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Increasing Doses of Purified Influenza Virus Hemagglutinin and Subvirion Vaccines Enhance Antibody Responses in the Elderly

WENDY A. KEITEL,[1]* THOMAS R. CATE,[1] ROBERT L. ATMAR,[1] CONNIE S. TURNER,[1] DIANE NINO,[1] CYNTHIA M. DUKES,[2] HOWARD R. SIX,[2] AND ROBERT B. COUCH[1]

Acute Viral Respiratory Disease Unit, Department of Microbiology and Immunology, Baylor College of Medicine Houston, Texas 77030,[1] and Connaught Laboratories, Inc., Swiftwater, Pennsylvania 18370-0187[2]

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The reactogenicities and immunogenicities of two influenza virus vaccines were compared in a placebo-controlled clinical trial among healthy ambulatory persons ≥65 years old (mean age, 72 years). Volunteers were assigned randomly to receive 15-, 45-, or 135-μg doses of monovalent influenza A/Taiwan (H1N1) hemagglutinin (HA) or subvirion (SV) vaccine intramuscularly or a placebo. Increasing doses of SV vaccine were associated with a higher rate of injection site discomfort ($P < 0.05$; chi-square test for linear trend), but all doses of both vaccines were well tolerated. Increasing the dose of the HA or the SV vaccine resulted in increasingly higher postimmunization levels of serum hemagglutination inhibition and neutralizing antibody levels ($P < 0.001$; multiple linear regression). Mean serum antibody titers at 1 month increased two- to threefold with a ninefold increase in dose; the frequencies of fourfold or greater rises in titer likewise increased. An increase in the dose of the HA or the SV vaccine also resulted in increased frequencies of rises in immunoglobulin A or G antibody titers in nasal wash specimens. The frequencies increased approximately twofold for each vaccine with a ninefold increase in the dose. These data suggest that increasing the HA vaccine dose is a promising approach to the development of improved influenza virus vaccines for use in elderly people.

Annual immunization with trivalent inactivated influenza virus vaccines (TIVs) is recommended for the prevention of influenza in persons at greatest risk for complications and death following influenza virus infection (2). The currently available vaccines in the United States contain 15 μg of hemagglutinin (HA) of each virus per dose; these commercial vaccines contain either whole virus or virus which has been disrupted chemically (subvirion [SV] vaccines). Although most healthy susceptible young adults develop an antibody response, the 15-μg dose may fail to elicit significant responses and/or protective antibody levels in elderly persons (1).

One approach to improving the antibody responses to and the levels of efficacy of inactivated vaccines is to increase the dose of antigen. In a previous study among young adults, we showed that high doses of purified influenza A virus HA are well tolerated and that increasing the dose improved anti-HA antibody responses in both serum and nasal secretions (7). In another study, purified HA produced by recombinant DNA technology was also shown to increase serum anti-HA antibody levels among young adults, and the response led to protection against infection (12). The purpose of the present study was to evaluate clinical and serological responses following the administration of increasing doses of purified influenza HA or a companion SV vaccine to a major target population for immunization with TIVs: persons ≥65 years old.

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MATERIALS AND METHODS

Vaccines. Purified monovalent influenza virus HA and SV vaccines were manufactured by Connaught Laboratories, Inc., Swiftwater, Pa. Both purified HA (lot 3D41054) and SV (lot 3D41051) vaccines were prepared from egg-grown influenza A/Taiwan/1/86 (H1N1) virus. The experimental HA vaccine was prepared as described previously (7). The placebo was sterile saline.

Clinical procedures. Ambulatory, medically stable persons ≥65 years of age were recruited from the Texas Medical Center and local volunteer groups and/or senior citizens' organizations between May and September of 1994. Those with acute or unstable medical problems or allergy to eggs were excluded. Written informed consent was obtained from each participant in accordance with protocols approved by the Baylor College of Medicine Affiliates Review Board for Human Subject Research. The subjects were randomized to receive either a 15-, 45-, or 135-μg dose of the HA or the SV vaccine or a placebo. Vaccine or placebo was injected as a single 0.5-ml dose into the deltoid muscle. Oral temperature and local and systemic symptoms and signs were recorded 6, 24, and 48 h after immunization. Blood and nasal wash (NW) specimens for antibody assays were collected prior to, at 2 to 3 weeks, and at 4 to 6 weeks after immunization. All subjects were offered a single dose of commercially available TIV in October of 1994. Blood and NW samples were also collected 8 to 16 weeks after immunization, but only from subjects enrolled early in the study period. The proportions of subjects providing samples at 2 to 3, 4 to 6, and 8 to 16 weeks after immunization were 100, 97, and 56%, respectively, of those providing at least one postimmunization sample.

