dose of DNA at week 4, week 8, and week 12. All animal procedures were done in accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. For ELISAs, mouse blood was collected by retro-orbital bleeding at 14 days after the fourth DNA immunization. Ninety-six-well plates were coated with purified bromelain-treated HA (BHA), purified His-tagged ORF RBD, and His-tagged ORF LEF (100 μl of 4 μg/ml in borate-buffered saline, pH 8.5) at 4°C overnight. After the plates were blocked in 2% BSA/PBST (phosphate-buffered saline, 0.1% Tween 20, 2% bovine serum albumin), 100 μl of serial dilutions of mouse serum were added to each well in duplicate and incubated at 37°C for 2 h. The plates were washed three times with 2% BSA/PBST and subsequently incubated in horseradish peroxidase-conjugated secondary antibody against mouse IgG for 2 h at 37°C. After a final wash, 50 μl of the substrate 3,3′,5,5′ tetramethylbenzidine (Sigma) in 20 mM citrate buffer, pH 4.5, with 0.03% H_2O_2 was added, followed by the addition of 50 μl of 0.1 N sulfuric acid to stop the reaction when color was developed. The data was quantified by spectroscopy at 450 nm.

Immunization with recombinant viruses. Female C57BL/6 mice (6 to 8 weeks old) purchased from Jackson Labs were used in the animal experiments. The mice (4 per group) were infected intranasally with WT, recombinant RBD/HA, or recombinant LEF/HA viruses at 5,000 PFU/mouse/30 μl. The mouse blood was collected by retro-orbital bleeding at 22 days after immunization. ELISA assays were done as described above.

RESULTS

HA-PA chimeric constructs. The PA subunit of the anthrax toxin mediates attachment to, and transport of, the LF and EF subunits into the cytosol (6). The PA contains 735 amino acids and folds into four separate functional domains, each of which plays a role in toxicity. Domain 1 (residues 1 to 258) contains a protease recognition site for furin and related proteases that are ubiquitously expressed in host tissues. Cleavage at this site yields an N-terminal product of approximately 20 kDa and a 63 kDa fragment. A region of domain 1 that remains associated with the 63-kDa fragment following cleavage, designated as domain 1′ (residues 168 to 258), is involved in binding to LF and EF. Domain 2 (residues 259 to 487) forms the core of the postcleavage heptamer that is involved in pore formation by the toxin. Domain 3 (residues 488 to 595) is thought to be involved in protein-protein interactions. Domain 4 (residues 596 to 735) is the RBD responsible for the attachment of the toxin to the cellular receptors (45). The receptors have been identified as tumor endothelial marker 8 (TEM8) and capillary morphogenesis protein 2 (CMG2) (3, 31, 46, 50). The RBD or LEF domain coding sequences were inserted between the HA signal peptide and the glutamine residue that is present at the N terminus of the protein following signal peptidase cleavage. The inserted sequence was followed by a Gly4-Ser (G-G-G-G-S) coding sequence to facilitate the proper folding of the inserted polypeptides as independent domains. The structures of HA and PA, including the sites of insertion and the identification of the PA domains that were used for construction of the chimeric proteins, are shown in Fig. 1A and B. Figure 1C also shows a linear diagram depicting the constructs that were made. The initial characterization of the chimeric PA/HA constructs containing the *B. anthracis* PA 1′ domain or domain 4 were carried out with proteins expressed using recombinant vaccinia viruses, as described by Blasco and Moss (2).

Protease analysis of chimeric proteins. During influenza infection of host cells, the HA is synthesized as a monomeric polypeptide of approximately 550 amino acids, depending on the virus strain. These polypeptides form trimers in the endoplasmic reticulum prior to transport through the Golgi apparatus and to the cell surface. The monomeric HA precursor (HA0) polypeptides must be cleaved into the disulfide-linked subunits HA1 and HA2 to activate membrane fusion potential and virus infectivity (26, 27). When the H3 subtype Aichi HAs utilized in our studies are expressed using recombinant vaccinia viruses, they are transported to the cell surface in the uncleaved HA0 form. Trypsin treatment of cell monolayers expressing such HAs cleaves the surface-expressed molecules into HA1 and HA2 and provides a useful assay for cell surface expression and for the capacity of the molecule to be cleaved and become competent for membrane fusion. In our initial trypsin cleavage experiments, WT and chimeric HAs were incubated either without trypsin or at a concentration of 5 μg/ml, an amount that is often used for HA0 cleavage analysis, and the migration patterns of the resulting polypeptides were determined by Western blot analysis (Fig. 2). The migration profiles of uncleaved HA0 proteins indicate that the chimeric proteins are larger than that of WT HA, as would be expected considering the size of the inserted segments. Upon trypsin cleavage, the apparent molecular weights of the HA2 subunits of RBD/HA and LEF/HA were indistinguishable from that of WT HA2. This is not surprising, as the inserted sequences were engineered at the N terminus of HA1, and HA2 is highly resistant to protease degradation. The RBD/HA chimeric protein displayed properties suggesting that the inserted domain is relatively stable to the action of the protease treatment required to cleave HA0, since the migration of a large proportion of cleaved HA1 was found to be slower than that of WT HA1. By contrast, the migration patterns of the HA1 polypeptide chains indicate that a majority of the inserted domain from the LEF/HA chimeric protein becomes degraded at 5 μg/ml trypsin and indicate that the postcleavage HA1 product is very similar to WT HA. The PA 1′ domain contains 13 lysine and arginine residues (55), so it is possible that some of these potential cleavage sites are as accessible to trypsin cleavage as the HA1-HA2 cleavage site normally recognized by activating proteases.

