Increased Immunogenicity of Inactivated Influenza Virus Vaccine Containing Purified Surface Antigen Compared with Whole Virus in Elderly Women

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Thirty-eight elderly female subjects (aged 80 ± 7 years, mean ± standard deviation) were randomized to immunization with trivalent inactivated influenza virus vaccine containing either purified surface antigen (n = 18) or whole virus (n = 20) components from A/Texas/36/91 (H1N1), A/Beijing/353/89 (H3N2), and B/Panama/45/90 strains. Humoral and cellular immune responses were assessed by measuring serum hemagglutination inhibition antibodies and cytotoxic T lymphocyte (CTL) activity at 0 and 3 weeks postvaccination. Serological responses to both of the type A vaccine strains following immunization with surface antigen vaccine (SAV) were significantly more frequent and greater in magnitude than those induced by whole-virus vaccine. Antibody responses to the B/Panama component were modest and did not differ significantly between the two vaccines. Persons given SAV, but not those given whole-virus vaccine, had a small but significant increase in mean percent specific lysis of influenza A (H1N1) virus-infected autologous targets by peripheral blood mononuclear cells which were stimulated in vitro with influenza A (H1N1) virus. The H1N1-stimulated cytotoxic effectors induced by SAV were CD8+ and were not cross-reactive against H3N2-infected targets. Influenza B virus-specific CTL responses were not observed with either vaccine. These results suggest that currently available subunit influenza virus vaccines may offer an advantage over inactivated whole-virus preparations for inducing humoral and cellular immune responses in the elderly, although the CTL response may be too limited to be of physiological significance.

The population aged 65 years or older includes most of the patients at highest risk for serious medical complications following influenza virus infection and is therefore targeted for annual influenza immunization (10). Attention is being given to defining vaccination strategies which can result in optimal immune responses and enhanced protection in the elderly (18). Influenza vaccine efficacy is best correlated with the production of antibodies directed against the hemagglutinins (HAs) of the vaccine strains (17). In addition to humoral responses, immunization also elicits antigen-specific T-cell-mediated immune responses which are measurable in terms of increased cell proliferation (4), lymphokine secretion (5, 12), and/or cytotoxicity (6, 13, 20) following secondary in vitro stimulation of peripheral blood lymphocytes. Commercially available inactivated influenza virus vaccines contain either whole virus, split virus, or purified surface antigen components. Current recommendations of the Immunization Practices Advisory Committee state that all three forms of vaccine are suitable for administration to adults, although limited evidence suggests that they may not induce equivalent serological responses in older persons (3, 15, 21, 22). Published data for humans are also inconsistent regarding whether subunit vaccines differ from whole-virus preparations in their respective abilities to elicit cytotoxic T lymphocyte (CTL) responses (6, 13). These observations prompted me to examine whether immunization of elderly adults with surface antigen vaccine (SAV) stimulates humoral and CTL responses which are comparable to those elicited by whole-virus vaccine (WVV).

MATERIALS AND METHODS

Subject selection, vaccination, and specimen collection. Study protocols were approved by the Institutional Review Board of St. Louis University. Elderly subjects aged ≥65 years were recruited from residential care facilities in the St. Louis metropolitan area. Volunteers were excluded from participation if they had a history of hematological malignancy, were taking antineoplastic or immunosuppressive medication, were allergic to influenza vaccine or eggs, or were acutely ill at the time of specimen collection. After giving informed consent, subjects were immunized by intramuscular injection of a 0.5-ml dose of licensed trivalent inactivated influenza virus vaccine which was available for the 1992-1993 season and which contained at least 15 μg each of the HAs from A/Texas/36/91 (H1N1), A/Beijing/353/89 (H3N2), and B/Panama/45/90 (B/Panama) viruses. The HA contents per vaccine dose for the specific lot of SAV used in this study were 15 μg of A/Texas (H1N1), 18 μg of A/Beijing (H3N2), and 15 μg of B/Panama (10a). Participants were randomly assigned to receive either SAV (Flu-Imune; Lederle Laboratories, Pearl River, N.Y.) or WVV (Fluzone; Connaught Laboratories, Swiftwater, Pa.). Vaccinations were performed during October and November 1992. Serum and heparinized whole-blood specimens were obtained from each subject at the time of vaccination and 3 weeks later. Specimens were given a coded identification number and processed in a blinded fashion.

