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Generation of Influenza A Viruses with Chimeric (Type A/B) Hemagglutinins

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To gain insight into the intertypic incompatibility between type A and B influenza viruses, we focused on the hemagglutinin (HA) gene, systematically studying the compatibility of chimeric (type A/B) HAs with a type A genetic background. An attempt to generate a reassortant containing an intact type B HA segment in a type A virus background by reverse genetics was unsuccessful despite transcription of the type B HA segment by the type A polymerase complex. Although a type A virus with a chimeric HA segment comprising the entire coding sequence of the type B HA flanked by the noncoding sequence of the type A HA was viable, it replicated only marginally. Other chimeric viruses contained type A/B HAs possessing the type A noncoding region together with either the signal peptide or transmembrane/cytoplasmic region of type A virus or both, with the remaining regions derived from the type B HA. Each of these viruses grew to median tissue culture infectious doses of more than 10^5 per ml, but those with more type A HA regions replicated better, suggesting protein-protein interactions or increased HA segment incorporation into virions as contributing factors in the efficient growth of this series of viruses. All of these chimeric (A/B) HA viruses were attenuated in mice compared with wild-type A or B viruses. All animals intranasally immunized with a chimeric virus survived upon challenge with a lethal dose of wild-type type B virus. These results suggest a framework for the design of a novel live vaccine virus.

The genomes of influenza A and B viruses each consist of eight single-stranded RNA segments of negative polarity. Both types of viruses have two envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA is responsible for viral binding to cellular sialic acid-containing receptors, followed by fusion between envelope and cell endosomal membranes, while NA is responsible for viral release from infected cells through removal of sialic acids from cellular glycoconjugates and viral glycoproteins (11). Type A viruses are further divided into subtypes based on HA (H1 to H15) and NA (N1 to N9) antigenicities. In cells infected with two different type A viruses, intratypic reassortants possessing various combinations of gene segments are produced (24). However, intertypic reassortants between type A and B viruses have not been detected in nature, although both viruses are cocirculating in human populations.

Attempts to generate reassortants between type A and B viruses have been unsuccessful (9, 15, 21). With reverse genetics, Muster et al. (17) produced a mutant type A virus whose NA noncoding regions were replaced with those of the nonstructural (NS) gene of type B virus. This result suggests that at least the noncoding regions of the type B NS segment are compatible with influenza A viral components at the level of RNA transcription and replication. However, this mutant virus replicated more slowly than wild-type WSN virus in cell culture and was attenuated in mice (17). Animals infected with the chimeric virus were resistant to challenge with the wild-type virus, suggesting that such manipulation of the influenza virus genome might be useful in producing live attenuated vaccine strains.

Recently, we established a plasmid-based reverse genetics system (18) that has provided a powerful tool for generating recombinant influenza viruses. To gain insight into the intertypic incompatibility between type A and B viruses, we used this technology to study the functionality of chimeric (A/B) HAs and then evaluated the potential of the chimeric (A/B) HA viruses as live vaccines.

MATERIALS AND METHODS

Cells. 293T human embryonic kidney cells and COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) with 5% newborn calf serum and antibiotics. Cells were maintained at 37°C in 5% CO_2.

Construction of plasmids. The generation of plasmid constructs for viral RNA production (pPolI), containing the HA genes of wild-type A/WSN/33 (H1N1, A/WSN) (pPolI-WSN-HA) and wild-type B/Lee/40 (B/Lee) (pPolI-B-HA) flanked by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator, was described in a previous publication (18). A series of chimeric (A/B) HA pPolI constructs (Fig. 1) were produced with these wild-type HA constructs, PCR amplification with ProofStart polymerase (Qiagen), and ligation. Primer sequences will be provided upon request. All constructs were sequenced to ensure the absence of unwanted mutations.

Biologic assays of HAs expressed in cell culture. Each chimeric (A/B) HA pPolI construct (1 μg) was transfected into COS-7 cells with Trans IT reagent (Mirus), together with four other pCAGGS-based plasmids (1 μg each) that