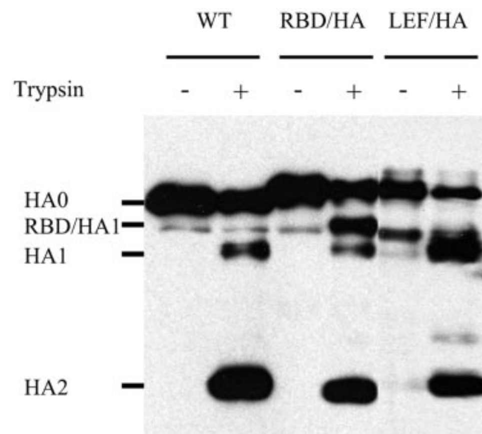

FIG. 2. Cell surface expression of WT, chimeric RBD/HA, and LEF/HA proteins. CV1 cells were infected with recombinant vaccinia viruses expressing the proteins, the cells were treated either in the absence (−) or presence (+) of 5 μg/ml TPCK trypsin, and the cell lysates were analyzed by Western blotting.

In order to find a trypsin concentration at which cleavage of HA0 into HA1 and HA2 was efficient without the concurrent digestion of the inserted PA domains, twofold dilutions of trypsin were assessed on CV1 cells expressing WT HA, LEF/HA, and RBD/HA chimeric proteins, as shown in Fig. 3. The results illustrate that we were not able to find conditions under which HA1-HA2 cleavage occurs without digestion of the LEF domain of the chimeric LEF/HA protein, as shown in Fig. 3A. This was supported by experiments showing that an anti-LEF antibody recognizes the HA0 form of LEF/HA but not the HA1 subunit that results following trypsin cleavage (data not shown). However, with the RBD/HA construct it was possible to titrate the trypsin concentration such that HA0 is processed without significantly digesting the inserted polypeptide (for example, 4 to 8 μg/ml trypsin, as shown in Fig. 3B). Overall, the data show that both of the chimeric proteins can be transported to the cell surface, but the PA 1′ domain is more susceptible than domain 4 to the proteolytic activity required to activate fusion potential and virus infectivity. At higher trypsin concentrations, both constructs displayed gel migration patterns that were indistinguishable from that of WT HA, showing that while the inserted domains were cleaved, the HA

