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Review

Principles Underlying the Development and Use of Live Attenuated Cold-Adapted Influenza A and B Virus Vaccines

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INTRODUCTION

THE COLD-ADAPTED (*ca*) influenza A and B viruses developed by Maassab show great promise for use as a live attenuated virus vaccine to protect against the upper and lower respiratory tract disease caused by the influenza viruses (8,52,58). Since influenza A and B viruses undergo continuous antigenic changes, the live attenuated virus vaccines, like the licensed influenza virus subunit vaccines, will need to be updated frequently to be able to protect against the newly emerged epidemic antigenic variants of influenza A and B viruses. There are 15 antigenically distinct hemagglutinins (H1–H15) and nine antigenically distinct neuraminidases (N1–N9) in influenza A viruses that infect birds or mammals (98). Currently, two antigenic subtypes of influenza A virus (H1N1 and H3N2) co-circulate with influenza B virus in humans. A trivalent *ca* vaccine is therefore needed to protect against each of these three influenza viruses.

The influenza A viruses possess a single-stranded, negative-sense RNA genome in eight segments that encode three polymerase proteins (98) (PB1, PB2, and PA); four membrane-associated proteins (the hemagglutinin [HA] glycoprotein, which mediates attachment and penetration; the neuraminidase [NA] protein, which mediates release of virus from the infected cell; the membrane protein [M], which lines the inner surface of the viral membrane and plays an essential role in virion structure and assembly, and the M2 protein, which forms an H^+ ion channel that is required for release of the nucleocapsid following viral penetration of the host cell); a nucleocapsid protein (NP); a nonstructural protein (NS1) that functions as an interferon antagonist; a nuclear-export structural protein (NEP); and a newly described PB1-F2 protein (for influenza A viruses) that induces apoptosis in macrophages and lymphocytes (12). The genome of the influenza B viruses is organized similarly, but the NA gene contains a second open reading frame that encodes a second protein, NB, and the M gene does not encode an M2 protein (50). The NB protein of influenza B virus is thought to have a similar function as the M2 protein of influenza A virus (50). The segmented nature of the influenza virus genome permits the exchange of viral genes between two viruses co-infecting the same cell (Fig. 1). Such reassortment occurs between two influenza A viruses or two influenza B viruses but not between influenza A and B viruses. Protective immunity to influenza A viruses is mediated primarily by serum IgG and mucosal IgA antibodies directed against the HA and NA, making it necessary that these two antigens are derived from the newly emerged antigenic variant virus (98) (Fig. 1). Heterosubtypic immunity, that is, resistance to infection with an influenza A virus conferred by previ-

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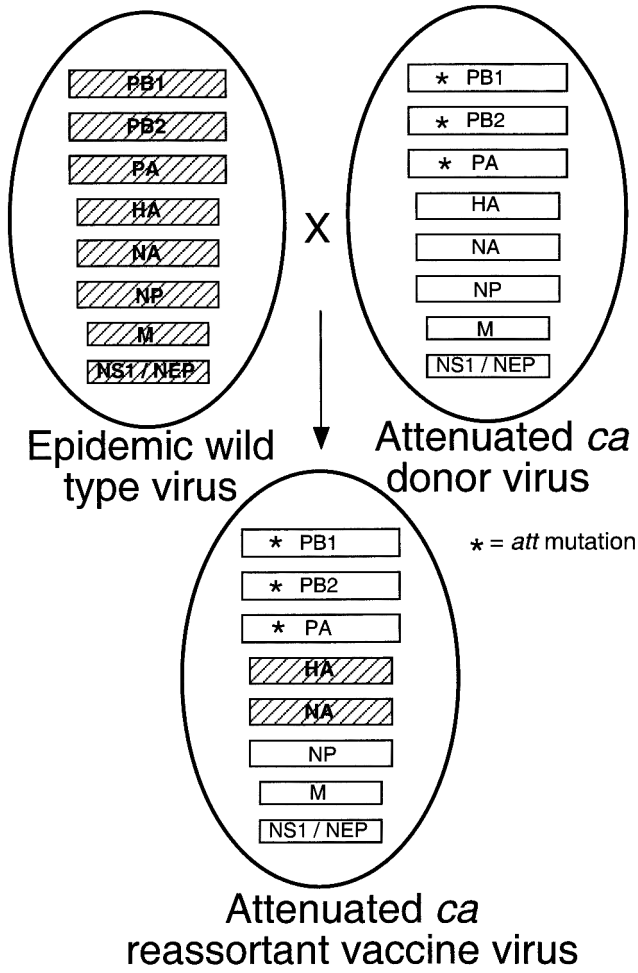

FIG. 1. Production of live attenuated influenza A or B reassortant virus vaccines that contain six genes from the *ca* donor virus bearing three hypothetical attenuating (*att*) mutations (*) and the HA and NA protective antigens from the newly emerged epidemic or pandemic wild-type parent. The epidemic wild-type and *ca* donor viruses dually infect a cell, and the attenuated reassortant virus is selected from amongst the progeny (58). These reassortants are referred to as 6/2 reassortants reflecting the number of RNA segments they inherit from the *ca* and wild-type parent viruses, respectively. Two *ca* donor viruses are needed, one to generate the live attenuated influenza A *ca* reassortants and another to generate the live attenuated influenza B *ca* reassortants.

ous infection with an influenza A virus belonging to another subtype, is weak in humans (61,80). Therefore, immunity is needed against each of the HA's and NA's of the viruses circulating in humans.

Two major concepts underlie the development of a live attenuated influenza virus vaccine. The first is that the attenuating mutations present in the attenuated donor virus must be on one or more of the six RNA segments that do not encode the HA and NA glycoproteins. This permits the ready transfer of these attenuating mutations to each newly emerged epidemic influenza A or B virus via gene reassortment (Fig. 1). In this process, a new *ca* reassortant virus is generated that contains the HA and NA genes of the new epidemic antigenic variant virus plus the remaining six RNA segments, which contain the attenuating mutations, from the *ca* donor virus. Such reassortant vaccine viruses are often referred to as 6/2 reassortants reflecting the number of segments that the reassortant receives from the attenuated donor virus and the newly emerged epidemic variant, respectively. Since gene reassortment does not occur between influenza A and B viruses, two donor viruses are needed, one to attenuate influenza A H1N1 and H3N2 viruses and one for

influenza B viruses. The *ca* virus vaccine differs from all previous live attenuated virus vaccines such as the trivalent poliovirus vaccine or the measles-mumps-rubella vaccine in that one or more of the three components of the trivalent *ca* vaccine likely will differ each year, a consequence of the need to update the HA's and NA's of last year's *ca* vaccine to protect against the new epidemic variants.

The second major concept underlying the development of the *ca* virus vaccine is that the transfer of the six RNA segments encoding internal viral proteins (i.e., those that do not encode the HA or NA) from the attenuated donor strain to the new epidemic virus must reproducibly confer upon each new reassortant vaccine virus a set of properties that include (a) a satisfactory level of attenuation; (b) stability of the attenuation phenotype following replication in humans; and (c) sufficient immunogenicity to protect against illness caused by wild type influenza virus. It is also necessary that the ability of the donor virus to confer this set of properties upon each new epidemic variant is highly reproducible and performed a sufficient number of times to preclude the need to extensively test each new reassortant vaccine virus as it is generated. This latter concept is practiced for the currently licensed subunit vaccine. Implicit in this approach is the assurance that the HA and NA genes in the new 6/2 *ca* reassortant vaccine virus do not significantly modify the level of attenuation specified by the attenuating mutations. Fortunately, this basic feature of the vaccine strategy has been substantiated by a large series of observations made over more than two decades (55,58).

This review includes five sections: (a) preclinical and clinical studies with monovalent and bivalent influenza A viruses; (b) preclinical and clinical studies with monovalent influenza B *ca* reassortant viruses and early formulations of multivalent influenza A and B *ca* reassortant viruses; (c) clinical studies with current formulations of trivalent influenza *ca* reassortant viruses; (d) suggested use of the live attenuated trivalent influenza *ca* vaccine; and (e) possible environmental issues arising from gene exchange between the *ca* viruses and other human, avian, or animal influenza A viruses that might occur during widespread use of the influenza A virus *ca* virus vaccines. This review will not provide the information summarized in a previous review (58), and the reader will be referred to that article for additional tabulated information.

PRECLINICAL AND CLINICAL STUDIES WITH MONOVALENT AND BIVALENT INFLUENZA A VIRUSES

Generation and characterization of the influenza A/AnnArbor(AA)/6/60 *ca* donor virus. The influenza A/AA/6/60 (H2N2) *ca* donor virus was generated by adaptation to replication at low temperature as indicated in Figure 2. The multiple passages at progressively lower temperatures in primary chicken kidney cell (PCKC) culture including biological cloning yielded the A/AA/6/60 *ca* donor virus that exhibited

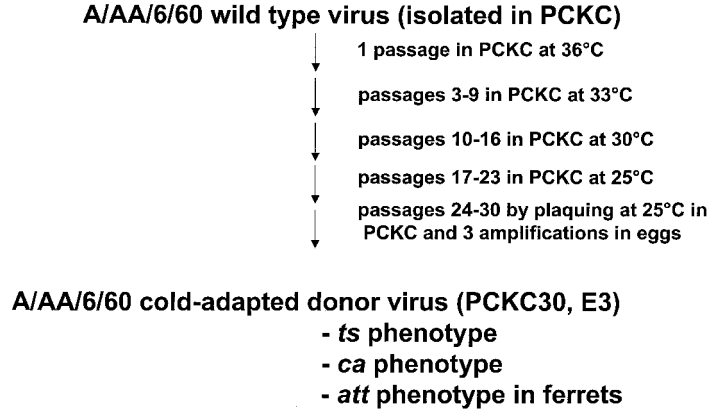

FIG. 2. Attenuation of the influenza A/AA/6/60 (H2N2) *ca* donor virus by passage in primary chicken kidney cells (PCKC) at successively lower temperatures (53). The influenza A/AA/6/60 (H2N2) *ca* donor virus exhibits three phenotypes, that is, the *ts*, *ca*, and *att* phenotypes. E indicates a passage in the allantoic cavity of embryonated chicken eggs.