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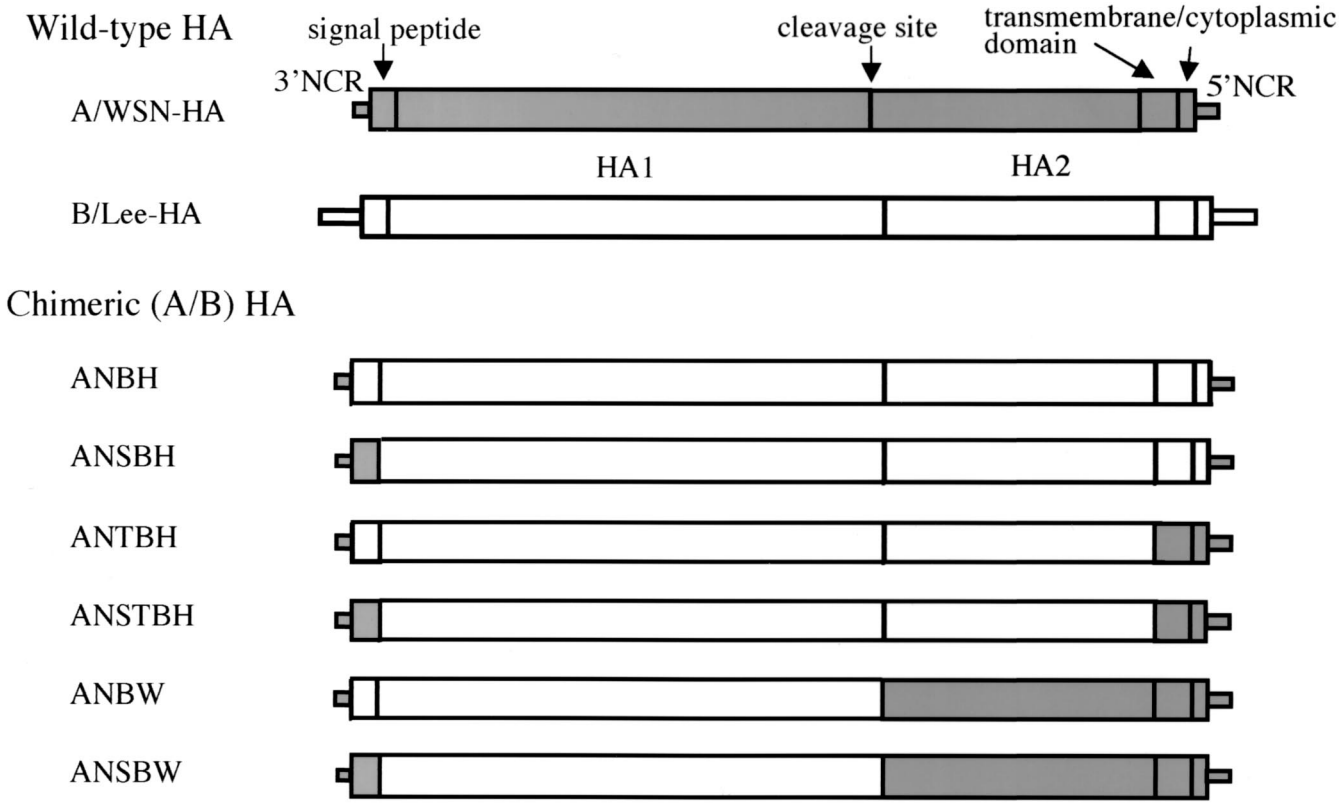

FIG. 1. Diagrams of chimeric (A/B) HA constructs. Chimeras were produced between the wild-type A/WSN virus HA (pPolI-WSN-HA) and the wild-type B/Lee virus HA (pPolI-B-HA) in a pPolI-based plasmid (pHH21) as described previously (18).

expressed three polymerase subunits (PA, PB1, and PB2) and the nucleoprotein (NP) of A/WSN virus (18). At 48 h after transfection, cells were treated with *Vibrio cholerae* sialidase (10 U/ml) and tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (2.5 μg/ml) at 37°C for 30 min. Cells were then fixed with 4% paraformaldehyde and immunostained with anti-B/HA antibody and a commercial ABC detection kit (Vector Laboratories). A hemadsorption assay was performed to assess the receptor-binding capacity of each HA. Briefly, transfected cells were incubated in a 1% chicken red blood cell suspension in phosphate-buffered saline at room temperature for 30 min and then washed five times before observation. A fusion assay in which transfected cells were incubated in HEPES buffer (pH 5.0) at 37°C for 5 min, followed by incubation in culture medium for 7 h, was also performed. After fixation with cold methanol, the cells were immunostained as described above.

Reverse genetics. Virus was generated by the plasmid-based A/WSN or B/Lee reverse genetics systems as described earlier (6a, 18). Viruses with wild-type genotypes produced from plasmids were designated A/WSN-R or B/Lee-R and used as controls in comparative analyses. To produce A/B chimeric viruses, we used chimeric HA-polymerase I constructs instead of pPolI-WSN-HA. Viruses produced from 293T cells were biologically cloned by limiting dilution, and stock viruses were produced in MDCK cells.