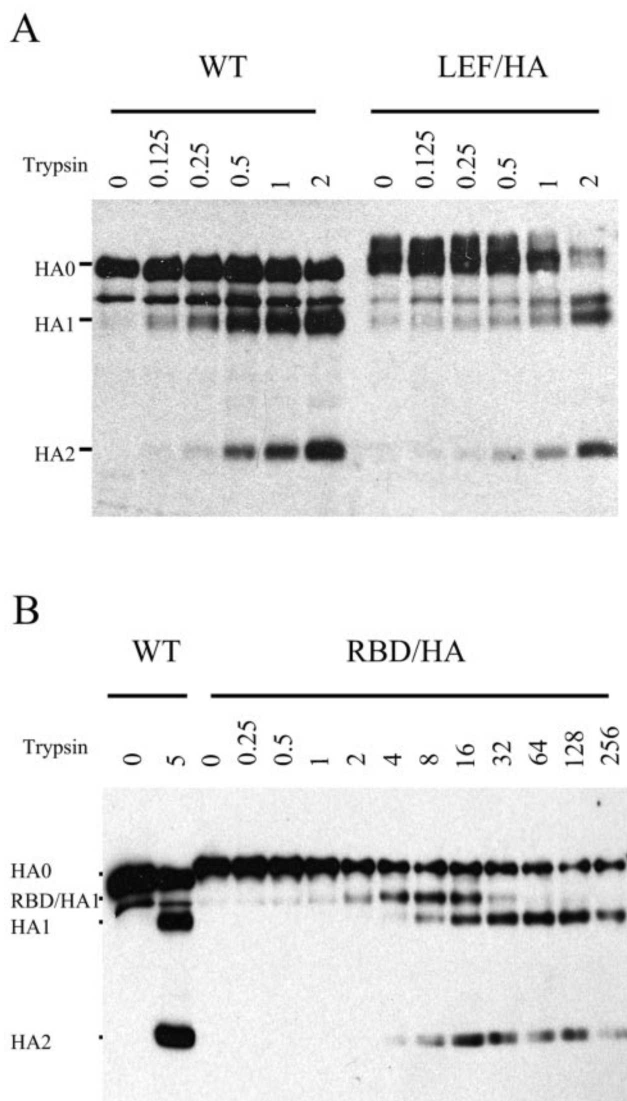

FIG. 3. Western blot analysis showing the titration of trypsin concentrations required to cleave HA0 into HA1 and HA2 and their effects on inserted sequences.

subunits of the chimeric proteins remained intact. We do not know the exact extent of the inserted domains that remain associated with the HA following cleavage of the chimeric proteins, but, as detailed below, they were able to produce immune responses.

TABLE 1. Analysis of cell surface expression and HA conformation by ELISA

HA	Antibody[a]					
	HC3	HC31	HC68	HC100	HC263	Jose
WT	100	100	100	100	100	100
RBD/HA	81	65	90	73	62	65
LEF/HA	79	43	79	66	50	85

[a] Values represent optical densities at 450 nm. The monoclonal antibodies (HC3, HC31, HC68, HC100, and HC263) recognize conformational epitopes on neutral-pH HA (8). Jose is a polyclonal rabbit serum.

Conformational changes of chimeric PA/HA proteins. Cell surface expression and HA1-HA2 cleavage do not dictate that the chimeric proteins will be functional. For HA, a useful analytical tool involves experiments on the reactivity of HA monoclonal antibodies that recognize conformationally intact structural regions of the molecule and the use of such antibodies to confirm that the protein can undergo the structural changes required for membrane fusion and virus infectivity. To analyze the conformation of the chimeric HAs, we used cell surface ELISA with a panel of well-characterized monoclonal antibodies that recognize nonoverlapping antigenic sites and a rabbit polyclonal antiserum. As shown in Table 1, the reactivity profiles of the RBD/HA and LEF/HA proteins were lower to various degrees relative to that of WT HA, but we have evaluated dozens of mutant HAs with similar values, and they have proven to be fully functional as expressed proteins and components of infectious viruses. We also analyzed the capacities of the chimeric proteins to undergo the acid-induced conformational changes required for membrane fusion activity. The monoclonal antibodies HC68 and HC3 are useful reagents for the analysis of conformational changes required for membrane fusion with the Aichi HA. HC68 recognizes only the native HA, whereas HC3 reacts with both the native and low-pH conformation of HA (8). Thus, the relative reactivities of these antibodies not only demonstrate that a particular HA can undergo the structural changes required for fusion but also reveal the pH at which this occurs. HeLa cells were infected with recombinant vaccinia viruses that express WT and chimeric HAs and were treated with trypsin to cleave the HA0 precursor, were incubated at a range of pH values, and were analyzed by ELISA using the HC3 and HC68 monoclonal antibodies to determine the pH at which conformational changes take place. As shown in Table 2, at a trypsin concentration of 5 μg/ml, both the RBD/HA and LEF/HA displayed characteristics of acid-induced conformational change similar to those of WT HA. The decrease in the ratio of HC68/HC3 reactivity suggests that RBD/HA does not undergo conformational changes as efficiently as WT or LEF/HA, possibly due to less-than-optimal cleavage under the conditions as described above. However, it is clear from the HC68-to-HC3 reactivity data that HAs containing additions of as many as 140 amino acids at the N terminus can be cleaved and undergo conformational changes required for fusion in fashions similar to that of the WT.

Heterokaryon formation by chimeric PA/HA proteins. The results of the conformational change experiments by the chimeric proteins indicate that they should be competent to mediate membrane fusion. This was analyzed by a fusion assay that assesses heterokaryon formation by protein-expressing cells following a reduction in pH of the tissue culture medium. WT and chimeric proteins were both shown to induce fusion at equivalent pH values. Results obtained at pH 5.0 are shown in Fig. 4. This suggested to us that such proteins are functional and that it could be possible to incorporate them into infectious influenza viruses using reverse genetics.

