Compromised B Cell Responses to Influenza Vaccination in HIV-Infected Individuals

Angela Malaspina,1,a Susan Moir,1,a Susan M. Orsega,3 Joshua Vasquez,1 Natalie J. Miller,1 Eileen T. Donoghue,1 Shyamasundaran Kottilil,1 Misrak Gezmu,2 Dean Follmann,2 Galina M. Vodeiko,4 Roland A. Levandowski,4 JoAnn M. Mican,2 and Anthony S. Fauci1

1Laboratory of Immunoregulation and 2Office of Clinical Research, National Institute of Allergy and Infectious Diseases, and 3Clinical Center, Nursing and Patient Care Services, National Institutes of Health, and 4Division of Viral Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland

Background. Yearly influenza vaccination, although recommended for human immunodeficiency virus (HIV)-infected individuals, has not received thorough evaluation in the era of antiretroviral therapy. We assessed the impact of HIV disease on B cell responses to influenza vaccination.

Methods. Sixty-four HIV-infected and 17 HIV-negative individuals received the 2003–2004 trivalent inactivated influenza vaccine. Frequencies of influenza-specific antibody-secreting cells (ASCs) were measured by enzyme-linked immunospot (ELISPOT) assay, and antibody responses were measured by hemagglutination-inhibition (HI) assay. Memory responses to influenza were measured by ELISPOT assay after polyclonal activation of B cells in vitro.

Results. Prevaccination HI titers were significantly higher in HIV-negative than in HIV-infected individuals. Peak HI titers and influenza-specific ASC frequencies were directly correlated with CD4+ T cell counts in HIV-infected individuals. Influenza-specific memory B cell responses were significantly lower in HIV-infected than in HIV-negative individuals and were directly correlated with CD4+ T cell counts.

Conclusions. HIV infection is associated with a weak antibody response to influenza vaccination that is compounded by a poor memory B cell response. CD4+ T cell count is a critical determinant of responsiveness to influenza vaccination, and the contribution of plasma HIV RNA level is suggestive and warrants further investigation.
The influenza vaccine recommended for HIV-infected individuals is an inactivated formulation for parenteral administration. Although both humoral and cellular responses are elicited by influenza infection, it is the antibodies directed against hemagglutinin (HA) and neuraminidase (NA) that are thought to protect against infection [16], whereas cell-mediated immunity is thought to be more important in recovering from rather than preventing infection [17]. The antibody response to influenza vaccination is thought to be critically dependent on CD4+ T cell help [18]. The most commonly reported correlate of protection after influenza vaccination is the serum titer of hemagglutination-inhibition (HI) antibodies, which peaks ∼3–4 weeks after vaccination [19]. Recently developed assays, such as the enzyme-linked immunospot (ELISPOT) assay, aimed at detecting early antibody-secreting cells (ASCs) [20] and later memory B cell responses [21] in peripheral blood, are proving to be powerful tools in evaluating the full scope of antigen-specific B cell responses.

In an effort to increase our understanding of the effect of HIV infection on the immune response to influenza vaccination, we undertook a comprehensive analysis of influenza-specific antibody responses to the vaccine within a cohort of HIV-infected individuals with a wide spectrum of CD4+ T cell counts and plasma HIV RNA levels and compared these with responses in HIV-negative control individuals. To evaluate peak effector and memory responses to influenza vaccination, we measured both HI titers and influenza-specific ASC frequencies before and at various time points after vaccination.

PARTICIPANTS, MATERIALS, AND METHODS

Participants. HIV-infected and HIV-negative volunteers were recruited between October 2003 and March 2004 to receive the 2003–2004 influenza vaccine (see below) and to donate blood at 4 time points: day 0 (D0; before vaccination), D7 (peak ASC effector response), D28 (peak HI titer), and D54 (memory ASC response). Eligibility criteria were identical for the HIV-infected and HIV-negative participants, with the exception of HIV status. We excluded participants <18 and >60 years of age, women who were pregnant, any HIV-infected individual whose antiretroviral drug regimen was recently changed or would change would likely affect CD4+ T cell count and/or plasma HIV RNA level during the course of the study, and any HIV-infected individual who was receiving cytokine-based therapies. All procedures were approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Procedures. Participants received a single intramuscular dose of licensed inactivated trivalent vaccine from a single lot (Fluzone; Aventis Pasteur), formulated to contain 15 μg of HA of each of the strains A/Panama/2007/99 (H3N2), B/Hong Kong/1434/2002, and A/New Caledonia/20/99 (H1N1). Influenza vaccination history was documented, in addition to any event during the course of the study that would be consistent with either influenza infection, as documented by an influenza-positive nasopharyngeal wash, or influenza-like illness [22]. For HIV-infected participants, CD4+ T cell counts and plasma HIV RNA levels (bDNA with limit of detection of 50 copies/mL; Bayer) were measured at each time point (except CD4+ T cell count at D7).