Experimental infections. The pathogenicity of the virus was tested in 4-week-old female BALB/c mice, anesthetized with sevoflurane and infected intranasally with A/B chimeric or wild-type viruses (10^5 tissue culture infectious doses [$TCID_{50}$]/50 μl). Survival and body weights were monitored for 14 days after infection. Three days later, some of the infected mice were euthanized for determination of virus titers in organs.

To evaluate the protective efficacy of each chimeric virus against wild-type challenge, we intranasally infected mice with chimeric or wild-type viruses (10^3 $TCID_{50}$/50 μl). Three weeks later, serum samples and tracheonasal washes were collected from subgroups of mice to detect virus-specific immunoglobulin A (IgA) or IgG antibodies. Four weeks after infection, the remaining mice were intranasally challenged with 50 50% lethal doses (LD_{50}) of wild-type virus (B/Lee-R) and monitored for survival and body weight over 14 days. Virus titers were determined in organs from some mice 3 days after challenge.

Detection of virus-specific antibody. Serum and tracheonasal wash samples were examined for IgA or IgG antibody by an enzyme-linked immunosorbent assay (ELISA) as previously described (10). Hemagglutination-inhibition (HI) antibody analysis of serum samples was performed after treatment with receptor-destroying enzyme (RDEII; Denka Seiken).

RESULTS

Construction of chimeric (A/B) HA genes. Figure 1 diagrams the series of chimeric constructs made with the HA genes from the A/WSN and B/Lee viruses. Since the noncoding sequences in both termini of the RNA segments are likely interchangeable between type A and B viruses during RNA transcription and replication (2, 3, 7, 17), we first made a chimeric HA gene (ANBH) that contains the noncoding sequences of type A virus and all coding sequences from type B virus. This construct produces an intact type B HA protein. We next made a chimeric gene containing a type A noncoding sequence, with the signal sequence of the type B coding region changed to that of type A virus (ANSBH). This construct produces an intact type B HA after removal of the type A signal peptide by the cellular signal peptidase. ANTBH is a chimeric gene in which the sequence encoding the transmembrane and cytoplasmic regions of the HA was changed from type B to type A, generating a chimeric (A/B) HA protein. In the ANSTBH chimera, sequences encoding both the signal and transmembrane/cytoplasmic regions were changed from type B to type A, so that the construct would produce the same chimeric HA protein as does ANTBH after removal of the signal peptide. ANBW

TABLE 1. Properties of chimeric HAs expressed in cells and viruses

HA construct	Property in cell culture[a]			Generation of virus possessing this gene[b]	Virus titer in supernatant of transfected cell[b] ($TCID_{50}$/ml)	Virus titer of stock[c] ($TCID_{50}$/ml)
	Cell surface expression	Hemadsorption	Fusion			
Wild type						
WSN-HA	+	+	+	+	3.2×10^7	6.3×10^7
B-HA	+	+	+	−	NA[d]	NA
Chimeric[e]						
ANBH	+	+	+	+	2.0×10	6.3×10^2
ANSBH	+	+	+	+	1.1×10^2	2.0×10^6
ANTBH	+	+	+	+	2.0×10^4	6.3×10^6
ANSTBH	+	+	+	+	1.1×10^6	3.6×10^6
ANBW	+	−	−	−	NA	NA
ANSBW	+	−	−	−	NA	NA

[a] Each HA construct was transfected into COS-7 cells together with type A polymerase- and NP-expressing plasmids. Biological assays were conducted at 48 h posttransfection.
[b] Virus possessing the wild-type or a chimeric HA gene together with other influenza A virus genes was generated by plasmid-based reverse genetics. At 48 h posttransfection, the supernatant of transfected 293T cells was harvested and titrated for infectivity.
[c] Virus stock was prepared with MDCK cells. Viruses were harvested when cytopathic effects became advanced.
[d] NA, not applicable.
[e] Descriptions of the A/B constructs are given in the first paragraph of Results.

designates a chimera in which all sequences upstream of the region corresponding to the HA cleavage site are from type B virus and all downstream regions are from type A virus. This construct produces a chimeric HA protein comprising the HA1 region of type B virus and the HA2 region of type A virus. Finally, we generated ANSBW, a chimeric gene in which the signal sequence within the ANBW construct was changed from type B to type A, yielding the same chimeric HA protein formed by ANBW.