Generation of recombinant influenza viruses containing chimeric RBD/HA and LEF/HA genes. By using the reverse genetics techniques developed by Neumann et al. (41), we were able to generate influenza viruses containing the WT Aichi/2/68 HA gene and the HA-PA chimeric HAs with other gene segments derived from WSN/1/33 virus (H3N1/WSN). The se-

TABLE 2. Determination of the pH of conformational change by ELISA

HA	Ratio of HC68-to-HC3 reactivity at pH[a]:										
	5.6	5.5	5.4	5.3	5.2	5.1	5.0	4.9	4.8	4.7	4.6
WT	0.67	0.64	0.62	0.63	0.58	0.54	0.45	0.40	0.32	0.21	0.22
RBD/HA	0.68	0.63	0.64	0.61	0.59	0.54	0.44	0.41	0.33	0.30	0.30
LEF/HA	0.71	0.68	0.62	0.65	0.65	0.47	0.32	0.24	0.09	0.03	0.06

[a] The values represent averages of two to four separate experiments.

quences of the rescued viruses were confirmed, and titers of the chimeric viruses were reproducibly found to reach levels of between 10^6 and 10^7 PFU/ml for viruses grown in either MDCK cells or embryonated chicken eggs. These titers were only marginally lower than the WT virus titers, which were generally found to attain titers of about 2×10^7 PFU/ml. To assess the genetic stability of the inserted segments, the rescued viruses were plaque purified and then passaged six times in either embryonated chicken eggs or MDCK cells. Titers of the viruses did not vary significantly upon passage. The replication kinetics of the viruses was assessed in MDCK cells infected at low multiplicity and monitored over a 5-day period by plaque assay. For most of the viruses, the titers peaked at levels between 10^6 and 10^7 PFU/ml at 2 days after infection (Fig. 5). The growth kinetics of the RBD/HA virus passaged in eggs was delayed relative to those of the other viruses but eventually reached titers comparable to those of the other viruses. The delay in replication kinetics with this virus can possibly be explained by suboptimal HA1/HA2 cleavage, as this large insert might possibly reduce the accessibility for HA-activating proteases.

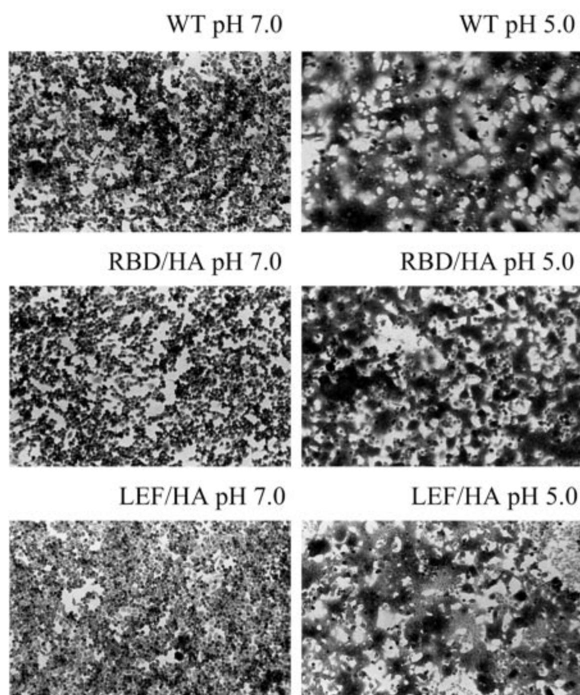

FIG. 4. Polykaryon formation by BHK 21 cells expressing WT, chimeric RBD/HA, or LEF/HA at pH 7.0 and pH 5.0.

Genetic stability of recombinant viruses containing chimeric PA/HA genes. To confirm whether the inserted sequences in the recombinant virus HA genes were maintained following serial passage, infected cells were analyzed by Western blotting and sequenced. As shown in Fig. 6A and B, Western blot analysis shows that at passage 6, the migration patterns of the HA0, HA1, and HA2 bands of each virus are not significantly different from those of the passage 1 viruses. The rescued viruses (H3/WSN backbone) showed that HA1 and HA2 cleavage could occur even without trypsin treatment. This is not unexpected in light of observations by Goto and coworkers pertaining to the capacity of WSN NA to sequester HA-activating proteases (19, 20). It is possible that proteases recruited by the NA also digest portions of the inserted sequences.

To examine the genetic stability of the chimeric PA/HA genes, the viral RNAs of rescued viruses after six passages were sequenced following reverse transcription-PCR. The inserted PA sequences remained genetically intact, but mutations in the HA portion of the molecules were detected, and these are shown in Table 3. These may be related to the selection of mutants with elevated pH values for membrane fusion and are discussed below.

