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Influenza B Virus With Modified Hemagglutinin Cleavage Site as a Novel Attenuated Live Vaccine

Jürgen Stech[1], Holger Garn[2], Astrid Herwig[3], Olga Stech[1], Bianca Dauber[4],[a], Thorsten Wolff[4], Thomas C. Mettenleiter[1] and Hans-Dieter Klenk[3]

[1]Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald, Insel Riems
[2]Institut für Labormedizin und Pathobiochemie–Molekulare Diagnostik, Medizinische Fakultät, Biomedizinisches Forschungszentrum
[3]Institut für Virologie, Fachbereich Medizin, Philipps-Universität Marburg
[4]Division of Influenza/Respiratory Viruses, Robert Koch-Institut, Berlin, Germany

Abstract

Background. *Both pandemic and interpandemic influenza is associated with high morbidity and mortality worldwide. Seasonal epidemics are caused by both influenza A and B virus strains that cocirculate with varying predominance and may give rise to severe illness equally. According to World Health Organization recommendations, current annual vaccines are composed of 2 type A and 1 type B virus-specific component.*

Methods. *As a novel attenuated live vaccine against influenza B virus, we generated a hemagglutinin cleavage site mutant of strain B/Lee/40 by replacing the common monobasic cleavage site recognized by trypsinlike proteases with an elastase-sensitive site, and we investigated the in vitro properties, attenuation, humoral responses, and efficacy in mice.*

Results. *This mutant virus replicated in cell culture equally well as the wild type but in a strictly elastase-dependent manner. In contrast to the mouse-pathogenic parental virus, the cleavage site mutant was fully attenuated in mice and not detectable in their lungs. After 1 intranasal immunization, the animals survived lethal challenge with wild-type virus without weight loss or any other signs of disease. Furthermore, no challenge virus could be reisolated from the lungs of vaccinated mice.*

Conclusions. *These findings demonstrate that proteolytic activation mutants can serve as live vaccine against influenza B virus*.

Among respiratory viruses, influenza A and B viruses pose one of the most significant public health problems worldwide, because they cause outbreaks of mild and severe respiratory disease each season on a global scale. Both type A and B viruses require interventions due to their effect on public health. Although the influenza B virus-associated mortality is lower than that observed with type A strains of the H3N2 subtype, it is still higher than with A/H1N1 strains. Moreover, influenza B strains become the major cause of the seasonal epidemics every 2–4 years [1].
Current established inactivated or live attenuated vaccines against seasonal influenza contain 2 type A and 1 type B component addressing the cocirculation of A/H1, A/H3, and type B virus strains. Although the hemagglutinin (HA) evolution rate of influenza B viruses is slower than in influenza A viruses, single amino acid changes can be sufficient for antigenic escape, most likely owing to a smaller number of nonoverlapping epitopes forming one continuous antigenic site [2–8]. Furthermore, beside antigenic drift, the emergence of new influenza B strains is driven by insertion-deletion mutations and reassortment [9]. In particular since the late 1980s, the coexistence of 2 alternating HA lineages named after their prototype strains B/Victoria/2/87 and B/Yamagata/16/88 complicates the epidemiologic assessment. In some years a single lineage predominates, whereas in others both cocirculate [3, 7]. Such unpredictable prevalences led to a vaccine mismatch in 5 of 10

seasons between 2000 and 2010, meaning that the predominant influenza B virus strain belonged to the opposite HA lineage of the selected vaccine strain, resulting in low cross-protection and reduced efficacy [10–12].

To resolve those problems, the inclusion of 2 type B components in the yearly influenza vaccine—that is, a quadrivalent seasonal vaccine instead of the conventional trivalent preparation—had been proposed [10]. However, this concept involves more complex production and licensing procedures plus a decrease in vaccination doses available. An alternative could be an attenuated live vaccine, such as the cold-adapted [13–15], the NS1-deleted [16, 17], or the HA cleavage site mutants [18, 19]. The HA cleavage site is a promising target for virus attenuation, because activation cleavage of the HA0 precursor into the HA1 and HA2 fragments by host proteases is an essential step in the replication cycle of all influenza A and B virus strains [20, 21].
Only the cleaved HA can undergo a conformational change in the acidic milieu of the endosome after receptor-mediated endocytosis to expose the hydrophobic N terminus of the HA2 fragment for mediating fusion between endosomal and virion membranes [22–25]. Attenuated influenza A viruses that contained in their HA an elastase cleavage motif, were tested successfully elsewhere in mice and swine, relevant influenza virus hosts [18, 19, 26–28].
Whereas overall structure and domain organization of the HA from type A and B viruses are very similar, their sequence identity is as low as ∼20% [6]. In contrast to influenza A viruses [29], influenza B viruses do not become susceptible to furin activation after introduction of a polybasic cleavage site into HA [30]. Thus, there seem to be differences between influenza A and B viruses in the interaction of HA and activating proteases. Correspondingly, it remained an open question whether a cleavage site alteration of the type B virus HA would completely switch the proteolytic activation to another protease, resulting in strict dependence on that alternative enzyme. In this study, we generated an elastase cleavage site mutant from strain B/Lee/40 by reverse genetics to investigate its in vitro replication and potential to serve as an attenuated live vaccine.

