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In Elderly Persons Live Attenuated Influenza A Virus Vaccines Do Not Offer an Advantage over Inactivated Virus Vaccine in Inducing Serum or Secretory Antibodies or Local Immunologic Memory

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In a double-blind, randomized trial, 102 healthy elderly subjects were inoculated with one of four preparations: (i) intranasal bivalent live attenuated influenza vaccine containing cold-adapted A/Kawasaki/86 (H1N1) and cold-adapted A/Bethesda/85 (H3N2) viruses; (ii) parenteral trivalent inactivated subvirion vaccine containing A/Taiwan/86 (H1N1), A/Leningrad/86 (H3N2), and B/Ann Arbor/86 antigens; (iii) both vaccines; or (iv) placebo. To determine whether local or systemic immunization augmented mucosal immunologic memory, all volunteers were challenged intranasally 12 weeks later with the inactivated virus vaccine. We used a hemagglutination inhibition assay to measure antibodies in sera and a kinetic enzyme-linked immunosorbent assay to measure immunoglobulin G (IgG) and IgA antibodies in sera and nasal washes, respectively. In comparison with the live virus vaccine, the inactivated virus vaccine elicited higher and more frequent rises of serum antibodies, while nasal wash antibody responses were similar. The vaccine combination induced serum and local antibodies slightly more often than the inactivated vaccine alone did. Coadministration of live influenza A virus vaccine did not alter the serum antibody response to the influenza B virus component of the inactivated vaccine. The anamnestic nasal antibody response elicited by intranasal inactivated virus challenge did not differ in the live, inactivated, or combined vaccine groups from that observed in the placebo group not previously immunized. These results suggest that in elderly persons cold-adapted influenza A virus vaccines offer little advantage over inactivated virus vaccines in terms of inducing serum or secretory antibody or local immunological memory. Studies are needed to determine whether both vaccines in combination are more efficacious than inactivated vaccine alone in people in this age group.

Influenza epidemics are a significant cause of severe illness and mortality in elderly people, even among those without chronic medical problems (4). Persons aged 65 years or older have therefore been targeted along with individuals in other high-risk groups to receive influenza vaccinations annually (22). Rates of protection against influenza illness afforded by commercially available inactivated virus vaccines have generally been lower in elderly individuals, particularly those who are institutionalized, than efficacy rates reported in studies of younger populations (3, 5, 14, 15, 18, 21, 30, 35). These observations, which suggest that the immune response to inactivated influenza vaccines may decline with advancing age, have prompted the search for alternative approaches to vaccination that will more effectively stimulate immunity to influenza in elderly individuals.

Immunologic factors, in addition to serum antibody, including the presence of local (secretory) immunoglobulin A (IgA) antibody in the upper respiratory tract, have been shown to correlate with resistance to influenza virus infection in children and young adults (10, 23). Optimal protection may therefore require both systemic and local components of the humoral immune response. Parenterally administered inactivated virus vaccines induce antibody in the serum, but they are less effective at stimulating the production of secretory IgA antibody (9, 11). On the other hand, intranasally administered, live attenuated cold-adapted (*ca*) influenza A reassortant virus vaccines effectively elicit both serum and secretory antibody responses in children and young adults (11, 27). In a previous study involving healthy elderly subjects (32), we reported that a monovalent live attenuated *ca* influenza A H1N1 virus vaccine induced infrequent and short-lived systemic and local antibody responses when administered alone, and it only marginally augmented the secretory antibody response to parenteral inactivated virus vaccine when the two vaccines were given in combination. Vaccines containing hemagglutinin and neuraminidase components from both H1N1 and H3N2 subtypes are likely to be more efficacious than monovalent preparations during influenza A epidemics. The present study was therefore undertaken in persons aged 60 years or older (referred to as elderly) to reexamine our previous findings with a bivalent live attenuated *ca* influenza A vaccine comprising H1N1 and H3N2 viruses.

In our earlier study, titers of local IgA antibody elicited by live, inactivated, or both influenza virus vaccines declined rapidly in elderly subjects and returned to near base-line levels by 3 months after vaccination (32). It is possible, however, that those volunteers immunized with live virus vaccine, despite having undetectable levels of antibodies in nasal secretions, may nonetheless have been primed to mount an anamnestic mucosal antibody response upon sub-

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sequent exposure to related antigens. This question was previously addressed in a study with children who were infected with attenuated influenza A virus vaccine but whose nasal antibody levels had returned to barely detectable levels 1 year later (38). These children were able to mount an anamnestic influenza virus-specific secretory IgA antibody response following intranasal administration of inactivated influenza virus vaccine. As part of the present investigation, we conducted a similar study by rechallenging the elderly subjects intranasally with inactivated virus vaccine 12 weeks after the initial immunization and then examining the local and systemic immune responses.