Immunization with plasmids and viruses expressing chimeric PA/HA proteins. As an initial step to address the potential that the chimeric PA/HA proteins might be effective immunogens, plasmids that express these proteins were used to immunize mice. These DNAs were injected intramuscularly as described in Materials and Methods, and immune responses to purified HA or purified RBD or LEF domains of the anthrax PA toxin subunit were analyzed. Immunization of the plasmids

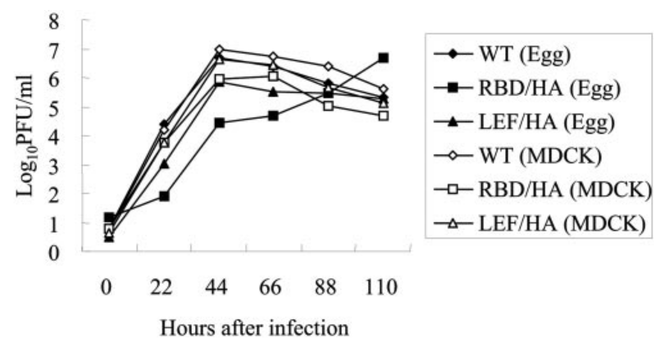

FIG. 5. Growth kinetics of MDCK-grown and embryonated chicken egg-grown viruses following six passages. The MDCK cells were infected with viruses at an MOI of 0.001, and the supernatants of these cells were titrated on MDCK cells following incubations at the indicated time points.

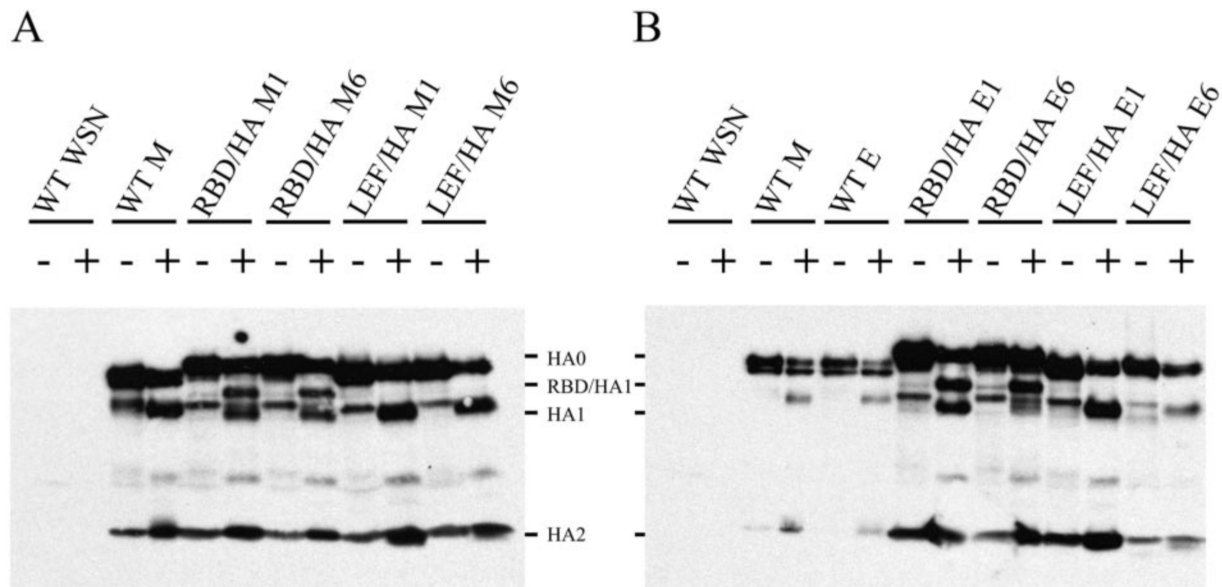

FIG. 6. (A) Expression of the chimeric proteins after one or six passages in MDCK cells. The analyses were done using MDCK-infected cells treated in the absence (−) or presence (+) of 5 μg/ml TPCK trypsin at 7 h after infection. (B) Expression of chimeric proteins after six passages in embryonated chicken eggs. The process is the same as that for panel A.

that express the chimeric RBD/HA and LEF/HA induced humoral immune responses to purified viral BHA (Fig. 7A). Furthermore, the chimeric plasmids induced antibody titers to the purified His-tagged RBD and LEF higher than those produced by plasmids that expressed only the LEF or RBD domains of PA as analyzed by ELISA (Fig. 7B and C). Western blot results shown in Fig. 7D and E indicate that the chimeric plasmids induced antibody responses to purified recombinant PA and His-tagged RBD and LEF. On the other hand, DNA immunization with the open reading frames of RBD and LEF did not induce significant antibody responses (data not shown).