Methods

Cells

The 293T human embryonic kidney cells were cultured in Dulbecco minimal essential medium (MEM) containing 10% fetal calf serum. Madin-Darby canine kidney (MDCK) cells were grown in MEM supplemented with 10% fetal calf serum.

Recombinant Viruses

For generation of recombinant influenza B viruses, we used for cotransfection the plasmids encoding all 8 genes of strain B/Lee/40, as described elsewhere [31]. Each gene segment is integrated into the pHW2000 plasmid vector [32] under control of the human RNA polymerase I promoter and mouse RNA polymerase I terminator and a truncated RNA polymerase II (immediate-early CMV) promoter. For rescue of wild-type B/Lee40-Wt, we used the original plasmids.
To engineer the elastase cleavage site, we replaced Arg-361 in the HA with valine or alanine by changing the nucleotides at positions 1114 and 1115 (corresponding to the published HA sequence with accession number FLBHAOA) from AG to GT or GC, respectively, using the QuikChange Kit (Stratagene). Primer sequences are available on request.

Virus Propagation and Titration

For propagation of B/Lee-Wt and the elastase cleavage site mutants on MDCK cells in MEM containing 0.2% bovine serum albumin, we used 2.0 µg/mL N-tosyl-l-phenylalanine chloromethyl ketone (TPCK)–treated trypsin (Sigma) or 5 µg/mL porcine pancreatic elastase (Serva Electrophoresis), respectively. We performed the plaque assays on MDCK cell monolayers essentially as described elsewhere [33]. For the elastase-dependent mutants, we used elastase instead of TPCK-

treated trypsin in the plaque overlay. For growth curves, we inoculated MDCK cells with virus at multiplicity of infection of 10^{-2} and determined the virus titer by plaque assay in the presence of the appropriate protease in 2 independent experiments. Formalin inactivation of virus was performed as described elsewhere [18].

Western Blot Analysis

We infected confluent MDCK monolayers, grown in 6-cm dishes, with virus at a multiplicity of infection of 10. All viruses were incubated in the presence of either TPCK-treated trypsin or elastase, or without any protease, for 16 hours in MEM containing 0.2% bovine serum albumin.
After sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10%) from pelleted supernatants, we used a monoclonal antibody to influenza B virus HA (Acris; 1:250; 1 hour at room temperature) and a mouse immunoglobulin (Ig) G–specific monoclonal antibody, conjugated with horseradish peroxidase as secondary antibody (Dako; 1:250; 1 hour at room temperature) followed by chemiluminescence (Supersignal West Pico Chemiluminescent Substrate Kit; Pierce).

Animal Experiments

Four-week-old female Balb/C mice (Charles River) were inoculated intranasally with 20 or 70 µL of B/Lee40-Ala or B/Lee40-Wt under anesthesia after an intramuscular injection of ketamine hydrochloride at a dosage of 200 mg/kg. At 12 hours or 1, 3, or 5 days after inoculation, we killed the animals by cervical dislocation and removed the entire organs (heart, lung, brain).
After homogenization in 1 mL of phosphate-buffered saline (PBS), we determined the organ titers by plaque assay in the presence of the appropriate protease in the overlay (333 µL of inoculum in plaque assay). Animal experiments were performed according to the German animal welfare legislation. All animal protocols had been approved by the relevant German authority.