TABLE 1. MUTATIONS PRESENT IN THE A/AA/6/60 *ca* DONOR VIRUS

RNA segment[a]	*Coding region*	*Other region*	*Mutation*	
			wt	ca
1	PB2		N 265[c]	S
2	PB1		K 391	E
			E 457	D
			E 581	G
			A 661	T
3	PA	3′ NCR[b]	K 613	E
			L 715	P
			T_{20}	–C
6	NP	5′ NCR	T 23	N
			D 34	G
			Insertion of A at 1550	
7	M2		A 86	S
8	NS1		A 153	T

[a]RNA segments 4 and 5 encode the HA and NA genes, respectively. These genes are not transferred from the *ca* donor virus to new reassortants.
[b]NCR, noncoding region of the indicated RNA segment. 3′ and 5′ are in vRNA sense.
[c]Number indicates the amino acid position in the indicated protein.
Silent nucleotide substitutions in the open reading frames of each gene are not included.

the temperature sensitive (*ts*), *ca*, and attenuation (*att*) phenotypes. A complete nucleotide sequence of the A/AA/6/60 *ca* donor virus has been reported and compared to that of its wild-type parent (24) (Table 1). The *ca* mutant virus sustained mutations in the coding region of six genes as well as in the noncoding region (NCR) of two of these genes. The *ca* and *ts* phenotypes of the *ca* donor virus and its reassortant viruses are illustrated in Figure 3. The *ca* donor and wild-type viruses each replicate efficiently at the permissive

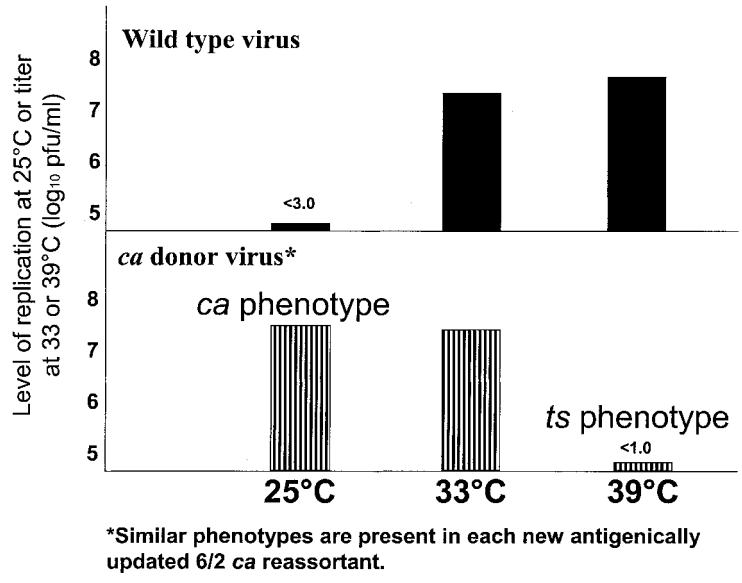

FIG. 3. The virus titer (log_{10} pfu/mL) of the *ca* donor and wild-type A/AA/6/60 H2N2 virus at 25°C, 33°C (permissive temperature), and 39°C identifies the *ts* and *ca* phenotypes of the *ca* donor virus. These phenotypes are also present in each of the 6/2 reassortants characterized to date. The level of replication of influenza A H1N1 and H3N2 wild-type viruses is variable at 25°C and at 39°C.

temperature of 33°C, but the *ca* donor virus is cold-adapted; that is, it also replicates efficiently at 25°C, a temperature restrictive for replication of many wild-type influenza A viruses. The *ca* donor virus and its reassortants are *ts*; that is, they are highly restricted in replication at 39°C, a temperature at which many wild-type influenza A viruses grow efficiently.

Level of replication and attenuation of the influenza A *ca* donor or its reassortant viruses in animals and humans. The influenza A *ca* donor virus and its H1N1 and H3N2 reassortants are restricted in replication in both the upper and lower respiratory tract of ferrets and rodents (mice and hamsters). A greater level of reduction of replication occurs in the warmer lower respiratory tract than in the cooler upper respiratory tract, a likely consequence of the temperature sensitivity of replication of the *ca* donor and its reassortant viruses. The reduction of replication of the *ca* donor virus and its reassortants in the upper respiratory tract of rodents and humans suggest that one or more of the attenuating mutations of the *ca* donor virus is a non-*ts* attenuating mutation operative at the low temperatures found in the upper respiratory tract. The details of the replication of the influenza A *ca* donor virus and its reassortants in animals will not be presented in this review, and the reader is referred to previous studies that provide this information (56,76). A *ca* reassortant was restricted in replication in the lower respiratory tract of chimpanzees, our closest primate relative that has a 37°C core body temperature similar to that of humans (79). The restriction of replication in the lower respiratory tract of the chimpanzee is consistent with the absence of symptoms of lower respiratory tract disease and the failure to detect significant alterations in pulmonary function (4) in adults administered *ca* reassortant viruses.

There is a direct correlation between the level of replication of wild-type influenza virus and the illness experienced by the host; replication to a peak titer of 10^5 to 10^7 is accompanied by high fever and lower respiratory tract disease, a peak titer of 10^3 to 10^4 is accompanied by mild disease in the upper respiratory tract (rhinitis and pharyngitis), and a peak titer below $10^{3.0}$ results in an asymptomatic infection (60,65). The level of replication and the illness experienced by adult volunteers selected to have a low serum influenza A virus antibody level following experimental infection with wild-type or *ca* H1N1 and H3N2 reassortants are illustrated in Figure 4 and Table 2 (14,18,20,63,67,74,76,82). During experimental infection of adult humans, wild-type virus replicates to a mean peak titer of about 10^4 to 10^6 $TCID_{50}$/mL and causes febrile or flu-like illness in 35–85% of subjects. In contrast, the *ca* reassortant viruses replicate to a peak mean titer of $10^{0.7}$ to $10^{2.6}$ $TCID_{50}$/mL and cause little febrile, systemic, or upper respiratory tract illness, the latter of which is seen in fewer than 10% of vaccinees. The level of replication of the *ca* reassortant viruses (Fig. 4) achieved in the vaccinees is, in most cases, below the threshold required for disease to develop even in the most susceptible subjects, namely, seronegative, immunologically naïve infants and young children (Fig. 4B).

The level of attenuation and the level of replication of the *ca* viruses was remarkably similar for each of the seven H3N2 and H1N1 reassortants tested (Table 2), indicating that the acquisition of the six internal RNA segments of the A/AA/6/60 *ca* donor virus reproducibly attenuates wild-type H3N2 and H1N1 viruses for humans. Actually, it was not until large-scale efficacy trials were performed that it became apparent that *ca* H1N1 and H3N2 reassortants retained a low level of reactogenicity for the upper respiratory tract with symptoms of mild rhinorrhea and mild sore throat occurring in 5–10% of vaccinees within the first 5 days after receiving a bivalent influenza A virus vaccine (28). The occurrence of otitis media, febrile disease, and lower respiratory tract symptoms were comparable in vaccinees and control subjects. The reactogenicity of the HIN1 and H3N2 *ca* reassortants was low even in very young infants and children who lack immunity from a previous infection with a related influenza A virus and in whom the *ca* reassortant virus replicates most efficiently. The low level of symptoms in this most susceptible group confirms the high level of attenuation of this vaccine for humans (18,81,97). Bivalent influenza A *ca* reassortant virus vaccines have reactogenicity profiles similar to that of the monovalent vaccine viruses (36). The *ca* reassortant viruses were also found to be safe in subjects with various high-risk conditions such as HIV infection and cystic fibrosis and in the elderly with underlying cardiopulmonary disease (32,35,47,49).

The level of restriction of replication of the *ca* H1N1 and H3N2 reassortants in the upper respiratory tract of humans reflects the overall immunological experience of the host (Fig. 4 and Table 2). In the upper two panels of Figure 4, the level of replication of an experimental wild-type influenza A virus in seronegative adults, that is, those with a low titer of serum hemagglutination-inhibiting (HAI) antibodies ($\leq$1:8) to the

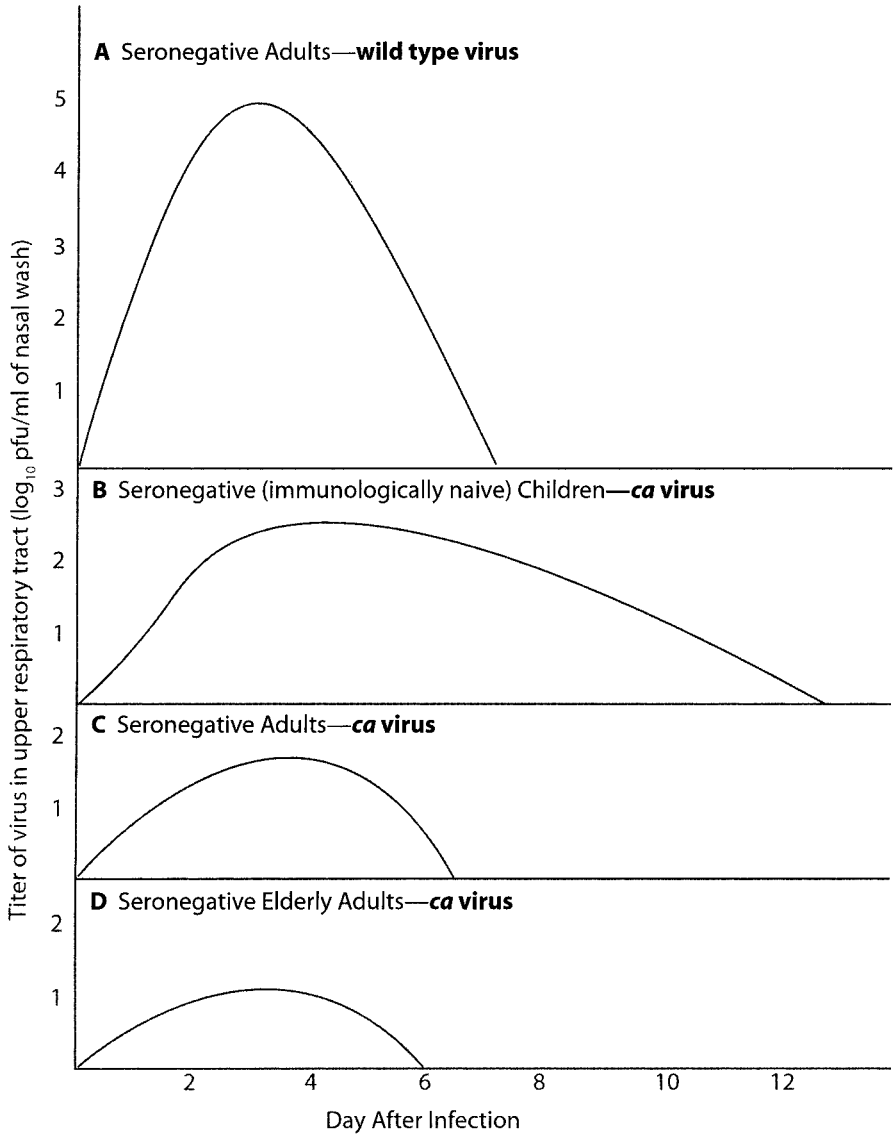

FIG. 4. (**A**) Level of replication of wild-type influenza A virus in the upper respiratory tract of adults is indicated. The level of replication of the *ca* influenza virus in seronegative infants and children not previously infected with an influenza A virus is indicated (**B**), that in seronegative but previously infected adults (**C**), and that in elderly adults (**D**).

infecting influenza A virus, is compared with that of a *ca* reassortant in an immunologically naive child or infant. It is clear that the wild-type virus replicates to a 100–1,000-fold higher peak titer than the *ca* virus. The *ca* virus is shed for a longer duration and in higher titers in immunologically naive, seronegative children (Fig. 4B) than in seronegative, but immunologically experienced adults (Fig. 4C). This seronegative adult would have been previously infected with a related influenza virus, that is, an antigenically drifted virus belonging to the same subtype. In comparison, the higher level and longer duration of replication of *ca* reassortant virus in very young vaccinees (Fig. 4B) reflects replication that is not restricted by prior immunity (97). In seronegative adults or children who were previously infected with a related influenza A virus, the *ca* reassortant replicates to lower titer and for a shorter duration than in an immunologically naive subject, reflecting the contribution of homotypic immunity induced by prior infection with a drift variant belonging to the same subtype. The *ca* reassortant virus is most restricted in replication in the elderly (31,71) (Fig. 4D).