Laboratory procedures. Tests for hemagglutination inhibition (HAI) and neutralizing (Neut) antibodies in serum samples were performed by previously described methods (4, 5). For HAI assays, sera were treated with receptor-destroying enzyme and were adsorbed with chicken erythrocytes to remove nonspecific inhibitors of hemagglutination. The concentrations of the reagents were altered to permit a starting dilution of 1:4. Serum dilutions were incubated with 4 hemagglutination units of virus before the addition of chicken erythrocytes to determine whether hemagglutination was inhibited. For the Neut assay, serum-virus dilutions were incubated for 1 h before inoculation into an MDCK tissue culture. On the second day, the plates were refed with trypsin-containing medium and were incubated for 4 more days; this was followed by the addition of chicken erythrocytes for the detection of hemagglutination. The test virus was influenza A/Taiwan/1/86 (H1N1). A fourfold or greater rise in serum HAI or Neut antibody titer was considered significant.

Antibody in NW specimens was determined by enzyme-linked immunosorbent assays, with influenza A/Taiwan/1/86 virus HA used as the test antigen. The procedure described previously was used, except that, before testing for antibody, NW specimens with a volume of at least 2 ml were concentrated two- to fivefold with Amicon Centriplus-50 concentrators (7, 8). All specimens were then treated with an equal volume of DNase (50 μg/ml) for 1 h at 37°C. Pairs of NW specimens suitable for analysis contained at least 4.0 μg of immunoglobulin A (IgA) and 0.5 μg of IgG per 0.1 ml in each specimen and total concentrations of IgA or IgG in paired specimens that were not more than 10-fold different from each other, characteristics required to detect and determine the specificity of an antibody increase (7). A fourfold or greater rise in the percentage of IgA or IgG in NW specimens that was virus specific was considered significant.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-4469. Fax: (713) 798-6802.

TABLE 1. Clinical responses following inoculation with ascending doses of influenza A/Taiwan (H1N1) virus vaccines or placebo

Treatment and dose (μg)	No. of subjects	No. (%) of subjects with injection site reaction		No. of subjects with systemic symptoms[a]
		Discomfort	Erythema and/or induration	
Placebo	20	2 (10)	0	0
SV vaccine				
15	9	3 (33)	0	1
45	11	4 (36)	1 (9)	0
135	9	6 (67)	1 (11)	0
Purified HA vaccine				
15	12	3 (25)	0	1
45	11	2 (18)	0	3
135	10	2 (20)	0	1

[a] Symptoms consisted of transient headache in five subjects and malaise in one subject who received a 45-μg dose of the HA vaccine.

Definition of vaccine reactions. Local symptoms or signs (pain or tenderness at the injection site) and systemic symptoms (feverishness, malaise, myalgia, and headache) were graded on a scale of from 0 to 3 (where 0 indicates no symptoms, 1 [mild] indicates an awareness of a sign or symptom but the sign or symptom is easily tolerated; 2 [moderate] indicates discomfort enough to cause interference with usual activity; and 3 [severe] indicates incapacitating, i.e., the subject is unable to perform usual activities). An oral temperature of 37.8°C (≥100°F) was considered fever.

Statistical methods. Differences in proportions were compared by the chi-square test or the two-tailed Fisher exact test. The dose-response for the incidence of a fourfold or greater rise in antibody titer was analyzed by the chi-square test for linear trend. Linear least-squares regression analysis was used to assess the dose-response of the antibody titers, and multiple linear regression analysis was used to assess directly whether the dose-response was affected by the prestudy antibody titer. Antibody titers were expressed as $\log_2$. The antibody levels elicited by the SV and the purified HA vaccines were compared at each dose by the two-tailed pooled variance *t* test or Wilcoxon's rank sum test. All analyses were performed with commercially available computer programs (Minitab, Inc., State College, Pa.).