Serum and whole-blood samples were collected immediately before vaccination (D0) and at D7, D28, and D54 after vaccination. HI titers were determined by a standard microtiter assay after treatment of the serum samples with neuraminidase to remove nonspecific inhibitors of HA, as described elsewhere [23]. Serum samples from all time points were tested against the 3 HA antigens of the vaccine as well as against A/Fujian/445/2003, an A/Fujian/411/2002-like strain representative of the predominant influenza A H3N2 viruses present in the United States during the 2003–2004 influenza season.

Whole blood was processed to obtain either peripheral blood mononuclear cells (PBMCs), by standard Ficoll density centrifugation, or B cells, by negative selection using a B cell enrichment cocktail (RosetteSep; StemCell Technologies), in accordance with the manufacturer’s specifications. The percentage of B cells in each preparation was established as reported elsewhere [24], to normalize ELISPOT counts. ASC frequency was measured by an ELISPOT assay [25] adapted to measure influenza-specific responses. Briefly, nitrocellulose-bottom 96-well filtration plates (MAHA S4510; Millipore) were coated with the influenza vaccine at a concentration of 5 μg/mL in PBS and were incubated overnight at 4°C. After washing with PBS containing 0.1% Tween 20 (PBS-T) and blocking with RPMI 1640 containing 5% fetal calf serum (FCS), the blocking media was replaced with 100 μL of RPMI–10% FCS containing 10-fold dilutions of B cells or PBMCs (D7) and was incubated for 5 h at 37°C. Plates were washed and incubated with 50 ng/mL biotinylated mouse anti-human pan-IgG Fc antibody (Southern Biotechnology Associates) overnight at 4°C. Plates were washed, incubated with alkaline phosphatase–conjugated streptavidin, and developed with substrate (ELISPOT Blue Color Module; R&D Systems). Spots were enumerated with an automated Immunospot Series 3A analyzer and software (Cellular Technology). Influenza-specific B cell memory responses were evaluated by ELISPOT assay at D54 after vaccination, after 5 days of polyclonal stimulation in vitro, similar to a recently published protocol [21]. Briefly, freshly isolated B cells were plated at 1 × 10^5 cells/well in 24-well plates containing RPMI–10% FCS supplemented with 1/10,000 *Staphylococcus aureus* Cowan (SAC; EMD Biosciences) and 5 μg/mL phosphorothiolated CpG oligodeoxynucleotide 2006 (Operon) [26]. After 5 days in culture, the cells were recovered, washed, and used in the ELISPOT assay described above.

Statistical analyses. Geometric mean titers (GMTs) for the HI assays were calculated as log10-transformed reciprocal HI
titers. Differences in HI titers and ASC frequencies between groups were analyzed using the Wilcoxon 2-sample test with the Bonferroni method to adjust P values for multiple testing. Correlations between variables were determined either by Spearman’s rank correlation with adjustment for multiple testing or by linear regression.

RESULTS

A total of 81 participants, including 64 HIV-infected patients and 17 HIV-negative healthy donors, were enrolled in the study. To ensure sufficient heterogeneity relative to CD4+ T cell count and plasma HIV RNA level, each HIV-infected participant was recruited from 1 of 4 prespecified groups: HIV-HH (CD4+ T cell count >350 cells/μL and plasma HIV RNA level >10,000 copies/mL), HIV-HL (CD4+ T cell count >350 cells/μL and plasma HIV RNA level <10,000 copies/mL), HIV-LL (CD4+ T cell count <350 cells/μL and plasma HIV RNA level >10,000 copies/mL), and HIV-NEG (CD4+ T cell count <350 cells/μL and plasma HIV RNA level <10,000 copies/mL) (table 1). The groups were defined by a CD4+ T cell count above or below 350 cells/μL, on the basis of recommendations for initiation of ART [27], and by a plasma HIV RNA level above or below 10,000 copies/mL, on the basis of our previous findings ([24] and authors’ unpublished data) and published literature on immunologic failure [28, 29]. Furthermore, to perform more-efficient data analyses, comparisons were made only if the different groups of participants (data not shown). However, when all HIV-infected individuals were grouped together, a significant direct correlation was observed between CD4+ T cell counts and D7 ASC frequencies (r = 0.30; P < .05) (figure 1A). By D28, influenza-specific ASC frequencies had returned to baseline levels in all patients, with the exception of 1 HIV-infected patient in group HIV-LL who received a diagnosis of influenza at around D19. The D28 influenza-specific ASC frequency for this patient was >1000-fold higher than the frequency at D7, indicating that a stronger anti-influenza response was mounted against the natural infection than against the vaccine.