Biologic properties of chimeric HAs in cell culture. To evaluate the functionality of the chimeric HAs, we transfected each pPolI HA construct into COS-7 cells together with type A virus PA-, PB1-, PB2-, and NP-expressing plasmids. All of the chimeric HA constructs were expressed on the cell surface. To test the receptor-binding activity of these HAs, we performed hemadsorption assays after treating the transfected cells with bacterial sialidase to remove terminal sialic acid from the HA oligosaccharide side chain, as the presence of this acid could interfere with the receptor-binding activity of the HA (12). ANBH-, ANSBH-, ANTBH-, and ANSTBH-expressing cells hemadsorbed, while those expressing ANBW and ANSBW did not (Table 1). Similarly, the HAs of all the chimeric constructs except those of ANBW and ANSBW induced cell fusion. These results indicate that all HA chimeras except ANBW and ANSBW were biologically functional. As anticipated from previous reports (2, 3, 7, 17), a functional type B HA was produced from the intact wild-type B HA segment by the type A polymerase complex and NP (Table 1), confirming compatibility between type B promoter structures and the type A polymerase complexes.

Production of viruses with chimeric HAs. To determine whether the chimeric HA genes function during influenza A virus infection, we attempted to produce a mutant WSN virus whose HA gene was replaced with a chimeric (A/B) HA gene. Plasmid-based reverse genetics allowed us to generate wild-type virus with a titer of approximately 10^7 $TCID_{50}$/ml (Table 1). When we used pPolI-B-HA instead of pPolI-WSN-HA, we were unable to generate an infectious virus. The four chimeric HA constructs that were biologically functional (Table 1) were successfully rescued in infectious type A virus, although with different efficiencies, as judged by virus titers in the supernatants of plasmid-transfected cells. The virus possessing the ANBH HA replicated only marginally, while that with the ANSTBH HA was produced at the highest efficiency and grew to more than 10^6 $TCID_{50}$/ml. The other two chimeric HA genes that did not express biologically functional proteins failed to support viral growth.

To confirm that the chimeric viral constructs indeed contained the type B HA ectodomain, we infected MDCK cells with these viruses and tested their reactivity with antibodies to the HA of type A and B viruses (Fig. 2). The cells infected with the viruses containing chimeric HA constructs reacted with anti-B HA as well as anti-A NP antibodies, but not with anti-A HA antibody, confirming the presence of type B HA ectodomains.

Growth characteristics of chimeric HA viruses in cell culture. To determine the replicative properties of the chimeric viruses, we infected cells at a multiplicity of infection of 0.01 and examined the kinetics of viral growth (Fig. 3). Although none of the viruses with the chimeric HAs grew better than wild-type A virus, ANSTBH and ANTBH grew to nearly 10^6 $TCID_{50}$/ml. In contrast to the results with both wild-type A and B viruses, each chimeric virus formed only pinpoint plaques by immunostaining (data not shown).

Replication of chimeric HA viruses in mice. The restricted replication of the chimeric viruses in cell culture suggested their attenuation in vivo. We therefore intranasally inoculated mice with the series of chimeric (A/B) viruses (10^5 $TCID_{50}$/50 μl). ANBH virus was not tested because the titer of the stock was too low (approximately 10^3 $TCID_{50}$/ml). None of the other three chimeric test viruses killed mice, whereas equivalent doses of wild-type A or B virus killed all or seven of eight infected mice, respectively (Table 2). Chimeric viruses were recovered from lungs and nasal turbinates on day 3 postinoculation, indicating their ability to replicate in mice. Chimeric viruses were restricted in lung tissue but less so in nasal turbinates by comparison with wild-type viruses, suggesting a link between viral replication capacity in the lung and lethality.