MATERIALS AND METHODS

Vaccines. Two vaccines containing antigenically similar influenza A subtype components were used in this study: a live attenuated bivalent virus vaccine comprising *ca* A/Kawasaki/86 (H1N1) (CR125, lot E-263) and *ca* A/Bethesda/85 (H3N2) (CR 90, lot E-254) reassortant viruses and a licensed trivalent subvirion vaccine comprising 15 μg of each of the HAs of A/Taiwan/86 (H1N1), A/Leningrad/86 (H3N2), and B/Ann Arbor/86 viruses (Wyeth Laboratories, Marietta, Pa.). The two *ca* reassortant viruses were derived by mating the attenuated *ca* A/Ann Arbor/60 (H2N2) donor with A/Kawasaki/86 (H1N1) or A/Bethesda/85 (H3N2) human wild-type influenza virus. The production, characterization, and safety testing of *ca* reassortant viruses have been described previously (28). Each reassortant virus suspension was grown in the allantoic cavity of specific-pathogen-free eggs and tested for the presence of adventitious agents by Louis Potash (Flow Laboratories, McLean, Va.). Live virus vaccination was performed by intranasal administration of $10^{7.5}$ 50% tissue culture infective doses of each virus in a total volume of 0.5 ml (0.25 ml per nostril). Vaccination with the inactivated vaccine was performed by injecting a 0.5-ml dose intramuscularly into the deltoid region. Challenge was performed by instilling 0.25 ml of the inactivated vaccine into each nostril.

Clinical studies. Study protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases and the Joint Committee on Clinical Investigations of the Johns Hopkins Medical Institutions. Ambulatory volunteers aged 60 years or older (37) were recruited from senior centers in Baltimore County, Md. Volunteers were screened by history, physical examination, complete blood cell count, and an SMA-12 biochemistry panel. Persons were excluded from the study if they had been immunized previously with live attenuated *ca* influenza A vaccine, had markedly abnormal results of any of the screening laboratory tests, had clinically unstable chronic obstructive pulmonary disease or cardiovascular disease, had end-stage renal failure, were taking immunosuppressive or antineoplastic medication, or were allergic to influenza vaccine, eggs, neomycin, amphotericin B, erythromycin, or amantadine. Participants in these studies gave written, informed consent. Serum samples collected from volunteers at the time of screening were tested for hemagglutination inhibition (HAI) antibodies to influenza A/Kawasaki/86 (H1N1) and A/Bethesda/85 (H3N2) viruses. To study the effect of preimmunization antibody status on response to vaccine, subjects were stratified into groups with low (≤1:16) or high (≥1:32) titers according to their prevaccination levels of serum HAI antibody to H1N1 virus. Persons within each group were then randomly assigned to receive one of the following in a blinded manner: (i) intranasal live virus vaccine and intramuscular saline placebo, (ii) intranasal saline placebo and intramuscular inactivated virus vaccine, (iii) intranasal live virus and intramuscular inactivated virus vaccines, or (iv) intranasal and intramuscular saline placebo. These preparations were administered between June and August 1988. Twelve weeks after inoculation, all subjects were challenged intranasally with inactivated influenza virus vaccine. At the conclusion of the study, participants were offered the commercially available inactivated influenza vaccine recommended for the 1988 to 1989 season. All subjects took and recorded their temperature four times daily for 4 days following inoculation. During this period they were questioned by telephone daily about the development of any symptoms; reported symptoms were confirmed by a physician. A volunteer was considered ill if he or she developed any of the following findings within 4 days after vaccination: oral temperature of ≥37.8°C (fever), myalgia alone or with chills or sweats (systemic illness), rhinitis or pharyngitis on 2 or more consecutive days (upper respiratory illness), a persistent cough or dyspnea on 2 or more consecutive days (lower respiratory illness), or local reactions at the injection site. Influenza wild-type viruses were not detected in the community during the course of this study.

Laboratory studies. Serum and nasal wash specimens for antibody measurements were collected before inoculation with vaccine or placebo and 4, 12, 13, 14, and 16 weeks afterward. Methods for collecting and concentrating nasal wash specimens have been described elsewhere (26). HAI antibodies in serial serum specimens were measured by standard techniques (29), using influenza A/Kawasaki (H1N1), A/Bethesda (H3N2), and B/Ann Arbor viruses as antigens. A significant response to vaccination was defined as a fourfold or greater rise in HAI titer between prevaccination and 4- or 12-week postvaccination serum specimens. An antibody response to challenge was defined as a fourfold or greater rise in HAI titer between the prechallenge specimen collected at 12 weeks and any of the postchallenge serum specimens collected at 13, 14, or 16 weeks. A kinetic enzyme-linked immunosorbent assay (KELISA) (34), with minor modifications, was performed, using A/Kawasaki (H1N1), A/Bethesda (H3N2), or B/Ann Arbor virus as antigens, to measure IgG antibodies in serum or IgA antibodies in nasal wash specimens. The sequence of reagents from the solid phase outward consisted of (i) whole virus (ii) human serum or nasal wash specimen, (iii) rabbit anti-human IgG (for serum) or IgA (for nasal wash) conjugated with alkaline phosphatase, and (iv) *p*-nitrophenyl phosphate disodium substrate. For each antigen, we used serum and nasal wash dilutions that were associated with prevaccination KELISA rates of 10 to 150 milli-optical density units per min, which were well within the dynamic range of the assay. The corresponding serum dilutions were 1:4,000 for A/Kawasaki (H1N1), 1:1,000 for A/Bethesda (H3N2), and 1:400 for B/Ann Arbor, whereas nasal wash dilutions were 1:8 for A/Kawasaki (H1N1) and 1:4 for A/Bethesda (H3N2). After addition of substrate, the initial rate of color development in each well of a 96-well plate was monitored with a Vmax Kinetic microplate reader (Molecular Devices, Palo Alto, Calif.). The minimum fold rise between KELISA rates that appropriately defined a significant antibody response was calculated for each of the test antigens as the mean plus 2 standard deviations of all possible ratios between prevaccination, 4-week postvaccination, and 12-week postvaccination KELISA rates in placebo recipients. This calculation should reflect both intrinsic assay variability and biologic but non-immunization-related variability in individuals over

