In order to evaluate the immunogenicity and the effect of a virosomal influenza vaccine on viral replication and T-cell activation in HIV-infected children receiving highly active antiretroviral therapy (HAART), 29 children infected with HIV-1 vertically (19 primed with a previous influenza vaccination and 10 who were not been immunized against influenza) were immunized with an intramuscular virome-adjuvanted influenza vaccine. According to the European Agency for Evaluation of Medical Products (EMEA) criteria, the immunogenicity of the vaccine was adequate against all three influenza strains (A H1N1, A H3N2, and B) in the primed children, and against A H1N1 and A H3N2 in the unprimed children. After in vitro stimulation with vaccine antigens, the IFN-γ levels in the peripheral blood mononuclear cells cultures increased significantly from a baseline level of $10^3.0 \pm 229.8$ pg/ml to a 30-day level of $390.7 \pm 606.3$ pg/ml ($P < 0.05$), with concentrations significantly higher ($P < 0.05$) in the primed children than in the unprimed children. No increase in plasma HIV-1 RNA or HIV-1 proviral DNA was observed in either subgroup, and the immunophenotype analyses demonstrated that the CD4+ cell counts and percentages, the CD4/CD8 ratio and activated lymphocytes remained stable in either group from baseline to 1 month after each vaccine dose. This study showed that the virosomal influenza vaccine does seem to be immunogenic in the majority of HIV-infected children receiving HAART and does not induce viral replication or T-cell activation. Given the possible influenza-related complications in children infected with HIV, these results support the use of this influenza vaccine in such patients.

**KEY WORDS:** influenza vaccine; immunogenicity; HIV; viral replication; T-cell activation; children

**INTRODUCTION**

Patients with chronic underlying diseases are considered at high risk for influenza-related complications and influenza vaccination is recommended every year [Neuzil et al., 2000; World Health Organization, 2002; Advisory Committee on Immunization Practices (ACIP), 2004; American Academy of Pediatrics, 2004; Principi and Esposito, 2004]. Children infected with HIV are usually included in this group, but the use of vaccines is debated in such patients because it has been associated with sub-optimal immune responses [Iorio et al., 1997; Lyall et al., 1997; Kroon et al., 2000; Zanetti et al., 2002], and there is also concern that vaccination may enhance HIV replication, activate T-cells and influence negatively disease progression [Ramilo et al., 1996; Jackson et al., 1997; Viganò et al., 1998; Keller et al., 2000; Rousseau et al., 2000; Sullivan et al., 2000; Pinto et al., 2001; Kolber et al., 2002].

Appropriate informed consent was obtained and the study was conducted in accordance with the guidelines for human experimentation specified by the authors' institutions. No author has a commercial or other association that might pose a conflict of interest.
Only a few studies have evaluated the immunogenicity and safety of influenza vaccination in patients infected with HIV receiving highly active antiretroviral therapy (HAART). Furthermore, they have consisted mainly of adults and have led to equivocal results concerning the potentially harmful effects of vaccine administration [Günthard et al., 2000; King et al., 2000; Amendola et al., 2001; Macías et al., 2001; Iorio et al., 2003]. Few data are available concerning children [Zucotti et al., 2002].

This study was planned to evaluate the immunogenicity and the effect of a virosomal influenza vaccine on viral replication and T-cell activation in children infected with HIV receiving HAART.

**MATERIALS AND METHODS**

**Study Population**

Twenty-nine children infected vertically with HIV-1 (19 females; mean age ± SD: 10.3 ± 4.3 years) treated with HAART (15 treated with lamivudine + stavudine + ritonavir, and 14 with lamivudine + stavudine + nelfinavir) were enrolled in October 2002: nine in class A (3 A1, 3 A2, and 3 A3), nine in class B (1 B1, 6 B2, and 2 B3), and 11 in class C (5 C1, 3 C2, and 3 C3). At the start of this study, five had baseline CD4⁺ cell counts of 200–500 cells/µl, and 24 had more than 500 cells/µl. All had been stable clinically, virologically and immunologically for 6 months. They had been treated with HAART for 3–5 years (median, 4.3 years) and before these patients began HAART they had baseline CD4⁺ cell counts of 70–410 cells/µl (median value, 210 cells/µl). Nineteen children had received at least one previous influenza vaccination (primed), and 10 had not been immunized against influenza (unprimed).