Serum IgG Enzyme-Linked Immunosorbent Assay and Virus Neutralization Assay

We detected serum IgG antibodies by enzyme-linked immunosorbent assay. First, we coated Maxisorp 96-well plates (Nunc) with total B/Lee40-Wt virus protein at 4°C overnight. Then we detected the bound antibodies by mouse-specific IgG labeled with horseradish peroxidase (BD Pharmingen) and BM Blue POD substrate (Roche Diagnostics). Finally, we expressed the titers as the reciprocal of the dilution that yielded an optical density of 0.1. The virus neutralization test was performed after heat inactivation of the mouse serum samples at 56°C for 30 minutes.
First, we prediluted the inactivated serum samples at 1:10 and then we diluted them 2-fold serially and incubated them with 50 plaque-forming units (PFU) of virus for 1 hour at 37°C. Afterward, we infected MDCK cells (grown on 48-well plates) with the virus-serum mixtures, incubated the cells for 2 days, and determined the hemagglutination titers of supernatants from all wells. The highest serum dilution at which the hemagglutination test still gave a negative result is the virus neutralization titer. For calculation of the geometric mean, each serum was titrated in quadruplicate.

Results

Generation of Recombinant Viruses by Reverse Genetics

Proteolytic activation of the HA of influenza B viruses involves the cleavage of the precursor HA0 into the HA1 and HA2 fragments by trypsinlike host proteases [21], which require a monobasic cleavage motif. Using site-directed mutagenesis, we generated 2 protease activation mutants of the mouse-adapted influenza virus strain B/Lee/40 by replacing the arginine at the HA cleavage site at amino acid position 361 (corresponding to nucleotides 1114–1115) with valine or alanine.
These amino acids, followed by the conserved glycine of the N-terminus of HA2, form a motif susceptible to porcine pancreatic elastase [34]. For generation of the recombinant viruses by reverse genetics, we cotransfected the respective HA plasmid encoding either the wild type or the mutant

together with plasmids expressing the other 7 gene segments [31], resulting in virus progeny B/Lee40-Wt and the 2 protease activation mutants B/Lee40-Val and B/Lee40-Ala (Figure 1*A*).

Replication of HA Cleavage Site Mutants In Vitro Is Strictly Dependent on Elastase

Proteolytic activation of the HA is essential for multicycle replication of influenza viruses [21], as indicated by plaque formation. To study the dependence of B/Lee40-Val and B/Lee40-Ala on exogenous proteases, we performed plaque assays on MDCK cells with elastase or trypsin or without any exogenous protease. Whereas the parental B/Lee40-Wt yielded visible plaques in the presence of trypsin, both mutants B/Lee40-Val and B/Lee40-Ala formed plaques only in the presence of elastase (Figure 1*B*). Correspondingly, Western blot analysis demonstrated the susceptibility of HA0 of B/Lee40-Val and B/Lee40-Ala to elastase (Figure 1*C*).
The appearance of cleavage products in the presence of trypsin can presumably be attributed to arginine or lysine residues in the vicinity of the cleavage site (positions 350, 352, 356, and 359). Likewise, the HA of the parental B/Lee40-Wt was partially cleaved by elastase, probably because of glycine residues adjacent to the cleavage site (positions 348, 362, 365, and 369). However, this cleaved HA is not fusion competent, as indicated by the plaque assay (Figure 1*B*). Cleavage of B/Lee40-Wt HA0 can be observed to a minimal extent in the absence of any exogenous protease, suggesting either detection of inoculum at minute amounts or low expression of a trypsinlike protease with monobasic specificity in MDCK cells (Figure 1*C*).
To study growth kinetics, MDCK cells were infected with B/Lee40-Wt in the presence of trypsin and with the cleavage site mutants B/Lee40-Val and B/Lee40-Ala in the presence of elastase. Whereas B/Lee-Val reached somewhat lower titers than B/Lee40-Wt, B/Lee40-Ala grew as well as the parental wild type in cell culture (Figure 1*D*). Taken together, the replacement of the monobasic cleavage site of the wild-type HA by an elastase motif leads to a strict dependence of viral growth on the addition of elastase to the culture medium. Furthermore, in the presence of elastase, the protease activation mutant B/Lee40-Ala demonstrated in vitro replication properties equivalent to its parental virus.

B/Lee40-Ala Is Attenuated in Mice

To assess the impact of the cleavage site alterations on virulence, we inoculated mice intranasally with 10^6 PFU (in 70 µL) of B/Lee40-Wt or B/Lee40-Ala. Animals infected with the former (n = 5) developed severe weight loss and died, whereas mice that had received the latter (n = 4) did not display any signs of disease or any weight loss (Figure 2*A*).
To assess the viral load, we determined viral titers in lungs at 12 hours or 1, 3, or 5 days after inoculation from 2 mice infected with 10^6 PFU (in 70 µL) by plaque assay, beginning with undiluted organ homogenate. The parent virus B/Lee40-Wt reached a titer of $\sim 10^7$ PFU/lung after 12 hours, which subsequently remained at this level. In contrast to its wild type, B/Lee40-Ala was not detected in the lungs of infected animals at any time point studied (Figure 2*B*). These observations support the notion that the protease activation mutant B/Lee40-Ala is severely restricted in in vivo replication and fully attenuated in mice.