At D54, influenza-specific memory B cell responses were measured by ELISPOT assay after a 5-day stimulation in vitro. As shown in figure 2, a significantly higher memory response was observed in HIV-negative individuals, compared with that in each of the groups of HIV-infected individuals. Furthermore, and consistent with D7 ASC findings, a significant direct correlation was observed between CD4+ T cell counts and D54 memory ASC frequencies (r = 0.36; P < .01) (figure 1B). A significant direct correlation (P = .001) was also observed between D7 and D54 ASC frequencies (data not shown), indicating that peak ASC frequencies in the peripheral blood are

Table 1. Baseline characteristics of participants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV-NEG</th>
<th>HIV-HL</th>
<th>HIV-LL</th>
<th>HIV-HH</th>
<th>HIV-LH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total participants, no.</td>
<td>17</td>
<td>20</td>
<td>19</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>CD4+ T cell count, mean, cells/μL</td>
<td>770</td>
<td>641</td>
<td>220</td>
<td>506</td>
<td>184</td>
</tr>
<tr>
<td>Plasma HIV RNA level, geometric mean, copies/mL</td>
<td>NA</td>
<td>146a</td>
<td>172b</td>
<td>33,104</td>
<td>47,074</td>
</tr>
<tr>
<td>Sex, no. male/female</td>
<td>11/6</td>
<td>15/5</td>
<td>16/3</td>
<td>9/3</td>
<td>10/3</td>
</tr>
<tr>
<td>2002 vaccinees, no.</td>
<td>15</td>
<td>17</td>
<td>17</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Influenza/influenza-like illness by D28, no.</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Influenza/influenza-like illness by D54, no.</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE. D28, day 28; D54, day 54; HIV-HH, HIV infected with CD4+ T cell count >350 cells/μL and plasma HIV RNA level >10,000 copies/mL; HIV-HL, HIV infected with CD4+ T cell count >350 cells/μL and plasma HIV RNA level <10,000 copies/mL; HIV-LL, HIV infected with CD4+ T cell count <350 cells/μL and plasma HIV RNA level >10,000 copies/mL; HIV-NEG, HIV-negative; NA, not applicable.

a Fourteen patients had plasma HIV RNA levels <50 copies/mL.

b Seven patients had plasma HIV RNA levels <50 copies/mL.

c The no. of 2003 participants who were vaccinated in 2002 (vaccines were identical).
predictive of memory B cell responses. Of note, however, the strong effector ASC frequency measured at D28 in the patient with a diagnosis of influenza did not translate into an equally strong memory response at D54 (figure 2). Taken together, and consistent with the strong T cell–dependent nature of antibody responses to influenza antigens [18], these findings demonstrate that B cell responses to influenza vaccination in HIV-infected individuals decrease with decreasing CD4+ T cell counts.

Whereas several studies have associated B cell defects with ongoing HIV replication [31–33], studies directly addressing the impact of plasma HIV RNA level on antigen-specific B cell responses in vivo are lacking. Although, in the present study, no significant correlation was observed between plasma HIV RNA level and frequencies of either D7 effector or D54 memory ASCs when all patients with detectable plasma HIV RNA levels were considered (figure 1C and 1D), a significant inverse correlation with frequencies of D54 memory ASCs was observed when plasma HIV RNA levels >1000 copies/mL was considered ($r = -0.43; P < .05$), and a similar trend was observed with frequencies of D7 effector ASCs ($r = -0.37; P = .07$). Of note, and with the caveat that no placebo group was included in the present study, plasma HIV RNA levels in patients with >50 copies/mL were not significantly different at D0 and at D7 after vaccination ($Z = -0.0125; P = .99$, Wilcoxon rank sum test), suggesting that the vaccine had no measurable effect on plasma HIV RNA level.