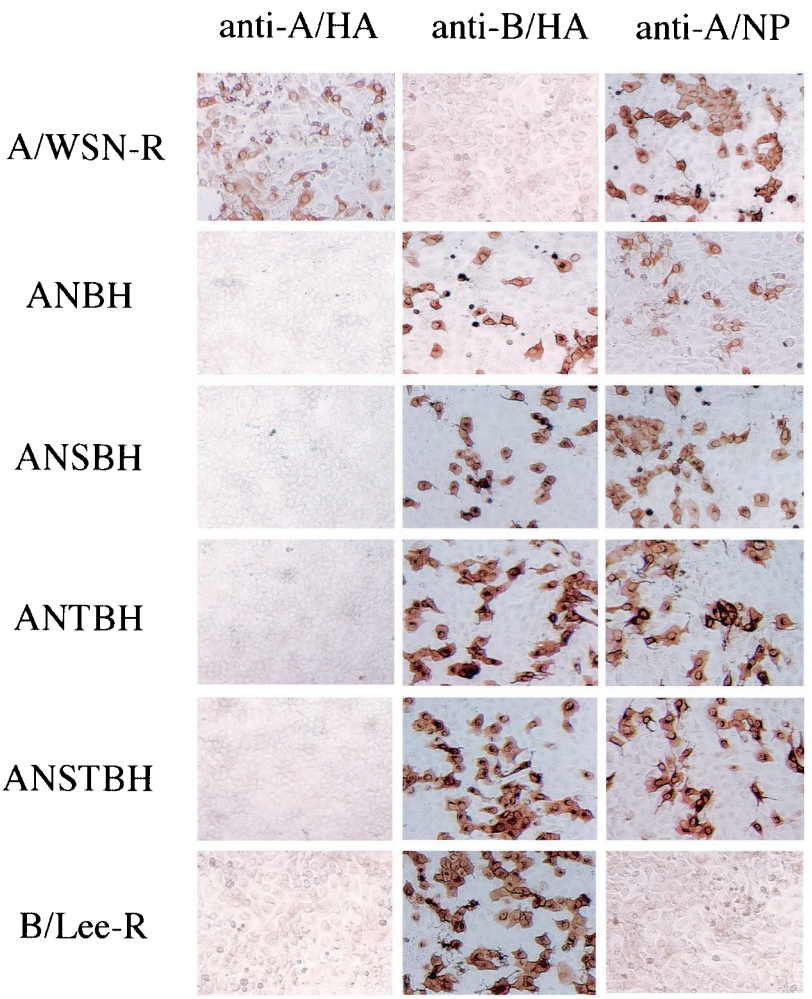

FIG. 2. Expression of type B HA by chimeric (A/B) HA viruses. MDCK cells infected with each virus were fixed 24 h postinfection and immunostained with anti-A HA, anti-B HA, or anti-A NP antibodies.

Mice infected with the ANTBH and ANSTBH chimeric viruses lost weight, though not to the same extent as mice infected with wild-type A viruses. Taken together, these data indicate attenuation of chimeric (A/B) HA viruses in mice.

Protection of mice immunized with chimeric HA viruses from wild-type virus infection. Since all of these chimeric viruses express an HA whose ectodomain was derived from type B virus, they were expected to provide a protective immune response against wild-type B virus infection. Before the challenge experiments, we evaluated whether chimeric virus infection elicited anti-B antibodies in mice. At 3 weeks postinoculation, ELISA demonstrated type B virus-specific IgA in tracheonasal wash samples and IgG antibodies in serum samples (Fig. 4A). HI antibodies were also detected in serum samples from chimeric virus-infected mice (Fig. 4B). Thus, all mice infected with a chimeric virus showed specific antibody responses, although the response to ANSBH virus was weaker than that to other viruses.

When challenged with 50 LD_{50} of wild-type B virus, all mice immunized with a chimeric virus survived, compared with none of the mock-immunized controls and only two of the eight mice immunized with WSN virus at a sublethal dose (10^3 $TCID_{50}$) (Table 3). These results indicate a specific protective effect of chimeric virus immunization against wild-type B virus infection. Moreover, type B virus was not recovered from the nasal turbinates or lungs of any mice immunized with a chimeric virus, excluding one animal given ANSBH virus at 3 days postchallenge (data not shown).

DISCUSSION

Attempts to generate A/B reassortants have not been successful (9, 15, 21). Here we demonstrate that reverse genetics can be used to construct an influenza virus possessing a type B HA gene in a type A genetic background. What are the constraints in generating such viruses? First of all, chimeric genes must be transcribed and replicated to confer viral infectivity. Although conserved among viruses of the same type, terminal sequences at both ends of noncoding regions, which contain promoter sequences needed for RNA transcription and replication (13), differ between type A and B RNA segments (2, 3). However, a previous study showed that a reporter gene flanked by a noncoding sequence of the type B virus NS segment was transcribed and replicated by a type A polymerase. Moreover,