RESULTS

Seventy-nine subjects were recruited between May and September of 1994; 3 of these were rerandomized in September following receipt of placebo early in the study. The mean age of the subjects was 72 years (range, 65 to 89 years). Fifty-nine percent (47 of 79) of the subjects were female, and 90% (71 of 79) of the subjects were Caucasian. Seventy-one of 79 (90%) of the subjects reported receipt of a commercially available influenza virus vaccine containing the antigenically related influenza A/Texas/36/91 (H1N1) virus during the previous year. Five of eight subjects who had not received vaccine the year before the present study were randomized to receive the 135-μg dose of HA vaccine. Four subjects withdrew from the study prior to the collection of postimmunization blood samples (one subject in the placebo group, one subject receiving the 15-μg dose of the HA vaccine, and two subjects receiving the 135-μg dose of the HA vaccine). None of these subjects reported adverse events during the 2 days after immunization.

The clinical responses during the 2 days following inoculation with various doses of the HA or the SV vaccine or placebo are provided in Table 1. No febrile or severe reaction was reported. A report of discomfort at the site of inoculation was common, and the frequency increased with increasing doses of the SV vaccine ($P = 0.05$; chi-square test for linear trend); a dose-related increase in injection site discomfort was not observed in the group receiving the HA vaccine. The severity of injection site discomfort was ≤1 for all except one subject in the group receiving the 135-μg dose of the HA vaccine; that subject experienced moderate tenderness. Erythema and induration at the site of immunization were reported by two subjects (one each in the groups receiving the 45- and 135-μg doses of SV vaccine); both subjects reported mild discomfort which resolved within 2 days. Occasional mild systemic symptoms including headache ($n = 5$) and malaise ($n = 1$) were noted; these were unrelated to the vaccine dose. Two subjects experienced upper respiratory symptoms, and one subject complained of diarrhea. Despite the dose-related increase in injection site discomfort in the SV vaccine group, all doses of both vaccines were well tolerated.

Antibody responses. The serum and NW antibody responses following vaccination are described in Table 2. The proportions of subjects with a fourfold or greater rise in serum HAI and/or Neut antibody levels after immunization increased in a dose-related fashion: a ninefold increase in dose approximately

TABLE 2. Antibody responses following immunization with ascending doses of influenza A/Taiwan (H1N1) virus vaccines or placebo

Treatment and dose (μg)	No. of subjects with antibody rise in serum[a]			GMT of antibody in serum[b]				No. of subjects with antibody rise in NW specimens[c]		
				HAI		Neut				
	HAI	Neut	Either[d]	Preimmunization	4–6 wk	Preimmunization	4–6 wk	IgG	IgA	Either[d]
Placebo	1	0	1/19 (5)	5.3 (1.6)	5.6 (1.6)	8.2 (2.0)	7.7 (1.9)	0	0	0/18 (0)
SV vaccine										
15	0	2	2/9 (22)	5.1 (1.2)	5.4 (1.1)	7.1 (2.1)	7.7 (1.6)	3	0	3/9 (33)
45	2	3	4/11 (36)	5.4 (2.1)	5.8 (1.1)	8.3 (2.6)	8.7 (2.1)	3	2	3/11 (27)
135	3	3	4/9 (44)	5.0 (1.5)	6.4 (1.7)	6.9 (1.7)	8.8 (1.6)	3	3	5/8 (63)
Purified HA vaccine										
15	3	1	4/11 (36)	5.2 (1.1)	5.4 (1.6)	7.5 (2.1)	7.8 (1.6)	0	2	2/11 (18)
45	4	7	7/11 (64)	5.2 (1.9)	6.4 (2.2)	7.0 (1.8)	9.2 (2.1)	3	0	3/11 (27)
135	5	5	6/8 (75)	4.5 (1.8)	6.9 (2.3)	6.6 (3.4)	9.2 (2.6)	3	1	3/7 (43)