**Vaccination**

All of the patients were given a single 0.5 ml intramuscular dose of 2002/2003 virosome-adjuvanted influenza vaccine (Inflexal V, Berna Biotech, Switzerland) in the deltoid muscle; the unprimed children received a second dose after 4 weeks. The vaccine contained 15 µg each of A/New Caledonia/20/99 (H1N1)-like, A/Moscow/10/99 (H3N2)-like, and B/Hong Kong/330/2001-like purified influenza surface antigens neuraminidase- and hemagglutinin-integrated into the lipid membrane of the virosome, and enough solvent to reach a volume of 0.5 ml [Mischler and Metcalfe, 2002].

**Study Design**

Blood samples (serum and whole blood) were collected immediately before, and 1 and 3 months after the administration of the vaccine in order to evaluate its immunogenicity and its effect on viral replication and T-cell activation. Immunogenicity was evaluated by measuring hemagglutination-inhibiting (HI) antibodies and interferon (IFN-γ) production after in vitro stimulation with vaccine antigens; viral replication was evaluated on the basis of plasma HIV-1 RNA and HIV-1 proviral DNA levels; T-cell activation was evaluated by means of immunophenotype analyses. The clinical condition of each child was also evaluated at each time-point.

The study was approved by the Ethics Committee of the University of Milan, Milan, Italy, and written informed consent was obtained from the child’s parents or legal guardians.

**Detection of Hemagglutination-Inhibiting (HI) Antibodies**

The serum samples of each patient were simultaneously examined for HI antibodies using standard microtiter assays for the influenza strains contained in the vaccine, as described previously [Amendola et al., 2001], and the HI antibody titer was expressed as the reciprocal of the highest dilution inhibiting agglutination. A minimum response was defined as seroconversion or a ≥four-fold increase in antibody titer in the post-immunization samples. Antibody titers of ≥1:40 were considered to protect against influenza infection. As described previously [Amendola et al., 2001], in order to allow the calculation of the HI geometric mean titers (GMTs), a titer of 1:5 was assigned arbitrarily to non-responders. The HI antibodies were evaluated using the criteria described in the guideline of the European Agency for the Evaluation of Medical Products (EMEA) [European Agency for the Evaluation of Medicinal Products (EMEA)—Committee for Proprietary Medicinal Products (CPMP), 1997]. As described previously [Kanra et al., 2004], because there are no EMEA-defined criteria for children, immunogenicity was evaluated on the basis of the criteria for adults aged 18–60 years, which require at least one of the following for each strain to confirm immunogenicity: (1) seroconversion: a ≥four-fold increase in HI antibody titer, with a titer of ≥1:40 being reached in >40% of the subjects; (2) seroprotection: an HI antibody titer of ≥1:40 in >70% of the subjects; and (3) GMT: a ≥2.5-fold increase in the HI antibody GMT.

**IFN-γ Production**

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood of the immunized children and cultured for in vitro stimulation, as described previously [McElhaney et al., 1998]. Briefly, 3 × 10⁶ PBMCs/ml were cultured in RPMI 1640 medium supplemented with 0.5% penicillin (100 U/ml), 0.5% streptomycin (100 µg/ml), 1% L-glutamine (100 µg/ml), and 10% human serum, and stimulated with 10 µg/ml phytohaemoagglutinin (PHA) or the vaccine antigens (A/New Caledonia/20/99 (H1N1)-like, A/Moscow/10/99 (H3N2)-like and B/Hong Kong/330/2001-like). After 6 days of incubation at 37°C in 5% CO₂, the culture supernatants were harvested and stored at −20°C, and their cytokine expression (pg/ml) was evaluated after in vitro stimulation by assessing IFN-γ production using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Human IFN-γ J. Med. Virol. DOI 10.1002/jmv
ELISA Kit, HybriDomus™, Nuclear Laser Medicine). The lower limit of the method was 4 pg/ml, and a value of 2 pg/ml was arbitrarily assigned to undetectable concentrations.