Serology. Hemagglutination inhibition (HAI) antibodies to influenza A/Texas (H1N1), A/Beijing (H3N2), and ether-extracted B/Panama virus antigens were measured in serum specimens by a standard microtiter assay, after removal of nonspecific inhibitors with receptor-destroying enzyme and of cold agglutinins by hemadsorption at 4°C. For each antigen, all
specimens were tested on the same day with identical reagents. The titer was defined as the highest serum dilution that completely prevented hemagglutination by four antigen units of virus, with 1:4 as the starting dilution.

**Cell separation.** Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density centrifugation and then washed in a cell culture medium consisting of RPMI 1640 (Sigma, St. Louis, Mo.) supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Following in vitro stimulation, effector cell suspensions were depleted of CD4+ and CD8+ T cells by immunomagnetic separation with commercially available beads coated with monoclonal antibodies to CD4 and CD8, respectively (Advanced Magnetics, Cambridge, Mass.). Undepleted effector cells (3 × 10^6 to 5 × 10^6) were incubated with prewashed beads, with an approximate bead-to-target-cell ratio of 40:1, at 34°C for 30 min in 2 ml of medium, and then the cells were placed in a magnetic field for 15 min before removal of the CD4+ or CD8+ supernatants. Using flow cytometric analysis, I have found that immunomagnetic negative selection routinely eliminates at least 95% of the targeted cell population.

**Viruses.** Infectious stocks of allantoic fluid had been previously prepared by growing influenza A/Taiwan/1/86 (H1N1) [A/Taiwan (H1N1)], A/Shanghai/6/89 (H3N2) [A/Shanghai (H3N2)], and B/Yamagata/16/88 (B/Yamagata) viruses in embryonated eggs and are characterized elsewhere (20). These viruses were used in place of strains homologous to the vaccine components for CTL assays because of the expectation that cytolytic effectors induced in bulk cultures would be cross-reactive between antigenically drifted variants.

**Influenza virus-specific CTL assay.** Experimental conditions for the induction and assay of influenza virus-specific CTL were exactly as previously described (20). In brief, freshly isolated responder PBMC (2 × 10^5) were cultured for 7 days in the presence of autologous stimulator cells (5 × 10^6) that had been infected with influenza A/Taiwan (H1N1) or B/Yamagata virus or that had been mock infected with allantoic fluid from uninfected eggs. PBMC to be used for target cells were incubated for 7 days in a complete medium to which 5 μg of phytohemagglutinin per ml was added 3 days after the initiation of culture. On the morning of the cytotoxicity assay, phytohemagglutinin-activated lymphoblasts were harvested and infected with influenza A/Taiwan (H1N1), A/Shanghai (H3N2), or B/Yamagata virus or were mock infected with allantoic fluid. Following infection, target cells were radiolabelled with 100 μCi of sodium [35S]chomate. The final target cell suspensions were then washed and resuspended at a concentration of 10^6/ml. Target cells (10^4 per well) were combined with equal volumes of undepleted, CD4-, or CD8- effector cells at various effector/target ratios in round-bottom wells on 96-microwell plates. For calculating effector/target ratios, the relative numbers of CD4- or CD8- effectors in negatively depleted cell suspensions were based upon cell counts of undepleted cell suspensions made prior to immunomagnetic separation. Control wells for determining the maximum and spontaneous release of radioactive label contained target cells together with 100 μL of 5% Tween 20 or complete medium, respectively. The cell suspensions were pelleted by centrifugation and incubated at 37°C for 6 h. Cell culture supernatants were then harvested, and the radioactivity was measured in a gamma counter. Percent specific lysis (%SL) was determined by the formula %SL = [(E - S)/(M - S)] × 100, where E, M, and S represent the measured radioactivity, in counts per minute, from experimental release, maximum release, and spontaneous release wells, respectively. The counts per minute (mean ± standard deviation) of maximum and spontaneous wells for all assays were 1,284 ± 632 and 209 ± 114, respectively. Spontaneous release ranged between 6 and 32% (16.3% ± 6.0%, mean ± standard deviation) of maximum release and was comparable between the two vaccine groups, as well as between pre- and postvaccination specimens.