time. The serum IgG KELISA rate ratios in placebo recipients were normally distributed, and 95% upper confidence limits were readily calculated. When determined in this manner, significant serum antibody responses were defined as post-KELISA/pre-KELISA rate ratios that were ≥1.34 for A/Kawasaki (H1N1), ≥1.46 for A/Bethesda (H3N2), and ≥1.37 for B/Ann Arbor. These ratios were used to compare serum IgG KELISA rates for each individual, first, between prevaccination and 4- or 12-week postvaccination specimens and, second, between the 12-week postvaccination (prechallenge) specimen and the highest of the three postchallenge specimens.

Because of the wide variability in concentrations between different nasal wash specimens, the nasal wash IgA KELISA rates were normalized to a total protein concentration of 350 μg/ml prior to further analysis by using the following formula: KELISA $rate_{corrected}$ = KELISA $rate_{sample}$ × [(350 μg/ml)/(protein $concentration_{sample}$)]. This total protein concentration of 350 μg/ml approximated the median among all specimens tested, as determined by a colorimetric assay (Pierce, Rockford, Ill.). Unlike the serum IgG data, the ratios between prevaccination, 4-week postvaccination, and 12-week postvaccination nasal wash IgA KELISA rates in placebo recipients were not normally distributed and contained multiple outlier values such that useful 95% upper confidence limits were not obtained. Cutoff ratios for defining significant rises in nasal wash antibodies were therefore arbitrarily determined as the minimum values associated with response rates of ≤10% among placebo recipients. This choice of values corresponded to post-KELISA/pre-KELISA rate ratios that were ≥1.40 for A/Kawasaki (H1N1) or ≥2.00 for A/Bethesda (H3N2). These cutoff ratios were used to compare nasal wash IgA KELISA rates for each individual, first, between prevaccination and 4-week postvaccination specimens and, second, between the 12-week postvaccination (prechallenge) specimen and the highest of the three postchallenge specimens.

Statistical analysis. Differences between various subject groups were analyzed by using several methods: (i) the Fisher exact test to compare proportions of antibody responses, (ii) the two-tailed unpaired Student *t* test to compare mean prevaccination antibody levels or mean increases in serum antibody levels, and (iii) the two-sample Wilcoxon (Mann-Whitney U) test to detect significant shifts in the distributions of post-KELISA/pre-KELISA rate ratios of nasal wash IgA antibody. The modified Bonferroni inequality was applied when appropriate to account for multiple possible comparisons (20). Differences within vaccine groups were analyzed two ways. We used the Fisher exact test to compare proportions of responders with low versus high prevaccination antibody titers, and the two-tailed paired Student *t* test to compare mean antibody levels from serially collected specimens within a group.

RESULTS

Study population. Volunteers aged 60 to 90 years (71 ± 6 years, mean ± standard deviation) were inoculated with live *ca* virus vaccine alone (n = 24), inactivated virus vaccine alone (n = 26), both vaccines (n = 26), or placebo (n = 26). Of the subjects in each group, approximately one-half had a prevaccination HAI antibody titer of ≤1:16 to A/Kawasaki (H1N1), and approximately one-third were immunized with inactivated influenza vaccine previously. There were no significant differences among the four vaccine groups in their mean age, gender, or prevaccination levels of serum HAI, serum KELISA IgG, or nasal wash KELISA IgA antibodies to any of the test antigens, with one exception. There was a higher mean serum IgG A/Bethesda (H3N2) KELISA rate in recipients of the vaccine combination than in recipients of the inactivated vaccine alone (55.8 ± 24.3 versus 34.2 ± 18.0; $P < 0.005$) (Table 1).

Safety. The live and inactivated virus vaccines were well-tolerated. None of the volunteers immunized with live *ca* viruses alone reported illness during the 4-day postvaccination period. Three inactivated vaccine recipients had a local reaction at the injection site. Among the subjects who received both vaccines, three had fever (37.9°C) (two with rhinitis), and two had local reactions.