TABLE 2. THE LEVEL OF ATTENUATION AND REPLICATION OF INFLUENZA A WILD-TYPE (WT) AND 6/2 ca REASSORTANT VIRUSES IN SERONEGATIVE (SERUM HAI[a] ANTIBODY TITER ≤1:8) ADULTS

ca *reassortant virus*	*Influenza A virus subtype*	*Percentage of volunteers with febrile or flu-like illness following infection with indicated virus*		*Mean peak titer of virus (log_{10} $TCID_{50}$/ml NP[b] specimen)*	
		wt[c]	ca[d]	*wt*	ca
A/Alaska/77	H3N2	50	10	4.5	1.0
A/Washington/80	H3N2	46	3	3.6	0.6
A/Korea/82	H3N2	36	0	3.4	0.7
A/Bethesda/85	H3N2	30	9	4.1	0.7
A/Hong Kong/77	H1N1	83	0	6.3	2.6
A/California/78	H1N1	56	4	3.9	1.2
A/Texas/85	H1N1	39	9	3.1	1.8

[a]Hemagglutination inhibiting.
[b]NP, nasopharyngeal wash.
[c]Illness includes, in large part, febrile and systemic symptoms.
[d]Illness is predominantly upper respiratory tract symptoms.

In seropositive adults and children (not shown), only a subset of the vaccinees become infected, generally only those persons with low preinfection nasal wash IgA anti-HA antibody titer; these individuals shed a very small quatitiy of virus (20,93).

Infectivity of the *ca* reassortants for humans and dose of vaccine. The attenuation of the *ca* virus by cold passage decreased its infectivity for humans. Influenza A wild-type viruses are highly infectious for humans with one to 10 median tissue culture infectious doses ($TCID_{50}$) of virus able to initiate an infection (68). The human infectious $dose_{50}$ (HID_{50}) of the *ca* reassortant viruses for seronegative adults is $10^{5.5}$ $TCID_{50}$, and it is about 10-fold less for immunologically naive subjects, indicating the greater infectivity of the *ca* vaccine for immunologically naive subjects (58). Within each of these two groups, the HID_{50}'s for a large set of H1N1 and H3N2 *ca* reassortants were very similar (58), indicating that the transfer of the full complement of six internal RNA segments from the *ca* parent reproducibly conferred a satisfactory level of infectivity upon each *ca* reassortant. Therefore, it is reasonable to expect this property in *ca* reassortant vaccine viruses generated in the future. Since each *ca* reassortant receives the same set of six internal genes from the *ca* donor virus and the HA and NA genes from a different wild-type virus, it is reasonable to conclude that the genetic determinants of reduced infectivity of the *ca* reassortant vaccine virus lie in one or more of the genes derived from the *ca* parent.

The dose of vaccine that has been found to be safe and immunogenic in all populations tested, including the elderly and young infants, is $10^{7.0}$ $TCID_{50}$ (18,58). This represents about 40 HID_{50}'s for seronegative adults and 400 for immunologically naive infants and children. When doses higher than $10^{7.0}$ $TCID_{50}$ are given, febrile systemic illness can occur in the absence of a high level of virus replication, indicating that there is an upper limit of dose that is acceptable for use in humans (9). Doses below $10^{7.0}$ $TCID_{50}$ are not optimally infectious for seronegative adults and also not optimally immunogenic for young infants.

The genetic basis of attenuation of the influenza A *ca* reassortant viruses for humans. For the influenza A/AA/6/60 *ca* donor virus, the six RNA segments transferred by the *ca* donor virus confer three phenotypes on the new reassortant virus, namely the *ca*, *ts*, and *att* phenotypes. To determine which RNA segment confers each of these phenotypes, six single gene reassortant viruses were produced that received one segment (PB1, PB2, PA, NP, M, or NS) from the influenza A/AA/6/60 *ca* donor virus and the remaining seven segments from the A/Korea/82 (H3N2) wild-type virus. Each of the six reassortant viruses was evaluated for its *ca* and *ts* phenotype *in vitro* and for the *att* phenotype in animals and humans (76,85).

In addition, other *ca* reassortants with only two to five genes from the *ca* parent virus were also evaluated in humans (77). The results of these analyses indicate that the PB1 and PB2 genes independently confer the *ts* and *att* phenotypes. The PB2 gene contains only one coding mutation, an amino acid substitution (Table 1), that specifies both phenotypes, a finding confirmed using the techniques of reverse genetics (84). The PA gene confers the *ca* and *att* phenotype. The NP and M2 genes also appear to contribute to the *att* phenotype in humans (76,77). Thus, the A/AA/6/60 *ca* donor virus has four to five genes that independently confer the *att* phenotype, two *ts* mutations (PB1 and PB2), and two to three non-*ts* attenuating mutations (PA, NP, M). Non-*ts* attenuating mutations present in a *ts* vaccine virus appear to greatly enhance the phenotypic stability of the *ts* mutations (66) and contribute to the restricted replication of the *ca* vaccine viruses in the upper respiratory tract of animals and humans. Importantly, a *ca* reassortant virus bearing two non-*ts* genes (the PA and M) was as attenuated in humans as the 6/2 *ca* virus containing all six internal genes from the *ca* donor virus (77). The polygenic basis of the attenuation of the A/AA/6/60 *ca* donor virus likely accounts for the high degree of phenotypic stability of its *ca* reassortant vaccine derivatives during their replication in humans.

Phenotypic stability of the influenza A *ca* reassortant viruses following replication *in vivo*. The *ts* and *ca* phenotypes of the A/AA/6/60 *ca* reassortants have been very stable following replication in humans (1,9,14,20,34,51,62,63,67,81,82) (Table 3). The observation that the *ts* and *ca* phenotypes of 11 H3N2 or H1N1 *ca* reassortants were phenotypically stable indicates that transfer of the six genes of the influenza A/AA/6/60 *ca* donor virus reproducibly confers these two phenotypes to its 6/2 gene *ca* reassortants. In addition, the phenotypic stability of the *ts* and *ca* phenotypes of reassortants viruses that do not contain the full set of six transferable genes from the *ca* donor virus has also been examined, and these have also been found to be phenotypically stable, even PB2 (*ts*) or PA (*ca*) single gene reassortants (64,76,77). A 6/2 *ca* reassortant virus that had undergone partial loss of its *ts* phenotype after replication in humans was administered to ferrets and was found to retain the *att* phenotype (97). Thus, the *ts* and *ca in vitro* surrogate markers of attenuation and the *att* phenotype itself are phenotypically stable following replication in humans. The four to five genes of the A/AA/6/60 *ca* donor virus that contribute to the *att* phenotype appear to reproducibly confer a satisfactory level of phenotypic stability on each new H1N1 and H3N2 A/AA/6/60 *ca* reassortant virus.

TABLE 3. A/AA/6/60 *CA* 6/2 REASSORTANTS RETAIN THE *TS* AND *CA* PHENOTYPES AFTER REPLICATION IN ADULTS AND CHILDREN

	No. with phenotype/ no. of specimens[a] *tested*	
ca *reassortant virus*	ca	ts
H3N2		
A/Alaska/77	16/16	54/54
A/Peking/79	3/3	3/3
A/Washington/80	29/29	29/29
A/Korea/82	21/21	21/21
A/Bethesda/85	NT[b]	21/21
A/Los Angeles/87	17/17	17/17
H1N1		
A/Hong Kong/77	62/62	62/62
A/California/78	29/29	29/29
A/Kawasaki/86	7/7	17/17

[a]Includes either an isolate or an original nasopharyngeal specimen.
[b]Not tested.

Transmissibility of the influenza A *ca* reassortants in humans. The *ca* reassortant viruses have been shown repeatedly to be poorly transmissible in adults and even in seronegative infants and children (14,23,62,67,82,96). At least two properties of *ca* reassortant vaccine viruses contribute to this low level of transmissibility. First, symptomatic infection of the upper and lower respiratory tract is seen very infrequently in vaccinees, thus decreasing the likelihood of spread of virus via coughing or sneezing. Second, the peak level of replication of the *ca* virus in the respiratory tract of children and adults ($\sim 10^{2.0}$) is consistently less than the HID_{50} for these two populations ($10^{4.5}$ to $10^{5.5}$), indicating that the amount of virus present in respiratory tract secretions is low and would spread to contacts very inefficiently.

Immunogenicity and efficacy of the influenza A reassortants. Immunity to influenza A viruses in humans is conferred primarily by antibodies directed at the HA and NA glycoproteins (68). Monoclonal antibodies directed against the HA or NA protected mice against challenge with virulent wild-type virus, whereas monoclonal antibodies specific for NP or M did not alter the course of disease (3). Among a set of recombinant vaccinia viruses each expressing one of the 10 known viral gene products of the influenza A virus, only recombinants expressing the HA or the NA glycoprotein induced resistance to subsequent challenge with wild-type virus (30). Influenza A virus undergoes antigenic drift and shift to evade immunity in the population conferred by antibodies specific for the HA and NA protective antigens (68). Both serum IgG and mucosal IgA antibodies to influenza A viruses can independently contribute to resistance to influenza A virus in humans (17), with serum IgG antibodies providing protection primarily to the lower respiratory tract and IgA antibodies providing protection primarily to the upper respiratory tract (57). Thus, the immunogenicity of influenza viruses can reasonably be assessed by examining the mucosal and systemic antibody responses to the HA and NA antigens.

The *ca* reassortant viruses efficiently induce both serum and mucosal antibodies in adult and pediatric populations (19,39), but the level of immunogenicity is a function of both age and prior immunological experience with related influenza viruses. The *ca* vaccine virus induces less antibody to the HA in infants younger than 6 months of age than in seronegative older infants and children despite similar levels of virus replication in both populations (42). Thus, there is an age-related restriction in antibody response to the HA in infants younger than 6 months of age that can be partially circumvented by administration of two or more doses of vaccine (18). Both age and immunosuppression by maternally acquired antibodies have been identified as major factors affecting the response of young infants to other respiratory viruses as well (41,59). In older infants and seronegative children, one dose of a *ca* reassortant vaccine virus efficiently induces a long-lasting and protective serum and mucosal antibody response (39). One dose of live virus vaccine is more efficient than one dose of inactivated virus in the induction of these antibody responses (39). However, use of two doses of live *ca* reassortant virus vaccine appears optimal both in the young infant for the reasons indicated above and in the older infant and child for reasons outlined below. Subunit vaccines induce a higher level of serum antibodies than *ca* vaccines in older children and young adults with prior experience with related influenza A viruses, but the *ca* vaccines more efficiently induce mucosal IgA antibody responses (19). In seropositive adults, the *ca* vaccine functions largely to immunize those individuals with low levels of preexisting IgA antibodies to the HA and to augment the mean titer of such antibodies following immunization (93). In the elderly, *ca* vaccines are less immunogenic than in young adults, reflecting the lower level of replication of the *ca* vaccine in this population (33,70,71). Importantly, the *ca* vaccine can infect this population and induce mucosal and serum antibodies, but the latter are induced more efficiently by subunit vaccine (33).

