Differential immunogenicity of *vaccinia* and HIV-1 components of a human recombinant vaccine in mucosal and blood compartments

Peter A. Anton, F. Javier Ibarondo, W. John Boscardin, Ying Zhou, Elissa J. Schwartz, Hwee L. Ng, Mary Ann Hausner, Roger Shih, Julie Elliott, Patricia M. Hultin, Lance E. Hultin, Charles Price, Marie Fuerst, Amy Adler, Johnson T. Wong, Otto O. Yang, Beth D. Jamieson

* Center for Prevention Research and the UCLA AIDS Institute, David Geffen School of Medicine at UCLA, USA
* Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine at UCLA, USA
* Department of Biostatistics, UCLA School of Public Health, Los Angeles, CA, USA
* Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA
* Department of Mathematics and School of Biological Sciences, Washington State University, Pullman, WA, USA
* Tower Cancer Research Foundation, Beverly Hills, CA, USA

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This paper is dedicated to Dr. Janis Giorgi, UCLA, whose vigor, insight and passionate commitment to understand why, and how, some individuals remain well in the setting of HIV infection inspired this study. These efforts toward a protective vaccine with effective mucosal responses owe much to her focused energy.

**Keywords:**
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**Abstract**

Mucosal immune responses induced by HIV-1 vaccines are likely critical for prevention. We report a Phase 1 safety and immunogenicity trial in eight participants using the *vaccinia*-based TBC-3B vaccine given subcutaneously to determine the relationship between HIV-1 specific systemic and gastrointestinal mucosal responses. Across all subjects, detectable levels of blood *vaccinia*- and HIV-1-specific antibodies were elicited but none were seen mucosally. While the *vaccinia* component was immunogenic for CD8+ T lymphocyte (CTL) responses in both blood and mucosa, it was greater in blood. The HIV-1 component of the vaccine was poorly immunogenic in both blood and mucosa. Although only eight volunteers were studied intensively, the discordance between mucosal and blood responses may highlight mechanisms contributing to recent vaccine failures.

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**1. Introduction**

Worldwide, sexual transmission is the leading cause of HIV-1 infection. As HIV-1 primarily infects across sexually exposed mucosae, preventive vaccines must provoke protective immune responses at these tissue frontiers that are rich in vulnerable cellular targets [1–6,35]. Given past experience in human vaccine trials, as well as mucosal studies of highly exposed yet persistently seronegative (HEPS) individuals, it is anticipated that having mucosal HIV-1-neutralizing antibody and cytotoxic T lymphocyte (CTL) responses would be preferable features of HIV-1 vaccines [11,13,15,16,20–25]. Human studies have suggested that induction of systemic (blood) immune responses do not always translate into comparable mucosal responses, while inducing mucosal responses often, but not always, predict detectable and stable systemic, peripheral blood responses [7,8].

While the window of opportunity for vaccine-generated immune responses to prevent establishment of infection following exposure remains unknown, having functionally effective and locally active mucosal immune responses seems intuitively essential. Inducing mucosal immune responses has been the focus of many efforts over the past 5–10 years, including comparisons of different immunization routes, often alternating prime-boost strategies mucosally and systemically, as well as comparisons of dif-
different immunization sites, with subsequent evaluation of induced mucosal immune responses [7–19]. While this human, Phase 1 trial aimed to secondarily assess the differential impact on induced mucosal responses with two systemic immunization sites (deltoid and inguinal) [10], it was halted after enrollment of eight subjects in favor of a larger trial concurrent to address the question using a canarypox vaccine. While not sufficiently powered to clarify the original question regarding route of administration, this report sheds critical first insights on discordances in detected mucosal and systemic immune responses with an HIV-1 vaccine that utilizes a replicating vector.