Protection Against Lethal Challenge

To investigate the potential of B/Lee40-Ala to serve as live vaccine, we inoculated mice intranasally with either PBS (n = 8; 20 µL), formalin-inactivated B/Lee40-Ala (n = 3; 10^6 PFU in 70 µL) before inactivation), or live B/Lee40-Ala at dosages of 10^3 (n = 6), 10^4 (n = 7), 10^5 (n = 8), or 10^6 PFU (n = 8) (in 20 µL for all dosages) and challenged them 4 weeks later with a lethal dose of B/Lee-Wt (10^6 PFU in 70 µL) Six of 8 mock-immunized animals died. No animal that had received the inactivated B/Lee40-Ala preparation survived.
Two of 6 mice survived after immunization with 10^3 PFU B/Lee40-Ala. The surviving animals had severe weight loss and recovered during the observation period to a negligible extent (Figure 3*A*). In contrast, all animals that had received 10^4, 10^5, or 10^6 PFU B/Lee40-Ala survived (Table 1) without weight loss (Figure 3*A*) or any other signs of disease. To study the viral load after challenge, we killed 2 mice from each group 3 days after challenge, and determined viral titers from their lungs beginning with undiluted homogenate. The mice that had received PBS, inactivated B/Lee40-Ala, or 10^3 PFU live B/Lee40-Ala demonstrated virus titers of $\sim 10^6$ PFU/lung. In contrast, animals that had been

immunized with 10^4, 10^5, or 10^6 PFU of live virus displayed no detectable challenge virus in their lungs (Figure 3*B*), corresponding with the absence of symptoms. Taken together, these data demonstrate that the attenuated protease activation mutant B/Lee40-Ala can prevent infection and disease after lethal influenza B virus challenge.

Induction of Neutralizing Serum Antibodies

To determine the induction of serum antibodies, we inoculated mice intranasally (20 µL) with either PBS (n = 1); 10^3 (n = 4), 10^4 (n = 3), 10^5 (n = 5), or 10^6 PFU (n = 5) of B/Lee40-Ala; or 10^4 PFU of B/Lee40-Wt (n = 4). Serum samples were obtained 4 weeks later. Mice immunized with 10^4 or 10^3 PFU of B/Lee-Ala or with PBS displayed low or no detectable serum IgG and virus neutralization titers, respectively.
However, mice that had received 10^6 or 10^5 PFU of B/Lee40-Ala had notable serum titers (Figure 4*A* and 4*B*). The considerably higher serum IgG and neutralization titers after immunization with 10^4 PFU of B/Lee40-Wt could be attributed to prolonged replication at higher titers compared with B/Lee40-Ala (Figure 2*B*).

Discussion

Besides influenza A viruses, which perpetuate annual epidemics and have pandemic potential, the type B viruses are a major cause for seasonal influenza [1, 8]. Thus, conventional inactivated and cold-adapted live vaccines [13–15] include 2 type A and 1 type B components [35, 36]. However, the influenza B virus HA has split up into 2 different antigenic lineages in the late 1980s [3, 7]. Since then, the frequent lineage mismatch of the vaccine to the predominant strain has led to considerably reduced efficacy and effectiveness, because the type B component selected did not induce complete cross-protection against both lineages [10, 11].
Moreover, substantial antigenic differences between vaccine and circulating viruses may result in enhanced disease as demonstrated with an inactivated vaccine against type A viruses and homosubtypic challenge in pigs [37, 38]; the relevance of this problem in humans remains to be investigated. Furthermore, the induction of heterosubtypic immunity in mice and ferrets against influenza A viruses was found to be diminished by previous application of an inactivated vaccine, in contrast to prior infection with live virus [39–41].
Alternatives may be attenuated live vaccines like the cold-adapted [13–15], NS1-deleted [16, 17], or HA cleavage site mutants [18, 19]. The altered HA cleavage motif results in attenuation by strict dependence on a protease not accessible to the virus in vivo, leading to an effective block in virus propagation. Protease activation mutants requiring elastase had been generated from influenza A viruses and investigated in mice (mouse-adapted H1N1, high-pathogenic laboratory H7N7 strains and corresponding reassortants) and in pigs (H1N1 swine influenza virus). Those attenuated viruses elicited protection against homologous, homosubtypic, and heterosubtypic challenges, probably because they strongly activated both humoral and cell-based immune responses [18, 19, 26, 42].