The influenza A *ca* virus vaccines have repeatedly been shown to be effective in protecting vaccinees against natural challenge or against experimental challenge with wild-type or live attenuated vaccine virus (the attenuated vaccine virus is used for experimental challenge in pediatric populations). Summary information on these results has been presented previously (52,58). Data on efficacy of *ca* vaccines in infants younger than 6 months of age is not available. In general, the efficacy of the *ca* vaccine reflects the level of replication of the vaccine in the host. The efficacy of live *ca* vaccine is greater than that of the inactivated vaccine for the young seronegative child (39), but is equivalent, or only moderately better, in the immunologically experienced older child or young adult (15,16,28). In the elderly, three trials of live intranasal *ca* virus vaccine administered simultaneously with parenterally administered subunit virus vaccine each improved efficacy by about 60% over that provided by subunit vaccine alone (10). Thus, despite the low level

of replication and the low level of antibody induced in this population by immunization with *ca* vaccine, enhanced protection of the elderly against influenza virus can clearly be achieved by co-administration of the *ca* and subunit vaccines (72). The combination of live and subunit vaccines takes advantage of the strength of each vaccine to optimally immunize the elderly, that is, the superior ability of the subunit vaccine to induce serum antibody in a immune host and the ability of the live virus to infect those with low levels of mucosal antibody and to induce a local IgA antibody response. The greater efficacy of the combined vaccine in the elderly clearly indicates that the *ca* vaccine is able to protect subjects who would otherwise be susceptible to infection with wild-type virus.

The immunological factors that affect the level of replication of the *ca* reassortant vaccine virus in older children and adults operate independently for each of the components of the vaccine (5) with each *ca* reassortant virus in a multivalent vaccine probing the host for susceptibility and infecting and immunizing those in whom immunologically specific resistance is low. This differential ability of the three components of a trivalent to replicate in human hosts with different levels of prior experience with related influenza viruses is an important factor to consider in the design of an optimal immunization regimen to protect against influenza and will be discussed further below.

PRECLINICAL AND CLINICAL STUDIES WITH MONOVALENT INFLUENZA B *ca* REASSORTANT VIRUSES AND EARLY FORMULATIONS OF MULTIVALENT INFLUENZA A AND B *ca* REASSORTANT VIRUSES

Generation and characterization of the influenza B/Ann Arbor(AA)/1/66 *ca* donor virus. The influenza B/AA/1/66 *ca* donor virus was generated by adaptation of the wild-type virus to replication at low temperature in PCKC culture as indicated in Figure 5. The multiple passages at progressively lower temperatures in PCKC and biological cloning yielded the influenza B/AA/1/66 *ca* donor virus that exhibited the *ts*, *ca*, and *att* phenotypes. A complete nucleotide sequence of the B/AA/1/66 *ca* donor virus was reported to differ from that of its wild-type parent virus at 105 nucleotide positions (25). This large number of nucleotide differences between the attenuated *ca* and B/AA/1/66 wild-type parent viruses far exceeds that seen in other attenuated viruses that have been passaged in tissue culture an even greater number of times (22,24,40,83). This suggests that the wild-type B/AA/1/66 was derived from a mixed population, and it seems unlikely that wild-type B/AA/1/66 was the immediate parent of the B/AA/1/66 *ca* reassortant. The mutations indicated in Table 4 are the unique nucleotide and predicted amino acid differences that exist between the influenza B/AA/1/66 *ca* donor virus and the sequence of both the B/AA/1/66 and a second influenza B wild-type virus (25). The *ca* and *ts* phenotypes of the B/AA/1/66 *ca* donor virus and its reassortant viruses are illustrated in Figure 6. The *ca* and wild-type viruses replicate efficiently at the permissive

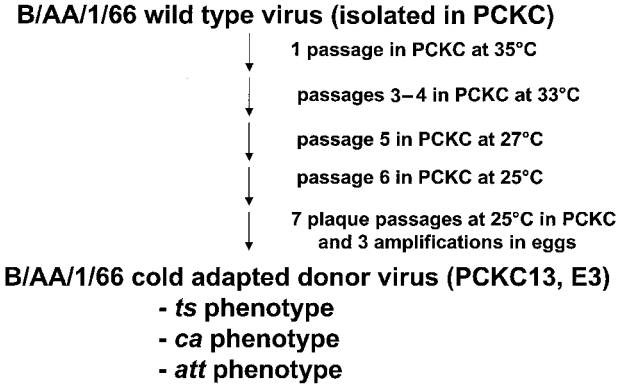

FIG. 5. Derivation of the influenza B/AA/1/66 *ca* virus by passage at progressively lower temperature in primary chicken kidney cells (PCKC) and by amplification in embryonated chicken eggs (E).

TABLE 4. UNIQUE MUTATIONS PRESENT IN THE B/AA/1/66 *ca* DONOR VIRUS

RNA segment	*Coding region*	*Other region*	*Mutation*	
			wt	ca
1	PB2		S630N	
		3′ NCR	*A2362G*	
2	PB1		I 651V	
3	PA		V431M (*ts* and *att*)	
			Y497H	
6	NP		T55A	
			V114A	
			P410H	
			A509T	
		3′ NCR	*C1804U*	
7	M		H159Q	
			M183V	

Nucleotide differences in 3′ and 5′ noncoding region are shown in positive sense and are in italics.

temperature of 33°C, but the *ca* donor virus is cold-adapted; that is, it also replicates efficiently at 25°C, a temperature restrictive for replication of many wild-type viruses. The *ca* donor virus and its reassortants are *ts* since they are highly restricted in replication at 37°C, a temperature at which many wild-type viruses grow efficiently. With the exception of the V431M mutation in PA that specifies a *ts* and *att* phenotype (27), other genes that specify the *ts*, *ca*, or *att* phenotypes of the influenza B/AA/1/66 *ca* donor have not been identified.

Level of replication and attenuation of the influenza B *ca* donor and its reassortant viruses in animals and humans. The influenza B/AA/1/66 *ca* donor and its B/Texas/84 *ca* reassortant virus were restricted in replication in the upper and lower respiratory tract of ferrets compared to that of the wild-type

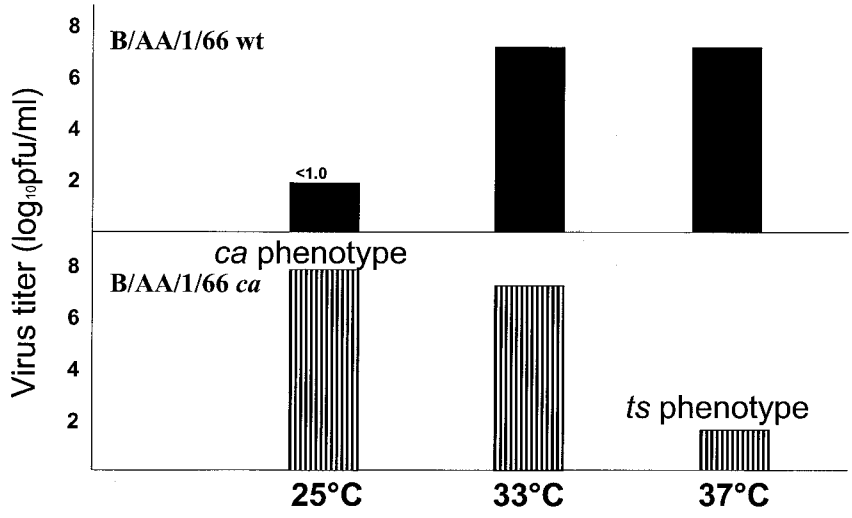

FIG. 6. The difference in the level of replication of the influenza B/AA/1/66 *ca* virus and its wild-type parent at 25°C in PCKC identifies the *ca* phenotype. The difference in the titer of virus (log_{10} pfu/mL) at 33°C and 37°C in PCKC identifes the *ts* phenotype of this virus. The influenza B/AA/1/66 *ca* virus and its 6/2 reassortants exhibit similar phenotypes.

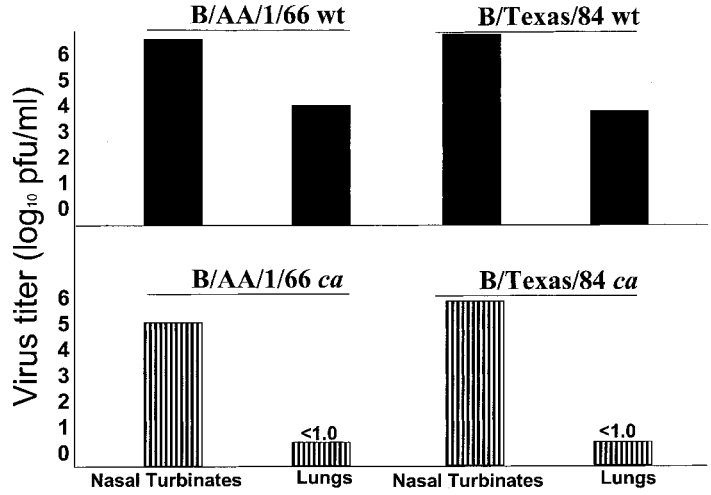

FIG. 7. The level of replication of the influenza B/AA/1/66 *ca* and wild-type viruses and the influenza B/Texas/84 6/2 *ca* reassortant virus and its B/Texas/84 wild-type parent in the upper and lower respiratory tract of ferrets.

virus parent (26,54) (Fig. 7). The level of restriction of replication is greater in the warmer lower respiratory tract than in the upper tract reflecting the temperature sensitivity of replication of influenza B *ca* viruses *in vitro.* The influenza B/AA/1/66 *ca* donor and its B/Texas/84 *ca* reassortant were also restricted in replication in both the upper and lower respiratory tract of hamsters and chimpanzees (78) (Fig. 8). These results clearly demonstrate that the six RNA segments of the influenza B/AA/1/66 *ca* donor virus reproducibly attenuate its 6/2 *ca* reassortant derivatives for animals, even for those with a core body temperature of 37°C (chimpanzees). These findings formed the basis for the subsequent evaluation of the B/AA/1/66 6/2 *ca* reassortants in humans.