Serum antibody responses after the initial vaccination. Antibody responses after the initial vaccination are summarized in Table 1. Only a few of the live *ca* virus vaccinees developed fourfold increases in serum HAI antibody or significant elevations of KELISA IgG antibody to either H1N1 or H3N2 virus antigen. In contrast, serum antibody responses were achieved by substantial numbers of subjects immunized with inactivated virus vaccine alone or with the vaccine combination. A greater proportion of recipients of either inactivated or combined vaccines mounted serum responses of HAI or KELISA IgG antibody to H1N1 antigen or of KELISA IgG antibody to H3N2 antigen than did those who were given live *ca* viruses along ($P < 0.05$ in all cases but one). The exception was the H1N1 KELISA IgG response in the live versus inactivated virus vaccine groups. Serum HAI response rates to H3N2 antigen were relatively low and did not differ significantly between the vaccine groups. Figure 1 depicts the time course of serum antibody responses. As expected, during the 12 weeks after inoculation of the placebo group, mean HAI titers and KELISA rates remained essentially unchanged from prevaccination levels. Mean serum antibody levels rose marginally, if at all, following immunization with the live *ca* viruses alone, and none of these changes were significant relative to those found in the placebo group. In contrast, 4 weeks after immunization, both the inactivated vaccine alone and the vaccine combination elicited substantial rises in mean serum levels of all antibodies, and the magnitudes of these increases were comparable between the two groups in all cases. Compared with the responses elicited by the live *ca* vaccine alone, mean increases in serum levels of both HAI and KELISA IgG antibodies to H1N1 and H3N2 viruses were significantly greater after immunization with inactivated vaccine alone ($P < 0.05$ in all cases) or the vaccine combination ($P < 0.01$ in all cases). Twelve weeks after the initial vaccination, mean serum antibody levels in both inactivated and combined vaccine recipients had declined modestly from the peak levels achieved 4 weeks postvaccination.

There were no significant differences in the frequencies or magnitudes of serum HAI and KELISA IgG influenza B antibody responses in persons immunized with inactivated vaccine compared with those in recipients of both vaccines (Table 2). This similarity suggested that the serum antibody response to the influenza B component of the inactivated virus preparation is not altered by the simultaneous administration of *ca* type A viruses.

Effect of prevaccination serum antibody levels on seroresponse to initial vaccination. Among subjects immunized with live *ca* viruses alone, HAI seroresponses were infrequent. Only two of these individuals (with prevaccination HAI titers of 1:4 and 1:512, respectively) had a fourfold or greater rise of HAI antibody to A/Kawasaki (H1N1), and no indi-

TABLE 1. Serum and nasal wash antibody responses in elderly persons inoculated with placebo, bivalent live attenuated influenza vaccine containing *ca* A/Kawasaki (H1N1) and *ca* A/Bethesda (H3N2) reassortant viruses, trivalent inactivated influenza virus vaccine, or both vaccines

Vaccine(s) administered	No. of subjects tested	Antigen tested	Serum HAI antibody				Serum KELISA IgG antibody				Nasal wash KELISA IgA antibody			
			HAI titer (reciprocal mean $\log_2$ ± SD)				KELISA rate (mean ± SD)				Median[b] KELISA rate			
			Before vaccination	Postvaccination		% with response[a]	Before vaccination	Postvaccination		% with response[a]	Before vaccination	Postvaccination		Overall % with any antibody response
				4 wk	12 wk			4 wk	12 wk			4 wk	12 wk	
Placebo	26	H1N1	4.65 ± 1.8	4.62 ± 1.7	4.68 ± 1.9	4	30.9 ± 18.6	29.3 ± 18.0	29.2 ± 17.2	4	98.7	76.7	86.1	8
Live	24	H1N1	5.08 ± 2.0	5.33 ± 1.9	5.45 ± 1.8	8	30.6 ± 16.5	31.7 ± 16.4	29.8 ± 15.2	16	95.1	93.2	75.7	21	42
Inactivated	26	H1N1	4.50 ± 2.1	6.00 ± 2.1	5.68 ± 2.2	35[c]	27.5 ± 13.3	36.0 ± 14.6	35.1 ± 15.2	42	76.0	69.4	67.5	31	65
Both	26	H1N1	4.96 ± 2.2	6.46 ± 1.9	5.27 ± 2.0	46[d]	30.7 ± 13.5	41.4 ± 17.5	37.8 ± 16.5	54[c]	62.5	81.0	73.1	46	81[d]
Placebo	26	H3N2	6.31 ± 2.2	6.27 ± 2.3	6.24 ± 2.1	8	51.3 ± 33.5	50.7 ± 33.5	41.0 ± 29.5	12	131.9	107.9	121.8	8	27
Live	24	H3N2	6.67 ± 1.5	6.54 ± 1.4	6.55 ± 1.5	0	47.2 ± 27.0	49.0 ± 26.2	48.0 ± 29.0	8	148.2	141.8	119.5	25	29
Inactivated	26	H3N2	5.73 ± 1.7	6.92 ± 2.0	6.68 ± 2.0	23	34.2 ± 18.0	52.8 ± 26.3	46.3 ± 22.6	46[c]	110.0	106.7	106.1	15	58
Both	26	H3N2	6.58 ± 2.2	7.50 ± 1.7	7.15 ± 1.6	19	55.8 ± 24.3	75.9 ± 28.5	67.3 ± 23.0	38[c]	108.6	121.1	104.4	23	62