2. Materials and methods

2.1. Study subjects

Eight subjects were enrolled on the basis of being vaccinia naïve by age (born after 1970) and history (travel, military service), HIV-1-seronegative, and at low risk for HIV-1 infection (6 males, 2 females; mean age 29.5 years with a range from 23–32 years). They were fully briefed on the infectious risks of vaccinia and TBC-3B vaccine safety in previous vaccine trials [5,26,27] as well as the potential for induced false positive HIV-1 serology [28]. Persons with immunological or gastrointestinal disorders were excluded. All subjects provided signed informed consent under University of California, Los Angeles (UCLA) IRB-approved protocols.

2.2. Vaccine

The vaccine used for these studies was a live recombinant vaccinia virus containing HIV-1env/gag/pol, TBC-3B (Therion Biologics Corporation, Cambridge, MA). This vaccine was produced under GMP conditions and provided, with IND support through the FDA, by Therion Biologics. Wild-type vaccinia (NYCBH) for laboratory studies was also provided by Therion Biologics.

2.3. Vaccination protocol

Participants were randomized (blinded to laboratory research personnel) to receive three SC deltoid (n = 4) or inguinal (n = 4) immunizations at weeks 0, 6, and 20, with clinical follow-up to week 72, all subjects received vaccine. The initial dose at week 0 was 10^8 PFU, followed by doses of 10^6 PFU at weeks 6 and 20. Inguinal vaccinations were administered as a modification of a previously described targeted iliac lymph node (TILN) protocol[10], by injection medial to the femoral vein to optimize delivery to the superficial inguinal, deep inguinal and external iliac lymph nodes. Both deltoid and inguinal vaccinations alternated between left and right extremities. While the data from the two sites is unique (meriting an IND with FDA for new site administration) and may be useful for others in the field, due to the small number of subjects studied, results are generally reported as “systemic”, not ‘deltoid’ or inguinal’. However, in figures, different legends clarify immunization sites.

2.4. Clinical laboratory safety monitoring

Routine clinical laboratory testing of complete blood counts, chemistries, HIV-1 ELISA, and plasma HIV RNA PCR (Roche Amplicor kit, Roche Diagnostics, Indianapolis, IN) were performed by the UCLA Medical Center clinical laboratories.

2.5. Blood sampling

Blood was obtained by standard venipuncture for plasma, serum separation and isolation of peripheral blood mononuclear cells (PBMC) by Ficoll-Hypaque gradient centrifugation.

2.6. Mucosal sampling

Mucosal sampling was performed as previously described [2,29,30] during two baseline visits (2 weeks prior, and immediately pre-vaccination at week 0), followed by two weeks after each vaccination (weeks 2, 8, and 22), and then again at 32 and 72 weeks after the first vaccination. During each sampling, anoscopy was first performed for placement of two, premoistened surgical sponges (Ultracell® Medical Technologies, North Stonington, CT) for 5 min to collect mucosal secretions for antibody quantification [31]. Flexible sigmoidoscopy was then performed with 20 biopsies acquired at approximately 30 cm from the anal verge as previously described [2,20,30], for isolation of mucosal mononuclear cells (MMC). Briefly, biopsies (8 mm × 2 mm × 1 mm from large-cup, endoscopic biopsy forceps (Microvasive Radical Jaw #1589, outside diameter 3.3 mm) were taken and immediately placed into 15 ml of tissue culture medium (RPMI 1640, Irvine Scientific).

2.7. Elution of mucosal antibodies from surgical sponges

Elution of antibody-containing fluid from the surgical sponges was performed with a protocol modified from previous reports [31]. Briefly, sponge samples for antibody quantification were immediately transported to the laboratory on ice and frozen at –80 °C for later batch processing. Absorbed rectal secretions were eluted twice with 250 μl cold PBS containing 0.25% BSA (Sigma Chemicals, St Louis, MO), 1% Igepal (Sigma Chemicals, St. Louis, MO) and 1× protease inhibitor cocktail (Sigma Chemicals, Louis, MO) from the sponges by centrifugation (10,000 rpm for 30 min at 4 °C). The recovered volume of secretion was calculated by subtracting the recovered volume from that recovered from negative control sponges that were run in parallel. Duplicate samples were pooled, frozen, and retrieved in batches for further analysis.