Three 6/2 *ca* reassortants (B/Texas/84, B/AA/86, and B/Yamagata/88) derived from the influenza B/AA/1/66 *ca* donor were found to be satisfactorily attenuated in adult or pediatric populations, indicating that the six RNA segments of the influenza B/AA/1/66 *ca* donor virus can reproducibly attenuate 6/2 *ca*

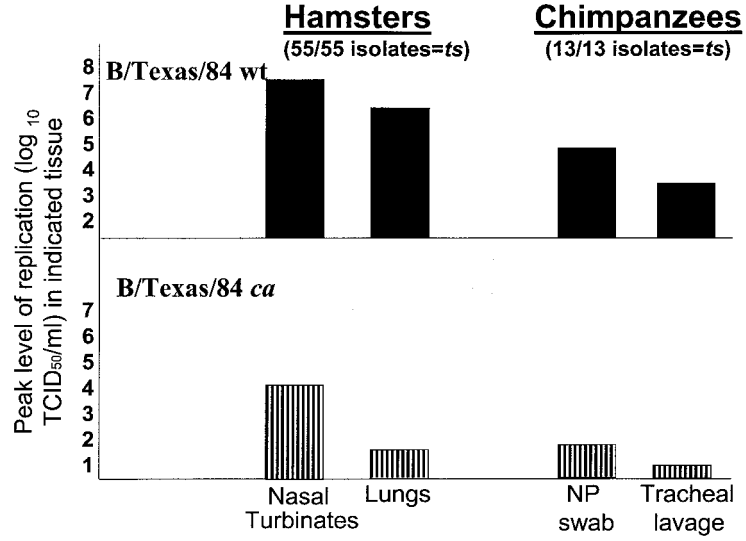

FIG. 8. The level of replication and genetic stability of the influenza B/Texas/84 6/2 *ca* reassortant virus and its B/Texas/84 wild-type parent in the upper and lower respiratory tract of hamsters and chimpanzees.

TABLE 5. THE LEVEL OF REPLICATION OF B/AA/1/66 *ca* REASSORTANTS IN SERONEGATIVE (SERUM HAI ANTIBODY TITER ≤1:8) ADULTS AND IN SERONEGATIVE NAIVE CHILDREN

	Mean peak virus titer[a] *(log_{10} $TCID_{50}$/mL)*		
	Adults		*Children*
ca *or wild-type virus*	*Wild-type*	ca	ca
B/Texas/84	2.2	0.8	1.4
B/AA/86	4.0	0.8	3.4[c]; 3.1[c]
B/Yamagata/88	NT[b]	1.1	NT

[a]Virus titer in upper respiratory tract.
[b]NT, not tested.
[c]Two separate studies.

reassortants for humans (2,5,21,29,37,43–45,90,91). Two *ca* reassortants were separately administered to seronegative infants and young children, and the illness observed was indistinguishable from that observed in placebo control subjects (2,29). This indicated that the influenza B *ca* reassortant viruses were satisfactorily attenuated for this fully susceptible population (2,29). In addition, the vaccine virus was recovered from the respiratory secretions of 60–90% of infants who received $10^{6.0}$ $TCID_{50}$ of virus, indicating that it was not overattenuated for this population (2,29). The level of replication of the influenza B/AA/1/66 *ca* reassortants in both adult (21,44,89) and pediatric (2,29,37) populations is summarized in Table 5, demonstrating that the level of replication of the influenza B virus *ca* reassortants, like their influenza A counterparts, is decreased compared to wild-type virus and that the peak titer of virus replication in seronegative children is greater than that in adults. The level of replication of the influenza B *ca* reassortants is similar to that of the influenza A *ca* reassortants (Table 2). The influenza B *ca* reassortants were also found to be safe, but only modestly immunogenic, in elderly and chronically ill adults and thus resembled their influenza A counterparts in these properties (89).

Infectivity of the influenza B *ca* reassortants for humans and dose of vaccine. The level of infectivity was assessed by determining the HID_{50} of the B/AA/1/66 *ca* reassortants viruses in adults and children (2,21,29,44) (Table 6). The level of infectivity of the influenza A and B *ca* reassortants for humans therefore appears similar (58). The dose of the influenza B *ca* reassortant that is being used in the trivalent vaccine is $10^{7.0}$ $TCID_{50}$ (8), which represents about 40 HID_{50}'s for seronegative adults and 3,000 HID_{50}'s for immunologically naive infants and children.

Genetic stability of the influenza B *ca* reassortant viruses following replication *in vivo*. The influenza B/AA/1/66 *ca* donor virus and its 6/2 *ca* reassortants retain the *ts*, *ca*, and *att* phenotypes following replication in animals and humans. The *ts* phenotype of B/Texas/84 was very stable following replication in hamsters and chimpanzees (Fig. 8) with 68 isolates obtained from the upper and lower respiratory tract retaining their *ts* phenotype (78). To further evaluate its phenotypic stability, the *ca* reassortant virus was administered to hamsters immunosuppresed with cyclophosphamide, and isolates were obtained from six ham-

TABLE 6. INFECTIVITY OF INFLUENZA BAA/1/66 *ca* REASSORTANTS VIRUSES FOR SERONEGATIVE (SERUM HAI ANTIBODY TITER ≤1:8) ADULTS AND CHILDREN

	HID_{50} (log_{10} $TCID_{50}$)	
ca *reassortant virus*	*Adults*	*Children*
B/Texas/84	5.4	4.5
B/AA/86	6.4	2.5

sters after 15 days of replication. These six isolates were tested for their *ts* phenotype *in vitro* and for their level of replication in immunocompetent hamsters (Fig. 9) and were found to retain their *ts* and *att* phenotypes even after prolonged replication *in vivo*. Thus, the influenza B/AA/1/66 *ca* donor virus can transfer the desired properties of phenotypic stability of the *ts* and *att* phenotypes to its *ca* reassortants.

The *ts* and *ca* phenotypes of the influenza B/AA/1/66 *ca* reassortants were stable in humans (2,21,29,44,88) (Table 7). Thus, the influenza B/AA/1/66 *ca* donor virus reproducibly transfers to its *ca* reassortants the desired property of stability of the *ts* and *ca* phenotypes following replication in humans, even after replication in fully susceptible children (2,29).

Efficacy and lack of transmissibility of the influenza B *ca* reassortants in humans. Only one study has been performed to evaluate the efficacy of an influenza B *ca* reassortant against experimental challenge with wild-type virus (21). The influenza B/AA/86 *ca* reassortant virus was administered at a dose of $10^{7.5}$ $TCID_{50}$ to 13 adult volunteers selected to have a low titer of serum antibodies prior to immunization. Six weeks later they were challenged with a high dose ($10^{7.0}$ $TCID_{50}$) of wild-type B/AA/86 virus. The response of the vaccinees was compared with that in 12 unimmunized controls (21). The influenza B/AA/86 *ca* reassortant virus completely protected against illness but did not protect against infection with this high dose of challenge virus.

The transmissibility of a monovalent influenza B/AA/86 *ca* reassortant virus from vaccinees to susceptible contacts has only been studied once, and the *ca* virus was not found to be transmissible (29).

Interference between influenza A and B *ca* reassortant viruses in multivalent formulations. It is possible for one component of a multivalent vaccine to interfere with the replication or immunogenicity of other components. The high level of replication of each of the three components of the trivalent influenza virus vaccine in a fully susceptible immunologically naive infant or young child would offer a greater opportunity for interference between components than would the lower level of replication seen in partially immune vaccinees. There is a paucity of information concerning the level of replication and immunogenicity of a monovalent influenza A or B *ca* reassortant virus given to immunologically naïve subjects at a dose of $10^{7.0}$ $TCID_{50}$ compared to that of the same virus given in a multivalent formulation. Most studies in this population compared the replication or immunogenicity of monovalent versus multivalent *ca* vaccines em-

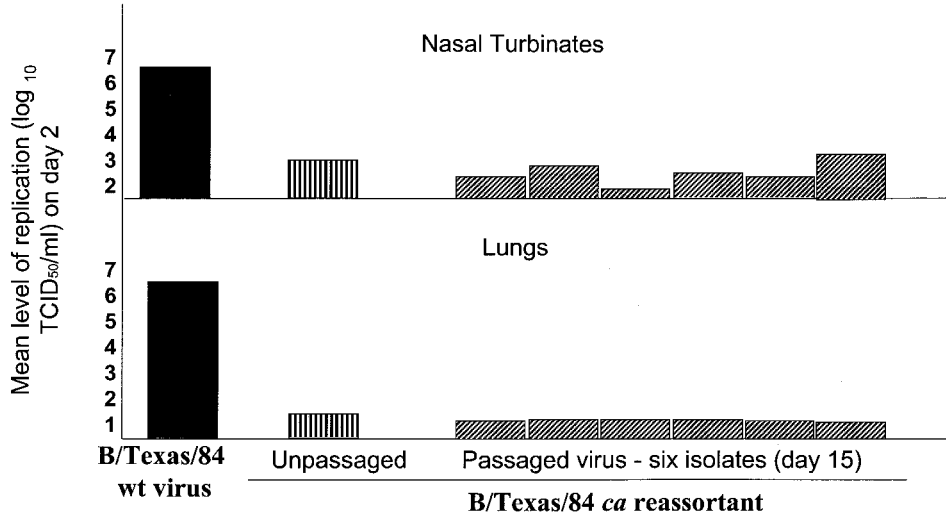

FIG. 9. Stability of the attenuation phenotype of B/Texas/84 *ca* reassortant virus in immunosuppresed hamsters. The B/Texas/84 *ca* reassortant virus was administered intranasally to immunosuppresed hamsters. Six isolates obtained after 15 days of replication were readministered to immunocompetent hamsters. On day 2, the level of replication in the immunocompetent hamsters of these six isolates was compared to that of the B/Texas/84 wild-type and *ca* viruses. The attenuation phenotype of the six isolates was maintained. Unpassaged virus is the B/Texas/84 *ca* reassortant virus that was administered to the hamsters and the passaged virus is the B/Texas/84 *ca* reassortant virus that was recovered from the hamsters after 15 days of replication.

TABLE 7. B/AA/66 *ca* 6/2 REASSORTANTS RETAIN THE *ts* AND *ca* PHENOTYPES AFTER REPLICATION IN SERONEGATIVE (SERUM HAI ANTIBODY TITER ≤1:8) ADULTS AND CHILDREN

	No. with phenotype/no. of specimens[a] *tested*	
ca *reassortant virus*	ca	ts
B/Texas/1/84	103/103	103/103
B/AA/1/86	202/202	202/202

[a]Includes either an isolate or an original nasopharyngeal specimen.

ploying a dose of $10^{6.0}$ or lower. However, four observations emerged from these studies in seronegative individuals that relate to the question of interference. First, the individual components of a trivalent *ca* virus vaccine are, for the most part, comparable in immunogenicity whether given singly or in combination (37). Second, in some instances, the immune response to one component of a bivalent or trivalent influenza *ca* reassortant virus vaccine can be lower than that of the same component given as a monovalent vaccine, indicating that interference in replication and/or immunogenicity can occur in multivalent formulations (34,37). Third, the component that is restricted in replication or immunogenicity can differ from one multivalent formulation to another; for example, in one case, the replication of the influenza B *ca* component was restricted in a trivalent formulation (35) and in another case the immunogenicity of the H1N1 *ca* component of a bivalent influenza A vaccine was decreased (36). This suggests that, in the fully susceptible child, the high level of replication of each component of the vaccine can interfere in an unpredictable fashion with that of another component. Fourth, decreased immunogenicity of multivalent formulations can be partially overcome by administration of two doses of vaccine 1 month apart (87). The lack of predictability of which component of a multivalent vaccine might manifest decreased immunogenicity is of concern especially since each component might have to be changed annually. However, considering the lack of time available to investigate the effect of vaccine dosage on immunogenicity on an annual basis, a practical solution to this problem is needed—for example, the administration of two doses of vaccine to populations that contain substantial numbers of young, fully seronegative subjects.