In the current study, we investigated the suitability of HA cleavage site mutants as attenuated live vaccine against influenza B virus. Accordingly, we replaced the arginine at the HA cleavage site of the strain B/Lee/40 with valine or alanine, resulting in an elastase cleavage site motif [34]. Both mutants were proved to undergo strictly elastase-dependent multicycle replication.
Because the alanine mutant replicated in cell culture to the same titer as the parental unmodified virus, we further studied its in vivo properties and immunogenicity. In contrast to the wild-type parental virus, which is pathogenic for mice, this mutant was fully attenuated and not detectable in the lungs after inoculation of mice in a dose escalation study. After a single intranasal immunization, the mice survived a lethal challenge with wild-type virus without weight loss or other signs of disease, and no challenge virus was detected in lungs.

A frequent concern against the use of an influenza live attenuated vaccine is reversion or pseudo-reversion to a virulent phenotype. Previous passaging experiments with an elastase mutant from the influenza A virus strain A/WSN/33 (H1N1) indicated a reversion frequency of $\sim 10^{-7}$ [18]. We expect a similar probability of influenza B virus revertants, because the mutation rates are of the same magnitude for type A and B viruses [43]. Hence, the modified HA cleavage site should be combined with other attenuating modifications, such as cold adaptation or NS1 deletion [16, 17].

Immune escape as a result of even minor antigenic HA changes is a typical feature of the influenza B virus [3–5, 7] and is largely attributed to a smaller number of nonoverlapping epitopes forming one continuous antigenic site [2, 6, 8]. A live vaccine delivering both HA and the more conserved internal proteins might counterbalance this major shortcoming by induction of strong cell-mediated immunity, especially in elderly people in whom the protection against influenza A virus infection is mediated by effector T cells rather than antibodies [44, 45].
However, the frequent HA lineage mismatch has led to decreased efficacy of both the inactivated vaccines and the established cold-adapted live vaccines [11, 12]. Therefore, reliable stimulation of a protective cell-mediated response by an attenuated live vaccine might require 2 subsequent applications, as demonstrated for the elastase HA cleavage site mutant from the swine influenza A virus [26–28]. Cross-protection against heterologous type B strains in mice has been demonstrated for 2 attenuated live vaccines: the NS1-deleted and the cold-adapted attenuated mutants [46, 47]. However, in humans, the established cold-adapted live vaccine proved to not be cross-protective in case of HA lineage mismatch [10, 11].
At first view, those 2 findings seem contradictory ; however, a common feature of the mouse model is protection against drifted and even heterosubtypic strains after previous infection with live virus [19, 48]. Therefore, the potential of a type B cleavage site vaccine to elicit HA lineage-independent protection remains to be investigated in suitable animal models or even in human volunteers. Another possibility to counteract the lineage mismatch would be the proposed quadrivalent seasonal vaccine [10]. To avoid a reduction in available vaccine doses, however, this concept is probably more feasible with live vaccines, because considerably less antigen is required per dose than with inactivated vaccine preparations [18, 49].

Taken together, our data demonstrate that a protease activation mutant of influenza B virus is strictly elastase dependent and replicates as well as wild-type virus in vitro but is fully attenuated and can confer protection against infection and disease after lethal challenge in mice. Furthermore, the strict dependence on elastase would prevent establishment of the HA gene from the vaccine strain in the gene pool of circulating viruses, thereby restricting the emergence of novel HA reassortants.
In conclusion, our study indicates that HA cleavage site alteration for generation of live vaccines is feasible for both influenza A and B viruses.

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Potential conflicts of interest.

J. S. and H. D. K. have filed a patent application at the German Patent and Trade Mark Office. All other authors: report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Tables and Figures

Table 1. Survival After Lethal Challenge

Inoculum	Surviving mice/total mice, no.
Phosphate-buffered saline	2/8
B/Lee40-Ala	
Formalin inactivated	0/3
10^3 PFU	2/6
10^4 PFU	7/7
10^5 PFU	8/8
10^6 PFU	8/8

Number of surviving mice and total number of mice challenged with 10^6 plaque-forming units (PFU) of B/Lee40-Wt 4 weeks after intranasal immunization.