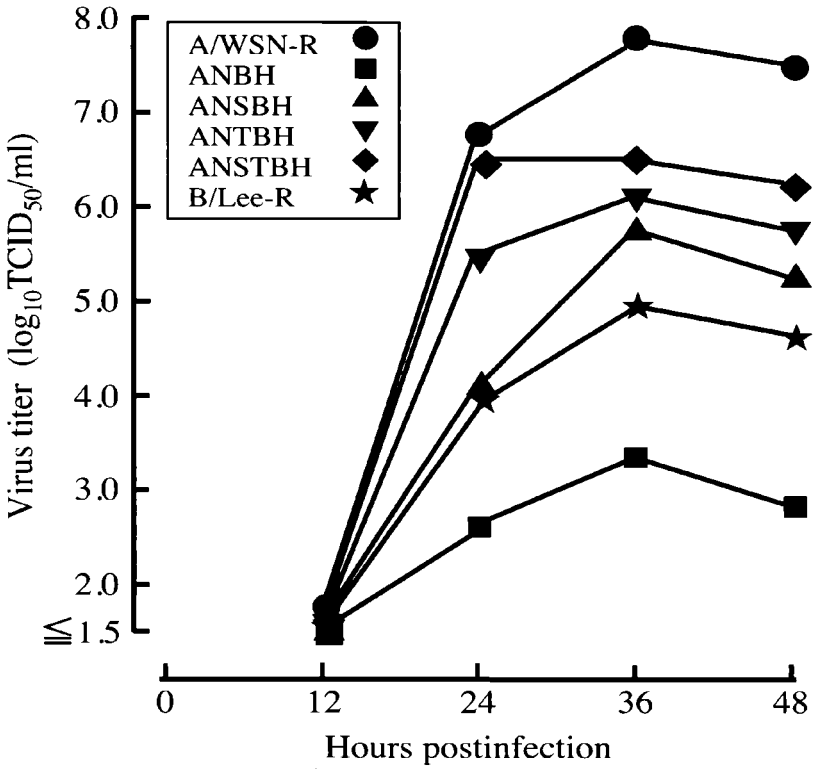

FIG. 3. Growth properties of chimeric HA viruses. MDCK cells were infected with each virus at a multiplicity of infection of 0.01 $TCID_{50}$ and monitored for virus growth. One of two independent experiments with similar results is shown.

a chimeric (A/B) influenza virus (NA/B-NS) containing a chimeric gene comprising the coding sequence of the type A virus NA and the noncoding sequence of the type B virus NS was produced (17). These data indicate that the type A polymerase complex recognizes the promoter sequence of the type B NS gene, although to a lesser extent than the homologous promoter of the type A virus gene.

The noncoding sequence of each RNA segment includes two structural regions, terminal sequences that are conserved among all eight RNA segments and inner segment-specific sequences. Since promoter activity is mainly determined by the former region (19), all type B gene segments are likely to be transcribed and replicated by the type A polymerase complex. In fact, this concept is supported by our data showing that the type B HA was expressed in cells cotransfected with pPolI-B-HA containing type B noncoding regions and type A polymerase complex- and NP-expressing plasmids (Table 1). Thus, the failure to generate a virus containing an intact type B HA segment (an HA intertypic reassortant) cannot be explained by the lack of RNA transcription and replication.

Alternatively, the restriction on generating a chimeric virus may originate at the level of RNA segment incorporation into virions; that is, the chimeric segment must be packaged into virions before an infectious virus can be generated. Although

TABLE 2. Pathogenicity of chimeric HA viruses in mice[a]

Virus	Mean virus titer ± SD		Mean change in body wt (%) ± SD		% dead (no. dead/8 tested)
	Nasal turbinates	Lungs	Day 5	Day 14	
Wild type					
A/WSN-R	5.0 ± 0.3	8.2 ± 0.1	−27.4 ± 1.1	NA[b]	100 (8)
B/Lee-R	4.7 ± 0.1	5.6 ± 0.1	−19.3 ± 7.9	(−11.7)	87.5 (7)
Chimeric					
ANSBH	4.0 ± 0.3	2.8 ± 0.3	2.6 ± 1.0	4.6 ± 1.2	0 (0)
ANTBH	5.3 ± 0.3	4.9 ± 0.1	−17.3 ± 0.7	−8.3 ± 0.4	0 (0)
ANSTBH	5.3 ± 0.4	4.6 ± 0.1	−20.9 ± 0.3	−6.2 ± 8.8	0 (0)
Control (PBS)	NA	NA	2.9 ± 1.3	7.1 ± 0.2	0 (0)

[a] Mice were intranasally inoculated with virus (10^5 $TCID_{50}$) and monitored for 14 days. Virus titers were determined as $\log_{10}$ $TCID_{50}$/g at 3 days postinoculation and reported as means ± standard deviation ($n = 3$). Changes in body weight are reported as means ± standard deviations ($n = 3$). The data in parentheses are from one mouse. Control mice were mock inoculated with phosphate-buffered saline (PBS).

[b] NA, not applicable.