In order to examine whether the recombinant viruses could induce immune responses, mice were infected intranasally by WT and recombinant viruses as described in Materials and Methods, and immune responses to purified HA or purified RBD or LEF domains of the anthrax PA toxin subunit were analyzed. Immunization of the WT and recombinant viruses RBD/HA and LEF/HA induced humoral immune responses to purified viral BHA (Fig. 8A). The lower levels of response to the chimeric proteins are possibly related to the large size of the inserts and the inhibition of access to antigenic sites. The recombinant LEF/HA and RBD/HA viruses induce specific immune responses against the purified His-tagged LEF and RBD (Fig. 8B and C).

TABLE 3. The virus titers and sequence changes of recombinant viruses

HA	Titer of recombinant virus at passage 6 (PFU/ml)	Sequence change of recombinant virus at passage 6:	
		In HA1	In HA2
WT (egg)	1.5×10^7	L226P[a], N246D	None
RBD/HA (egg)	4.5×10^6	None	D132V
LEF/HA (egg)	8.0×10^6	None	S113L
WT (MDCK)	2.0×10^7	L226P	None
RBD/HA (MDCK)	7.0×10^6	S107F	D132V
LEF/HA (MDCK)	8.0×10^6	None	S113L, M115I

[a] Indicates H3 numbering.

DISCUSSION

Influenza A viruses cause epidemics nearly every year due to the evolving nature of the viral glycoproteins. Although this antigenic drift can cause problems with vaccine design, during most years the vaccine strains that are chosen are well matched with the strains that circulate during the subsequent influenza season and are very effective, providing protective immunity to 52 to 80% of vaccinees (43). For decades, the influenza vaccines have been composed of inactivated reference strains of H1 and H3 subtype influenza A viruses and of an influenza B virus. More recently, attenuated viruses based on cold-adapted strains have been licensed for use as vaccines for certain segments of the population. In contrast to that for influenza viruses, vaccine development for several viruses, bacteria, and parasites has proven problematic. There are various reasons for this, but it is clear that the techniques and approaches that have been successful for some pathogens are not directly applicable to others and that for some of these, novel systems will be required to provide immune protection.

There are several reasons to consider the potential advantages of influenza viruses as vectors for the development of vaccines against other pathogens. (i) The virus stimulates strong mucosal and systemic immune responses. (ii) The influenza vaccine is produced in large scale on an annual basis. (iii) The structures and functions of the HA and NA surface proteins are well characterized, and it has been shown that these proteins can be genetically manipulated without compromising their functions. (iv) Highly efficient reverse genetics systems have been developed for influenza. (v) Mice and ferrets provide well-characterized animal model systems for the analysis