[a] Definitions of response for antibodies measured by HAI and KELISA are given in the text.
[b] Median values are given due to the nonnormal distribution of nasal wash IgA KELISA rates.
[c] $P < 0.05$. All significance levels given here were by comparison with recipients of live *ca* virus alone (Fisher exact test with application of the modified Bonferroni inequality [20]).
[d] $P < 0.01$.

viduals had HAI seroconversions to A/Bethesda (H3N2). Serum H1N1 HAI antibody responses to inactivated virus or combined vaccines occurred significantly more often among vaccinees with low prevaccination titers (≤1:16) than it did among those with high prevaccination titers (≥1:32). In those immunized with the inactivated virus vaccine, the response occurred in 8 of 14 subjects with low prevaccination titers, whereas it occurred in 1 of 12 subjects with high prevaccination titers ($P < 0.05$). In those immunized with both vaccines, it occurred in 9 of 11 versus 3 of 15 subjects ($P < 0.005$). Similarly, a higher proportion of volunteers who had A/Bethesda (H3N2) HAI antibody titers of ≤1:16 before vaccination developed fourfold rises in H3N2 HAI antibody than did those who had titers of ≥1:32. In the inactivated vaccine group, the fourfold or greater rise occurred in 3 of 5 versus 3 of 21 subjects ($P = 0.062$); in the combined vaccine group, the fourfold or greater rise occurred in 4 of 6 versus 1 of 20 subjects ($P < 0.005$). Serum H3N2 IgG KELISA antibody responses to vaccination were also more frequent among volunteers with low (≤1:16) versus high (≥1:32) prevaccination titers of H3N2 HAI antibody: 5 of 5 versus 9 of 21 subjects ($P < 0.05$) in the inactivated vaccine group and 5 of 6 versus 7 of 20 subjects ($P = 0.052$) in the combined vaccine group.

Nasal wash and overall antibody responses following the initial vaccination. Four weeks after immunization, the proportions of subjects who mounted H1N1 or H3N2 nasal wash IgA responses were not significantly different among the live, inactivated, and combined vaccine groups (Table 1). The same result was apparent when a nonparametric (two-sample Wilcoxon) test was used to compare these groups on the basis of their 4-week postvaccination with their prevaccination nasal wash IgA KELISA rate ratios (data not shown).

Compared with live virus vaccinees, a higher proportion of recipients of the vaccine combination mounted any antibody response to H1N1 ($P < 0.01$) and H3N2 ($P = 0.053$) viruses. Inactivated vaccine alone also elicited higher rates of overall antibody response than did live *ca* viruses, but these differences were not statistically significant.

Serum and nasal wash antibody responses following intranasal inactivated virus challenge. It is most likely that all the elderly subjects, even those with low levels of serum HAI antibody, were previously infected with influenza A H1N1 and H3N2 viruses, and were thus immunologically primed for secondary responses to related strains. To distinguish between anamnestic responses induced by previous infection and those induced by vaccination, we compared the postchallenge antibody responses of placebo recipients (who served as immunologic controls) with those of vaccinees. Whereas fourfold or greater rises of serum HAI antibody were rarely elicited by the 12-week challenge, significant increases of serum H1N1 and H3N2 IgG antibody were detected by the more sensitive KELISA in approximately 20 to 30% of placebo recipients (Table 3). Similarly, the placebo group was found to have little, if any, change in mean serum HAI antibody titers but a statistically significant peak rise in mean KELISA rates for both H1N1 ($P < 0.05$, paired Student *t* test) and H3N2 ($P < 0.05$, paired Student *t* test) serum IgG antibody (Fig. 1). In the groups immunized with live *ca* viruses alone or the vaccine combination, the frequency and magnitude of serum antibody responses to the intranasal inactivated virus challenge were generally less than, although not statistically different from, those observed in the placebo group. Persons who were initially immunized with inactivated virus vaccine alone, however,