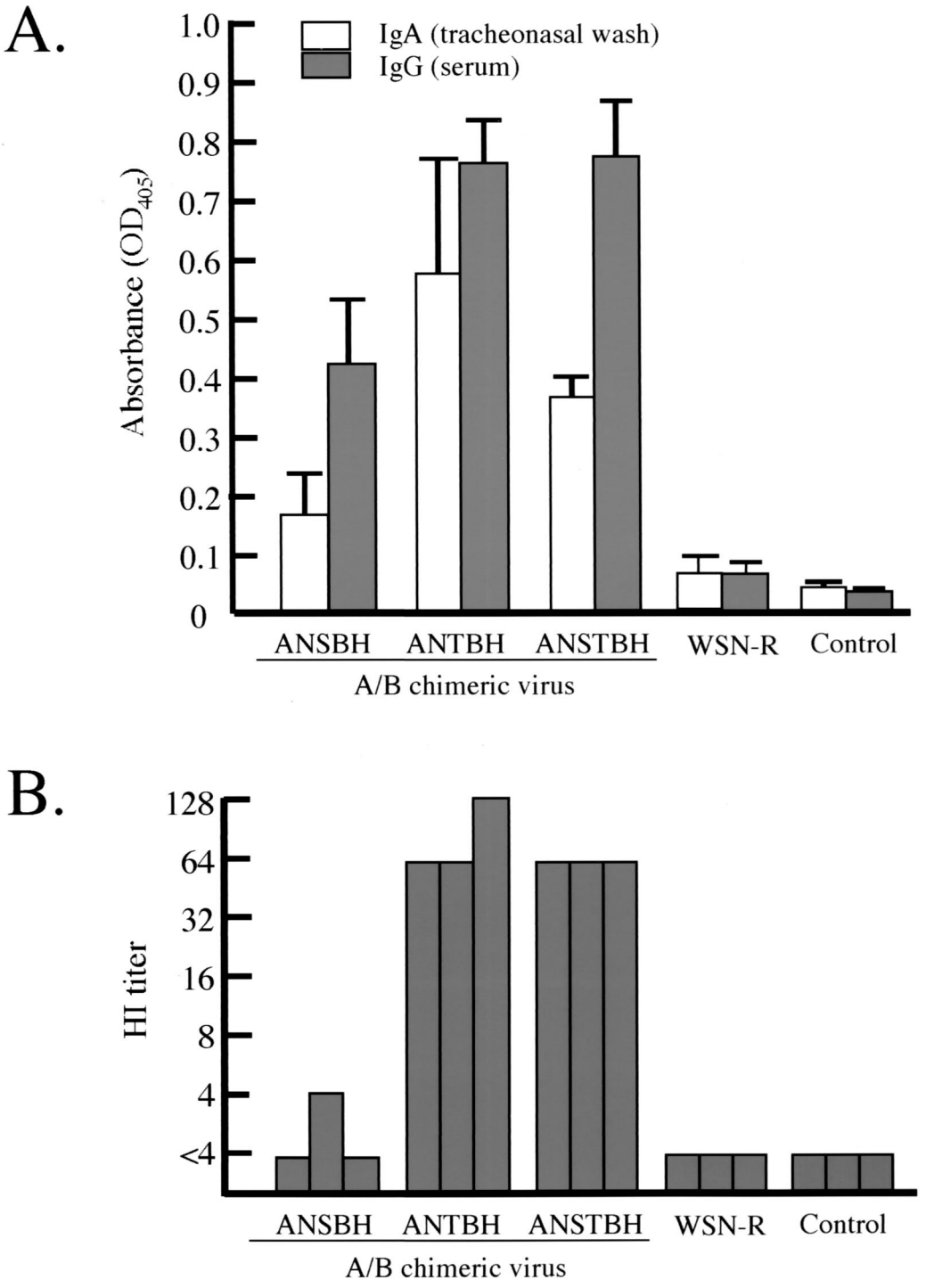

FIG. 4. Antibody response to type B virus in mice inoculated with chimeric HA viruses. (A) Mice (three/group) were intranasally inoculated with each virus (10^3 $TCID_{50}$). Three weeks postinoculation, tracheonasal washes and serum samples were taken from mice and tested for anti-B virus-specific IgA (tracheonasal wash) and IgG (serum) antibodies in an ELISA. Absorbance values (optical density at 405 nm [OD_{405}]) are reported as means ± standard deviation ($n = 3$). (B) HI titers were also tested in serum samples. Each bar indicates the titer for an individual mouse infected with the indicated chimeric virus.

the noncoding region of the type A NS segment was reported to contain an RNA packaging signal (13), the packaging mechanism has not been fully elucidated. Although the sequences or structural features of the RNA segments required for their incorporation into virions remain largely unknown, we recently showed that the type A NA RNA segment possesses virion incorporation signals at both ends of the coding regions (5).