Plasma HIV-1 RNA

HIV-1 RNA was measured using a commercially available quantitative assay (Cobas Amplicor HIV-1 Monitor™ Test, 1.5 version, Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The plasma samples collected from each patient during the follow-up were tested simultaneously. The lower limit of detection for this assay was 400 cp/ml; undetectable levels were assigned a value of 200 cp/ml.

HIV-1 Proviral DNA

HIV-1 proviral DNA was assessed by an “in-house” quantitative PCR as previously described [Amendola et al., 2001]. Briefly, DNA was extracted from PBMCs using a commercially available kit (QIAamp DNA Mini Kit, QIAGEN, Germany), and sample competence was determined by amplifying the human β-globin fragment. One microgram of proviral DNA was then amplified in the gag region of HIV-1 using nested-PCR with reference to an external standard curve (Gene Amplimer HIV-1 Control, Perkin Elmer, Oak Brook, IL) in the range of 10–10^4 cp/μg genomic DNA. The amplified products were quantified densitometrically (Digital Science 1D Image Analysis Software, Kodak). A value of 5 cp/μg was arbitrarily assigned to the samples with less than 10 cp/μg.

Immunophenotype Analyses

Lymphocyte subsets were evaluated using an Epics XL flow-cytometer (Coulter Electronics, Miami Lakes, FL) and 100 μl of EDTA peripheral blood incubated with fluorochrome-labeled monoclonal antibodies at 4°C for 30 min. Erythrocyte lysis was obtained after incubation with the Immuno-Prep Epics Kit (Coulter Electronics) and Q-prep Work Station (Coulter Electronics). The lymphocytes were analyzed using forward- and side-scatter properties. For each sample, multiparametric data were acquired for 5,000 events, as described previously [Clerici et al., 2000].

Statistical Analysis

The seroconversion rates and the prevalence of vaccinated individuals with HI antibody titers considered to be protective (≥1:40) in both primed and unprimed children were compared using the Chi-square or Fisher’s test. Student’s t-test was used to compare the GMTs of HI antibodies, plasma HIV-1 RNA and HIV-1 proviral DNA levels, the lymphocyte subset counts, and cytokine production. A P-value of <0.05 was considered significant for all of the statistical tests.

<table>
<thead>
<tr>
<th>TABLE I. Hemagglutination Inhibition (HI) Antibody Responses to Virosomal Influenza Vaccine in HIV-Infected Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>A H1N1</td>
</tr>
<tr>
<td>A H3N2</td>
</tr>
<tr>
<td>B</td>
</tr>
</tbody>
</table>

Seroconversion rates, number (%)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primed (n = 19)</th>
<th>Unprimed (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A H1N1</td>
<td>16 (84.2)</td>
<td>10 (100.0)</td>
</tr>
<tr>
<td>A H3N2</td>
<td>16 (84.2)</td>
<td>8 (50.0)</td>
</tr>
<tr>
<td>B</td>
<td>8 (42.1)</td>
<td>4 (40.0)</td>
</tr>
</tbody>
</table>

Seroprotection rates, number (%)

*P < 0.01 versus unprimed after 60 and 90 days; no other significant between-group differences.

<table>
<thead>
<tr>
<th>TABLE II. Geometric Mean Titers (GMTs) of Hemagglutination Inhibition (HI) Antibody Before and After Virosomal Influenza Vaccine in HIV-Infected Children</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>A H1N1</td>
</tr>
<tr>
<td>A H3N2</td>
</tr>
<tr>
<td>B</td>
</tr>
</tbody>
</table>

Mean increases in parentheses.