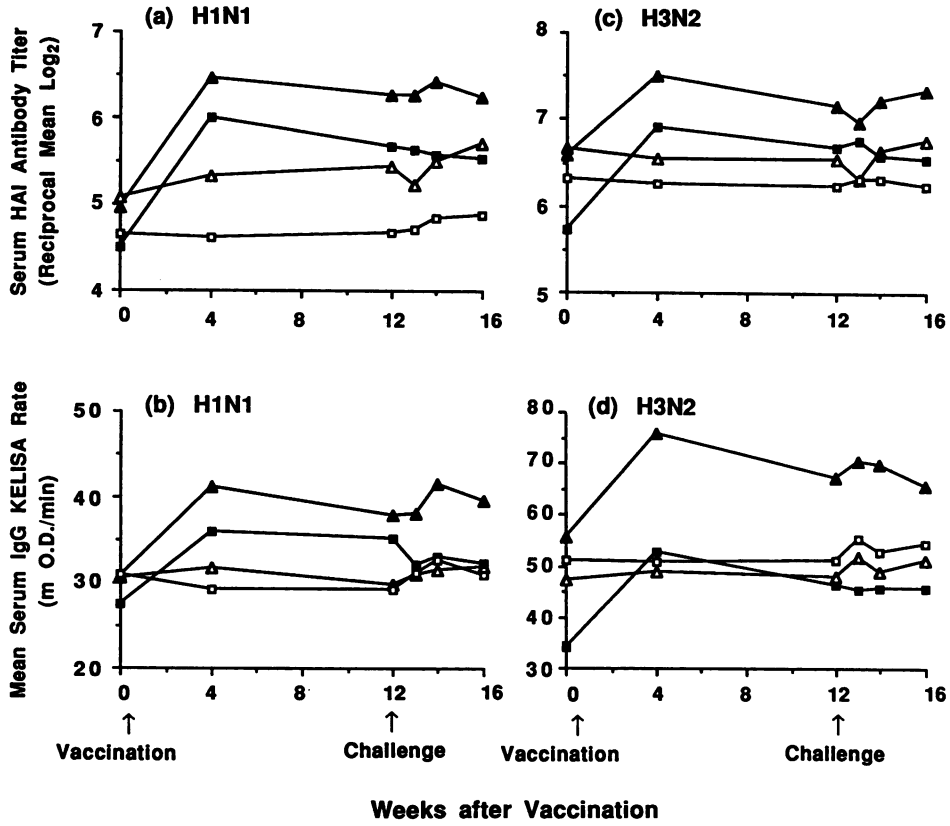

FIG. 1. Time course of serum HAI and KELISA IgG antibody responses to A/Kawasaki (H1N1) (a and b) and A/Bethesda (H3N2) (c and d) in elderly subjects inoculated at 0 weeks with placebo (□), intranasal live attenuated *ca* influenza virus vaccine alone (△), parenteral inactivated influenza virus vaccine alone (■), or both vaccines (▲) and challenged intranasally 12 weeks later with the inactivated virus vaccine. The live attenuated vaccine contained *ca* A/Kawasaki (H1N1) and *ca* A/Bethesda (H3N2) reassortant viruses; the inactivated virus vaccine contained A/Taiwan (H1N1), A/Bethesda (H3N2), and B/Ann Arbor components. Standard errors of the means were similar among the four vaccine groups and ranged from 0.25 to 0.46 (reciprocal $\log_2$) for HAI antibody and from 2.6 to 6.6 (milli-optical density units per min) (m O.D./min) for KELISA IgG antibody.

failed to mount any appreciable serum antibody responses to the subsequent challenge. Compared with placebo recipients, inactivated virus vaccinees had a significantly lower KELISA H3N2 IgG seroresponse rate ($P < 0.05$), as well as a significantly lower change in mean KELISA H1N1 IgG levels ($P < 0.01$, unpaired Student t test), after the intranasal inactivated virus challenge.

Modest nasal wash IgA responses were observed after challenge in all four volunteer groups (Table 3). There were no significant differences in the postchallenge rates of local antibody production between those people who did and those who did not mount antibody responses in serum, nasal washes, or both after their initial immunization (data not shown). Moreover, neither of the previously cited methods of analysis of nasal wash data (viz., comparison of response rates by the Fisher exact test or comparison of post-KELISA/pre-KELISA rate ratios by the two-sample Wilcoxon test) could identify a significant difference in

TABLE 2. Lack of effect in elderly volunteers of simultaneous immunization with bivalent live attenuated *ca* influenza A (H1N1 and H3N2) virus vaccine on serum antibody responses to the B/Ann Arbor component of trivalent inactivated influenza virus vaccine

Vaccine(s) administered	No. of subjects tested	Serum HAI antibody response			Serum IgG KELISA antibody response		
		HAI titer (reciprocal mean $\log_2$ ± SEM)		No. (%) with ≥fourfold rise in antibody titer	Mean KELISA rate (mOD[a]/min ± SEM)		No. (%) with significant rise in KELISA rate[b]
		Before vaccination	4 wk after vaccination		Before vaccination	4 wk after vaccination	
Inactivated[c]	24	4.31 ± 0.3	5.19 ± 0.4	5 (19)	84.4 ± 9.8	108.6 ± 8.8	11 (42)
Both[d]	26	5.19 ± 0.3	5.65 ± 0.3	4 (15)	107.0 ± 11.0	124.2 ± 11.4	10 (38)

[a] mOD, Milli-optical density units.
[b] A significant rise was calculated as a ≥1.37-fold increase in KELISA rates between prevaccination and 4- or 12-week postvaccination serum specimens.
[c] Trivalent inactivated subvirion vaccine containing influenza A/Taiwan (H1N1), A/Leningrad (H3N2), and B/Ann Arbor components.
[d] Inactivated virus vaccine plus bivalent live attenuated influenza virus vaccine containing *ca* A/Kawasaki (H1N1) and *ca* A/Bethesda (H3N2) reassortant viruses.