**Statistical analyses.** Reciprocal HAI antibody titers were logarithmically transformed for statistical analysis. A significant antibody response was defined as a fourfold or greater rise in titer between the prevaccination and postvaccination specimens. Differences within vaccine groups between paired pre- and postvaccination %SL were analyzed by the Wilcoxon signed ranks test. Differences between vaccine groups were analyzed by Fisher’s exact test to compare proportions of vaccinees with significant antibody responses and by the Mann-Whitney U test to compare mean increases in reciprocal log\(_2\) HAI antibody titers and %SL.

**RESULTS**

**Subject characteristics.** Thirty-eight elderly female volunteers aged 67 to 96 (80 ± 7, mean ± standard deviation) years were immunized with either SAV (n = 18) or WVV (n = 20). The mean ages of subjects were identical between the two vaccine groups. Inactivated influenza vaccine had been received at least once within the past 5 years by 16 SAV recipients and 18 WVV recipients and within the past 12 months by 13 SAV recipients and 18 WVV recipients.

**Antibody responses to vaccination.** Study participants had elevated levels of serum antibodies to influenza A and B viruses, presumably secondary to repetitive priming by natural infection and prior vaccination. Baseline titers of serum HAI antibody to A/Texas (H1N1), A/Beijing (H3N2), and B/Panama virus antigens were ≥1:32 in 87, 97, and 100% of the 38 subjects, respectively.

A higher proportion of SAV recipients than WVV recipients had fourfold or greater rises in serum HAI titers to A/Texas (H1N1) and A/Beijing (H3N2) (P < 0.025 in both cases) (Table 1). Similarly, SAV elicited greater mean increases in HAI titers to A/Texas (H1N1) (P = 0.0051) and A/Beijing (H3N2) (P < 0.05) than did WVV. Serological responses to the B/Panama strain were reduced relative to those observed with the influenza A virus components in both vaccine groups, but the responses were nonetheless greater in both magnitude and frequency among SAV recipients than among WVV recipients.

**CTL responses to vaccination.** Secondary in vitro stimulation of PBMC with influenza virus was necessary for the detection of cytolytic activity, since the mean %SL of autologous infected targets by effectors from unstimulated control cultures was <3%. Immune lysis was largely abrogated by depletion of CD8+ T cells from effector cell populations by immunomagnetic beadings, whereas CD4+ cell depletion had relatively little effect (Table 2). Cytotoxic effectors displayed reciprocal specificity for the influenza virus type (A or B) with which they were sensitized in vitro. At an effector/target ratio of 50:1, the mean %SL of influenza B virus-infected targets by A (H1N1)-stimulated effectors was 6.7 ± 1.6, while that of influenza A (H1N1) virus-infected targets by B-stimulated effectors was 7.1 ± 1.4.

Overall, the CTL response to vaccination was quite modest. A small but significant (P < 0.05) increase in the mean %SL of A/Taiwan (H1N1)-infected targets by A/Taiwan (H1N1)-stimulated effectors was observed among subjects given SAV (Table 2). Cell depletion experiments revealed that most of the
TABLE 1. Serum HAI antibody responses in elderly volunteers immunized with inactivated influenza WVV or SAV