*P < 0.01 and **P < 0.05 versus primed after 30 and 90 days; †P < 0.01 versus unprimed after 60 days and 90 days; ‡P < 0.05 versus unprimed after 30, 60, and 90 days; no other significant between-group differences.

RESULTS

Immune Response

On the basis of the EMEA criteria, the immunogenicity of the virosomal influenza vaccine was adequate for all three influenza strains in the primed children, and for strains A H1N1 and A H3N2 in the unprimed children.

Table I shows the HI antibody responses to the vaccine. The EMEA criteria for seroconversion were fulfilled for A H1N1 in the primed children and for A H3N2 in the unprimed children, whereas criteria for seroprotection were reached for all three strains in the former, and for A H1N1 and A H3N2 (but not B) in the latter. The seroconversion and seroprotection rates were similar 3 months after vaccination.

The HI antibody GMTs before and after vaccination are summarized in Table II. The related EMEA criteria were fulfilled for A H1N1 in the primed children and for A H1N1 and A H3N2 in the unprimed children; however, the increase in GMT levels after vaccination was significant for all three influenza strains in the former, and for A H1N1 and A H3N2 (but not B) in the latter. The GMTs against each antigen were similar 3 months after vaccination.

After in vitro stimulation with vaccine antigens, the IFN-γ levels in the PBMCs cultures significantly increased from a baseline level of 103.0 ± 229.8 pg/ml to a 30-day level of 390.7 ± 606.3 pg/ml (P < 0.05), but did not change significantly during the same period after stimulation with PHA (162.4 ± 279.6 vs. 114.5 ± 217.8; P > 0.05). One month after the first vaccine dose, the concentration of IFN-γ was significantly higher (P < 0.05) in the primed children (413.1 ± 293.1 pg/ml) than in the unprimed children (114.9 ± 160.3 pg/ml). There was no significant change in IFN-γ levels after administration of the second dose in the unprimed children (82.6 ± 64.5 pg/ml).

Viral Replication

Plasma HIV-1 RNA and HIV-1 proviral DNA levels are shown in Table III. The fact that they remained similar after vaccination indicated the absence of viral replication in both groups.

T-Cell Activation

The immunophenotype analyses demonstrated that the CD4⁺ cell counts and percentages, and the CD4/CD8 ratio, remained stable from baseline to 1 month after each vaccine dose (Table IV). No significant post-vaccination difference was observed also in the percentages of activated lymphocytes in either group (Table V).

DISCUSSION

The vaccination of patients infected with HIV has been associated with sub-optimal immune responses and inadequate levels of protection [Iorio et al., 1997; Lyall et al., 1997; Kroon et al., 2000; Zanetti et al., 2002],
as well as with possible viral replication, T-cell activation and consequent disease progression [Ramilo et al., 1996; Jackson et al., 1997; Viganò et al., 1998; Keller et al., 2000; Rousseau et al., 2000; Sullivan et al., 2000; Pinto et al., 2001; Kolber et al., 2002]. This means that immunization may not only be useless against a specific disease, but also dangerous in terms of HIV status.

However, the present study demonstrates that the use of the virosomal influenza vaccine is safe and moderately immunogenic in HIV-infected children. In this regard, although there are currently no EMEA immunogenicity guidelines for children, a previous study has shown that the administration of the virosomal influenza vaccine in healthy children met the adult EMEA criteria on seroconversion, seroprotection and increase in HI antibody GMTs, thus indicating an appropriate immunogenic profile in such subjects [Kanra et al., 2004]. A control group of healthy children and a comparison with a conventional inactivated vaccine were not included. However, on the basis of the EMEA criteria, the humoral immune responses recorded in this study were adequate for all three influenza strains in primed children and adequate for the two A strains in the unprimed children, although the seroconversion rate and increase in GMTs were less than those observed in healthy children [Kanra et al., 2004]. These differences were particularly evident in the case of the influenza B strain and in the group of unprimed children.