Figure 1. Recombinant influenza B viruses, B/Lee40-Wt, B/Lee40-Val, and B/Lee40-Ala, with their cleavage sites and in vitro phenotypes. *A*, Scheme of the hemagglutinin (HA) cleavage site region. *B*, Plaque assay on Madin-Darby canine kidney (MDCK) cells in the presence of either elastase or trypsin or in the absence of an exogenous protease. *C*, Western blot analysis of lysates from MDCK cells infected with the indicated viruses in the absence of an exogenous protease or in the presence of either trypsin or elastase. *D,* Growth curves of B/Lee40-Wt (*circles*) in the presence of trypsin and of B/Lee40-Val (*diamonds*) or B/Lee40-Ala (*squares*) in the presence of elastase. Abbreviation: PFU, plaque-forming units.

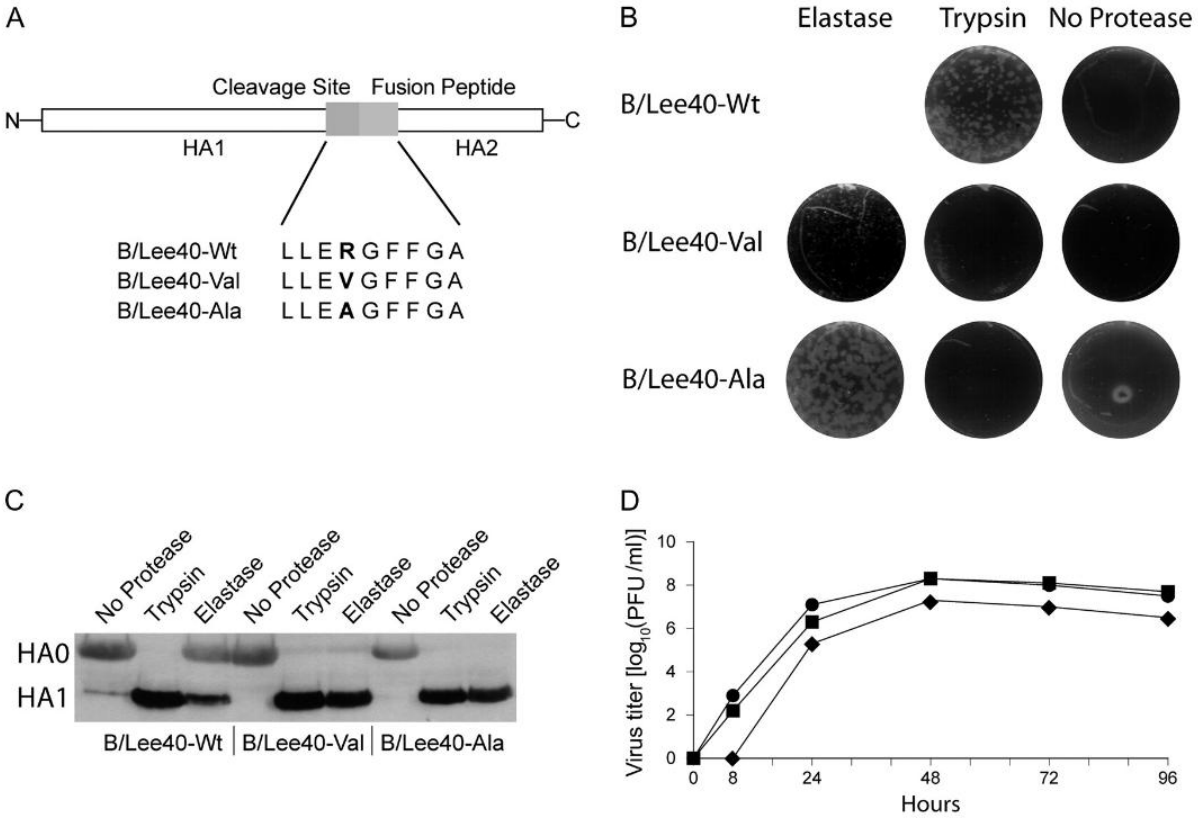

Figure 2. Viral pathogenicity and lung titers in mice. *A*, Weight curves from mice inoculated intranasally with 10^6 plaque-forming units (PFU) of either B/Lee40-Wt (*circles*) or B/Lee40-Ala (*squares*). *B*, Plaque assays from lungs taken 12 hours and 1, 3, and 5 days after intranasal inoculation with 10^6 PFU of B/Lee40-Wt or B/Lee40-Ala (titration beginning with undiluted lung homogenates).