2.8. Evaluation of HIV-1-specific antibody responses

Total HIV-1 specific immunoglobulin was quantified in plasma and eluted rectal secretion samples from concurrent visits throughout the trial (weeks 0, 2, 8, 22). Quantification of HIV-1-specific antibodies was performed with a modification of previously reported protocols using the Vironostika® HIV-1 MICROELISA system (Organon Teknika Corp, Durham, NC) [20,32,37,38]. Samples were run according to the manufacturer’s instructions with the addition of a standard curve generated using serial dilutions (10–3000 ng/ml) of human anti-gp-120/160 IgG (Immunodiesagnostics, Inc Woburn, MA). Total IgG and total IgA were quantified in the eluted rectal secretions or plasma by ELISA previously reported [20,31]. In brief, 96-well plates (Corning Inc., Corning, NY) were coated overnight at 4 °C with rabbit anti-human IgG or IgA (Dako Corp, Carpenteria, CA) diluted 1/6000 in bicarbonate buffer (pH 9.6). Serially diluted standard curves utilized purified human immunoglobulin (IgG or IgA) ranging from 78–500 ng/ml (Jackson Immunoresearch Laboratories, West Grove, PA). Samples were run in duplicate, along with a positive control sample, for which performance characteristics and acceptable ranges had been previously established. Plates were incubated for 60 min at 37 °C, and washed five times in wash buffer prior to the addition of 100 μl of peroxidase conjugated rabbit anti-human IgG or IgA (Dako Corp, Carpenteria, CA). Absorbance was read at 492 nm using a Bench-
mark Plus ELISA plate reader (Biorad, Hercules, CA) equipped with Microplate Manager® software. Values were expressed in ng/ml as extrapolated from standard curves, and the means were calculated for each sample. Final results were expressed in units of anti-HIV-1/µg of total IgG + IgA.

2.9. Evaluation of vaccinia-specific antibody responses

Vaccinia-specific antibodies in blood and rectal secretions were detected by ELISA at the same time-points. Wells were coated with 50 µl of inactivated vaccinia virus (2 × 10^9 pfu/ml in 0.1% Tween 20 in PBS) for 60 min at 37 °C. After blocking for 60 min at room temperature with 1% BSA in PBS, wells were layered with serial dilutions of plasma. Plates were incubated for 90 min at 37 °C. Bound vaccinia-specific antibodies were detected with specific peroxidase-conjugated anti-human IgG, anti-human IgA, and anti-human IgM. Standard serial dilutions of human IgG, IgA, and IgM were used to enable comparisons of plasma and rectal secretion readings. Plasma and rectal fluids from weeks 2, 8, and 22 were compared to baseline levels from week 0.

2.10. Isolation of mucosal mononuclear cells

Colonial MMC were isolated from the sigmoid colon biopsies as previously reported [2,30]. Briefly, the biopsy fragments were washed, collagenase digested, and disrupted into single cell suspensions in medium containing piperacillin-tazobactam antibiotic (Zosyn, Wyeth Co., Philadelphia, PA) and amphotericin B (Fungizone, GIBCO Invitrogen, Carlsbad, CA). Typically, this procedure yielded between 2 and 5 million viable CD3+ T lymphocytes per 17 biopsies. Cell yield and phenotypes were quantified with Multitest staining and TRILCount counting beads (Becton Dickinson Immunocytometry Systems, San Jose, CA) respectively, following the manufacturer’s instructions. The remaining biopsies were used for histology and banking.

2.11. Polyclonal expansion of CD8+ T lymphocytes from PBMC and MMC

To obtain adequate numbers of CD8+ T lymphocytes (CTLs) for measurements of vaccine responses, CTLs from MMC and PBMC preparations were polyclonally expanded using a CD3/CD4 bi-specific monoclonal antibody as previously described [20]. Briefly, the cells were cultured for 14 days with the antibody and IL-2, which inhibits CD4+ T lymphocyte growth and stimulates CTL growth. This procedure has been shown to produce polyclonally expanded CTLs allowing quantitative measurement of antigen-specific cells reasonably approximating those in non-expanded lymphocytes [20,33,34]. Average yield of expanded CD3+ T lymphocytes was roughly 20 million expanded cells from 1 million fresh MMC [20], providing sufficient CTLs to use in the 53-pool ELISpot assays. Verification of expanded CTL numbers was confirmed using 3-color flow cytometry (CD3/CD4/CD8) and routinely demonstrated >85% purity of expanded CTLs from MMC and >95% from PBMC.