<table>
<thead>
<tr>
<th>Virus strain used as antigen</th>
<th>Vaccine type (no. in group)</th>
<th>Serum HAI titers (reciprocal mean log$_2$ ± SEM)</th>
<th>% with ≥4-fold rise in titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prevaccination</td>
<td>Postvaccination</td>
</tr>
<tr>
<td>A/Texas (H1N1)</td>
<td>SAV (18)</td>
<td>6.6 ± 0.4</td>
<td>8.8 ± 0.6$^a$</td>
</tr>
<tr>
<td></td>
<td>WVV (20)</td>
<td>6.7 ± 0.5</td>
<td>7.6 ± 0.5</td>
</tr>
<tr>
<td>A/Beijing (H3N2)</td>
<td>SAV (18)</td>
<td>6.7 ± 0.4</td>
<td>8.9 ± 0.4$^c$</td>
</tr>
<tr>
<td></td>
<td>WVV (20)</td>
<td>7.1 ± 0.3</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>B/Panama</td>
<td>SAV (18)</td>
<td>6.8 ± 0.2</td>
<td>7.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>WVV (20)</td>
<td>6.8 ± 0.2</td>
<td>7.5 ± 0.2</td>
</tr>
</tbody>
</table>

$^a$ P = 0.0051, comparing mean rises in antibody titers between vaccine groups (Mann-Whitney U test).
$^c$ P < 0.0025, comparing proportions of antibody responses between vaccine groups (Fisher's exact test).
$^d$ P < 0.05, comparing mean rises in antibody titers between vaccine groups (Mann-Whitney U test).

increase in cytolysis after vaccination was accounted for by the CD4$^-$/CD8$^+$ T-cell subset. There were no significant increases in mean %SL by influenza A/Taiwan (H1N1) virus-stimulated effectors among WVV recipients or by influenza B/Yamagata virus-stimulated effectors among persons in either vaccine group. The specificity of CTL induced by vaccination was examined by testing each population of effector cells against a panel of autologous targets that had been either infected with influenza A/Taiwan (H1N1), A/Shanghai (H3N2), or B/Yamagata virus or mock infected with allantoic fluid alone. As shown in Fig. 1a, the enhanced cytotoxicity of A/Taiwan (H1N1)-stimulated effectors from SAV recipients was primarily directed against targets infected with the same influenza A virus subtype rather than exhibiting heterosubtypic cross-reactivity against H3N2-infected targets. The difference between SAV and WVV groups in the magnitude of the H1N1-specific CTL response to immunization was not statistically significant. Increases in the mean levels of cytolysis by effector cells from influenza B virus-stimulated or unstimulated bulk cultures were generally negligible (Fig. 1b and c).

DISCUSSION

Using commercially available inactivated influenza virus vaccines, I have observed that elderly volunteers mount significantly greater humoral immune responses to SAV than they do to WVV. These data corroborate several previously published studies that have likewise found subvirion vaccines to be more immunogenic for serum antibody responses in older adults than are whole virus vaccines (3, 15, 21), although the differences have not always been statistically significant. In contrast, split-virus vaccines are less immunogenic than WVVVs in immunologically unprimed subjects (1, 7). It is noteworthy that unlike the split-virus vaccines used in all of the previous reports referenced above, the subunit vaccine in the present study contained purified surface antigen. Others have observed better serological responses to split-virus vaccine than to SAV in both middle-aged and elderly adults, particularly among seropositive individuals (22).

The mechanisms which underlie these effects are not known, although there is clear substantial variability between vaccines and subject populations which may confound interstudy comparisons. Since inactivated influenza vaccines are standardized according to HA content, variability in antigen dose is not likely to account for the differences in serum HAI antibody responses between SAV and WVV recipients. Moreover, the administration of higher-than-usual doses of inactivated influenza vaccine has been previously shown to have little if any effect on serum HAI antibody responses in elderly subjects (9, 14). On the other hand, disruption of intact virions and further purification of envelope glycoproteins may induce HA aggre-

TABLE 2. Peripheral blood CTL responses of elderly volunteers immunized with inactivated influenza WVV or SAV