In adults with prior experience with related influenza viruses, the immunogenicity of each component of a trivalent *ca* virus vaccine ($10^{7.1}$ $TCID_{50}$) was comparable to that in vaccinees given the monovalent viruses (5). This suggests that the more restricted replication of the components of a multivalent vaccine in an immunologically experienced population minimizes interference between the components of the vaccine. Thus, one dose of vaccine should be sufficient for such populations.

Summary of studies with monovalent, bivalent, and early trivalent preparations of influenza A and B *ca* reassortant viruses. The studies summarized above in which 16 distinct 6/2 influenza A *ca* reassortants and three influenza B *ca* reassortants were studied indicate that the six internal RNA segments of the influenza A/AA/6/60 and B/AA/1/66 *ca* donor viruses are able to reproducibly confer a satisfactory level of attenuation, immunogenicity, and genetic stability on new wild-type viruses as they appear in nature. The vaccine viruses are poorly transmissible but are highly effective in protecting against illness caused by experimental or natural challenge with wild-type influenza virus. Although the accumulated data are much more extensive for the influenza A virus than for the influenza B virus, it is reasonable to predict that both *ca* donor viruses should be able to reproducibly confer on each new wild-type virus the above set of properties, and recent studies with trivalent vaccines given at about $10^{7.0}$ $TCID_{50}$ per component prove that this prediction is correct.

CLINICAL STUDIES WITH CURRENT FORMULATIONS OF TRIVALENT COLD-ADAPTED INFLUENZA VACCINE

Strategy for clinical development of CAIV-T. As described above, monovalent, bivalent, and early trivalent cold-adapted influenza vaccine (CAIV) formulations were demonstrated to be attenuated, im-

munogenic, and efficacious in children and adults. The current trivalent CAIV (CAIV-T) and early CAIV formulations are biologically comparable in that both consist of 6/2 vaccine strains, derived by a process of genetic reassortment using the same type A and B master donor viruses (MDV), and both express the *ca*, *ts*, and *att* biological phenotypes. Because of this biological comparability, the strategy for clinical development of CAIV-T was to build upon the safety, immunogenicity, and efficacy results obtained with CAIV.

Pivotal clinical trials of CAIV-T utilized vaccine that was formulated to contain approximately 10^7 $TCID_{50}$ of each of the three current vaccine strains since earlier studies with CAIV had indicated that this dosage level was safe and infectious for a high proportion of children and adults. Although CAIV had been administered by intranasal drops, CAIV-T was administered intranasally as a large particle spray. In most trials, children up to the age of 9 years, who had not previously been vaccinated against influenza, received two doses of CAIV-T separated by at least 1 month, whereas adults were vaccinated with a single dose of CAIV-T. The rationale for two doses of vaccine for younger children was based on previous experience with CAIV, which had indicated that two doses resulted in an 80%, 93%, and 67% cumulative antibody response rate to the H1N1, H3N2, and B components, respectively (87).

The immunogenicity of 19 different CAIV strains was studied over a period of 25 years at various investigative sites and in different populations, using different immunological assay methods. In spite of these differences, the overall immunological responses measured were generally consistent across the studies and demonstrated that serum or nasal antibodies specific for either the viral HA or NA proteins were associated with protection against influenza. Studies with CAIV-T built upon this experience, and focused primarily on the serum and nasal antibody response following vaccination.

Trials were conducted to evaluate the safety, immunogenicity, and efficacy of CAIV-T in adults and children. In addition, a clinical study was conducted to evaluate the transmissibility of CAIV-T among young children, and laboratory studies were conducted to evaluate the genetic and phenotypic stability of CAIV-T during manufacture and during replication in vaccine recipients. To date, over 20,000 children and adults have received CAIV-T vaccine.

Safety of CAIV-T in healthy children, healthy adults, and high-risk individuals. Prior to initiation of efficacy trials, the safety and immunogenicity of a single dose of CAIV-T delivered by intranasal spray or intranasal drops to children 18–71 months of age was evaluated (48). No significant difference in reactogenicity, immunogenicity, or "take rate" (i.e., immune response and/or vaccine virus shedding) was detected between spray or drop recipients (48). These results supported delivery of CAIV-T by intranasal spray in subsequent studies.

Reactogenicity of CAIV-T in healthy children or adults has been assessed using fever and symptomotology data obtained from daily dairy cards completed by parents of pediatric study participants or by adult study participants, and serious adverse events following vaccination were collected by study personnel (6–8,48,69,92,100). Placebo consisted of normal allantoic fluid obtained from uninfected eggs. Reactogenicity events in children were generally collected for 10 days following receipt of vaccine or placebo, and in adults for 7 days. Serious adverse events were generally collected for 28 days following receipt of vaccine or placebo by adults and for 42 days for children. Among trials completed to date, no serious adverse events have been reported to be vaccine-associated.

In general, data from trials in healthy children indicated that, during the 10 days following the first dose of CAIV-T, runny nose/nasal congestion, sore throat, irritability, headache, vomiting, muscle aches, and decreased activity was reported more frequently by vaccine recipients than by placebo recipients. The largest absolute differences observed between CAIV-T and placebo recipients reporting any individual event following Dose One was 12.1% for runny nose/nasal congestion and was 4.6% for fever of >100°F (MedImmune files). Reactogenicity events following the second dose of CAIV-T occurred with similar frequency for vaccine and placebo recipients.

In general, data from healthy adults indicated that cough, runny nose, sore throat, headache, chills, muscle aches, and tired/weak were reported more frequently by CAIV-T recipients than by placebo recipients during the 7 days after a single dose. The largest absolute differences observed between CAIV-T and placebo recipients reporting any individual reactogenicity event following a single dose was 16.6% for runny nose,

9.3% for sore throat, and 3.9% for tired/weak (MedImmune files). Fever of >100°F was similar in CAIV-T and placebo recipients (1.3% vs. 1.5%, respectively) after a single dose (MedImmune files).

Currently, data regarding the safety of CAIV-T in high-risk individuals is limited. A study of CAIV-T in 24 mildly symptomatic or asymptomatic HIV-infected children indicated that reactogenicity and vaccine-related adverse events occurred at a rate similar to those in non–HIV-infected children (46). Furthermore, following vaccination vaccine-attributable changes in HIV RNA concentrations and in CD4 counts were not observed compared to placebo recipients and the quantity and duration of CAIV-T shedding was not increased (46). Similar results were obtained in a study of CAIV-T in 57 relatively asymptomatic HIV-infected adults (49). Results of a study in children and adolescents with moderate to severe asthma indicated that reactogenicity was similar for CAIV-T and placebo recipients, and that vaccination with CAIV-T did not reduce pulmonary function or worsen clinical features of asthma (73).

Immunogenicity of CAIV-T in children and adults. Results of immunogenicity and efficacy studies with CAIV-T in children indicated that two vaccine doses were generally more immunogenic than a single dose, particularly for the H1N1 strain. For example, among a subset of seronegative children 15–71 months of age enrolled in the first year of a field efficacy trial, 16%, 92%, and 88% developed a serum HAI antibody response to the H1N1, H3N2, and B vaccine components, respectively, after the first dose of CAIV-T. After the second dose, 61% had developed serum antibodies to the H1N1 strain and 96% had developed antibodies to the H3N2 and B strains (8). Furthermore, 62%, 69%, and 85% of children tested developed a mucosal IgA response to the H1N1, H3N2, and B strains, respectively, following two doses of CAIV-T (11). These findings using CAIV-T at $10^{7.0}$ $TCID_{50}$ confirm the importance of using two doses of vaccine for seronegative children as suggested above using a lower dose of vaccine. In the second year of the study, revaccination resulted in an 82% seropositivity rate to the H1N1 strain and 100% seropositivity to the H3N2 and B strains in the immunogenicity subgroup (6). Overall efficacy against H3N2 and B strains circulating during the 2 years of the efficacy trial was 92% (6). These data suggest that high serum antibody and mucosal antibody response rates have high positive predictive value for vaccine efficacy.

Results of previous studies of CAIV have suggested that antibodies specific for the viral HA and NA proteins were associated with protection, and that protective antibodies were present in either serum or nasal secretions. None of the previous studies of CAIV in children or adults have identified a single type of immune response that has both high *positive* and high *negative* predictive value for efficacy. To extend these findings, the correlates of immune protection induced by CAIV-T were examined in a study in which children were challenged with monovalent H1N1 CAIV vaccine 5–8 months after their second year of participation in a 2-year field efficacy trial (7). Of the 222 children challenged, 144 had previously received CAIV-T and 78 had previously received placebo. The results indicated that CAIV-T generally induced serum and nasal IgA antibodies that persist over an interval of 5–8 months following vaccination with CAIV-T and that CAIV-T efficacy against challenge was 83%. In general, the presence of either serum HAI antibody or any nasal wash IgA antibody correlated with the absence of shedding of the H1N1 vaccine virus used as the challenge. However, among 16 of 144 prior CAIV-T recipients with no detectable serum HAI or nasal IgA antibodies (measured by ELISA), efficacy was 45% (95% confidence interval [CI] of 22–79%). Twelve of these 16 children had detectable serum neutralizing antibody, which may have contributed to the observed efficacy in the absence of detectable serum HAI or nasal IgA antibody. These data suggest that vaccination with CAIV-T, in addition to inducing serum HAI antibody or nasal IgA antibody, also induces immune factors such as neutralizing antibody, which also may provide protection.

Results of immunogenicity and efficacy studies in adults indicated that CAIV-T induces a relatively modest rate of serum HAI responses, but is highly efficacious (92). These results are consistent with those obtained in previous studies with CAIV in adults, and suggest that a low serum HAI response does not have high negative predictive value for efficacy of CAIV-T.

In summary, the data support the conclusion that there was no single correlate of protection for CAIV-T, and that serum HAI, serum neutralizing, and nasal IgA antibodies played an important role in protection. Furthermore, it appears that vaccination with CAIV-T stimulated multiple immunological mediators of protection, some of which are currently not identified. Identification of multiple mechanisms of protection induced in children by CAIV-T was not surprising in light of previous results with CAIV, in which

both serum and nasal antibodies correlated with protection. The complex nature of the protective immune response to immunization with CAIV-T resembled that induced by natural infection with influenza.

Efficacy and effectiveness of CAIV-T in children. Efficacy and effectiveness of CAIV-T in preventing influenza disease in children was demonstrated in a 2-year, double-blind, placebo-controlled efficacy field trial in healthy children 15–71 months of age. Overall, during the 2 years of the study, vaccine was 92% efficacious against culture-confirmed influenza.