2.12. Evaluation of vaccinia- and HIV-1-specific CD8+ T lymphocyte responses

Standard IFN-γ ELISpot assays using the expanded CTLs from MMC and PBMC were utilized to measure both vaccinia- and HIV-1-specific CTL responses as previously reported [20,30,34,35]. For vaccinia-specific responses, autologous PBMC were infected with wild-type vaccinia virus (NYCBH) at a multiplicity of 3 PFU/cell for 16 h. These cells were then washed and utilized as antigen presenting cells at a ratio of 3 × 10^5 expanded MMC or PBMC CTLs with 3 × 10^4 vaccinia-infected PBMC. For HIV-1-specific responses, a library of HIV-1 peptides (consecutive 15-mers overlapping by 11 amino acids) spanning all HIV-1 proteins was added directly to the expanded MMC or PBMC. These were obtained from the NIH AIDS Research and Reference Reagent Repository (Gag catalog # 8116, Pol #6208, Env #9487, Nef #5189, Tat #5138, Rev #6445, Vpr #6447, Vpu #6444, Vif #6446, all Clade B consensus sequences with the exception of Env). The peptides were screened in 53 pools of 12–16 peptides each. Triplicate negative controls included expanded CTLs alone, and a positive control included expanded CTLs with anti-CD2/CD2R and anti-CD28 monoclonal antibodies (Becton Dickinson, San Jose, CA). After counting using an automated ELISpot counting system (Cellular Technologies Limited, Cleveland, OH), results were expressed as spot-forming cells (SFC) per million cells after subtracting the background mean of the negative controls (generally <50 SFC/well, usually <20 SFC/well).

2.13. Statistical analysis

As this Phase 1 trial was not powered for statistically significant endpoints, formal statistical inference was de-emphasized. Means and standard deviations of available data are reported for antibody and CTL data with estimated means and standard errors at each time point, computed using a mixed effects linear model. Specifically, the log-transformed values were modeled using a separate mean for each time period (baseline, 8, 22, 32, and 72 weeks) and a random subject effect. To confirm these observations, more complex models were also fit and similar results obtained. The resulting time trajectories are intended to visually convey the average response each week, and are not used for formal inference.

3. Results

3.1. Systemic (inguinal and deltoid) vaccinations with a recombinant vaccinia virus and mucosal biopsies were well tolerated in the study subjects

The eight individuals examined in this study were HIV-1-uninfected, vaccinia-naïve volunteers. These participants were vaccinated via either deltoid or inguinal SC inoculations of the TCB-3B vaccine at weeks 0, 6, and 20, with the rationale that inguinal delivery might better access deep inguinal lymph nodes, antigenically stimulating lymphocytes that would preferentially home to the colonic mucosa [10]. All eight were vaccinated, six of eight subjects completed the full vaccination series, and two had incomplete vaccination schedules due to mild adverse events (AEs). All eight subjects completed the follow-up biopsy protocol. There were no Grade 3 or 4 AEs, procedure-related events or HIV-1 infections during the trial. There were a total of 107 Grade 1 and 2 events (58 Grade 1 AEs for inguinal versus 42 in deltoid group; 5 Grade 2 AEs in inguinal versus 2 in deltoid group). Almost half of these Grade 1 and 2 events were mild vaccinia-related injection site related events (27 for inguinal and 27 for deltoid group). Of the reported AEs, 26 were Grade 1 constitutional symptoms post-vaccination such as malaise, myalgia, arthralgia, and headache. Overall, vaccination by both routes was well tolerated, with mild AEs as expected for vaccinia exposure.