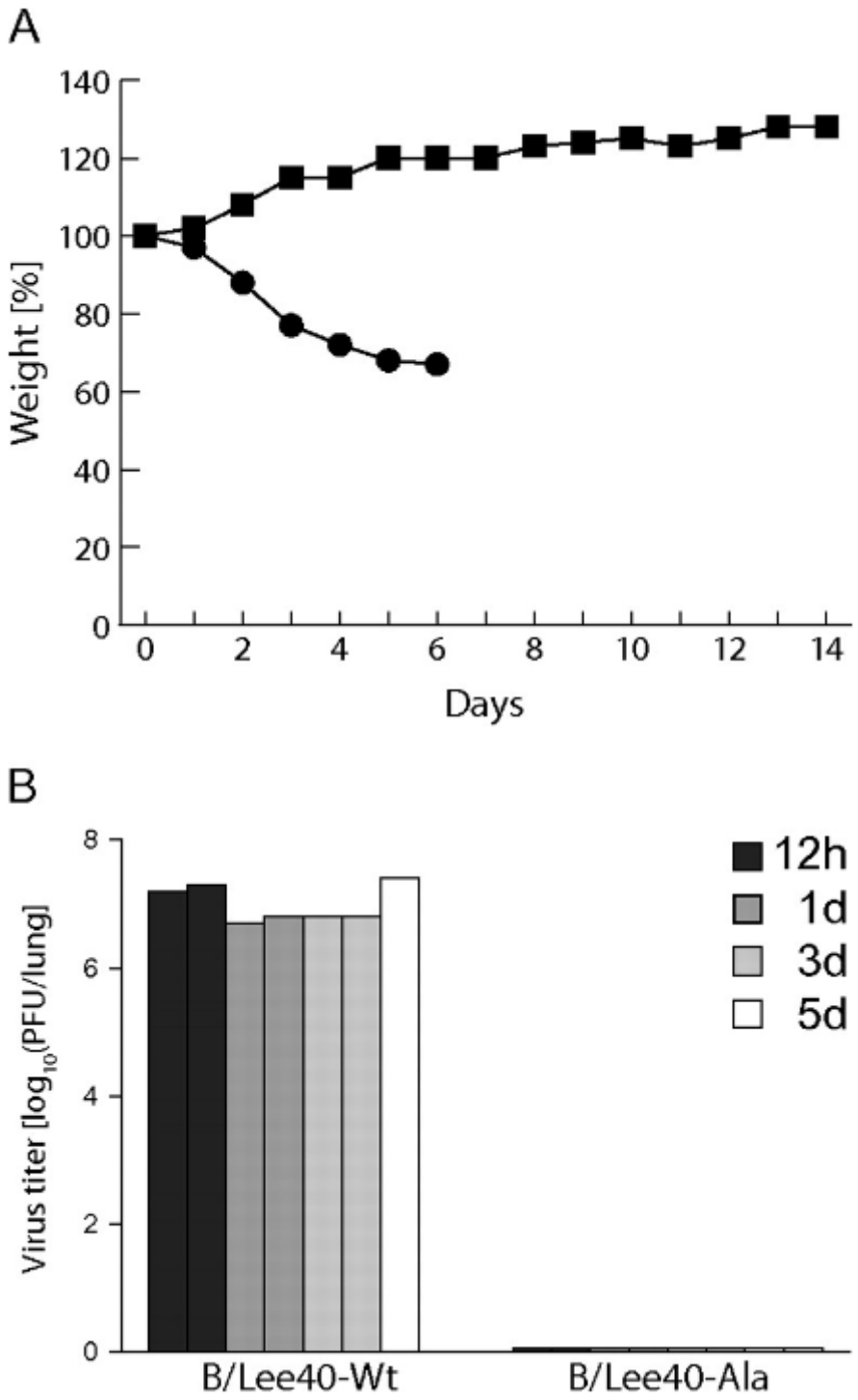

Figure 3. Immunization with B/Lee40-Ala confers protection against lethal challenge with B/Lee40-Wt. *A*, Average weight loss of mice challenged with 10^6 plaque-forming units (PFU) of B/Lee40-Wt 4 weeks after immunization with B/Lee40-Ala at dosages of 10^6 (*squares*), 10^5 (*diamonds*), 10^4 (*upside-down triangles*), or 10^3 (*right-side-up triangles*) PFU; formalin-inactivated B/Lee40-Ala (*filled circles*); or phosphate-buffered saline (PBS) (*open circles*). *B*, Virus titers in lungs from 2 mice in each group at day 3 after challenge, as determined by plaque assay.