A total of 1,602 children were randomized 2:1 (vaccine:placebo) during the first year of the study to evaluate efficacy of two doses of CAIV-T (8). A total of 1,358 of the original 1,602 children (85%) returned for the second year of the study, remained in their original treatment groups, and received a single dose of CAIV-T or placebo (6). The primary endpoint of the trial during both years was prevention of culture-confirmed influenza illness. In the first year of the study, both influenza type A/H3N2 and type B strains circulated. Vaccine efficacy against culture-confirmed influenza was 93% (95% CI of 88–96%) (8), and efficacy was demonstrated against both the influenza type A and B strains. Effectiveness of CAIV-T was also demonstrated, in that vaccinated children had significantly fewer febrile illnesses, including 30% fewer episodes of febrile otitis media (95% CI of 18–45%; $p < 0.001$).

During the second year of the trial, the predominant epidemic strain was an antigenically drifted H3N2 strain (A/Sydney/05/97), and vaccine efficacy was 86% (95% CI of 75–92%) against culture-confirmed influenza due to the A/Sydney/05/97 variant (6). Protection against influenza A–associated febrile otitis media was 94% (95% CI of 78–99%), and protection against lower respiratory tract disease associated with culture-confirmed influenza was 100% (95% CI of 77–100%).

H1N1 influenza viruses did not circulate in the US during the two years of the efficacy trial. As a surrogate test of efficacy against wild-type H1N1 viruses, a subset of 222 children (who received CAIV-T or placebo following the second year of the efficacy trial) was challenged with a monovalent H1N1 vaccine virus. Efficacy, measured as protection against shedding of the challenge H1N1 vaccine strain, was 83% (95% CI 60–93%).

Efficacy and effectiveness in adults. Efficacy of CAIV-T was demonstrated in a randomized, double-blind, placebo-controlled challenge trial in healthy adults 18–40 years of age that were "serosusceptible" (i.e., had serum HAI antibody titers of $\leq$1:8) to wild-type influenza viruses representative of the vaccine components. Ninety-two individuals were vaccinated with CAIV-T ($n = 29$), licensed trivalent inactivated influenza vaccine ($n = 32$), or placebo ($n = 31$), and subsequently challenged with a wild-type influenza virus to which they had been initially serosusceptible (92). Efficacy of CAIV-T or inactivated vaccine against laboratory-documented influenza illness (respiratory symptoms with either isolation of challenge wild-type virus or a serum HAI antibody response following wild-type virus challenge) was 85% (95% CI of 28–100%) and 71% (95% CI of 2–97%), respectively.

Effectiveness of CAIV-T in adults was demonstrated in a randomized, double-blind, placebo-controlled trial in 4,561 healthy working adults 18–64 years of age who were followed for influenza-like illness, associated absenteeism, and health care utilization during a community outbreak of influenza (69). The trial was randomized 2:1 (vaccine/placebo), and the outbreak periods for influenza-like illness were based on community surveillance. Three febrile influenza-like illness definitions were prospectively assessed: any febrile illness (AFI), severe febrile illness (SFI), and febrile upper respiratory illness (FURI). Adults were characterized as having AFI if they had symptoms for at least 2 consecutive days with fever on at least 1 day and if they had two or more symptoms (fever, chills, headache runny nose, sore throat, cough, muscle aches, tiredness/weakness) on at least 1 day. SFI was defined as having at least 3 consecutive days of symptoms, at least 1 day of fever, and two or more symptoms on at least 3 days. FURI was defined as at least 2 consecutive days of upper respiratory symptoms (runny nose, sore throat, or cough), fever on at least 1 day, and two symptoms on at least 1 day.

During influenza outbreak periods, CAIV-T recipients and placebo recipients were equally as likely to experience one or more febrile illnesses (69). However, CAIV-T recipients experienced a 23% reduction in days of illness for AFI, a 27% reduction in days of illness for SFI, and a 25% reduction in the days of illness for FURI compared to placebo; each reduction was significant. Illness-associated days of missed work were significantly decreased for SFI and FURI. Days of healthcare provider visits and prescription antibiotics were significantly decreased across all three febrile illness definitions.

A trial in 2,215 older adults with chronic obstructive pulmonary disease was conducted to evaluate whether CAIV-T provides added protection against culture-documented influenza when administered concurrently with subunit influenza vaccine (32a). The results indicated that, in comparison to vaccination with subunit vaccine alone, vaccination with CAIV-T and subunit vaccine provided addition efficacy of 0.21 (95% Cl of −0.23–0.49), 0.26 (95%) Cl of −0.17–0.53), and −0.05 (95% Cl of −1.13–0.48) against H1N1, H3N2, and B influenza strains. The results indicated that there was a trend toward additional efficacy following vaccination with both CAIV-T and subunit vaccine, and that a much larger study would be required to confirm this finding.

Genetic and phenotypic stability during manufacture and during replication in humans. Nucleotide misincorporations, such as substitutions, deletions, or insertions, can occur due to the lack of proofreading function of RNA viral polymerases. It has been estimated that type A influenza virus RNA polymerases have an error rate of 10^{-4} to 10^{-5} misincorporations per nucleotide position per genome (75). Based on this estimated error rate, it would not be unexpected if CAIV-T vaccine strains were to undergo a certain degree of genetic change while replicating in embryonated hens' eggs during the manufacturing process. Therefore, it was of interest to compare the genetic sequences of CAIV-T vaccine viruses throughout the manufacturing process in eggs. In addition, since specific gene segments and sequences of influenza viruses have been associated with host-cell restriction and since the attenuating mutations in the CAIV-T viruses could undergo genetic modifications, it is possible that CAIV-T vaccine strains could accumulate nucleotide misincorporations during replication in vaccinated human hosts. Therefore, the genetic sequences of vaccine viruses isolated from vaccinated young children were determined and compared to those of the vaccine strains manufactured in eggs.

The three manufacturing lineages studied were the A/Sydney/05/97 (H3N2), A/Beijing/262/95 (H1N1), and B/Harbin/7/94-like vaccine strains. Vaccine intermediates in each lineage consisted of the master virus seed (MVS), manufacturer's working virus seed (MWVS), and three production lots of bulk vaccine harvest (VH). The complete genomic sequence of each available vaccine intermediate in the three manufacturing lineages studied was determined using high capacity consensus sequencing techniques. In addition, two gene segments encoding HA and NA proteins of the wild-type parental viruses and the six gene segments encoding nonglycoprotein "internal" proteins of the A/AA/6/60 and B/AA/1/66 master donor viruses (MDV) were sequenced. The results demonstrated that the live, attenuated viruses comprising CAIV-T vaccine are genetically stable and can be manufactured consistently (99a). These results suggested that the biological cloning steps utilized to prepare vaccine virus reassortants yielded a virus clone containing a genomic sequence found within the attenuated parental MDV population. Importantly, this nucleotide sequence was substantially stable throughout the production of MVS, MWVS, and VH. All vaccine intermediates had a 6/2 genotype and maintained the cold-adapted (*ca*), temperature-sensitive (*ts*), and attenuated (*att)* phenotypes of the MDV. In addition, the HA and NA protein sequences of vaccine intermediates (MVS, MWVS, and VH) were identical with those of the original wild-type virus parent. Taken together, these results demonstrated that each of the three components of the CAIV-T vaccine are highly consistent with respect to the *ca*, *ts*, and *att* phenotypes and the nucleotide sequence.

Genetic and phenotypic stability of CAIV-T during replication in vaccine recipients was assessed by genomic sequence analysis of virus isolates obtained from children who were vaccinated with CAIV-T in a daycare study. In order to maximize the likelihood of detecting changes in genetic sequence, samples originating from swabs taken at later times postvaccination were chosen for sequence analyses, since nucleotide misincorporations would be expected to accrue with increased replication cycles. Genetic stability of vaccine strains during replication in the human host was assessed by comparing the genomic sequences of the viruses contained in the CAIV-T vaccine to those of 56 (21 H1N1, 12 H3N2, 1 H3N1, and 22 type B) virus isolates obtained from young children following vaccination.

The results demonstrated that limited sequence alterations occur in vaccine viruses during replication in children (S. Udem and T. Zamb, personal communication). The minor changes detected were not at the genetic loci associated with attenuation as indicated above, did not disrupt the *ca*, *ts*, and *att* phenotype, and did not affect attenuation in young children. These minor changes in sequence appeared to be stable during transmission to and replication in another human host.

Transmissibility. To detect transmission of CAIV-T, healthy children aged 8–36 months were enrolled

in a randomized, double-blind, placebo-controlled trial in a daycare setting during the 1999–2000 influenza season (94). The daycare centers were organized such that independent playgroups were each composed of approximately 15 children. Within each playgroup, the subset of the children who were participating in the trial was randomized approximately equally between vaccine and placebo recipients. In total, 98 children received CAIV-T and 99 children received placebo. Nasal swabs were taken every other day for 21 days following intranasal administration of a single dose of either vaccine or placebo. The vaccine administered was formulated from the same VH lots that were studied in the genetic stability study, which allowed an assessment of the genetic and phenotypic stability of any transmitted vaccine virus.

B/Harbin-like vaccine virus was shed by a total of 70 subjects, while A/Beijing vaccine virus was shed by 34 subjects. Fewer subjects shed A/Sydney vaccine virus (12 of the 98 vaccine recipients), presumably due to preexisting immunity (wild-type H3N2 viruses had circulated in the community during the two previous influenza seasons). Transmission of vaccine viruses from vaccinees to placebo controls was a rare event in this study. Type B vaccine virus was detected in a single specimen obtained on day 15 from a single placebo recipient during the course of the study (calculated transmission rate of 1.75%). Sequence analysis of the transmitted virus and of viruses shed by other study participants indicated that the influenza type B vaccine virus was transmitted within the playgroup from a vaccinee to a placebo recipient (MedImmune files). The transmitted virus retained the *ca*, *ts*, and *att* phenotypes and was attenuated in humans, since both the vaccinated child and the placebo recipient who shed the transmitted virus experienced clinical signs and symptoms that were similar to those of other participants (MedImmune files).

Summary. In summary, nine unique CAIV-T vaccines consisting of 13 different 6/2 vaccines strains have been administered in clinical trials. The results indicate that CAIV-T is safe and well-tolerated, induces both serum and mucosal antibodies, and is efficacious and effective in healthy children and adults. Genomic sequencing studies support the conclusion that CAIV-T is genetically and phenotypically stable throughout the manufacturing process and during replication in young children.

SUGGESTED USE OF THE LIVE ATTENUATED TRIVALENT INFLUENZA *ca* VACCINE

Since influenza viruses infect and cause serious disease in humans of all ages, there is a need for a vaccine to protect people of all ages including the very young infant (98). The live attenuated trivalent *ca* vaccine should not be thought of as a simple replacement for the licensed subunit influenza virus vaccine. More likely, the two vaccines may be used in conjunction in certain populations and differentially in other populations to achieve optimum protection. The major differences in the properties of the *ca* reassortant virus and the licensed subunit virus vaccines will dictate how they can most effectively be used in the different age groups.