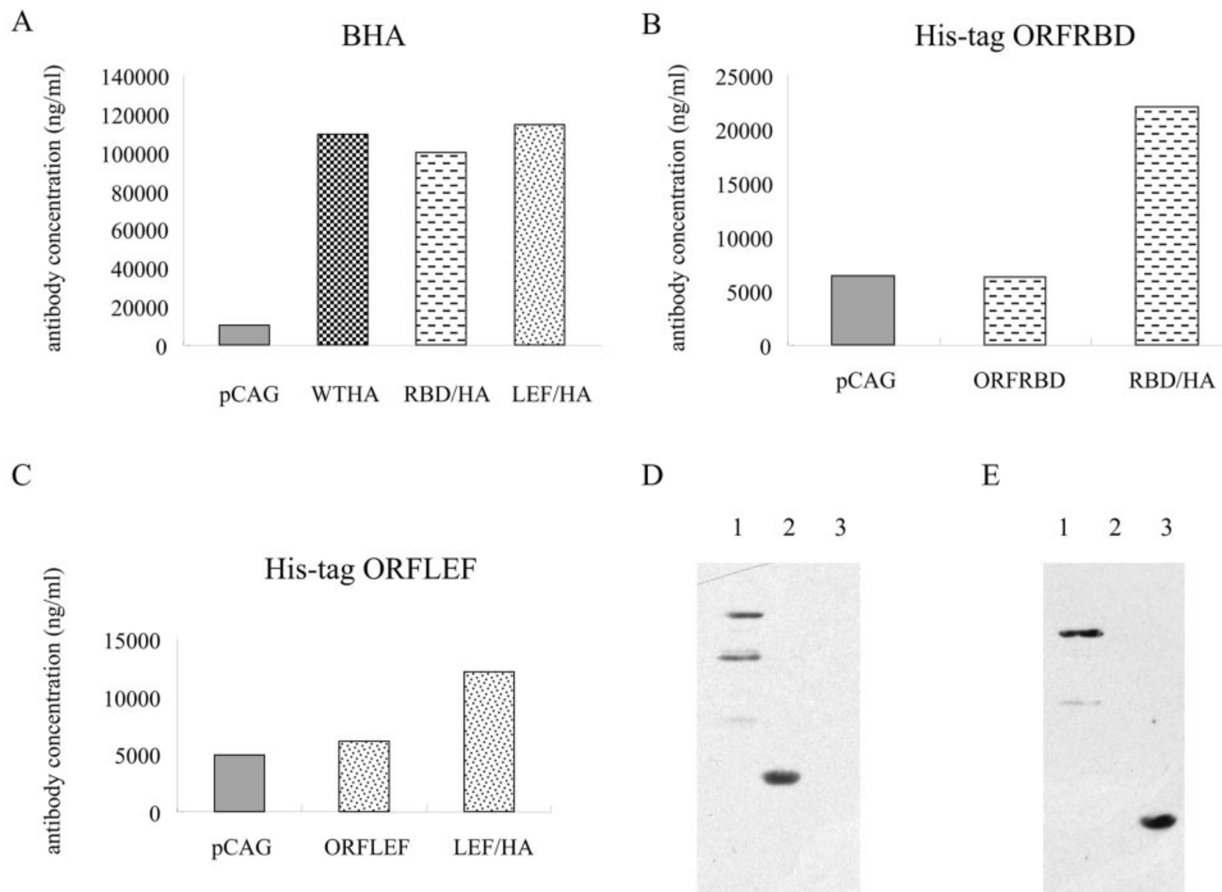

FIG. 7. Induction of immune responses following DNA immunization of mice with plasmids expressing WT HA and the chimeric RBD/HA and LEF/HA proteins. The pooled sera from mice immunized with the different DNA constructs were collected 2 weeks after the fourth immunization and analyzed for antibodies specific for different proteins by ELISA. Antigens utilized were as follows: (A), bromelain-released HA ectodomains (BHA); (B), His-tagged RBD; (C), His-tagged LEF. The antibody concentrations were calculated according the standard curve for absorbance and the amount of mouse IgG. (D), RBD/HA-immunized mice; (E), LEF/HA-immunized mice. The pooled sera from mice immunized with the RBD/HA and LEF/HA constructs after the fourth immunization were used for Western blot analysis against whole PA (lanes 1), His-tagged RBD (lanes 2), and His-tagged LEF (lanes 3).

of immune responses to potential vaccine candidates involving influenza virus.

For these reasons, several approaches have been utilized to genetically modify influenza viruses for the expression of foreign epitopes. Among the many examples documented to date, these various strategies have involved the induction of immune responses in mice against human immunodeficiency virus 1 envelope protein antigenic regions (9, 17, 36, 37), simian immunodeficiency virus Gag protein sequences (38, 39), cytotoxic T lymphocyte epitopes from lymphocytic choriomeningitis virus (4) and herpes simplex virus 2 (1), an antigenic region of the outer membrane protein of *Pseudomonas aeruginosa* (49), and polypeptides from the malaria parasite *Plasmodium yoelii* (18, 28). However, the previous studies involving the influenza virus HA have involved only the insertion of small peptides into antigenic loop regions. The studies described here follow approaches used by Hatziioannou and colleagues (24), who showed that polypeptide domains as large as 246 amino acids can be added at the N terminus of the HA of A/Rostock/34 virus, an H7 subtype, and that such chimeric HAs can express at the cell surface and be incorporated into pseudotyped retroviruses. Our work shows that large fragments of the *B. anthracis* PA protein can also be incorporated into functional HA molecules as insertions at the N-terminal regions of H3 subtype HAs. The PA domains that we analyzed were the 1′ domain responsible for binding to the LF and EF subunits of the toxin and domain 4, which is involved in the binding of the toxin to cell surface determinants. Chimeric HA proteins containing each of these domains were shown to express on the cell surface and mediate membrane fusion. Moreover, we were able to demonstrate that they can act as functional components of infectious influenza viruses with regard to virus infectivity and replication. They grow as well as viruses with WT HA in both MDCK cells and embryonated chicken eggs (Fig. 5). The PA sequences of the chimeric HAs of the recombinant viruses were stably maintained following six passages in MDCK cells or eggs, though several mutations were detected in the HA portions of these proteins (Table 3). The significance of the S107F and N246D mutations in the HA1 region is not known, but the L226P mutation is known to confer a high pH fusion phenotype, and other mutations that were detected, such as D132V, S113L, and M115I in the HA2 region, are very close in