The finding of a reduced humoral immune response in children infected with HIV is in line with the results of previous studies demonstrating that vaccinations may not induce protective antibody titers in immunocompromised patients, and that a second vaccine dose does not improve their immune response [Rousseau et al., 2000; Advisory Committee on Immunization Practices (ACIP), 2004; American Academy of Pediatrics, 2004]. On the other hand, a previous study using the same virosomal influenza vaccine in HIV-infected children demonstrated optimal immunogenicity, similar to that observed in healthy children [Zuccotti et al., 2002]. The difference in the results of that and the current study may be explained by the different degree of viral replication and immunosuppression in the enrolled children: all of our patients were receiving HAART but, as shown by the high levels of plasma viremia, the HAART response of some was poor and this may have led to impaired lymphocyte functional activity and a reduced antibody response after vaccination. However, the use of the vaccine seemed to be potentially effective in the majority of these study patients.

Furthermore, in an attempt to clarify further the immune response of children infected with HIV to influenza vaccination, the production of IFN-γ was studied in cultured PBMCs after in vitro stimulation with vaccine antigens and it was found that it increased 1 month after the administration of the first dose. This suggests that vaccination may also lead to a cell-mediated immune response, which, together with the humoral response, may enhance the protection offered by immunization.

On the other hand, the importance of recommending influenza vaccination for patients infected with HIV is supported by the fact that influenza may cause a transient increase in plasma viremia and lower CD4 cell counts in HIV-infected children [Marchisio et al., 1998], whereas influenza vaccination does not induce viral replication or T-cell activation. The finding that the plasma levels of HIV-1 RNA and HIV-1 proviral DNA, and the number and characteristics of lymphocytes, remained stable after each vaccine dose highlights the fact that the antigen stimulation induced by the vaccine is safe in HIV-infected children. These results are in agreement with those of some previous studies [Fowke

### TABLE IV. Immunophenotype Analyses of CD4+ Cells Before and After Virosomal Influenza Vaccine in HIV-Infected Children

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Primed (n = 19)</th>
<th>Unprimed (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After 30 days</td>
</tr>
<tr>
<td>CD4+ cell count, cells/µl</td>
<td>667 ± 225</td>
<td>610 ± 258</td>
</tr>
<tr>
<td>CD4+ percentage, %</td>
<td>29 ± 7</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>0.6 ± 0.3</td>
<td>0.7 ± 0.4</td>
</tr>
</tbody>
</table>

Mean value ± SD. No significant difference between the groups.

### TABLE V. Immunophenotype Analyses of Percentages of Activated Lymphocytes Before and After Virosomal Influenza Vaccine in HIV-Infected Children

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primed (n = 19)</th>
<th>Unprimed (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After 30 days</td>
</tr>
<tr>
<td>CD4/DRII</td>
<td>0.210 ± 0.116</td>
<td>0.282 ± 0.210</td>
</tr>
<tr>
<td>CD8/DRII</td>
<td>0.567 ± 0.367</td>
<td>0.422 ± 0.482</td>
</tr>
<tr>
<td>CD4/CD25</td>
<td>0.694 ± 0.116</td>
<td>0.864 ± 0.661</td>
</tr>
<tr>
<td>CD8/CD25</td>
<td>0.113 ± 0.222</td>
<td>0.049 ± 0.119</td>
</tr>
</tbody>
</table>

Mean value ± SD. No significant difference between the groups.
Influenza Vaccine in HIV-Infected Children

et al., 1997; Jackson et al., 1997; Keller et al., 2000; Zucconi et al., 2002], but not with others [Ramilio et al., 1996; Viganò et al., 1998], once again possibly because of differences in the response to antiretroviral therapy.

In conclusion, this study of children infected with HIV shows that, although the virosomal influenza vaccine is less immunogenic than in healthy subjects, it does seem to be immunogenic in the majority of cases and does not induce viral replication or T-cell activation. Given the possible influenza-related complications in HIV-infected children [Marchisio et al., 1998; Advisory Committee on Immunization Practices (ACIP), 2004; American Academy of Pediatrics, 2004], these results support the use of this influenza vaccine in such patients.

REFERENCES