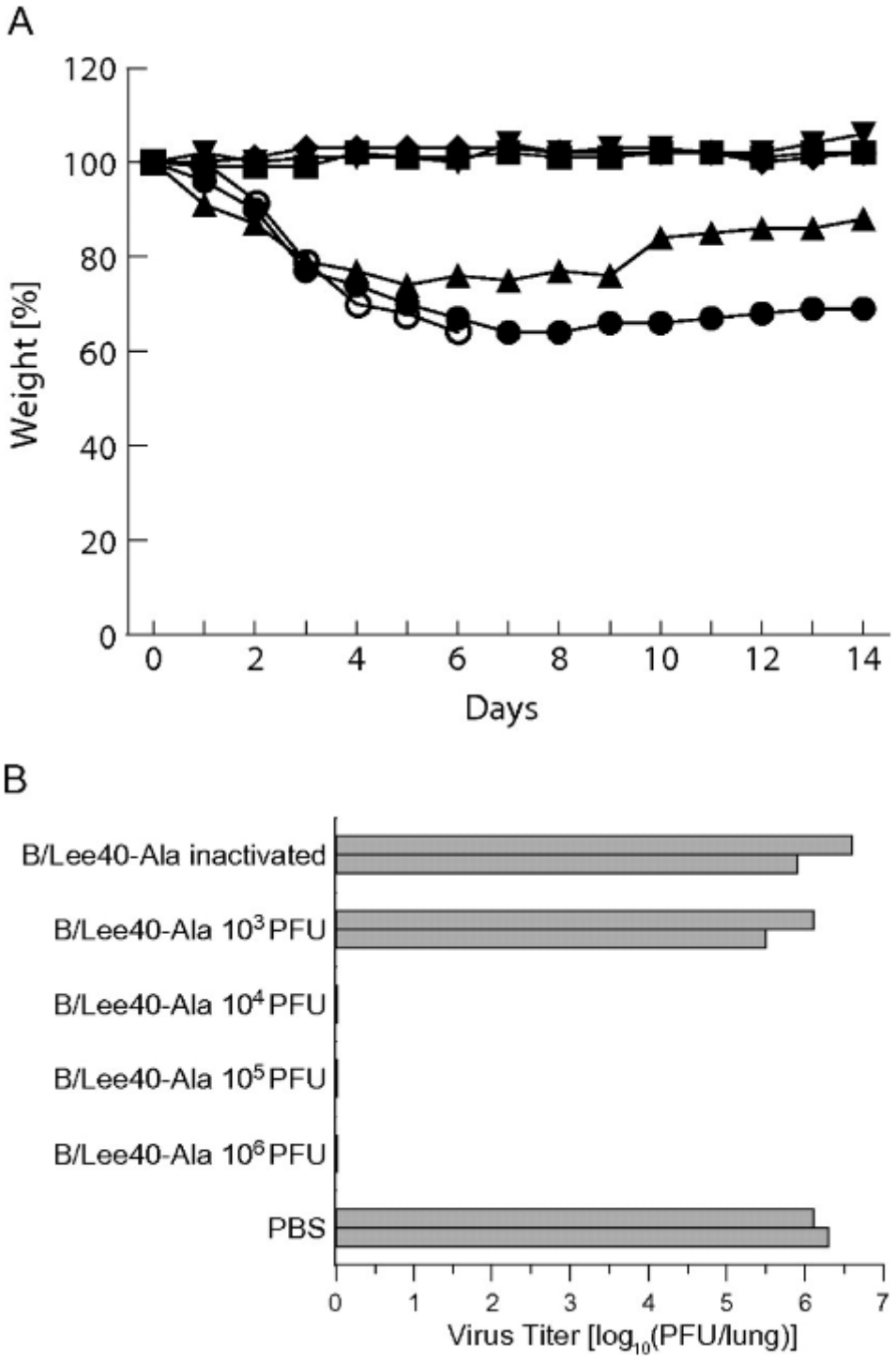

Figure 4. Humoral responses in mice after immunization, including immunoglobulin (Ig) G (*A*) and virus neutralization (*B*) titers from mice serum samples 4 weeks after mock-immunization with phosphate-buffered saline (PBS) or immunization with B/Lee40-Ala at dosages of 10^3, 10^4, 10^5, or 10^6 PFU or B/Lee40-Wt at 10^4 PFU.