[a] Fourfold or greater rise in serum HAI or Neut antibody titer between pre- and postimmunization serum samples.
[b] Mean titer ($\log_2$); standard deviations are given in parentheses.
[c] Fourfold or greater rise in percent HA-specific antibody between preimmunization NW sample and the NW samples obtained 2 to 3 or 4 to 6 weeks postimmunization.
[d] Data indicate number of subjects with antibody rise/total number of subjects (percent).

doubled the frequencies of the responses in both vaccine groups ($P < 0.025$; chi-square test for linear trend). All but one rise in serum antibody titer occurred within 1 month after immunization; in one subject, a slow rise was observed over an 8-week period. The frequencies of response were consistently higher with the HA vaccine when equivalent doses of the HA and SV vaccines were compared, but the differences were not statistically significant. Four of eight evaluable subjects in the 135-μg dose HA vaccine group had no history of vaccination against influenza virus in 1993. Of these, three responded after receipt of the HA vaccine. Likewise, three of four previously vaccinated subjects given the 135-μg dose of HA vaccine responded. The mean and median ages of the subjects with a serum antibody response were similar to those of the nonresponders, and the overall frequencies of fourfold or greater rises were similar in males and females (data not shown). The geometric mean titers (GMTs) of HAI and Neut antibodies in serum prior to immunization were not significantly different between the vaccine groups ($P > 0.05$; analysis of variance). The GMTs of antibody in serum specimens collected 4 to 6 weeks after vaccination increased in a dose-related fashion in recipients of both the SV and the HA vaccines (dose range, 0 to 135 μg): serum HAI and Neut levels increased two- to threefold with a ninefold increase in dose ($P \leq 0.001$; multiple linear regression). In addition, the postimmunization titers varied directly with preimmunization titers ($P \leq 0.002$; multiple linear regression). Significant dose-response relationships for serum antibody levels were observed in both the HA and the SV vaccine groups up to 16 weeks after immunization (data not shown). Mean titers at 4 to 6 weeks were not significantly different in groups given equivalent doses of vaccine (SV versus HA vaccine) at all doses administered ($P > 0.05$; pooled variance *t* test or Wilcoxon rank sum test).

HA-specific IgA and IgG antibody responses in NW specimens collected 2 and 4 weeks after immunization are given in Table 2. No significant increases in antibody levels were seen in NW specimens from subjects given placebo. No clear dose-related pattern for the frequencies of the response of either IgA or IgG anti-HA antibody was observed. However, when a response for either antibody was considered, a dose-response pattern was apparent for both the SV and the HA vaccine groups ($P < 0.01$ for each; chi-square test for linear trend). Since a dose-related response frequency also was seen for serum antibody, a relationship between a serum antibody response and an NW antibody response was sought, but none was apparent. Four of 16 (25%) of those who were given 15- to 135-μg doses of the HA vaccine and who developed a significant antibody rise in serum also developed an antibody increase in NW specimens, while an antibody increase in NW specimens was also detected in 4 of 13 (31%) of those who did not develop an antibody increase in serum. The corresponding figures for those given the SV vaccine were four of seven (57%) for those with an antibody increase in serum and 7 of 21 (33%) for those without an increase. However, when the preimmunization serum antibody titer was considered, there was a trend toward a greater likelihood for an antibody rise in NW specimens among those with lower preimmunization levels. The trend was statistically significant only for preimmunization Neut antibody levels among those given the SV vaccine ($P < 0.05$; logistic regression controlling for vaccine dose). All persons with an antibody increase in their NW specimens and no antibody rise in serum had two- to threefold increases in serum HAI and/or Neut antibody levels. NW specimens were available 12 weeks after vaccination from 13 of the 19 subjects with an earlier rise, and 9 (69%) of these subjects still exhibited significant increases in antibody levels over the baseline levels.