TABLE 3. Serum and nasal wash antibody responses following intranasal inactivated virus challenge[a] in elderly persons immunized 12 weeks earlier with influenza virus vaccine(s) or placebo

Vaccine group	No. of subjects tested[b]	% with specified antibody response to[c]:					
		A/Kawasaki (H1N1)			A/Bethesda (H3N2)		
		Serum		Nasal wash KELISA IgA	Serum		Nasal wash KELISA IgA
		HAI	KELISA IgG		HAI	KELISA IgG	
Placebo	25	4	20	36	4	32[d]	16
Live	22	5	18	32	0	18	18
Inactivated	24	0	4	29	0	0[d]	33
Both	26	4	8	31	0	8	23

[a] A 0.5-ml dose of trivalent inactivated subvirion vaccine containing A/Taiwan/86 (H1N1), A/Leningrad/86 (H3N2), and B/Ann Arbor/86 components was administered intranasally.
[b] Postchallenge specimens from five vaccinees were unavailable for testing.
[c] Definitions of response for antibodies measured by HAI and KELISA are given in the text.
[d] $P < 0.05$ (Fisher exact test with application of the modified Bonferroni inequality [20]).

responses between the recipients of any vaccine regimen and recipients of placebo.

DISCUSSION

The present study with a bivalent *ca* reassortant influenza A virus vaccine confirms our previous finding with a monovalent preparation that live attenuated viruses, although well-tolerated, are poorly immunogenic in a population of healthy elderly individuals. Prior influenza infections in elderly persons may result in an inverse correlation between serum antibody levels before vaccination and seroconversion rates after vaccination (19, 25, 31). To control for preexisting antibody, we stratified our subjects on the basis of screening titers of HAI antibody to the H1N1 vaccine component prior to randomization into vaccine groups. The low to moderate frequencies of HAI antibody response among our elderly subjects were similar to those reported previously for noninstitutionalized older persons after vaccination with inactivated or live influenza virus vaccines (1, 2, 7, 8, 16, 24, 25). The present results also corroborate earlier observations by us and others (16, 32) that older adults mount greater seroresponses to inactivated influenza virus vaccines than they do to live attenuated viruses. In this study, the KELISA was more sensitive than HAI in detecting antibody responses in serum. Better detection was expected since the minimum fold rise in antibody levels that defined a significant response was smaller for KELISA than it was for the HAI antibody. Since KELISA with whole virus antigen presumably detects antibodies to external glycoproteins as well as to other more conserved viral proteins, this assay may not be expected to discriminate well between heterologous responses. It was therefore noteworthy that the patterns of antibody production detected by HAI and KELISA were quite similar in the present study. Seroresponses to both H1N1 and H3N2 viruses were meager in recipients of live *ca* viruses alone. In contrast, recipients of inactivated vaccine alone or the vaccine combination demonstrated significant serum antibody responses relative to those in the live virus group, but neither regimen was superior to the other.

In a previous study with healthy elderly subjects (32), we reported that the frequency of a fourfold or greater rise in HA-specific nasal wash IgA was not different between recipients of monovalent *ca* A/Kawasaki (H1N1) or inactivated A/Taiwan (H1N1). Because of unexplained rises of nasal wash IgA KELISA rates in three placebo recipients in the present study, satisfactory cutoff rate ratios for defining a significant response could not be derived in the same manner as they were for serum antibodies. We therefore used two alternative methods to analyze the nasal wash data and found that both approaches yielded similar results (viz., comparable responses between live and inactivated vaccine groups). Despite the technical difficulties encountered, KELISA appears to be much more sensitive than the traditional HAI or endpoint titration ELISA for detecting antibody responses. The present data confirm our previous findings, and they extend our observations to a live attenuated bivalent vaccine containing a *ca* influenza A H3N2 virus component. Among older adults with chronic diseases, Gorse and colleagues (17) compared local hemagglutinin-specific IgA antibody response rates following immunization with *ca* A/California (H1N1) or *ca* A/Washington (H3N2) virus to those elicited 1 year later in the same persons by inactivated virus vaccine. They found no significant difference between the live and inactivated virus vaccines in the H1 response, but they did observe a more frequent H3 response in the live *ca* virus recipients (17). The results presented here (i.e., the relative abilities of live attenuated and inactivated virus vaccines to stimulate a local antibody response in older adults) may differ from those of Gorse et al. (17) because of inherent differences in immunogenicity between *ca* viruses derived from different wild-type parent strains. Likewise, the different ages or background immunity to influenza of study populations could account for the contrasting results. Geometric mean titers of prevaccination serum HAI antibody to both H1N1 and H3N2 vaccine viruses were approximately two to three times higher among our healthy elderly subjects compared with those among the older adults in the other study (17). It has been shown previously (12, 13) that the susceptibility of seropositive young adults to infection with *ca* influenza A virus is inversely correlated to prevaccination levels of serum or nasal wash antibody. In our studies, healthy elderly volunteers with any level of preexisting serum HAI antibody were relatively resistant to infection by live attenuated influenza viruses at the doses used, presumably because of acquired immunity resulting from previous infections. It is possible that immunologic interference between simultaneously administered *ca* viruses in a bivalent vaccine may have attenuated the responses to live virus vaccination in this study. Likewise, differences between vaccine groups in terms of their mean prevaccination levels of serum and local antibodies, although not statistically significant, may have partially