Serum HI titers against the 3 HA antigens of the vaccine plus 1 derived from a Fujian-like strain were measured before vaccination (D0) and at 3 time points after vaccination (D7, D28, and D54) and are reported as kinetics of GMTs for each group (figure 3). The kinetics of antibody responses revealed several notable patterns: overlapping response curves for Panama and Fujian antigens, suggesting high cross-reactivity between the 2 strains; generally high D0 GMTs, followed by modest responses (see below); and peak GMTs that did not always occur at D28 in the HIV-infected groups. Because GMTs against Panama and Fujian (the strain associated with most cases of influenza during the season in question)—but not against Hong Kong and New Caledonia—continued to increase past D28 in groups HIV-HL and HIV-LH (figure 3), we suspected that cases of influenza between D28 and D54 were contributing to the increasing GMTs. When such cases were excluded from the analyses, the GMTs against the Panama and Fujian strains decreased as expected after D28 for the HIV-HL group but not for the HIV-LH group (data not shown). The continued increase or slow leveling off in GMT past D28 was a pattern consistently observed in both low–CD4+ T cell count groups (figure 3).
Prevaccination (D0 in figure 3) GMTs were significantly higher in HIV-negative individuals than in those in the entire HIV-infected cohort (P values ranging from <.001 to <.05 for the 4 antigens). Because the vast majority of participants in the study were vaccinated in 2002 (table 1) and repeated annual influenza vaccination has been associated with higher prevaccination HI titers and lower fold increases in titer at peak than in the HIV-negative group, with several patients in the high–HIV RNA level groups: HIV-LL (1/4 antigens), HIV-HH (2/4 antigens), and HIV-LH (4/4 antigens), with P values ranging from <.005 to <.05. Furthermore, and consistent with peak ASC frequencies, a significant direct correlation was observed (data not shown) between CD4+ T cell counts and peak HI titers at D28 for HA antigens Fujian (r = 0.31; P <.05), Hong Kong (r = 0.34; P <.05), and New Caledonia (r = 0.31; P <.05). Of note, peak ASC frequencies were also directly correlated with peak HI titers (P values ranging from <.05 to <.0005; data not shown), indicating that frequencies of antigen-specific ASCs in the peripheral blood at D7 were predictive of the serum levels of antigen-specific antibodies 3 weeks later.

**DISCUSSION**

We investigated multiple parameters of B cell responses to influenza vaccination in a cohort of HIV-infected individuals representing a wide range of disease stages and activity and compared these responses to those in HIV-negative healthy individuals. Whereas most studies of B cell responses to influenza in immunosuppressed/immunodeficient individuals have focused primarily on HI titers [16], we used an ELISPOT assay to evaluate both the early effector and the later memory phases of B cell responses to influenza. In doing so, we have gained further insight into immune dysfunction in the setting of HIV disease. Consistent with past [4–8, 14] and present findings on peak antibody responses, the peak effector anti-influenza B cell response measured by ELISPOT assay at D7 was directly correlated with CD4+ T cell count. Furthermore, the memory anti-influenza B cell response measured by ELISPOT assay at D54 was also directly correlated with CD4+ T cell count, with several patients in the 2 low–CD4+ T cell count groups unable to mount a response of >10 ASCs per million B cells. Taken together, our findings indicate that the loss of CD4+ T cells in HIV-infected individuals is associated with a weak influenza-specific antibody response, as measured by HI titers and ASC frequency, as well as with a diminished memory B cell response. These observations are quite consistent with the high dependency of influenza-specific B cell responses on CD4+ T cell help [18] and with the results of numerous studies indicating that HIV infection leads to an irreversible loss in T cell–dependent antigen-specific B cell responses [36].