In the present study, the ANSBH virus replicated more

TABLE 3. Protection of mice immunized with chimeric HA viruses against wild-type B virus challenge[a]

Virus used for immunization	Mean change in body wt (%) ± SD		Survival rate (%) (no. surviving/8 tested)
	Day 5	Day 14	
Wild type			
A/WSN-R	−17.5 ± 3.6	(−8.1 ± 2.5)	25 (2)
B/Lee-R	1.8 ± 0.9	1.4 ± 0.6	100 (8)
Chimeras			
ANSBH	−5.6 ± 0.8	−0.7 ± 0.7	100 (8)
ANTBH	0.9 ± 0.9	1.9 ± 0.9	100 (8)
ANSTBH	1.5 ± 0.2	2.9 ± 0.7	100 (8)
Control (PBS)	−20.8 ± 0.5	NA[b]	0 (0)

[a] Mice were intranasally infected with each virus (10^3 $TCID_{50}$). Four weeks postimmunization, mice were intranasally challenged with wild-type B/Lee-R virus (50 LD_{50}) and monitored for 14 days after challenge. Changes in body weight are reported as means ($n = 3$). The data in parentheses are from two mice. Control mice were mock immunized with phosphate-buffered saline (PBS) and challenged with wild-type B/Lee-R virus.

[b] NA, not applicable.

efficiently than ANBH virus (Fig. 3 and Table 1). Since the HA proteins expressed in these two viruses should be identical, the difference in replicative efficiency may reflect different RNA packaging efficiencies. A structural feature required for efficient RNA packaging may reside in the signal-encoding region of the HA gene. The difference in replicative efficiency between the ANTBH and ANSTBH viruses (expressing identical HA proteins) might be explained by the same reason. In fact, recent data from our laboratory indicate that the packaging signal for the type A HA segment resides at both ends of the coding region (T. Watanabe and Y. Kawaoka, unpublished data). Interestingly, a chimeric NA segment containing the noncoding sequence of the type A virus NA and the coding sequence of the type B NA was not rescued into type A virus (6). This failure may be explained by the lack of a type A NA coding region that contains an RNA packaging signal, in accord with our recent findings (5).

The generation of chimeric (A/B) influenza viruses may also entail critical interactions at the protein level to ensure that chimeric proteins are packaged into virions and can function in viral replication. In one study, a type B NA protein supplied in *trans* was incorporated into type A virions and replaced the function of a deleted type A NA, supporting multiple cycles of replication of an NA-defective type A virus in cell culture (6); however, a type A virus containing the type B NA was not generated. Although we were able to generate chimeric (A/B) HA viruses, they did not replicate as well as the wild-type virus, possibly because of suboptimal HA receptor binding or fusion activity or a suboptimal balance between the receptor-binding activity of the type B HA and the sialidase activity of the type A NA (16, 22). Finally, the transmembrane/cytoplasmic domains of the type A HA may be required for proper interactions with the type A M1 protein, leading to efficient virion assembly (1, 4, 8, 25).

The chimeric viruses in this study not only were attenuated in mice, showing restricted replication in lung tissue, but also conferred protective immunity to mice against wild-type B virus infection, suggesting a novel approach to the development of influenza vaccines. The current trivalent inactivated influenza vaccines, administrated by subcutaneous injection, are used worldwide, yet their efficacies remain suboptimal, primarily because of inadequate stimulation of mucosal immunity in the upper respiratory tract, the site initially invaded by influenza viruses (23). Hence, these vaccines do not prevent viral infection; they only lessen the severity of the illness. Unlike inactivated vaccines, live vaccines induce both mucosal and cytotoxic T-cell-mediated immune resposes. Our study suggests that judicious manipulation of the HA gene can generate chimeric (A/B) viruses with various degrees of attenuation, allowing one to produce live vaccine strains with an appropriate balance between attenuation and immunogenicity. Alternatively, one could incorporate the chimeric HAs into a cold-adapted A virus whose attenuating mutations are well characterized (14). Current cold-adapted live vaccines are mixtures of type A and type B viruses, raising concern that interference between the components of these two viruses might affect vaccine efficacy, although this possibility has been addressed by fine tuning the ratio of virus doses (20). A type A virus possessing a chimeric (A/B) HA protein would enable the production of live influenza vaccines based on a single attenuated virus rather than two attenuated viruses, eliminating the risk of interference between type A and B viruses.

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