accounted for the lower response rates among volunteers given live vaccine alone. Despite these considerations, the available data indicate that intranasal immunization with live *ca* influenza A viruses elicits local IgA antibody less often in older adults than it does in seronegative young adults and children as reported in previous studies (6, 9, 11, 23, 27, 33, 39).

We previously reported that nasal wash IgA antibody responses in elderly volunteers are more frequent and of greater magnitude after the simultaneous administration of live attenuated and inactivated influenza virus vaccines than they are after administration of inactivated vaccine alone (32). These findings may have been due to differences between prevaccination titers of local antibody in the two vaccine groups. By contrast, in the present study prevaccination nasal wash IgA antibody levels were comparable between groups, and no significant differences were found between H1N1 or H3N2 local IgA antibody responses elicited by the live, inactivated, or combined vaccine regimens. Overall, the data from this and earlier investigations support the contention that live attenuated *ca* influenza A viruses enhance the immunogenicity of inactivated virus vaccines only marginally, if at all, when the two are administered in combination.

Our earlier investigation suggested that vaccine-induced secretory antibody responses are short-lived in the elderly. To determine whether vaccination of this age group establishes memory in the local compartment of the humoral immune system such that anamnestic responses are elicited upon exposure to influenza virus, we conducted a challenge study similar to a previous one conducted in children (38). Wright and colleagues (38) found that naturally infected children and *ca* virus vaccinees, but not inactivated vaccine recipients, mounted an anamnestic nasal wash IgA antibody response as long as 1 year after primary immunization. This antibody response demonstrated the ability of live virus infection to induce local memory in previously immunologically naive children. We challenged our elderly vaccinees by administering inactivated vaccine intranasally 12 weeks after vaccination and used a KELISA to measure nasal wash IgA antibodies. Local antibody responses were comparable among the live, inactivated, and combined vaccine groups, and within each group they did not differ in frequency between responders and nonresponders to the initial vaccination. Antibody rises induced by the challenge were no greater in frequency or magnitude than those observed among subjects initially inoculated with placebo. These results suggest that virus-specific secretory anamnestic responses in older adults are not measurably boosted above the preexisting immunologic background by any of the vaccine regimens. The failure of live attenuated virus to elicit a heightened state of immunologic recall to related influenza antigens in persons either with or without a response to the initial vaccine indicates that such priming did not occur. An unexpected finding of this study was the inability of persons initially immunized with inactivated vaccine alone to mount any appreciable serum antibody response following intranasal challenge. This phenomenon cannot be explained on the basis of high serum antibody levels at the time of the challenge: combined vaccine recipients had slightly higher mean antibody levels than inactivated vaccine recipients did, yet combined vaccine recipients mounted an anamnestic response. Other investigators have also reported that, even after controlling for base-line antibody levels, seroresponses to influenza vaccines are diminished in persons with a history of prior immunization (7, 31). The mechanism that accounts for this observation is unclear.

The present results, considered together with those of our earlier investigation, fail to demonstrate any major advantage of live attenuated *ca* influenza A viruses as an alternative to commercially available inactivated virus vaccines in healthy elderly subjects. It remains possible that those elderly persons who are infected with live *ca* virus represent a subset that is at increased risk of serious illness following wild-type virus infection. If so, live attenuated virus vaccines may be efficacious in a population of elderly subjects. A recent report suggested that *ca* virus vaccines may be efficacious in seropositive adults, although it should be noted that vaccine-induced protection was associated with a significant local antibody response in the majority of subjects (36). The results presented here also suggest that live virus vaccine does not adversely affect the immunogenicity of inactivated virus vaccine when both are administered in combination. Future studies need to explore the potential benefit of using live attenuated viruses as a coimmunogen with inactivated virus vaccine, particularly during influenza epidemics when there is a major antigenic difference between the vaccine component and the circulating wild-type strain. Based on the immunogenicity data from our studies, *ca* influenza viruses do not appear to be a suitable alternative to inactivated virus vaccines for immunization of people in this age group. Greater attention should be directed to the development of better adjuvants or immunomodulators to improve the immunogenicity and efficacy of live and inactivated influenza virus vaccines for populations of elderly subjects.

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