3.2. Significant peripheral blood antibody responses to both HIV-1 and vaccinia vaccine components were observed

The vaccines were assessed for their HIV- and vaccinia-specific peripheral blood antibody responses by ELISA (Fig. 1A, C) at mul-
multiple time points after vaccinations with TCB-3B. HIV-1-specific antibody responses developed in all vaccines’ plasma, becoming detectable after the second or third vaccination (Fig. 1A). Vigorous vaccinia-specific antibody responses were observed in most vaccines after the first vaccination (Fig. 1C), peaking earlier than to the HIV-1 component (compare Fig. 1A to C). Of note, one subject (B01) had detectable vaccinia antibodies at baseline suggesting this individual had been exposed/vaccinated despite screening questions (all results were batch-processed at trial’s end). The blood HIV-1-specific antibody responses remained detectable out to 72 weeks, and peaked around week 22 at $351 \pm 266$ units/ml IgG + IgA. This level was lower than that observed in chronically HIV-1-infected individuals on antiretroviral drug treatment, who demonstrated mean blood levels of $17,256 \pm 8838$ units/ml IgG + IgA [20]. Thus, humoral responses against the HIV-1 component of the vaccine appeared to be less vigorous than natural responses against HIV-1 infection or the vaccinia component of the vaccine [20,32,35].

3.3. No mucosal antibody responses against either vaccinia or HIV-1 were observed

Mucosal antibody responses to vaccinia and HIV-1 were evaluated in the vaccines at the same time points. In contrast to the clear blood humoral responses against both vaccinia and HIV-1 after TCB-3B vaccination, mucosal antibodies against HIV-1 and vaccinia were essentially absent in all participants (Fig. 1B and D). By comparison, we previously measured significant HIV-1-specific antibody levels in the mucosa of chronically HIV-1-infected individuals on antiretroviral drug treatment [20], with average values of $38,464 \pm 44,441$ units/μg IgG + IgA (Fig. 1B inset). Thus, this vaccine failed to induce mucosal vaccinia-or HIV-1-specific antibodies comparable to natural infection.

3.4. Vaccinia-specific CTL responses were detected in blood and mucosa

Given the global CTL activation observed after vaccination with TCB-3B and the known immunogenic potency of vaccinia as a smallpox vaccine, the blood (Fig. 2C) and mucosa (Fig. 2D) compartments were assessed for CTL responses against the vaccinia component of TCB-3B. There was early evidence of a vaccinia-specific CTL response in both compartments. Across all vaccines, a rapid rise in blood vaccinia-specific CTL was noted after the first vaccination, and this response appeared to peak after the third vaccination (Fig. 2C). There was a similar pattern in the mucosa, although overall frequencies appeared lower than the blood (Fig. 2D). These results suggest that vaccinia did promote CTL responses that trafficked through both blood and mucosa compartments.

3.5. Minimal HIV-1-specific CTL responses were detected in blood and mucosa

At the same time-points, CTL responses against the HIV-1 component of TCB-3B were evaluated in both blood (Fig. 2A) and mucosa (Fig. 2B) of the vaccines. HIV-1-targeted CTL responses were modest or absent in the blood, with a few individuals showing possible low level responses but the majority demonstrating no detectable HIV-1-specific CTL (Fig. 2A). Within the mucosal compartment, there were no clearly discernable patterns of reactivity, and the majority of vaccines had no detectable HIV-1-specific CTL (Fig. 2B). Background levels were much higher in mucosal assays, and it was thus unclear whether these represented true specific activity. Overall, these data suggested that vaccinia was immunogenic in both compartments, with higher reactivity in the blood, while the HIV-1 component of the vaccine was minimally immunogenic in either compartment.
4. Discussion

The recent failure of the STEP trial, which tested a promising candidate for generating HIV-1-specific cellular immunity, raises important questions about the mechanisms of HIV vaccine protection. One potential explanation for recent observed failures would be that mucosal humoral and CTL responses were lacking at the site where HIV-1 transmission occurs. Mucosal immunity in response to vaccines remains a poorly understood area.