First, since the live attenuated influenza virus vaccines are more immunogenic and efficacious than subunit vaccines in seronegative subjects (39), the live virus vaccines may be used preferentially in young children and infants. Two or more doses of vaccine will be required in early infancy to overcome the decreased immunogenicity of the HA in this age group. Since this immunization requires several doses administered over a period of several months to overcome the age-related decrease in immunogenicity, it might be incorporated into the routine immunization schedule at 2, 4, and 6 months of age rather than initiation of immunization in the fall. Incorporation into the routine childhood immunization schedule would, of course, be dependent upon important practical considerations such as demonstration that the childhood vaccines are not interfered with, and that the influenza vaccine viruses are available throughout the year. In older seronegative infants and young children, the vaccine should be given annually in two doses at least 1 month apart. The purpose of two doses in this age group is to overcome interference that might occur between the three components of the vaccine. The upper age limit for this group can be determined experimentally pre- or postlicensure, but probably would be between 4 and 9 years of age.

Second, although live *ca* and nonliving vaccines appear equally efficacious in immunologically experienced older children and young adults, the ease of administration of the live virus vaccine would favor its use in these age groups, as well as in younger children and infants. A single dose of vaccine given annually in the fall should be sufficient.

Third, older adults, >65 years of age, and children and adults of all ages who are at increased risk for serious consequences of influenza virus infection, should receive optimal protection by immunization. Since the live virus vaccine preferentially induces influenza-specific mucosal IgA antibodies in all age groups and subunit vaccine preferentially induces influenza-specific serum IgG antibodies in immunologically experienced populations, optimal immunization for elderly and high-risk patients is likely best achieved by simultaneous administration of one dose each of live attenuated and subunit vaccine given annually in the fall. The increased efficacy of live *ca* reassortant plus subunit vaccine versus subunit vaccine alone in the elderly needs to be confirmed in phase 3 trials prior to the combined use of the two vaccines.

ENVIRONMENTAL ISSUES CONCERNING WIDESPREAD USE OF *ca* VIRUS IN HUMANS

Does exchange occur between the HA and NA genes of the influenza H1N1 and H3N2 *ca* reassortant viruses, and does this represent a significant concern? Reassortant viruses containing mixed constellations of HA and NA genes, that is, H3N1 and H1N2 reassortants, are readily detected in vaccinees given bivalent influenza A H1N1 and H3N2 vaccines with up to 25% of virus plaque populations isolated directly from original nasal wash specimens being antigenic hybrid viruses (95). These antigenic hybrids, of course, would contain all six of the internal genes of the A/AA/6/60 *ca* donor virus and therefore would be highly attenuated and poorly transmissible. Gene exchange between the influenza A and B components in the vaccine would not be expected to occur and has not been observed.

Does exchange between the HA and NA genes of H1N1 and H3N2 *ca* reassortant viruses pose a particular or unique problem? Gene exchange between wild-type H1N1 and H3N2 viruses has been observed repeatedly in humans since 1977 when the two viruses began co-circulating (99). The antigenic hybrid viruses were identified as a part of a routine surveillance program to genetically characterize human influenza A viruses and not because they possessed unique clinical signatures. In the past, the antigenic reassortant wild-type viruses circulated in nature only transiently and were subsequently replaced by nonreassortant wild-type H1N1 and H3N2 viruses, but currently wild-type H1N2 viruses are circulating more widely. Since the antigenic hybrid viruses are not associated with unique epidemiological or clinical properties and they occur following co-infection with circulating wild-type viruses, it is reasonable to conclude that the generation of H1N2 and H3N1 antigenic hybrid *ca* reassortant viruses does not pose a unique or significant risk to the vaccinee or to the rest of the population.

Does the widespread introduction of any of the genes present in the influenza A and B *ca* reassortant viruses by immunization of the human population raise any specific concerns? The HA's and NA's of the influenza A and B components of the trivalent vaccine are derived from the recently emerged epidemic influenza A and B viruses that are already widely distributed in nature, and therefore the reintroduction of these genes into the population by immunization does not represent a unique risk.

The currently circulating influenza B viruses are descendants of viruses related to the influenza B/AA/1/66 wild-type virus, and thus their internal genes are highly related genetically and functionally to those of the B/AA/1/66 *ca* donor virus. Since the internal genes of the B/AA/1/66 *ca* donor virus were once widely distributed in humans, they are considered not to pose a specific epidemiological or environmental risk when administered to the human population as part of a vaccine.

The six internal RNA segments of the H1N1 and H3N2 *ca* reassortant vaccine viruses are derivatives of a 1960 H2N2 virus. In 1968, when the H3N2 virus replaced the circulating H2N2 viruses, all genes of the H2N2 virus, except the HA and PB1, were derived from the human H2N2 parent virus, the HA and PB1 genes coming from an avian influenza A parent virus (98). The PB2, PA, NP, M, and NS genes of the H1N1 and H3N2 *ca* reassortant vaccine viruses are thus highly related to those of circulating H3N2 wild-type viruses, which are also direct descendants of the genes that were present in H2N2 viruses. Therefore, the shared presence of these highly related genes in the influenza A *ca* vaccine viruses and in the circulating wild-type H3N2 viruses suggest that they do not pose a specific epidemiological or environmental risk when administered to the human population as part of the CAIV-T.

The PB1 gene of the currently circulating H3N2 viruses was derived in 1968 from an avian influenza A

parent virus different from the H2N2 donor of the PB1 gene in 1957. Thus, the PB1 genes of the currently circulating H1N1 and H3N2 wild-type viruses are considered to be from a different lineage than that in the A/AA/6/60 *ca* donor virus. However, the percent similarity of the amino acid sequence of the A/AA/6/60 *ca* PB1 gene and that of current H1N1 and H3N2 wild-type viruses are 94% and 98%, respectively, indicating a high degree of functional and genetic relatedness of the three PB1 genes. Since each of the three PB1 genes has been widely distributed in the human population, it is reasonable to suggest that the PB1 gene of the H1N1 and H3N2 *ca* reassortants does not pose a specific epidemiological or environmental risk when administered to the human population as part of the trivalent vaccine.

Does the widespread use of the trivalent *ca* reassortant vaccine present specific agricultural concerns? Influenza A viruses naturally present in humans or birds have gained access to pigs, horses, or domestic poultry with significant adverse agricultural consequences (38,86,98). Such interspecies transfer of wild-type influenza viruses will continue to occur. Influenza B viruses have not been found in birds or farm animals and are not considered to be an agricultural concern.

How likely is it that the H1N1 and H3N2 *ca* reassortants will also spread from humans to animals? Would this transfer pose unique hazards that are not shared with the transfer of the currently circulating wild-type H1N1 and H3N2 viruses? It is considered unlikely that the H1N1 and H3N2 *ca* reassortants will spread from humans to animals and cause disease in animals for several reasons. First, the findings outlined above indicate that the *ca* reassortants are poorly transmissible to their preferred host, humans. Second, the factors that restrict transmission from human to human, namely, low level of replication in the human host and the reduced level of sneezing and coughing, should theoretically act to restrict their transmission from vaccinees to animals or birds. Third, birds, swine, and horses have body temperatures considerably higher than that of humans, and this would serve to further restrict the replication of the temperature-sensitive attenuated H1N1 and H3N2 *ca* reassortants in these animals. For each of these reasons, it is much more likely that a currently circulating H1N1 and H3N2 wild-type virus would be transmitted from a human to an animal or bird than a H1N1 or H3N2 *ca* reassortant. The specific genetic makeup of the H1N1 and H3N2 *ca* reassortants should not represent a unique threat to the animals or birds since there is great diversity of all influenza virus genes in birds, and they are continually being transferred to animals and humans. Thus, environmental risks for poultry and farm animals inherent in the use of the H1N1 and H3N2 *ca* reassortants would not be considered unique to these viruses. In the context of the greatly restricted transmissibility of the *ca* reassortant virus vaccines, it is therefore reasonable to conclude that specific agricultural concerns are not raised by the widespread use of a trivalent *ca* vaccine in humans.

Are there concerns that the widespread use of *ca* reassortant vaccines could lead to the generation of new reassortant viruses that are pathogenic for humans. Avian influenza viruses intermittently gain access to the human population (38,86), and during the time they are replicating in humans, they have the opportunity to undergo genetic reassortment with a co-infecting human influenza A H1N1 or H3N2 wild-type virus. In addition, avian and human influenza viruses can infect and be maintained in the swine population. Reassortment between human and avian influenza viruses can occur in swine, and reassortant viruses can be subsequently transmitted from swine to humans (13). It appears unlikely that an influenza A *ca* reassortant virus would become established in the swine population, and we therefore consider the possibility for reassortment between an avian influenza A virus and a *ca* reassortant occurring in swine to be remote. Since swine are preferentially infected with H1N1 and H3N2 viruses, any reassortant formed in swine between an avian influenza A virus and a *ca* reassortant would most likely generate a H1 or H3 reassortant virus; that is, viruses that are already prevalent in the human population and whose transmissibility within the human population would likely be restricted by homotypic immunity.

We will only further consider the possibility that a pathogenic reassortant virus could be generated by the simultaneous infection of a human with an influenza A avian virus and a *ca* reassortant vaccine virus. It is possible that a human could be simultaneously infected both with a novel avian influenza A virus, for example, H5N1 or H9N2 (38,86) and with a *ca* reassortant vaccine virus and that a reassortant virus bearing the novel avian HA and NA genes and a set of human internal genes derived from the *ca* parent could be generated in such a vaccinee. Since the *ca* reassortant virus is attenuated by mutations in up to five of its six internal genes, it is very unlikely that a reassortant virus bearing the novel avian HA and NA genes and the human internal genes from the *ca* vaccine virus would be a highly transmissible virulent virus with

pandemic potential. Generation of such a pandemic virus is much more likely to occur when the avian influenza A virus infects a human co-infected with a wild-type influenza A H1N1 or H3N2 virus. Importantly, the use of a trivalent *ca* reassortant virus vaccine does not present a novel opportunity for the generation of an avian–human pandemic reassortant since this opportunity occurs everyday somewhere in the world as the avian influenza A viruses continuously probe the human population directly or via their intermediate hosts.

However, it is clear that there are times when an influenza A *ca* reassortant vaccine virus should not be used in open populations. One such circumstance is in context of an abortive epidemic. In 1997, an avian H5N1 virus infected humans in a mini-epidemic, but this virus failed to spread efficiently in the human population and resulted only in an abortive epidemic. In anticipation of the continued spread of the H5N1 virus, the preemptive introduction outside of Asia of a H5N1 6/2 *ca* reassortant vaccine virus into an open population in which the H3N2 and H1N1 wild-type viruses are circulating could have posed a significant risk. Under these circumstances, it would have been possible to generate a highly transmissible H5N1 virus bearing one or more wild-type human influenza A virus internal genes in place of the H5N1 avian genes. Such a reassortant virus could be generated by co-infection of a recipient of H5N1 *ca* reassortant vaccine with a wild-type influenza A virus.

The H5N1 avian–human influenza A hybrid virus could then spread efficiently in humans and potentially initiate a pandemic that might never have arrived naturally. Therefore, only in the face of a unequivocal pandemic threat should a *ca* reassortant vaccine be considered for introduction into the population in advance of a new pandemic virus since the small risk of its reassortment with concurrently circulating H1N1 or H3N2 wild-type viruses in such a situation would be more than balanced by the significant benefit of the protection achieved by the use of the vaccine.

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