We also found evidence that plasma HIV RNA level impedes influenza-specific B cell responses at viral burdens >1000 copies/mL. These data are consistent with those from a study, con-
Figure 3. Kinetic representation by geometric mean titer (GMT) of hemagglutination-inhibition (HI) antibody responses to influenza vaccination. The GMTs of HI titers to the 4 hemagglutinin antigens were calculated for the HIV-negative group (HIV-NEG) and for each of the 4 HIV-infected groups (HIV-HH [CD4+ T cell count >350 cells/μL and plasma HIV RNA level >10,000 copies/mL], HIV-HL [CD4+ T cell count >350 cells/μL and plasma HIV RNA level ≤10,000 copies/mL], HIV-LH [CD4+ T cell count <350 cells/μL and plasma HIV RNA level >10,000 copies/mL], and HIV-LL [CD4+ T cell count <350 cells/μL and plasma HIV RNA level ≤10,000 copies/mL]) before vaccination (day 0 [D0]) and at D7, D28, and D54 after vaccination. The 95% confidence interval for each GMT is indicated by the vertical and horizontal bars.

ducted when the limit of detection of HIV RNA plasma levels was 10,000 copies/mL, that found an inverse correlation between plasma HIV RNA level and HI titers [14]. These data are also consistent with recent findings suggesting that ongoing HIV replication may have a dichotomous effect on CD4+ T cell responses to HIV [37]; although low plasma HIV RNA levels appear to stimulate immune responses, there is a critical level above which plasma HIV RNA level begins to have a negative impact that overrides any stimulatory effect. It remains to be determined whether such a dichotomous effect of plasma HIV RNA level on B cell responses also occurs. To fully address this possibility, a larger cohort representing a wide range of viral burdens needs to be studied.

By introducing the ELISPOT assay to measure early and memory B cell responses against influenza antigens, we have expanded the scope of assays that can be used to evaluate responsiveness to influenza vaccination. We also found a good correlation between peak frequencies of influenza-specific ASCs at D7 and peak HI titers at D28, indicating that the burst of actively secreting plasma cells detected by D7 in the peripheral blood predicts the levels of influenza-specific antibodies that will later be detected in the serum. However, as described above, these 2 assays should be viewed as complementary. Whereas the HI assay provides a well-established correlate of protection [16], the ELISPOT assay, whose readout reflects ASC responses to the entire vaccine, was not designed to measure neutralization potential. Although it remains to be determined whether the ELISPOT assay can be successfully adapted to measure frequencies of influenza-specific neutralizing ASCs, such an assay would be highly desirable, because the high prevaccination responses often detected with HI assays, which can render the interpretation of HI titers difficult, are negligible with the ELISPOT assay.

A somewhat unique and ultimately very informative feature of the ELISPOT assay is its amenability to evaluating influenza-specific memory B cell responses. Antigen-specific B cell frequencies in humans are notoriously difficult to measure by flow cytometry. Hence, the ELISPOT assay remains the assay of choice for measuring antigen-specific human memory B cell responses.
By employing such an assay to measure influenza-specific memory B cell responses, we have been able to elucidate yet another defect associated with HIV infection—namely, the perturbation of memory B cell responses—as well as the fact that perturbations are observed to varying extents in patients at all stages of disease. However, since most of the patients enrolled in the present study were chronically infected and none would be defined as acutely infected, the temporal loss of anti-influenza B cell responses after HIV infection remains to be defined.

Finally, the low and possibly delayed nature of the B cell responses to influenza vaccination observed in HIV-infected individuals with low CD4+ T cell counts and high plasma HIV RNA levels, as well as the paucity of influenza-specific memory B cells elicited by the vaccine, indicate that the recommendation to vaccinate HIV-infected individuals annually [3] may be especially important for this category of patients. The strikingly higher prevaccination HI titers that were observed irrespective of vaccination history in HIV-negative individuals, compared with those in HIV-infected individuals, suggest that healthy individuals maintain a certain level of protection that is absent in the HIV-infected population. Although it remains to be determined whether the differences in prevaccination HI titers reflect differences in frequencies of memory B cells, there are indications that memory B cells contribute to the pool of long-lived plasma cells that are responsible for maintaining serum antibody titers [38]. In light of these defects, it may also be desirable to target both effector and memory B cell responses when considering strategies to enhance anti-influenza responses in the HIV-infected population. Although booster vaccination schemes have not proven successful in immunocompromised individuals [5, 39], recent strategies that employ adjuvants may prove to be effective at enhancing influenza-specific responses in HIV-infected individuals [40, 41]. There are also indications that live attenuated influenza vaccines may elicit a broader and more potent immune response (reviewed in [42]), an advantage over the current inactivated formulas that could improve the breadth and degree of protection in high-risk populations such as HIV-infected individuals. Although the theoretical potential
for adverse effects associated with the administration of a live vaccine needs to be addressed with clinical trials, findings in a small group of healthy HIV-infected individuals [43] suggest that a live attenuated influenza vaccine may be suitable for high-risk groups.

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