Short Communication: Parallel Analyses of Systemic and Local Vaccinations with Envelope Formulated in Adjuvant for Induction of HIV-Specific Antibodies in the Vaginal Mucosa

Robert E. Sealy¹ and Julia L. Hurwitz^{1,2}

Abstract

To prevent HIV-1 infections in females, vaccines that elicit antibodies in vaginal secretions are much desired. To induce these antibodies, intravaginal vaccinations are sometimes recommended. However, the benefit of intravaginal vaccination remains a topic of debate, and parallel studies of intravaginal and intramuscular vaccination routes are rarely performed. Here we describe tests of mucosal and systemic antibodies after mouse vaccinations by several routes with HIV-1 envelope protein formulated in adjuvant. Response magnitudes were as follows: intraperitoneal > intramuscular = intravaginal tissue ≥ subcutaneous. We found that the well-accepted and logistically feasible intramuscular immunization was similar to intravaginal tissue immunization for the induction of antibodies in blood and vaginal secretions. Results suggest that a routine comparison of intravaginal and intramuscular immunizations may serve as a beneficial gatekeeper for the development of new intravaginal HIV-1 vaccines.

Keywords: intravaginal, intramuscular, intraperitoneal, route of immunization, vaccine

HIV-1 INFECTIONS and AIDS have persisted for decades in the human population, and there remains no successful licensed vaccine. Because HIV-1 infections often initiate in vaginal tissues in females, a vaccine that can induce antibodies at this site is much desired. To induce such antibodies, researchers sometimes advocate vaccination by the intravaginal route, but other researchers argue that this site is noninductive.^{1–3} To address the debate, we designed experiments in which side-by-side comparisons of immunization routes were performed: intramuscular (i.m.), intraperitoneal (i.p.), subcutaneous [s.c. (beneath the skin of the back or leg)], or intravaginal tissue [ivag-t (by superficial injection into vaginal tissue)]. Our studies were with an affinity-purified HIV-1 gp140 envelope protein (named 1007⁴) in female adult C57BL/6J animals.

In a preliminary experiment, we compared adjuvants. We mixed 5 µg HIV-1 envelope protein with CFA/IFA [Sigma, Freund's adjuvant complete or incomplete; envelope/phosphate-buffered saline was emulsified in CFA (for the first injection) or IFA (for booster injections) using equal volumes of antigen and adjuvant], alum (Imject® Alum, Pierce Cat #77161; equal volumes of antigen and adjuvant were mixed per the manufacturer's recommendations),

nonoxynol (Options Conceptrol Vaginal Contraceptive Gel with 4% nonoxynol-9, Ortho, 10 µl/dose⁵), or mannan (Sigma M7504, 0.6 mg/dose, prepared as described previously⁶). Vaccines were injected superficially into vaginal tissue (ivag-t, 50 microliters per mouse injection) to ensure retention of product at the site. We found that CFA/IFA generated the best response among the tested adjuvants (data not shown). The next best response was with alum or nonoxynol, and because alum is a licensed product, it was selected for continued study.

Parallel tests of different injection routes were then performed with the envelope protein/alum vaccine. In this case, animals received priming and booster immunizations separated by a 3- to 4-week interval. As before, 5 µg protein in alum were used per injection in a 50 µl final volume. Animals were not pretreated to synchronize estrus cycles before vaccinations.

Figure 1A and B shows the results from an experiment in which i.m. (in the hind leg) and ivag-t immunizations were compared. Animals either received their priming and booster dose by the same route (homologous, i.m. followed by i.m., or ivag-t followed by ivag-t) or by different routes (heterologous, i.m. followed by ivag-t, or ivag-t followed by i.m.),⁷

¹Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee.

²Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee.

FIG. 1. Comparing antibody responses toward HIV-1 envelope protein/alum after immunizations by different routes. HIV-1 envelope-specific antibody responses are shown in sera (**A**) and vaginal washes (**B**) following prime–boost strategies using ivag-t, i.m., or alternating routes. Each set of *bars* represents a different mouse. Experiments were repeated to ensure reproducibility. A preliminary analysis of longitudinal responses is also shown. Sera were analyzed during a prime–boost protocol with homologous i.m. (**C**), i.p. (**D**), ivag-t (**E**), and s.c. (**F**) immunizations. In the same mice, envelope-specific antibodies in vaginal washes were tested 6 months after the boost (**G**). For serum analyses, *bars* represent sample dilutions of 1:100 (*black bars*), 1:1,000 (*gray bars*), and 1:10,000 (*white bars*). For wash analyses, *bars* represent sample dilutions of 1:5 (*black bars*) or 1:50 (*gray bars*). *Methods:* Adult female C57BL/6J mice were used. Animals received two injections with 5 μ g HIV-1 envelope protein in alum with a 3- to 4-week interval. Inject alum contains an aqueous solution of aluminum hydroxide (40 mg/ml) and magnesium hydroxide (40 mg/ml) plus inactive stabilizers. Mice were anesthetized with isoflurane before injections and sampling. Vaginal samples were collected by washing the lumen twice with 50 μ l PBS, followed by a brief microcentrifugation of combined washes to remove debris. Ninety-six-well ELISA plates were coated with 1 μ g/ml purified HIV-1 (1007, 50 μ l/well) envelope protein in PBS. Plates were incubated overnight at 4°C. Plates were washed 2 $\times$ with PBS and then blocked with 100 μ l 1% BSA in PBS for 2 h at room temperature. Mouse sera or vaginal samples were serially diluted in 1% BSA in PBS. After removing blocking buffer from wells, 50 μ l of sample were added per well in replicate and incubated for 1 h at room temperature. Plates were washed 6 $\times$ with PBS. Next, alkaline phosphatase-conjugated goat anti-mouse IgG [Southern Biotechnology Associates, Inc. (SBA)] was diluted to 1:1,000 and added to plates at 50 μ l per well. Plates were incubated for 1 h at room temperature and then washed 6 $\times$ with PBS. To each well, 50 μ l of p-nitrophenyl phosphate (Sigma-Aldrich) at 1 mg/ml in diethanolamine buffer was added. OD 405 nm readings were recorded. Statistical tests were conducted using the GraphPad Prism Software. BSA, bovine serum albumin; i.m., intramuscular; i.p., intraperitoneal; ivag-t, intravaginal tissue; PBS, phosphate-buffered saline; s.c., subcutaneous.