<table>
<thead>
<tr>
<th>Vaccine type (no. in group)</th>
<th>Effector cells</th>
<th>E/T ratio$^a$</th>
<th>% SL (mean ± SEM) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Influenza A (H1N1)-virus infected targets$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prevaccination</td>
</tr>
<tr>
<td>SAV (18)</td>
<td>Undepleted</td>
<td>50:1</td>
<td>21.1 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>Undepleted</td>
<td>17:1</td>
<td>15.3 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Undepleted</td>
<td>6:1</td>
<td>10.2 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>CD8$^-$/CD4$^+$</td>
<td>17:1</td>
<td>5.4 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>CD4$^-$/CD8$^+$</td>
<td>17:1</td>
<td>11.1 ± 2.7</td>
</tr>
<tr>
<td>WVV (20)</td>
<td>Undepleted</td>
<td>50:1</td>
<td>19.2 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>Undepleted</td>
<td>17:1</td>
<td>15.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Undepleted</td>
<td>6:1</td>
<td>10.2 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>CD8$^-$/CD4$^+$</td>
<td>17:1</td>
<td>2.1 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>CD4$^-$/CD8$^+$</td>
<td>17:1</td>
<td>12.0 ± 2.0</td>
</tr>
</tbody>
</table>

$^a$ E/T ratio, effector/target ratio.
$^b$ Lysis of autologous targets by A (H1N1)-stimulated effectors.
$^c$ Lysis of autologous targets by B-stimulated effectors.
$^d$ P < 0.05, compared with prevaccination value at respective effector/target ratio (Wilcoxon signed ranks test).
gation and/or conformational changes which enhance the antigenicity of a subunit preparation in primed subjects. It is also possible that host factors, including the latency and/or multiplicity of a prior vaccination(s) with similar virus antigens, influence the relative immunogenicity of SAV compared with that of WVV. Even after baseline antibody titers have been taken into account, antecedent immunization appears to be associated with some blunting of subsequent vaccine responses in elderly adults (2, 16) and may account for the relative advantage of subunit influenza virus vaccines in this population. Further studies to examine these possibilities are warranted.

The CTL response to vaccination was of low magnitude and was apparent only in the group of subjects given SAV. These data contrast with earlier reports in which virus-stimulated effector cells from young adult recipients of inactivated whole-virus preparations had clear-cut rises in mean %SL of targets infected with homotypic virus (6, 13). The poor CTL response to WVV in the present study relative to responses in the two previous studies may have been due to inherent differences in the immunogenicity of the vaccines that were used, as well as to the advanced age of my volunteers. Older adults have nevertheless been shown to mount influenza virus-specific CTL responses to subvirion vaccines (8, 20), as did the SAV group in this study.

Immunization with SAV resulted in significantly enhanced cytotoxicity of influenza A (H1N1) virus-stimulated effector cells against autologous target cells infected with homologous virus but not against target cells infected with heterosubtypic virus. Cell depletion experiments demonstrated that CD8+ T cells accounted for most of the measurable immune lysis. Gorse and Belshie similarly observed that human leukocyte antigen class I-restricted CTL elicited by a monovalent influenza A H1N1 subvirion vaccine were not cross-reactive against targets infected with an H3N2 subtype virus (8). In both cases, the target antigens for these CTL most likely included subtype-specific epitopes on the HA and/or neuraminidase glycoproteins of the H1N1 vaccine component strains. While the trivalent SAV administered in the present study may have also stimulated CTL with specificity for H3N2 virus, I was unable to examine this possibility because insufficient numbers of PBMC were available to include influenza A (H3N2) virus-stimulated bulk cultures. The failure to detect an influenza B virus-specific CTL response to vaccination may have reflected the poor immunogenicity of the B virus component also observed with serum antibody responses.

In conclusion, the present results demonstrate that alternative forms of commercially available influenza virus vaccine cannot be assumed to stimulate equivalent serological responses when administered in standard doses to immunologically primed adults. Specifically, vaccines which contain purified influenza virus surface antigens may offer an advantage over whole-virus preparations for immunization of the elderly. On the other hand, the overall weight of published evidence, including this study, suggests that CTL responses to conventional inactivated influenza virus vaccines are quite limited. Given the demonstrated role of CTL in promoting viral clearance and host recovery following influenza virus infection, future efforts will need to be directed toward the design of vaccines which are better able to elicit CTL memory. Such vaccines may be particularly important for use in high-risk elderly patients, who have diminished levels of cellular immunity to influenza virus (11, 19).

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