This is the first Phase 1 trial investigating mucosal immune responses to the previously studied HIV-1 vaccine TBC-3B, a live recombinant vaccinia virus containing HIV-1IIIB env/gag/pol. Low risk HIV-1 seronegative subjects were immunized via deltoid versus inguinal routes to evaluate human response correlates of previously reported macaque data using targeted inguinal lymph node vaccination [1,10]. Eight subjects completed the trial. This number was too few to provide initial insights into the question of whether deltoid versus inguinal site of systemic immunization induces more pronounced mucosal immune response, which will be addressed in a recently completed, larger, similarly designed trial using a non-replicating, canarypox recombinant vaccine (Anton et al., manuscript in preparation). This small vaccinia trial demonstrated safety for both routes of vaccination, the safety and blood immune responses are similar to other reported vaccinia-based HIV-1 vaccine trials [26–28,36].

As expected, the vaccines demonstrated detectable blood humoral responses to vaccinia and HIV-1 antigens after vaccination. These blood responses were durable, providing evidence that both vaccinia and HIV-1 vaccine components were antigenically available to generate immune responses. However, it was observed that the peak levels of vaccine-induced blood HIV-1-specific antibodies were significantly lower than in natural HIV-1 infection [20].

Disappointingly, there were no detectable mucosal antibodies against either vaccinia or HIV-1. These data, using an assay that consistently detects HIV-1-specific mucosal antibodies in HIV-1-infected subjects [20], suggest that vaccine access to the mucosal immune compartment might be a limiting factor. During pilot studies, we compared rectal lavage collection methods, as reported by others [37,38], but found tremendous variability in recovery, often relating to the participant’s state of hydration. Using the same method with surgical sponges, we have reported vigorous mucosally secreted HIV-1 specific antibodies in HIV-1 seropositive subjects, indicating the ability of this technique to detect such...
responses [20,39]. However, comparative data on mucosal antibody responses against other vaccines that successfully protect against other mucosally acquired infections, such as hepatitis B virus, are lacking.

CTL responses to the both vaccinia and HIV-1 components were contrasting. Consistent with its known immunogenicity and efficacy as a vaccine, vaccinia-induced a CTL response in most vaccines. Blood frequencies of vaccinia-specific CTL appeared to be somewhat higher than in mucosa. In contrast, HIV-1-specific CTL responses were sporadic and generally absent in both compartments. This finding may correlate, in part, with the lesser immunogenicity of the HIV-1 component of the vaccine (subdominance) compared to the replicating vaccinia portion.

The discordance of responses between blood and mucosal compartments may be instructive. It has long been known that adaptive immunity is compartmentalized between the mucosa and systemic circulation, and that systemic immunization does not ensure mucosal protective responses, while the converse is more often true [7,8]. Insufficient immunogenicity, particularly of the vaccine's HIV-1 component, may have contributed to the observed greater response in blood versus gut mucosa; the same was observed for responses against the vaccinia component. However, while vaccinia is a historically effective vaccine against smallpox, the usual CTL responses against wild type vaccinia in the mucosae and their contribution to vaccine efficacy against smallpox are unknown.

In the absence of vigorous CTL responses against either vaccinia or HIV-1 detected in the mucosal compartment, it is difficult to know whether immunodominance of one vaccine component over the other could play a role in the observed paucity of HIV-specific incurred CTL responses. In summary, this first attempt to quantify mucosal immune responses to a systemically delivered HIV-1 vaccine demonstrated the safety and feasibility of targeted inguinal delivery of this vaccine.

The highly immunogenic vaccinia component of the vaccine generated blood but not mucosal antibody responses, and elicited vaccinia-specific CTL responses in both blood and mucosa compartments. The HIV-1 portion of the vaccine was more weakly immunogenic, generating delayed blood antibody responses and no mucosal antibody responses, with low or absent CTL responses in both blood and mucosa compartments. The results suggest that generating mucosal HIV-specific, humoral and CTL responses via systemic immunization with this vaccine is difficult. These observations underscore the need to investigate mucosal responses to future HIV-1 vaccine candidates at an early time-point in product pipeline development.

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