and anti-envelope antibodies were tested 2 weeks after the booster by ELISA.

As demonstrated in Figure 1A and B, the homologous i.m. immunizations were similar to homologous ivag-t immunizations or heterologous immunizations (i.m. followed by ivag-t, or ivag-t followed by i.m.) for the induction of serum (Fig. 1A) and vaginal wash (Fig. 1B) antibody responses. One-way analysis of variance (ANOVA) with the Tukey Multiple Comparisons Test was performed and showed no significant differences between i.m. and ivag-t immunizations, but showed that antibodies in vaccinated animals were significantly improved compared with controls ($p < .05$).

When i.p. and s.c. immunizations were added to comparisons, i.p. immunizations were found to be the best at inducing antibody responses in blood and vaginal wash, and s.c. immunizations were weak. Analyses were routinely conducted 2 weeks after the boost, but a preliminary longitudinal experiment was also conducted, in which all four routes of immunization (i.m., i.p., ivag-t, and s.c. by homologous prime and boost) were compared over an extended time course.

As shown in Figure 1C, the i.m. injections elicited responses postboost that waned considerably, but incompletely, by 6 months after the boost. This response was not as strong as that achieved following i.p. injections (Fig. 1D). Responses to ivag-t injections (Fig. 1E) were no better than i.m. injections, and s.c. injections (Fig. 1F) yielded only weak responses. The i.p. vaccination route was the only route that maintained detectable responses in several, but not all, animals in vaginal washes 6 months after the boost (Fig. 1G).

A one-way ANOVA with the Tukey Multiple Comparison Test was conducted using serum results from the 2-week postboost time point for each of the vaccination regimens in the longitudinal experiments (Fig. 1C–F) and demonstrated a significant difference between the i.p. group versus all other groups at each of the three tested serum dilutions ($p < .05$). The i.p. route was also significantly better than all other routes for the maintenance of antibody responses in vaginal washes 6 months after the boost (Fig. 1G).

Altogether, our results showed that the ivag-t-tissue injection was not superior to i.m. immunizations for the induction of local immunity with envelope protein/alum formulations. Intraperitoneal injections were best of all. These conclusions are made with the understanding that our experiments were conducted only in mice and that numerous untested variables (e.g., the use of a replication-competent vaccine) could have improved immunization outcomes. A recent study by Stary *et al.*⁸ identified an attractive new adjuvant and route that may improve upon old systems for the induction of immune responses in the female reproductive tract. The new vaccination regimen was compared with s.c. vaccinations and was proven superior. A side-by-side comparison between the new strategy and i.m. immunizations is now encouraged.

As stated previously, the vaginal route of immunization has been described as noninductive by some researchers.³ In some cases, a heterologous prime-boost strategy using a combination of local and systemic routes of immunization might improve outcome,^{6,7} but in our study, we found that the homologous i.m. prime-boost was comparable to the heterologous prime-boost for the induction of systemic and local immune responses.

Other vaccination sites [e.g., intranasal (i.n.) or sublingual] were not tested as part of our analyses and may have improved outcome, particularly for the induction of both IgA and IgG (our preliminary tests revealed poor IgA responses in vaginal washes, as predicted by previous literature³). In theory, the engagement of common mucosal lymphoid tissue should assist immune responses in vaginal secretions.⁶ We note, however, that Barnett *et al.*⁹ showed that when macaques were immunized by homologous i.m. immunizations, animals were protected from a vaginal challenge with a chimeric simian/human immunodeficiency virus, but homologous i.n. immunizations were not protective.

In conclusion, our results show that the i.m. vaccination route is comparable to the ivag-t route for the induction of systemic antibodies and antibodies in vaginal washes. The i.p. route was better than all others, and the s.c. route was weak. Data suggest that a routine comparison of ivag and i.m. immunizations may serve as a beneficial gatekeeper for the development of new ivag HIV-1 vaccines. The i.m. vaccination route is more attractive because it is simple and is a well-accepted route for vaccine delivery. Experience with the licensed papillomavirus vaccine (GARDASIL, a multivalent vaccine representing nine different virus types) serves as precedent, showing that an i.m. vaccination regimen protects humans from a sexually transmitted disease.¹⁰ Possibly, i.m. injections of a multi-envelope HIV-1 vaccine⁷ will eventually protect humans from HIV-1 infections and AIDS.

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Author Disclosure Statement

JLH is named on a patent describing a multi-envelope HIV-1 vaccine. RES reports no conflicts of interest.

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Address correspondence to:

Julia L. Hurwitz, PhD

Department of Infectious Diseases

St. Jude Children's Research Hospital

262 Danny Thomas Place

Memphis, TN 38105

E-mail: julia.hurwitz@stjude.org