DISCUSSION

The present study demonstrates that purified influenza virus HA and SV influenza virus vaccines are well tolerated when they are administered intramuscularly to elderly persons in doses containing up to 135 μg of HA. Increasing the dose of either the HA or the SV vaccine resulted in significant increases in serum antibody titers. The frequencies of fourfold or greater serum antibody titer rises increased about twofold and GMTs increased two- to threefold with a ninefold increase in dose for both types of vaccine. Increasing doses of HA and SV vaccines also resulted in increasing frequencies of NW antibody responses. The frequencies of the NW antibody response increased twofold with a ninefold increase in dose in a dose-response fashion. Postimmunization levels of antibody in serum were directly correlated with preimmunization levels as well as with dose. These data are similar to the results obtained following immunization of healthy young adults with comparable highly purified HA preparations (7), indicating that a significant relationship between increasing dose and the likelihood of an antibody response in both serum and respiratory secretions also characterizes responses in a highly immunized population at higher risk for complications and death following influenza virus infection.

Of some concern in the present study was the detection of anti-HA antibody increases in NW specimens without a concomitant rise in antibody in serum. Two subjects had NW antibody responses without a rise in serum in our earlier study among young adults given HA vaccine and both were in a group with high prevaccination serum antibody levels (7). That the increases in the present study are real and not artifacts is supported by the absence of increases in the placebo group and the fact that rises occurred in a group with lower preimmunization serum antibody titers. The factors influencing the occurrence of an NW antibody response without a simultaneous response in serum are unknown. Nevertheless, the data suggest a greater benefit of inactivated vaccines to elderly individuals than is apparent with testing for serum antibody only.

The likelihood of an NW antibody response increases with increasing doses of both vaccines, as was demonstrated previously in healthy young adults (7), suggesting that optimization of parenteral immunization could elicit protective antibody responses in the respiratory tracts of most persons. Powers et al. (11) immunized elderly subjects with $10^{7.5}$ median tissue culture infective doses of live attenuated influenza A/Kawasaki/9/86 CR125 intranasally or a 15-μg dose of commercially available inactivated influenza A/Taiwan/1/86 subvirion vaccine intramuscularly; significant IgA HA responses were elicited in NW specimens from 4 of 25 (20%) subjects given vaccine intramuscularly compared with 13 of 27 (48%) subjects given vaccine intranasally, although higher titers of antibody were elicited in the sera of the group given vaccine intramuscularly. As shown in the present study, the administration of a single high dose of vaccine intramuscularly may accomplish both the systemic and the topical immunization goals simultaneously by stimulating both high levels of antibody in serum and a high frequency of antibody responses in respiratory secretions. Improved responses in the upper respiratory tract should enhance protection against upper respiratory tract replication of virus, and increased serum antibody responses should enhance protection against lower respiratory infection (3).

One goal of the present study was to compare directly the occurrence of adverse clinical reactions in persons given high doses of commercially available SV vaccine with those seen in persons given the more highly purified HA vaccine. In contrast

to the HA vaccine groups, a statistically significant increase in the frequency of pain and/or tenderness at the injection site occurred with increasing dose in groups given the SV vaccine. No significant trends in the occurrence of systemic symptoms were noted for individuals given either vaccine. The purified HA vaccine may offer an advantage in this regard over the SV vaccine when it is administered at high doses, but both preparations were well tolerated. Reductions in minor injection site reactions have also been reported for young and elderly adults given up to 135 μg of a recombinant DNA-produced influenza A/Beijing/92 (H3N2) virus HA vaccine compared with the reactions for those given a standard dose of SV trivalent influenza vaccine (14). Although a 405-μg dose was not tested in the present study, it was shown in our earlier study to be well tolerated by young adults. Therefore, a trivalent vaccine containing 135 μg of each HA (405 μg total) may offer an advantage over an SV vaccine containing a similar amount of viral antigen on the basis of the HA content with regard to injection site symptoms.

Numerous studies have documented the beneficial effect of increasing the dose of various influenza virus vaccines (whole-virus, SV, or purified or recombinant HA vaccines) on serum antibody responses (7, 9, 12, 13), although large increments in dose may be required to elicit a significant enhancement of serum antibody responses (6, 10). Of interest was the observation that both the frequency of serum antibody titer rises and the GMT elicited by equivalent doses of the HA and the SV vaccines tended to be higher for recipients of the HA vaccine. However, the frequency of NW antibody responses tended to be higher in the SV vaccine group, although neither of these trends were significantly different for the two vaccine types. This study confirms and extends findings reported previously and provides further support for increasing the HA content of influenza virus vaccines in order to improve antibody responses in elderly people.

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