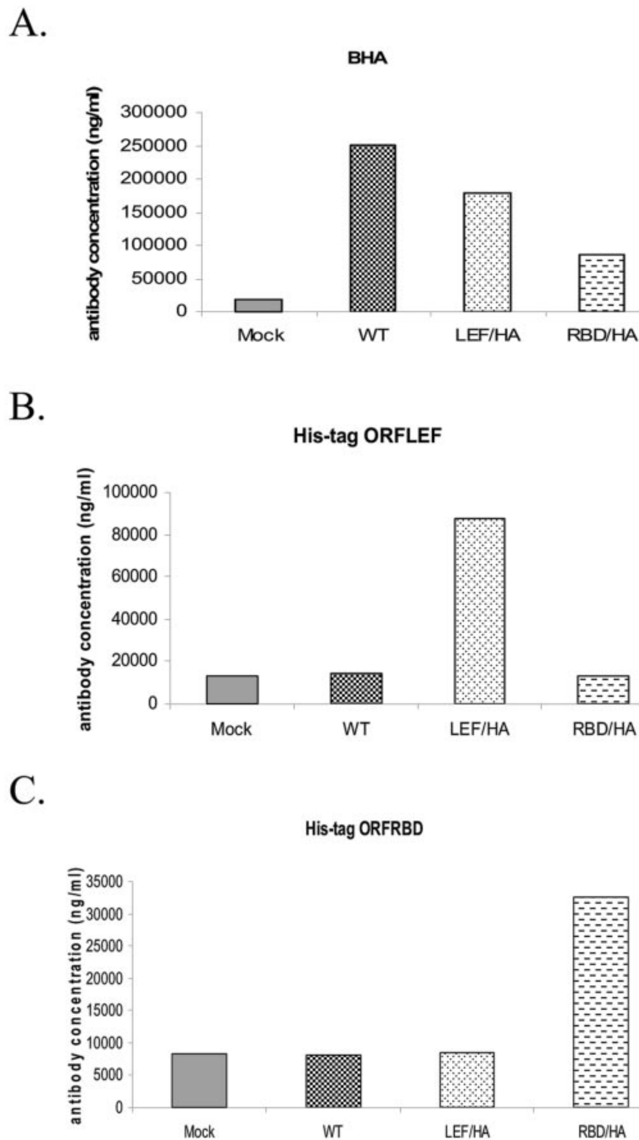

FIG. 8. Induction of immune responses following the immunization of mice with WT and recombinant RBD/HA and LEF/HA viruses. The pooled sera from each group of mice immunized intranasally with 5,000 PFU per mouse were collected at day 22 postinfection and analyzed by ELISA for antibodies specific for different proteins. Antigens utilized were as follows: (A), BHA; (B), His-tagged LEF; (C), His-tagged RBD. The antibody concentrations were calculated according to the standard curve for absorbance and the amount of mouse IgG.

terms of molecular structure to high-pH fusion mutations that have been reported in previous studies (29, 51). The significance that can be attributed to the selection of high-pH fusion mutants when HA mutant viruses are generated using reverse genetics techniques and propagated is not known. Perhaps the various well-utilized laboratory strains with histories of passage in eggs can select for higher-pH fusion mutants when tissue-culture-based techniques involved in the reverse genetics process are utilized (29).

The currently utilized vaccine against anthrax is composed of a preparation of bacterial supernatant containing the PA protein (11) and requires multiple vaccinations over several months. Our DNA and virus vaccination experiments using mice show that immune responses to both the HA and the PA components of the chimeric proteins are generated. The mouse immunization experiments with recombinant viruses demonstrate that immune responses against both HA and PA domains can be elicited following a single inoculation with a low dose of virus. Furthermore, the immune responses to the PA components of the chimeric HA-PA proteins were greater than those detected for the domain 1′ and domain 4 PA regions expressed on their own. This suggests that chimeric HAs may provide a useful tool for the expression of foreign protein domains for vaccine purposes. With the chimeric proteins described in this study, the expressed molecules were completely or partially degraded when HA1-HA2 fusion activation cleavage occurred, depending on conditions. This was also observed for the influenza viruses generated by reverse genetics. However, as DNA or subunit vaccine components or as live attenuated vaccine candidates, this might not be a negative factor. The degradation products of the PA domains may be capable of binding to major histocompatibility complex proteins and contributing to effective immune responses. As inactivated vaccine candidates, further modification of the inserted sequences and sites of insertion will be required, and these studies are being pursued.

With the approaches described here and other approaches involving influenza virus-based DNA, protein, or virus vaccines, the issue of preexisting immunity in vaccinees must be considered. This will require further study, but with our expanding structural knowledge of the 15 HA antigenic subtypes and the mechanisms of virus neutralization, it is not difficult to envisage strategies to circumvent the potential difficulties. We anticipate that there will be progress on these fronts and that it will be possible to utilize approaches such as those described here for the design of vaccines against a variety of pathogens.

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